

THE CELLULAR ROLE OF THE SPECIFIC IMMUNE SYSTEM IN FRACTURE REPAIR

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Declaration

The cellular role of the specific immune system in fracture repair

I confirm that the work within this thesis and its composition is my own and that it has not been submitted for any other degree or professional qualification

Mark Gaston

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Abstract

The cellular role of the specific immune system in fracture repair

This thesis embodies a novel investigation into the cellular role of the specific immune system in fracture repair. McKibbin first described in detail the stages of fracture healing in the early 1970s and while much work has been done since, particularly on the molecular aspects of this process, little work has investigated the roll of the cellular components of the acquired or 'specific' immune system. An estimated 10% of patients have problems with fracture healing. Initial studies have revealed that it is likely that both the innate and specific immune systems play a role in fracture repair, but this has not been attributed to particular components, cells or their products. Studies from soft tissue wound healing have shown the importance of T cells and suggest an overall inhibitory role. It is also known that T cells play a large role in the maintenance of normal bone via their effect on osteoclast activation (via RANKL) and inhibition (via the release of interferon γ). This study used a validated murine model of bone healing, employing a reproducible closed tibial fracture. This had the advantage of not requiring internal fixation thereby preventing any foreign body response which could alter the immune response. The specific immune system in fracture repair was examined in several ways.

Firstly, the cells of the specific immune system were identified using immunohistochemistry in the fracture callus and shown to be actively retained there after organisation of the haematoma. Secondly, an immuno-deficient mouse, with no functioning T or B cells (SCID) was subjected to the fracture model and showed enhanced fractured repair with quicker healing times compared to the wild type with equivalent genetic background. This was assessed histologically, radiographically, with measures of bone density and bio-mechanically. To ensure this animal had no inherent enhancement of fracture repair it underwent reconstitution of the cellular specific immune system which was ensured by measuring an antibody response. This reconstituted animal then underwent testing of fracture repair and showed that the enhancement was lost. Comparison of the immune-cellular immunohistochemistry between the immune-competent and SCID animals revealed selective recruitment of macrophages to the active areas of fracture healing in both animals. In wild type animals T cells were also actively recruited suggesting an active role for both these cells in the normal process. Lastly, examination of the same cells *systemically* showed mobilisation of the specific immune cells followed by rapid depression and then recovery. It is likely that these cells were mobilised for early availability to the fracture callus.

These studies suggested an inhibitory role for the specific immune system in fracture healing, in particular T cells. In fracture repair it may be that T cells act to counter the effects of the macrophage to give a more ordered bony healing process. It is thought they act in this way systemically following major trauma to 'dampen' the inflammatory response. Further research is required to determine which particular T cell or ratio of T cells and the factor/s which they produce that is causing this inhibition of fracture healing. Ultimately this could result in novel therapies directed toward avoidance of non/delayed-union.

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Abbreviations

BMP	Bone Morphogenetic Protein
DAB	Diaminobenzidine
DNEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic Acid
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
H&E	Haematoxylin and Eosin
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
mm	millimetre
M	Molar
Mpa	Megapascal
N	Newton
NaCl	Sodium Chloride
PBS	Phosphate Buffered Saline
RANK(L)	Receptor Activator of Nuclear Factor Kappa Beta (Ligand)
SCID	Severe Combined Immuno Deficient
TBS	Tris Buffered Saline
TNF	Tumour Necrosis Factor
TRANCE	(TNF)-Related Activation-Induced Cytokine
TGF-β	Transforming Growth Factor Beta

Chapter 1

Introduction

Section one: General introduction

1.1 Background

Bone is a dynamic tissue that provides mechanical support, physical protection, and enables movement. However the physical capacity of bone to withstand force is often overcome and this is when a fracture occurs. Over 1 million fractures occur each year in the UK and with the ageing population this figure is known to be increasing. Bone also serves as a storage site for minerals and is where blood cells are produced. Bone remodelling refers to the constant state of turnover with formation and resorption being carried out by osteoblasts and osteoclasts respectively. An estimated 10% of adult total bone is remodelled every year through the action of bone forming and resorbing cells (MacDonald and Gowen 1993). It has recently become appreciated that the skeletal system is greatly influenced by components of the immune system. For example, some pathological bone resorption observed under inflammatory conditions has been shown to be due, in part, to direct and indirect effects of activated T cells on osteoclasts (Rho, Takami *et al.* 2004).

The discovery that activated T cells express tumour necrosis factor (TNF)-related activation-induced cytokine (TRANCE), a member of the TNF superfamily, coupled with the subsequent finding that TRANCE is a key differentiation factor for osteoclasts *via* receptor activator of nuclear factor kappa beta (RANK), provides evidence that a link exists between normal immune cell function and bone metabolism (Walsh, Kim *et al.* 2006). During their lifetimes, mammals are challenged with various infectious

agents which result in a gradual change in the composition of the T cell compartment toward an accumulation of TRANCE-expressing memory cells that preferentially reside in bone. Hence, with age the immune system might exert a greater influence on bone homeostasis. It is from this work that the research field termed “osteimmunology” has emerged.

Despite this few studies exist which have investigated the role of the immune system on fracture repair. At fifty years of age the lifetime risk of a fracture is 53.2% and 20.7% for women and men respectfully (van Staa, Dennison *et al.* 2001) and this increases with age. With the elderly population set to rise the social and economic burden of fracture treatment will become greater. Fracture repair is a wound healing process that in young healthy patients usually proceeds to uncomplicated union. However, the healing cascade is delayed with increasing age, certain diseases such as rheumatoid arthritis and osteoporosis and some medications (Bogoch and Moran 1999). Apart from the known effects of NSAIDs (non-steroidal anti-inflammatory drugs) little research has been done to determine the role of the immune system in fracture repair or into the area of immunosuppression and fracture healing and how this might be modulated to improve bone repair (Beck, Salem *et al.* 2005). Andrew *et al.* showed infiltration of T cells into the fracture callus early in human fracture repair and they concluded that T cells ‘may control and coordinate fracture healing’ (Andrew, Andrew *et al.* 1994). However there are no studies reported which manipulate the specific (adaptive) immune system in fracture repair. The contribution that the cells of the specific immune system

make to soft tissue healing is well documented (and is discussed further later) while this is not so in the field of bony fracture repair.

Section one of this chapter reviews the current literature in the field of fracture repair and how the immune system interacts with this. It will place in context the experimental content of this thesis. This includes a review on the most current understanding of the biology and function of bone and an in depth review of the current knowledge on fracture healing and osteoimmunology.

Section two outlines the aims of this thesis as well as stating the hypothesis.

1.2 Bone biology:

1.2.1 Skeletal development:

The skeleton is one of the largest organs in the body. It is composed of a mineralised framework that is maintained by a complex cellular network (Raisz, Kream *et al.* 2002).

The skeleton is made up of specialised connective tissues, namely bone and cartilage and serves several functions within the body. It provides structural integrity and is protective for vital organs and bone marrow. It is a storehouse for calcium and contains 99% of body calcium, a critical ion for metabolic processes, and contains 85% of body phosphorus. It is the site of haematopoiesis and has major mechanical functions including joint provision and muscle attachment which allow for mobility.

Bone development occurs along two pathways. The first type is *via* the production of endochondral bone, which includes the long bones and vertebrae and begins in the embryo as a cartilaginous template (Ross, Kaye *et al.* 2003). In such bone the condensed embryonic mesenchyme transforms into cartilage, which reflects in both position and shape, the eventual bone to be formed at that site. In the central part (or diaphysis) of such a bone, there is linear, interstitial proliferation of columns of chondrocytes. These cells undergo progressive hypertrophy, and mineralisation of the intercolumnar cartilage matrix in the long axis of the bone (primary ossification centre). The persistence of mineralised cartilage after death of its cells (by apoptosis) acts as a scaffold for deposition of subchondral (metaphyseal) bone (Hunziker 1994).

Concomitant neovascularisation occurs, and osteoclasts and osteoblasts are recruited to replace the cartilage scaffold gradually with bone matrix and to excavate the bone marrow cavity. Longitudinal bone growth takes place through a similar pattern of endochondral ossification in the growth plates (physis) of long bones. At the physis the calcified, hypertrophic cartilage provides a scaffold for the formation of new trabecular bone. Ultimately, all remaining cartilage is replaced by bone except at the articular surfaces of the joints (Erlebacher, Filvaroff *et al.* 1995; Ferguson, Miclau *et al.* 1998). See Figure 1.1.

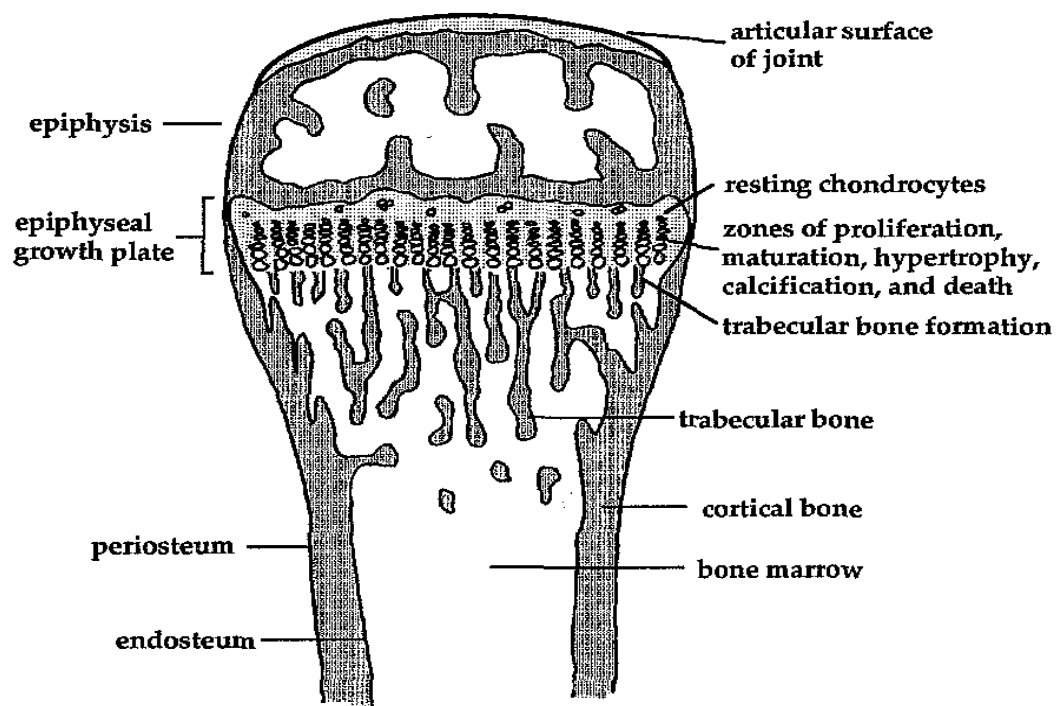


Figure 1.1: Long Bone Growth through Endochondral Ossification: Longitudinal bone growth occurs at the epiphyseal growth plate in which chondrocytes proliferate and mature, and the calcified matrix of the hypertrophic cartilage provides a scaffold for new trabecular bone formation. Above the growth plate, the secondary ossification centre has already replaced the epiphyseal cartilage with bone and bone marrow. Modified from Erlebacher *et al Cell.* 1995 Feb 10; 80(3):371-8.

The second type of bone, intramembranous bone, is responsible for production of the flat bones, *e.g.* skull, scapula and ileum. Intramembranous ossification occurs within a highly vascularised area during embryonic development by the direct transformation of a condensation of mesenchymal cells into preosteoblasts which differentiate into osteoblasts. This process not only generates the flat bones but also adds bone to the outer surface of the long bones, thereby contributing to width (appositional growth). The bone matrix in intramembranous bone formation characteristically has collagen fibres which are not preferentially oriented but appear as irregular bundles. Intramembranous bone formation has osteocytes which are large and numerous and shows calcification which is delayed and does not proceed in an orderly fashion but as irregularly distributed patches. The initial type of bone produced is the disorganised, disordered woven bone into which blood vessels are incorporated between the trabeculae and will form the haemopoietic bone marrow. Later this woven bone is remodelled and progressively replaced with mature, organised, layered lamellar bone (Kerr 1987).

1.2.2 Cells of bone

Bone is composed of four main types of cell osteocytes, osteoblasts, osteoclasts and bone lining cells. Osteoblasts, osteoclasts and the bone lining cells are present on the bone surface, whereas osteocytes reside in the mineralised interior. Osteoblasts, osteocytes and bone lining cells originate from local osteoprogenitor cells, whereas

osteoclasts arise from the fusion of mononuclear precursors, which originate in haemopoietic tissues (Marks and Hermey 1996).

Osteoprogenitor cells arise from mesenchymal stem cells which can also give rise to adipocytes and myoblasts. A common myeloid precursor gives rise to mononuclear precursors which differentiate to osteoclasts but can also give rise to macrophages and dendritic cells (Aubin, Liu *et al.* 1995). A stem cell is defined as “a cell type which, in the adult organism, can maintain its own numbers in spite of physiological or artificial removal of cells from the population” (Owen 1988). The high capacity for regeneration and formation of bone tissue suggest that these normally quiescent stem cells supply the cellular requirements for extensive new bone formation. See Figure 1.2.

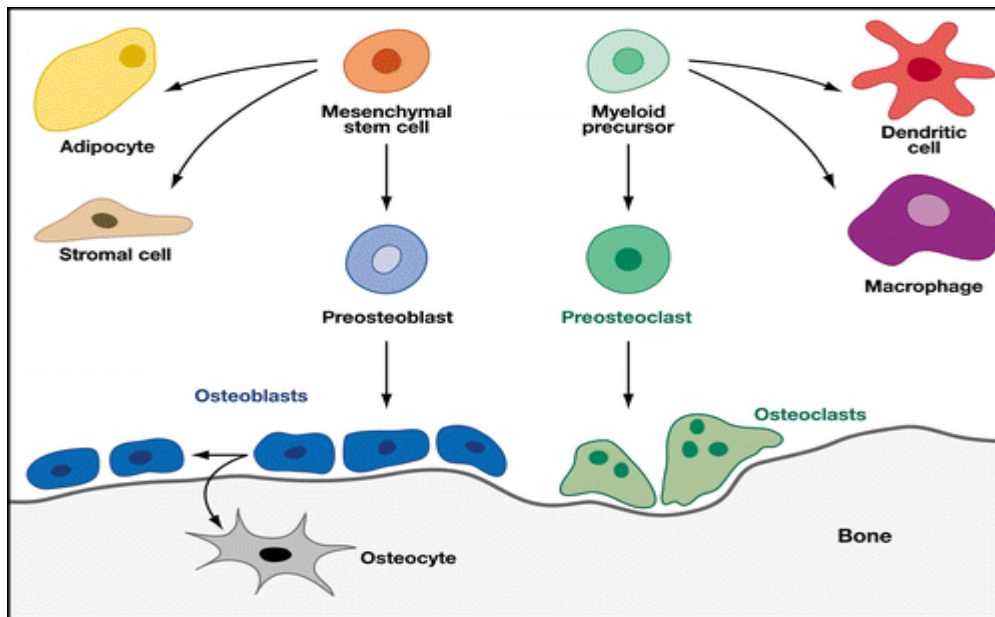


Figure 1.2 Schematic summary of bone cell differentiation. Mesenchymal stem cells, which also give rise to myoblasts, adipocytes, chondrocytes, and some as yet uncharacterized stromal cells, differentiate into preosteoblasts and then become osteoblasts on the bone surface. Osteoblasts either incorporate into bone as osteocytes or remain on the surface as lining cells. A common myeloid lineage precursor, which can also give rise to macrophages and dendritic cells, commits to becoming a preosteoclast and then fuses to become a mature, multinucleated osteoclast. Modified from Walsh MC *et al.*, 2006 Annu. Rev. Immunol. 24: 33-63

Osteoblasts:

Osteoblasts are fully differentiated bone forming cells responsible for the production of matrix and regulation of its mineralization. They are derived from a local mesenchymal stem cell. The signals that regulate the decision of mesenchymal stem cells to form osteoblasts are not fully understood. However, a number of critical paracrine signals and transcription factors have been identified. These include the transcription factors Runx2 and osterix, which when absent prevent osteoblast formation, and members of the bone morphogenetic protein family (particularly 2 and 7), which initiate the signals for osteoblast differentiation (Little, Carulli *et al.* 2002). Most recently, it was demonstrated that Wnt signalling pathways are involved in the decision of the mesenchymal stem cell to become either an adipocyte or an osteoblast (Bennett, Longo *et al.* 2005).

The osteoblast is a typical protein producing cell with a prominent Golgi apparatus and well-developed rough endoplasmic reticulum. It secretes Type 1 collagen and non-collagenous proteins of the bone matrix. It is characterised by a round nucleus at the base of the cell opposite the bone surface and a basophilic cytoplasm. Osteoblasts are post-proliferative (*i.e.* non-dividing), cuboidal, strongly alkaline phosphatase positive cells, lining bone matrix at sites of active matrix production (MacDonald and Gowen 1993). The collagen and proteoglycan matrix secreted by osteoblasts is not mineralised immediately. Unmineralised matrix is referred to as osteoid (Remedios 1999). In woven bone, mineralisation is initiated away from the cell surface in matrix vesicles that bud from the plasma membrane of osteoblasts. This is similar to the well documented role

of matrix vesicles in cartilage mineralisation (Barckhaus and Hohling 1978). In lamellar bone, the mechanism of mineralisation begins in the region between overlapped collagen molecules where there are few if any matrix vesicles (Landis, Song *et al.* 1993) and appears to be initiated by components of the collagen molecule itself or noncollagenous molecules at this site. Whatever the mechanisms of mineralisation, collagen is at least a template for its initiation and propagation and there is always a layer of osteoid on the surface of osteoblasts. Matrix deposition is usually polarised towards the bone surface, but periodically becomes generalised, surrounding the osteoblast, entrapping it in calcified matrix. These osteoblasts then persist in bone as unique cells called osteocytes (10% of osteoblasts are thought to become osteocytes (Remedios 1999)). Deposition of mineral (mostly calcium hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) makes the matrix impermeable to almost all nutrients and metabolites. To ensure a metabolic lifeline, osteocytes establish numerous cytoplasmic connections with adjacent cells before mineralisation (Marks and Hermey 1996).

Osteocytes:

The osteocyte is a mature osteoblast within bone matrix and is responsible for bone maintenance. These cells have capacity not only to synthesise, but also to resorb matrix to a limited extent. They do not provide a net increase in matrix but are vital for its routine turnover and therefore maintenance (Buckwalter, Glimcher *et al.* 1996). Each osteocyte occupies a space, or lacunae, within the matrix and extends filopodial processes through canaliculi in the matrix to contact processes of adjacent cells by

means of gap junctions (Palumbo, Palazzini *et al.* 1990). Because diffusion of nutrients and metabolites through the mineralised matrix is limited, filopodial connections permit communication between neighbouring osteocytes, bone lining cells and with pericytes of blood vessels traversing the matrix. However, osteocytes cannot survive more than 0.2mm away from a blood vessel (Cross and Mercer 1993). Osteocytes modulate signals arising from mechanical loading and so direct the appearance and disappearance of bone tissue at the microscopic level, which allows bone as an organ both to grow and to adapt efficiently to the body's mechanical needs for strength with lightness. They may also direct the removal of damaged or redundant bone through mechanisms linked to their own apoptosis or *via* the secretion of specialised cellular attachment proteins such as osteopontin (Noble, Stevens *et al.* 1997; Noble and Reeve 2000). More recently the release of fibroblast growth factor 23 by osteocytes as been shown to have an important role in renal control over bone metabolism (Schiavi 2006). Therefore the importance of this encased cell in bone biology should not be underestimated. Figure 1.3

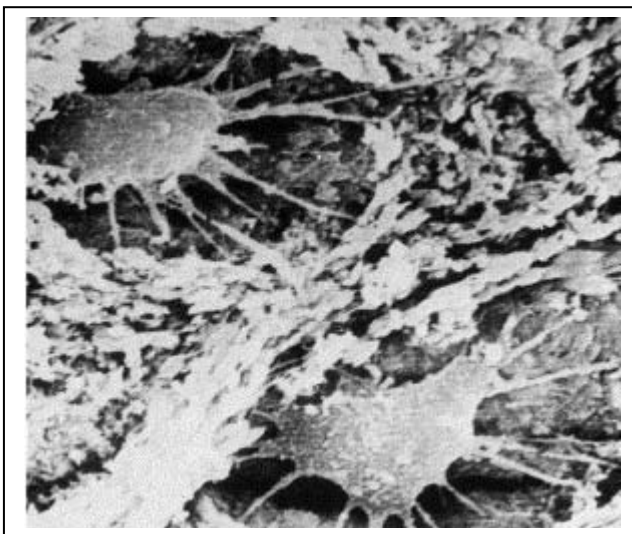


Figure 1.3
Osteocyte
embedded in
calcified bone
matrix.
Magnification
1000x
From: (Klein-
Nulend, Bacabac
et al. 2005)

Osteoclasts:

Osteoclasts are specialised multinucleated giant cells that resorb bone. The signals that stimulate osteoclasts to form and resorb bone involve a series of transcription factors and paracrine cytokines, such as TRANCE (TNF-related activation-induced cytokine) also known as RANK-L. Osteoclasts are attached to bone through a specialized structure, the sealing zone. This allows them to create a resorption space that is isolated from the extracellular space. Osteoclasts can acidify the resorption space to dissolve the mineral component of bone (Teitelbaum 2000). Osteoclasts produce lysosomal enzymes, including cathepsin K, that are released into the resorption space to remove the organic components of bone. Osteoclasts facilitate resorption by polarising their structure to form a unique element, the ruffled border, which provides an increased surface area available for active transport of H^+ ions through a unique vacuolar proton pump. The products of resorption traverse the osteoclast by transcytosis and leave the osteoclast through the basolateral membrane opposite the resorption space. Osteoclasts are highly motile, move across the bone surface, and resorb relatively large areas of bone. The osteoclast expresses many features of phagocytic cells such as the presence of a large number of lysosomes and mitochondria (reflecting the high energy demand of these processes) (MacDonald and Gowen 1993). Osteoclasts are usually found in contact with a calcified bone surface and within Howship's lacunae, which are the result of its own resorptive activity. Osteoclasts die by apoptosis that is regulated by paracrine-acting cytokines and possibly by factors in the bone matrix (Teitelbaum 2000).

The activity of the osteoclast is unique due to the exocytosis of lysosomal enzymes whose action takes place outside the cell instead of inside. The large amounts of calcium released during osteoclast digestive activity would be incompatible with intracellular functioning. Inorganic and organic products of the osteoclastic activity enter capillaries and are recycled to other locations (Roodman 1999). See Figure 1.4.

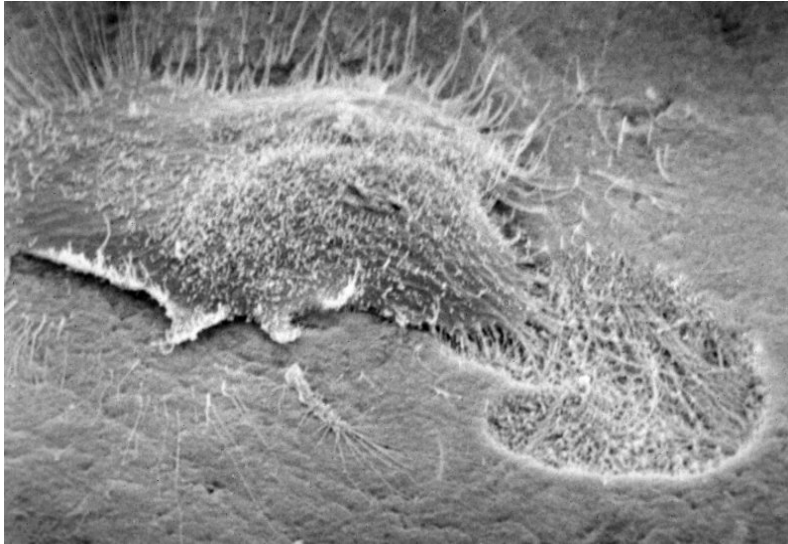


Figure 1.4
Scanning
Electron
Microscope of
an Osteoclast
resorbing bone
(From
www.brsoc.org.uk/gallery)

Bone lining cells:

Bone lining cells are flat, elongated cells that cover bone surfaces undergoing neither bone formation nor resorption. They are thought to be another type of end form cells of the osteoblastic lineage (Marks and Hermey 1996) along with osteocytes and are in the quiescence phase (Noble and Reeve 2000). Because these cells rarely multiply, they have few cytoplasmic organelles. Little is known regarding the function of these cells, although some evidence suggest they can revert back to their previous osteoblastic phenotype (Leaffer, Sweeney *et al.* 1995).

1.2.3 The anatomy of bone

Flat bones like the skull and sternum are derived from intramembranous ossification and consist of mature lamellar bone. The long bones have wider extremities (epiphyses), a cylindrical tube in the middle (diaphysis) and a progressive passage from one to the other (metaphysis), formed by endochondral ossification, as previously discussed. Endochondral bone is further organised into cortical and cancellous (or trabecular) bone. Cortical bone forms the outer surface of endochondral bones and provides the structural integrity for many of the long bones. It forms up to 80% of the skeleton and is typically dense bone with a well organised pattern of collagen fibrils that are aligned along stress lines to provide bone with maximum strength. Trabecular bone is thinner and less well organized, and primarily is found traversing the bone marrow space. However, in some bones with a high degree of trabecular bone such as vertebrae, trabecular bone provides much of the structural integrity. A major function of trabecular bone is to provide a large surface area for metabolism such as in calcium haemostasis. Bone turnover, which consists of bone resorption and its replacement with new bone, occurs much more frequently in trabecular bone than in cortical bone (Bilezikian, Raisz *et al.* 1996). See Figure 1.5.

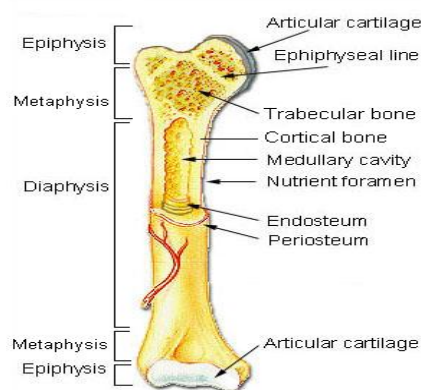


Figure 1.5: Diagram Representing Long Bone Anatomy

Bone consists of organic and mineral fractions. Of the organic fraction, the major structural protein is type I collagen (approximately 95%), which provides tensile strength. The remaining 5% is composed of proteoglycans and non-collagenous proteins. The mineral crystal of bone is hydroxyapatite, a calcium-phosphate salt, containing hydroxyl ions. Calcium hydroxyapatite constitutes 65 - 75% of the dry weight of bone and provides the compressive strength of bone. The hydroxyapatite crystal is imperfect and substitutions are found frequently as carbonates (Marks and Popoff 1988) & (Boskey and Posner 1984).

There are two surfaces of a long bone where bone is in contact with non-osseous tissues. The external surface is the periosteum and the internal surface the endosteum (see Figure 1.5). Ligaments, tendons and joint capsule attach to the periosteum and can also provide a source of cells that can form new bone or cartilage (Buckwalter 1994). The endosteum also has cells which have osteogenic potential and is where the majority of long bone turnover takes place. Periosteum and endosteum can be activated to proliferate by trauma, retroviruses, tumours, and lymphocyte mitogens, resulting in the production or destruction of cartilage and bone (Wlodarski 1989).

Periosteum consists of two main layers, an osteogenic sleeve known as the cambium layer and an outer fibrous sleeve (Remedios 1999). The outer layer is composed of fibroblast like cells embedded in a collagenous matrix and carries the vascular and nerve supply to the surface of the cortical bone. The cambium layer is composed of elastic fibres which are synthesised by osteogenic cells. These elastic fibres lie parallel to the bone and form five to six layers. It is thought that the innermost part of the elastic

fibre layer contains undifferentiated cells that can differentiate into osteoblasts or elastoblasts. The cambium layer provides the osteogenic cells necessary for appositional growth in the immature skeleton and is essential for fracture healing.

The endosteum is a single cell layer that surrounds the bone marrow tissue and trabeculae of the spongiosa. This endosteal envelope is composed of cuboid osteoblasts or resting lining cells that contain long nuclei and a small amount of cytoplasm. These cellular linings of bone are responsible for physiological modelling and remodelling (Yee 1983).

Bone has a highly developed vascular system to support its metabolic activity. There is an elaborate system of vessels which penetrate even the most dense cortical bone, with no cell lying more than 300µm from a blood vessel (Buckwalter 1994). The afferent vascular network comprises the nutrient artery, proximal and distal metaphyseal arteries, and epiphyseal and periosteal vessels which provide a rich supply to adult bone. There are corresponding efferent veins which drain the bone and the intermediate vessels consist of medullary sinusoids, cortical capillaries and periosteal capillaries, where nutrient and gaseous exchange take place (Remedios 1999).

Cortical bone is organised into lengthwise bony columns (Haversian systems or osteons) in which concentric bony lamellae surround a neurovascular central channel, a Haversian canal. Haversian canals are interconnected by perpendicular Volkmann's canals that also bring the blood supply from the surface of the bone. Osteocytes are trapped within the bone lamellae in spaces known as lacunae. Canaliculi are tiny

channels interconnecting osteocytes in lacunae and Haversian canals. In remodelling, osteoclasts bore a tunnel called a resorption canal which carries capillaries and osteoblasts that lay down concentric layers of bone to form a new osteon. Between osteons are interstitial lamellae that are remnants of the previous osteons. In cancellous bone, the vascular function of Haversian canals is replaced by blood sinusoids in the marrow and bone remodelling occurs at the surfaces of the trabeculae (Cooper, Turinsky *et al.* 2003).

See Figure 1.6

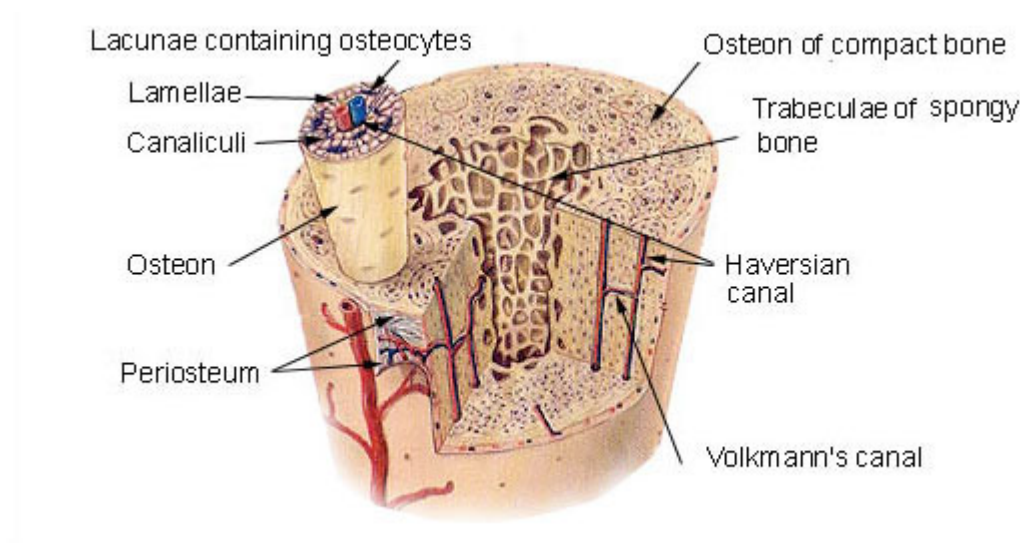


Figure 1.6: Diagram illustrating Haversian organisation and blood supply within cortical bone (From heidimatchembiol3500.blogspot.com)

1.2.4 Mechanical forces and bone remodelling

Bone is a living tissue that is in a constant state of turnover. Under normal conditions, bone resorption and bone formation are intimately linked and always occur in the same sequence. This is executed by a group of osteoclasts and osteoblasts that form the so-called 'basic multicellular unit'. This remodelling allows bone tissue to adapt its internal structure and mass to mechanical demands (functional adaptation) to ensure maximal strength with minimal bone mass (Klein-Nulend, Bacabac *et al.* 2005). Julius Wolff originally described how bone could maintain the relationship between external load and architecture in a variable functional environment (Wolff 1869). During remodelling, alignment of new bone is along the dominant local loading direction, suggesting local regulation of bone formation by mechanical stimuli (Petrtyl, Hert *et al.* 1996). It has become clear over the last several years that the osteocytes are the mechanosensory cells of bone (Nijweide, Burger *et al.* 2002). It has been shown that mechanical load induces fluid flow in the canalicular network (Knothe Tate, Steck *et al.* 2000). This fluid flow has been suggested as a physical mediator of mechanosensing by osteocytes *in vivo* as has physical deformation of the canalicular extensions themselves (Weinbaum, Cowin *et al.* 1994). The osteocytes respond to mechanical stimuli with the production of signalling molecules which modulate the activities of osteoblasts and osteoclasts, thus converting mechanical stimuli into cellular signals (Burger and Klein-Nulend 1999) See Figure 1.7.

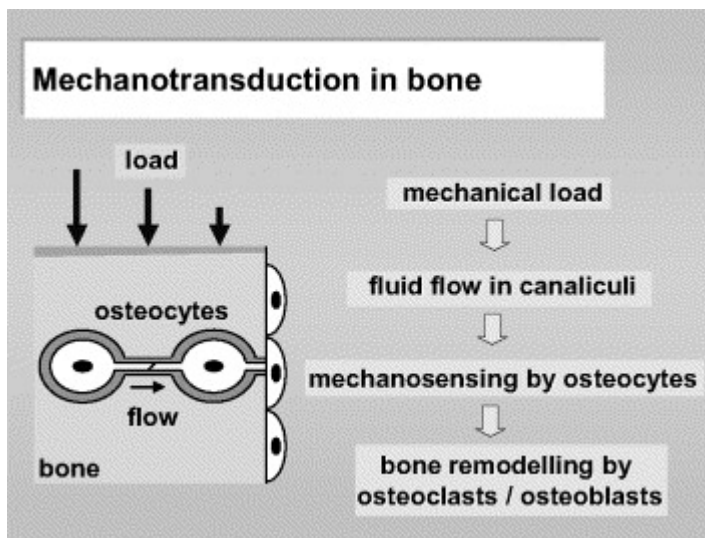


Figure 1.7. Model demonstrating the conversion of mechanical strain into osteocyte signalling in bone

Modified from: (Klein-Nulend, Bacabac et al. 2005)

Apart from mechanical signals, the rate of bone remodelling and the activity of bone cells are mainly regulated by hormones such as oestrogen and parathyroid hormone. The large supplies of calcium and phosphorus within bone can be called upon when dietary supplies are insufficient and in this way bone serves as a “metabolic bone bank”. Therefore net turnover of bone is dependent upon both mechanical and metabolic requirements.

1.2.5 Bone regeneration after defects

Defects in bone secondary to trauma and other causes all require reestablishment of bony continuity along the lines of mechanical stress. Bone remodelling as described above is insufficient alone to re-establish integrity to long bone defects. Consequently a separate process of bone healing, known as bone regeneration must occur to restore continuity. There are three separate components required in bone regeneration, namely osteoinduction, osteoconduction and osteoprogenitor cells.

1. Osteoinduction: This is the first step in bone regeneration and involves the recruitment of immature cells (undifferentiated mesenchymal cells) and the stimulation of these cells to develop into preosteoblasts (Albrektsson and Johansson 2001). This is initiated and sustained by an inductive factor which can be a chemical, humoral or a physical signal. The inductive factors currently receiving much interest are bone morphogenetic proteins (BMPs), originally described by Urist as a chemical inductive factor in 1979 (Urist, Mikulski *et al.* 1979). BMPs belong to the transforming growth factor (TGF)- β -family of growth factors. There are at least 15 different BMPs, of which BMP-2 and BMP-7 are thought to be most important in bone induction (Solheim 1998). BMPs are naturally released from damaged tissues in response to trauma and are known to stimulate an undifferentiated mesenchymal cell to transform into a preosteoblast (Lind 1996). However, several different factors may be involved at different stages of bone regeneration: the initial inductive phenomenon following an injury may be quite distinct from the inductive phenomenon that guides ultimate bone remodelling along the lines of stress. Other physical modalities have also been shown to be capable of osteoinduction. For example direct current stimulation, pulsed electromagnetic fields and low-intensity ultrasound have all been suggested as agents to speed normal fracture healing and manage delayed unions (Nelson, Brighton *et al.* 2003). How these factors act in physiological healing is not understood and debate continues over their efficacy. When fractures occur, the bone marrow and soft tissue injury triggers the subsequent repair by sensitising different types of surviving cells. The injury releases local messengers that induce the differentiation and organisation of precursor cells. This starts immediately after the injury and is very active during the first week thereafter,

even though the action of the newly recruited preosteoblasts is not obvious until several weeks later, in the callus stage (Frost 1989; Frost 1989).

2. Osteoconduction: This refers to the establishment of an appropriate environmental template upon which osteoprogenitor cells, when properly stimulated can produce bone. In most situations this is provided by a three dimensional bony scaffold which provides continuity between the two fractured ends of bone (Csongradi and Maloney 1989). In larger bone segmental defects an artificial osteoconductive scaffold may need to be surgically added. There are a number of natural (*e.g.* morselised autologous bone graft) and fabricated (*e.g.* bone graft substitute such as calcium phosphate paste) substances which can create an optimal environment for bone formation (Szpalski and Gunzburg 2002).

3. Osteoprogenitor cells: These are the third component necessary for bone regeneration. They are present within bone marrow and represent a very small fraction of the total bone marrow cells. Undifferentiated mesenchymal cells are also present within the adjacent tissue of bone, particularly the cambium layer of periosteum (Ashton, Allen *et al.* 1980) and muscle (Levy, Joyner *et al.* 2001). These primitive mesenchymal cells are attracted to the site of injury (Diaz-Flores, Gutierrez *et al.* 1992) and under the appropriate conditions of osteoinduction and osteoconduction are activated to initiate and sustain the bone regeneration process. They will differentiate and proliferate into osteoblasts and eventually osteocytes. Pre-existing osteoblasts may

contribute to bone formation but it is thought they only contribute a minor portion of the new bone needed in fracture-healing (Frost 1989).

The process of bone regeneration is best exemplified by fracture repair.

1.3 Fracture healing

1.3.1 Introduction

Fracture healing is a complex physiological process in which bone heals for the purpose of transferring mechanical loads. A remarkable feature is, unlike other tissues which heal by the formation of a poorly organised scar, fracture healing reconstitutes the injured tissue into its original form. Although complete restoration of the original anatomy occurs only in the skeletally immature, particularly young children, remodelling of the newly formed bone in adults also produces a mechanically stable lamellar structure. Fracture healing involves a series of distinct cellular responses that are under the control of specific paracrine and autocrine intercellular signalling pathways and it can be viewed as a ‘well-orchestrated series of biological events’ (Einhorn 1998). Thus a discontinuity in osseous structure will initiate what is known clinically as “Fracture Healing”, but which is more closely aligned to the term “Regeneration” (replacement with a tissue which is qualitatively similar to the original tissue). The synthesis of osseous tissue requires the conversion of undifferentiated

osteochondral progenitor cells to mature osteoblasts (in both endochondral and intramembranous bone formation) and chondrocytes (in endochondral bone formation only). Both intramembranous and endochondral bone formation are critically dependent upon cell growth and differentiation. This, in turn, is dependent on biochemical factors such as hormones and growth factors and on the mechanical environment.

Over 1 million fractures occur each year in the United Kingdom. As the aged population increases, fragility fractures, such as neck of femur (hip) fracture will be an increasing problem. The consequences of such fractures are devastating with 80% of patients reporting that they would rather die than suffer the consequences of a hip fracture (Neuner, Binkley *et al.* 2006). The death rate following hip fracture exceeds the combined annual death rate from ovarian, uterine and breast cancer (Chang 2004).

Furthermore the cost to the National Health Service of fractures in patients with osteoporosis, which are more common in the elderly, is £1.7billion per annum (Seaggar, Howell *et al.* 2004).

1.3.2 Fracture treatment

Fractures occur when there is a break in the continuity of the bone. They are caused when the load imposed on the bone is larger than its material properties can withstand, leading to failure. They normally occur in normal bone exposed to sudden injury but can occur with repetitive injury (stress fractures) and in bone with previous alteration of its material properties such as by a tumour (pathological fracture). Most fractures are treated with immobilisation, in a position which realigns the bones as close to their normal anatomical position as possible. Fractures in general can be immobilised in

splints, plaster of Paris, external fixation, internal fixation with intramedullary nailing or internal fixation with plates and screws and more recently locked plates. All methods allow some micromotion at the fracture site (and secondary healing) except for standard plates which provide absolute stability and permit primary healing (as opposed to some locking plates which do allow micromotion) (Apley 2001).

1.3.3 Fracture healing biology:

Fracture healing can be considered in several ways. McKibbin studied the various stages of repair by reviewing mainly observational clinical and radiological studies in humans (McKibbin 1978). Einhorn described the different processes involved at a cellular and molecular level, mainly from animal studies (Einhorn 1998). He described fracture healing in terms of four distinct responses. These four responses include those that take place in the bone marrow, cortex, periosteum, and external soft tissues.

Depending on the type of fracture, its location, and the way it is treated, one or several of these responses can occur simultaneously. See Figure 1.8.

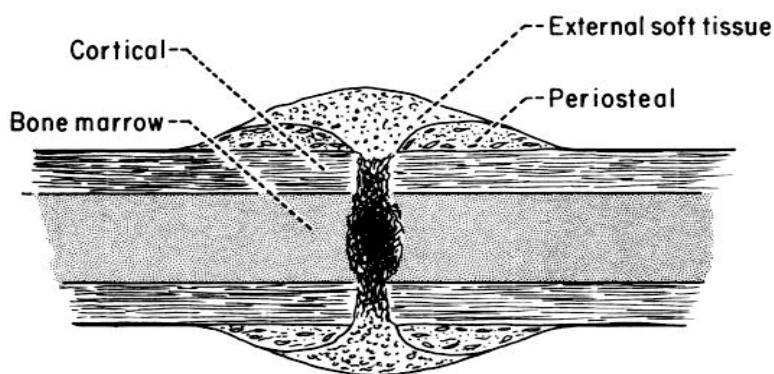


Figure 1.8. Tissue types that contribute to the four main fracture healing responses.
From: Einhorn: Clin Orthop, Volume (355S) Supplement, October 1998.S7-S21

In the following sections a comparison of primary and secondary healing will be made, followed by a review of the stages of fracture healing as originally described by McKibbin. A review into the current knowledge of the contribution of the surrounding tissues and their cells to fracture healing as well as the molecular aspects of fracture healing is then described.

1.3.4 Primary versus secondary healing:

In established histological terms, fracture healing has been divided into primary fracture healing and secondary fracture healing. Primary healing, or primary cortical healing, involves an attempt by the cortex to re-establish itself by direct repair once it has become interrupted. Secondary healing involves responses in the periosteum and external soft tissues and the subsequent formation of callus. In primary cortical healing, bone on one side of the broken cortex must unite with bone on the other side of the cortex to re-establish mechanical continuity. This occurs only when there is anatomical restoration of the fracture fragments and absolute stability of fracture reduction is ensured by rigid internal fixation (McKibbin 1978). Under these conditions, bone resorbing cells on one side of the fracture undergo a tunnelling resorptive response whereby they re-establish new Haversian systems by providing pathways for penetration by blood vessels. These new blood vessels are accompanied by endothelial cells and perivascular mesenchymal stem cells which are the osteoprogenitor cells for osteoblasts (Doherty, Ashton *et al.* 1998). These events result in the formation of

discrete remodelling units known as cutting cones (Schenk and Willenegger 1977). This is the same as normal remodelling that occurs physiologically in bone for microcracks (microscopic 'stress' fractures that occur in normal bone) and randomly (Parfitt 2002). Therefore primary healing, which is normally achieved by open reduction and internal fixation, utilises the cells already present in bone with little contribution from the surrounding tissues. Most fractures sustained worldwide are not treated in this way. Most are immobilised by non-rigid means (*e.g.* sling immobilization, cast immobilization, external fixation). This majority of fractures therefore heal by secondary fracture healing in which micromotion occurs at the fracture site and this involves a combination of intramembranous and endochondral ossification. Secondary healing involves the formation of callus potentially with contribution from all four tissue types mentioned above. Secondary healing has conveniently been divided into three main stages which describe the main events that take place, namely inflammation, angiogenesis and cartilage formation and remodelling. There is however considerable overlap in each stage. These will now be described.

1.3.5 Stages of fracture healing:

1. Stage of inflammation and immediate reaction:

Initially there is bleeding from the damaged bone ends and from the associated soft tissues. A haematoma forms between the fragments, usually contained by the periosteum (although it can become stripped or torn by the bone ends causing it to seep into the soft tissue and be contained by the muscles, fascia and skin). The soft tissues in

the region show changes of acute inflammation and vasodilation and the exudation of plasma and leucocytes. This was shown originally in a closed tibial fracture model in dogs (Wray and Schneider 1969). Polymorphs, histiocytes and mast cells soon make their appearance and phagocytosis of debris begins. This was demonstrated originally in a rat femoral fracture model, representing a well designed early study (Lindholm, Lindholm *et al.* 1969).

The first evidence of increased cell division was demonstrated by autoradiographic studies of cell proliferation in the periosteum of fractured femora in mice utilizing DNA labelling with tritiated H3-thymidine (Tonna and Cronkite 1961). This occurs within about eight hours of the injury, reaching a maximum in twenty-four hours. This activity is first seen in the periosteum and tissues immediately around it and at first extends throughout the whole length of the injured bone. However, within a few days this generalised activity declines and eventually becomes confined to the area immediately adjacent to the fracture where the rate of cell division remains above normal for several weeks.

A significant observation at this stage is that the ends of the broken bones die back by a few millimetres. This was originally documented in a histological study of human fractures which showed the presence of empty osteocyte lacunae which can extend for a variable distance from the fracture. This is most likely due to the anatomical arrangements of the blood vessels in cortical bone (Ham 1930). This work implies that the bone ends do not unite directly with one another but probably play only a passive role in the bridging process between the more distant regions of living bone in

secondary bone healing (McKibbin 1978). This is an important concept for understanding secondary fracture repair. See Figure 1.9.

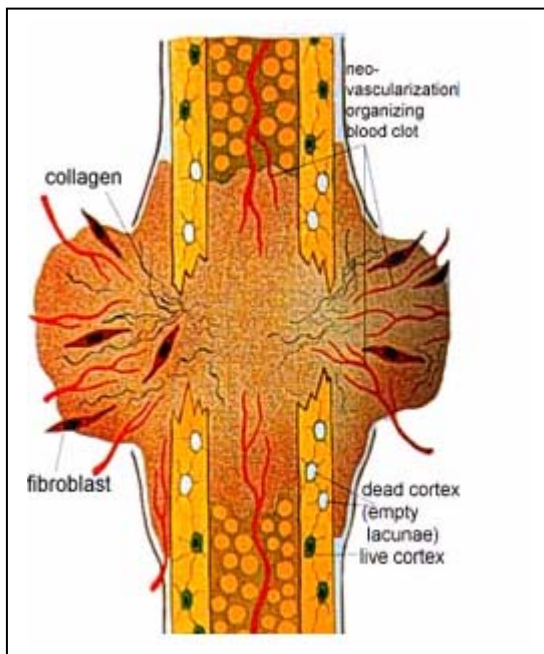


Figure 1.9
Diagram representing
the Inflammatory
Phase of fracture
healing

2. Stage of Angiogenesis and Cartilage Formation

This is the stage during which organisation of the fracture haematoma occurs. The haematoma is invaded by fibrovascular tissue, which replaces the clot and lays down the collagen fibres and matrix components, which will later become mineralised to form woven bone. This is known as the 'provisional' or 'primary' callus. The most active cell division takes place in the centre of the callus, leaving the more mature tissue closer to the bone ends to differentiate. It is in this region that the first bone is seen anchoring the newly formed mass of callus to the fragments, as described in an un-splinted rib fracture in a sheep model (McKibbin 1978). More recent evidence from well controlled studies using a rat femur fracture model suggests the function of the haematoma is a source of signalling molecules that have the capacity to initiate the cascade of cellular events that

are critical to fracture healing (Bolander 1992). The authors postulated regulatory roles for PDGF, aFGF, bFGF, and TGF-beta in the initiation and the development of fracture callus. Inflammatory cells that secrete cytokines such as IL-1 and IL-6 may also be important in regulating the early events in fracture healing, again demonstrated in a rat femur fracture model with organ culture expansion of the cytokines of interest (Einhorn, Majeska *et al.* 1995). Degranulating platelets in the clot have been demonstrated to release signalling molecules such as TGF- β and PDGF that are known to be capable of regulating cell proliferation and differentiation of mesenchymal stem cells (Bolander 1992). These cytokines and signalling molecules may also be involved in chemotaxis, angiogenesis, and may serve as competence and progression factors in many of the cellular responses. The role of such factors will be discussed further in Section 1.3.8, reviewing the molecular regulation of fracture repair.

These signalling molecules cause proliferation of osteoblast precursors from the deep surface of the periosteum close to the fracture site. Light and electron microscopic studies of a rib fracture in rabbits has demonstrated that these cells form a collar of active tissue that surrounds each fragment (Brighton and Hunt 1991). The periosteum undergoes an intramembranous bone formation response and this histological evidence shows formation of woven bone opposed to the cortex a few millimetres from the site of the fracture. At the same time, as demonstrated by Einhorn in the rat femur histologically, callus overlying the fracture site, which has arisen from the adjacent periosteum and external soft tissues, begins to take on the appearance of cartilage (chondrogenesis) (Einhorn 2005). A short time following this, abundant cartilage overlies the fracture site and this chondroid tissue initiates biochemical preparations to

undergo calcification. At this time, the callus can be divided into two components: hard callus, where intramembranous ossification is taking place, and soft callus, where the process of endochondral ossification is proceeding (Einhorn 1998). During this stage the haematoma is pushed aside by proliferating tissue and is eventually resorbed.

Gothman demonstrated in a rat tibial fracture model that there is also cellular differentiation and proliferation within the medullary canal, where the proliferating cells appear to be derived from the endosteum. The activity in the medullary region was reported as being slower in response to that on the periosteal side (Gothman 1962).

Medullary callus formation is the principal method of union in cancellous bone but can also form in cortical bone (McKibbin 1978).

See Figure 1.10

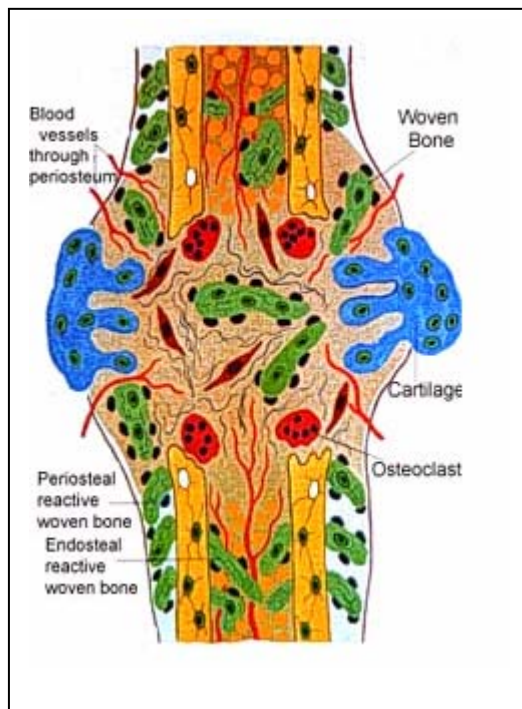


Figure 1.10:
Diagram representing the
Reparative Phase of
fracture healing

3. Stage of Remodelling:

a) Cartilage Calcification:

The calcification of fracture callus cartilage occurs by a mechanism almost identical to that which takes place in the growth plate. In this phase of fracture healing, again demonstrated by Einhorn in rat, there is an abundance of elongated proliferative chondrocytes that undergo mitosis. Later, cell proliferation declines and hypertrophic chondrocytes become the dominant cell type in the chondroid callus. Electron microscopic examination of the hypertrophic chondrocytes shows budding of membrane structures to form vesicularised bodies (Brighton and Hunt 1991). These matrix vesicles, migrate to the extracellular matrix where they participate in the regulation of calcification. An investigation using potassium pyroantimonate histochemistry has shown that the mitochondria in these cells store and release calcium for transport by matrix vesicles (Brighton and Hunt 1986). It has been shown that these matrix vesicles possess the enzyme complement needed for proteolytic degradation of the matrix, a necessary step in the preparation of the callus for calcification (Einhorn, Hirschman *et al.* 1989). In addition, matrix vesicles possess phosphatases needed to degrade matrix phosphodiesterases to release phosphate ions for precipitation with calcium. Expression of all types of neutral proteases peaks at approximately 14 days after fracture in the rat and the peak in alkaline phosphatase occurs approximately 3 days later (Einhorn, Hirschman *et al.* 1989). The temporal distribution of enzymes in the callus demonstrates that large proteins and proteoglycans in the extracellular matrix of callus may inhibit calcification until they are degraded sufficiently.

b) Cartilage Absorption and Bone Formation:

Once cartilage is calcified, it becomes a target for the in-growth of blood vessels. These vessels bring with them perivascular cells that are the progenitors of osteoblasts. The calcified cartilage in fracture callus, as described in rat femur, is nearly identical to the primary spongiosa found in the growth plate (Gerstenfeld, Cullinane *et al.* 2003). As it becomes resorbed by calcified tissue resorbing cells (chondroclasts), the woven bone that replaces the calcified cartilage is nearly identical to the secondary spongiosa of the growth plate. The removal of calcified cartilage includes not only resorption of the mineralised matrix but also removal of the chondrocytes themselves. This resorption by the chondroclasts is closely followed by the laying down of woven bone by osteoblasts, again demonstrated by Einhorn in rat femur (Einhorn 1998). The formation of intramembranous bone (hard callus) by the periosteum begins immediately after fracture but the proliferative activities in the cells slow down as the activity in the soft callus is progressing. By the time the soft callus has reached the stage of cartilage formation, a substantial amount of woven bone already has been formed adjacent to the fracture site. Endochondral calcification then takes place as described forming more “hard” callus from the soft callus, which is then replaced with woven bone. Once the fracture is united by bone bridging the gap, the callus, now fully composed of woven bone, undergoes remodelling to form a mechanically competent lamellar structure (Einhorn 1998).

This transition from cartilage to bone involves a highly programmed series of events for cell removal and matrix modification (Einhorn 2005). This transition can be a key restriction point in the progress of healing (Lee, Choi *et al.* 1998). The gritty material

that is often found during surgery on a non-union is thought to be calcified cartilage that has not made the conversion step to bone, according to these authors. At this point in the lineage, a signal is necessary for removal of the cartilage and the chondrocytes. Lee demonstrated (using a triphosphate-biotin nick end labelling (TUNEL) staining technique) in fractures of rat femora that chondrocytes undergo programmed cell death (apoptosis) during endochondral fracture healing. The multinucleated chondroclasts degrade the calcified cartilage by binding to anchorage sites in the extracellular matrix. Osteoblasts then form osteoid which becomes mineralised. It is thought that they also express a glycoprotein, osteopontin, which binds with multinucleated osteoclasts, which can then remodel the tissue (Lee, Choi *et al.* 1998).

Replacement and repair (*i.e.* remodelling - see Figure 1.11) are no different in fracture healing than in the normal skeleton. There are differences, however, depending on whether it is occurring in compact or cancellous bone. Both involve osteoclastic bone removal and osteoblastic bone replacement, together with accompanying blood vessels. In cancellous bone the cells are never very far away from blood vessels and so the process of bone apposition or replacement can take place on the surface of the trabeculae, a phenomenon often referred to as “*creeping substitution*” (Bourne 1971; McKibbin 1978). In cortical bone, the deeper placement of the cells require the presence of the Haversian system and here the osteoclasts ream out a tunnel into the bone down which a blood vessel follows, bringing the osteoblasts which lay down the lamellar bone of the new osteon. Remodelling here represents the same phenomenon as healing with ‘cutting cones’ in primary bone healing (Schenk and Willenegger 1977). Figure 1.12 describes the relative time scale of the stages of secondary fracture healing.

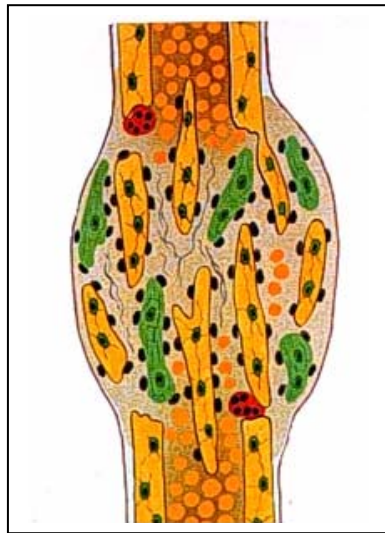


Figure 1.11:
Remodelling phase of
fracture repair

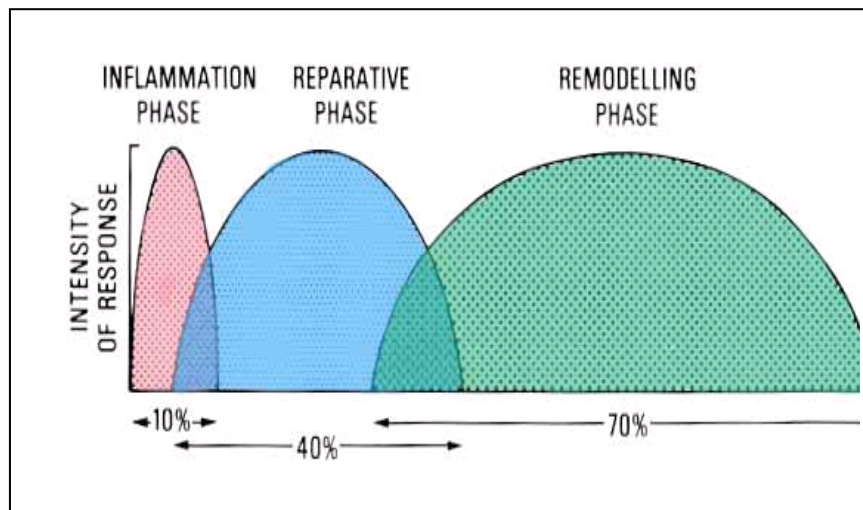


Figure 1.12
The relative
time taken
for each
stage of
fracture
repair.

1.3.6 Contribution of tissues to fracture healing

1. The Periosteum

The periosteum provides one of the most important responses in fracture healing.

Committed osteoprogenitor cells and uncommitted undifferentiated mesenchymal cells derived from the inner cambial layer contribute to fracture healing by recapitulation of

embryonic intramembranous ossification and endochondral bone formation. The response from the periosteum is a fundamental reaction to bone injury and is enhanced by motion and inhibited by rigid fixation (McKibbin 1978). It has been shown to be rapid, capable of bridging gaps as large as half the diameter of the bone, and to be independent of the external soft tissues. The bone that forms by intramembranous ossification is found farther from the site of the fracture, results in the formation of a hard callus, and forms bone directly without first forming cartilage. The structural proteins associated with bone matrix appear very early in this tissue. The bone that forms by endochondral ossification is formed adjacent to the fracture site, involves the development of a cartilaginous template which becomes calcified and is replaced by bone, and is characterized by the production of molecules that are related to the different musculoskeletal tissue types. The importance of the periosteum and in particular the cambial layer is demonstrated by stripping experiments, where stripping of the full thickness of the periosteum in animal models can lead to atrophic non-union (Brownlow, Reed *et al.* 2000; Brownlow and Simpson 2000).

2. The External Soft Tissues:

The external soft tissue (muscle, fascia) response involves rapid cellular activity and the development of an early bridging callus that stabilises the fracture fragments. This depends heavily on mechanical factors and may be depressed by rigid immobilisation. The type of tissue that is formed from the external soft tissue evolves through a process of endochondral ossification in which undifferentiated mesenchymal cells are recruited,

attach, proliferate, and eventually differentiate into cartilage and bone forming cells.

The phenomenon by which un-associated soft tissues are recruited in this way is known as osteogenic induction, originally described by Urist (Urist and McLean 1952).

3. The Bone Marrow:

Within a few hours after a fracture is sustained, there is loss of normal architecture of the bone marrow elements, disappearance of blood vessels in the region adjacent to the fracture callus clot, and a reorganisation of the cellular complement of the bone marrow into regions of high and low cellular density (Brighton and Hunt 1991). In the region of high cellular density, there appears to be a transformation of endothelial cells to polymorphic cells and those cells, within 24 hours after fracture, express an osteoblastic phenotype and begin to form bone. Therefore the bone marrow makes a direct contribution to the formation of bone during the early phase of healing (Pritchard 1963). Activities in the bone marrow during fracture healing are independent of gross mechanical influences (Pritchard 1963).

4. Callus:

Callus formation is of utmost importance in mechanical stability in secondary fracture healing (Charnley 1970). It can be considered as external callus and medullary callus (McKibbin 1978). The external callus acts as a bony bridge between bone fragments and since this involves joining hard tissue it follows that the whole system must be stabilised at least temporarily. The callus does not spread indiscriminately in all

directions but tends to orientate itself towards the opposite fragment. The more interfragmentary motion at the fracture site, the larger the size of the external callus, as it attempts to increase the moment arm to abolish this movement (Gardner, Hardy *et al.* 1997). As soon as the gap is bridged the proliferative activity in the callus decreases. The development of rigid fixation for fractures has demonstrated that this external callus response can be eliminated entirely, leading to complete healing by primary bone union (Anderson 1993).

Healing by medullary callus is quite similar to that of the external callus. It is distinguished primarily by its location, although it has been observed that the cartilaginous intermediate is much less prominent (Pritchard 1963). It is under the same controlling influences as external callus, especially in the earlier stages of fracture healing when in a translated fracture the medullary callus can unite directly with the external callus. The main difference is the origin of the osteogenic cells which are from the endosteal surface of the bone itself, as opposed to the periosteum in external callus. It is often the method of healing relied on when healing by external callus has failed (McKibbin 1978).

1.3.7 The vascular response to a fracture

Periosteal Circulation:

The periosteal blood vessels are considered to be of critical importance in the event of a fracture, illustrating the importance of this tissue overall. The periosteal arteries are thought to supply only the outer third of the cortex in long bones, although the

periosteal capillary bed is of more importance in the event of damage to the medullary blood supply (Macnab and De Haas 1974; Rhinelander 1974). A careful investigation into the blood supply of the periosteum resulted in the discovery of a system of new vessels. The blood supply of periosteum was found to be derived from four sets of vessels: (1) Intrinsic periosteal system. (2) Musculoperiosteal system. (3) Fascioperiosteal system. (4) Cortical capillary anastomoses. The intrinsic system of vessels lies within the fibrous layer of the periosteum. According to the pattern of these vessels they could be divided into: (a) a short vessel pattern, where there were many small vessels with no predominant direction; (b) a circular pattern, where the vessels encircled the bone; (c) a longitudinal pattern, where the vessels ran parallel to the long axis of the bone. The musculoperiosteal system consisted of connections between the muscle circulation and the periosteal vessels at the sites of muscle origin. The fascioperiosteal system consisted of branches from a limb artery that ran in a fascial plane between muscles to supply the periosteum. The cortical capillary anastomoses consisted of capillaries that ran in the bone cortex between the intramedullary circulation and the periosteal vessels (Simpson 1985). This work revealed the intricacies of periosteal blood supply which showed similarities to blood supply of skin. This is important as medullary supply (normally the dominant blood supply of bone, flow being centripetal) is often compromised during fracture.

Blood Supply and Fracture:

The ability of bone to undergo repair is dependent on its blood supply. Healing can be delayed or cease due to damage to the blood supply at the time of the fracture or

through poor surgical technique in fracture fixation. A complete fracture of a long bone cannot occur without causing variable disturbance of both the cortical and periosteal vasculature of the affected bone, as well as the damage to the nutrient artery (Trueta 1974). When the nutrient (medullary) artery is damaged either by trauma or surgery, the metaphyseal and intact periosteal arteries can hypertrophy rapidly to assume dominant cortical blood supply (Remedios 1999). However, the response of normal vasculature within bone to injury is limited, and in most cases a supplementary blood supply, external to the bone develops. This is often referred to as periosteal due to its origin outside of the bone. However, the source of this new blood supply is from within the soft tissues surrounding the bone and should be referred to as the extraosseous blood supply. This develops immediately after fracture arising first from torn blood vessels in the vicinity of the fracture. Where fascia has been detached from bone, the ruptured periosteal arterioles combine locally with the ruptured capillaries and arterioles of torn muscle in supplying the external callus (Rhineland 1974). This phenomenon revascularises bone that has been deprived of its normal medullary circulation. The extraosseous supply persists only during fracture healing and once the normal components of bone blood supply have been restored, this supplemental vasculature subsides (Wilson 1991).

1.3.8 Molecular regulation of fracture healing

An understanding of the cellular and molecular pathways that govern the events of fracture healing is thought to be critical to the future advancement of fracture treatment. Studies on the factors that initiate and control the responses, the cells that participate in

those responses, and the molecules that are synthesized by those cells will lead to new insights and eventually new therapies for treating fractures (Einhorn 1998).

The signalling molecules that are known to control bone healing can be categorized into four groups: (1) the pro-inflammatory cytokines, (2) the transforming growth factor-beta (TGF- β) superfamily and other growth factors, (3) the angiogenic factors and (4) systemic hormones (Dimitriou, Tsiridis *et al.* 2005).

1. Pro-inflammatory cytokines:

Interleukin-1 (IL-1) and Interleukin-6 (IL-6) as well as tumour necrosis factor-alpha (TNF- α) have been shown in culture expanded callus tissue from rat femoral fractures to play a role in initiating the repair cascade (Einhorn, Majeska *et al.* 1995). They are secreted not only by macrophages and inflammatory cells but also by cells of mesenchymal origin present in the periosteum. They carry out central functions in the induction of downstream responses to injury by having a chemotactic effect on inflammatory cells, enhancing extracellular matrix synthesis, stimulating angiogenesis, and recruiting endogenous fibrogenic cells to the injury site (Kon, Cho *et al.* 2001). They show peak expression within the first 24 hours after fracture, depressed levels during the period of cartilage formation, and their levels increase again during bone remodelling (Gerstenfeld, Cho *et al.* 2003).

2. Transforming growth factor-beta (TGF- β) superfamily and other growth factors

The TGF- β superfamily is a large family of growth and differentiation factors including bone morphogenetic proteins (BMPs), transforming growth factor-beta (TGF- β), growth

differentiation factors (GDFs), activins, inhibins and the Mullerian inhibiting substance (ten Dijke, Fu *et al.* 2003). They originate from high molecular weight precursors and are activated by proteolytic enzymes. They act on serine/threonine kinase membrane receptors on target cells (Massague, Attisano *et al.* 1994). This ligand–receptor interaction activates an intracellular signalling pathway which ultimately affects gene expression in the nucleus. Specific members of this superfamily including BMPs 1–8, GDF-1, 5, 8, 10 and TGF- β 1, - β 2, - β 3, promote the various stages of intramembranous and endochondral bone ossification during fracture healing. The temporal expression of these members of the superfamily has been investigated using a murine tibial fracture model (Cho, Gerstenfeld *et al.* 2002). By using molecular probes for all of the relevant BMPs known to be expressed in human patients, the investigators were able to demonstrate the normal timing of expression of these proteins in the mouse. Within 24 hours after the fracture was sustained, there was an increase and a peak in activity of BMP-2, which is considered an early response gene. GDF-8 also showed maximal expression, peaking within 24 hours after fracture. GDF-8 is a negative regulator of cells in the myogenic lineage, and its expression therefore suggests that it may similarly regulate cell differentiation early in fracture healing. For example, GDF-8 may function to direct undifferentiated mesenchymal cells toward a chondro-osteogenic lineage, i.e., to heal bone, rather than to a myogenic pathway. By seven days post fracture in the mouse model, when type II collagen expression peaked during cartilage formation, there was maximal expression of other genes: GDF-5, TGF- β 2, and TGF- β 3. In contrast, BMP-3, BMP-4, BMP-7, and BMP-8 showed a restricted period of expression from day 14 to day 21, the central part of fracture healing when the resorption of

calcified cartilage and osteoblastic recruitment were most active (Cho, Gerstenfeld *et al.* 2002; Einhorn 2005). More recently a mouse knock out model lacking the ability to produce BMP-2 showed inability to initiate fracture healing (Tsuji, Bandyopadhyay *et al.* 2006). Therefore several members of the TGF- β superfamily seem to be important in fracture healing. Although they are closely related both structurally and functionally, each has a distinct temporal expression pattern and a potentially unique role in fracture healing

Other Growth Factors:

Platelet derived growth factor (PDGF) is released by platelets during the early phases of fracture healing and it is a potent chemotactic stimulator for inflammatory cells and a major proliferative and migratory stimulus for mesenchymal stem cells and osteoblasts, as demonstrated *in vitro* (Canalis, McCarthy *et al.* 1989; Lieberman, Daluiski *et al.* 2002).

Fibroblast Growth Factor (FGFs) promote growth and differentiation of a variety of cells such as fibroblasts, myocytes, osteoblasts, and chondrocytes. FGFs are identified during the early stages of fracture healing and they play a critical role in angiogenesis and mesenchymal cell mitogenesis, as demonstrated in a rat calvarial defect model (Rodan, Wesolowski *et al.* 1987). Alpha-FGF mainly effects chondrocyte proliferation and is probably important for chondrocyte maturation, whilst β -FGF is expressed by osteoblasts and is generally more potent than α -FGF (Lieberman, Daluiski *et al.* 2002).

Insulin like growth factors (IGFs): IGF-I promotes bone matrix formation (type I collagen and non-collagenous matrix proteins) by fully differentiated osteoblasts and is more potent than IGF-II, as demonstrated in culture of 21 day foetal rat calvaria

(Canalis 1980). IGF-II acts at a later stage of endochondral bone formation and stimulates type I collagen production, matrix synthesis, and cellular proliferation, as demonstrated by bone formation studies after intramuscular implantation of demineralised bone matrix in rat muscle (Prisell, Edwall *et al.* 1993)

3. Angiogenic Factors:

Optimal bone regeneration requires adequate blood flow. During the final stages of endochondral ossification as well as during the remodelling phase, specific matrix metalloproteinases degrade cartilage and bone, allowing the invasion of blood vessels (Gerstenfeld, Cullinane *et al.* 2003). The vascular in-growth into the developing callus is regulated by FGF, vascular endothelial growth factor (VEGF) and angiopoietin 1 and 2 (Lieberman, Daluiski *et al.* 2002). Angiopoietin 1 has been suggested to be induced during the initial periods of fracture healing whereas VEGF occurs later, mainly during endochondral ossification (Gerstenfeld, Cullinane *et al.* 2003). Street demonstrated the inherent angiogenicity of human fracture samples. Subcutaneous transplantation of human fracture haematoma into a murine wound model resulted in new blood vessel formation after haematoma resorption. This was thought to be mediated by the significant concentrations of VEGF found in the haematoma (Street, Winter *et al.* 2000).

4. Systemic Hormones:

Although the cellular events during fracture healing seem to be predominantly regulated by local factors and cytokines, systemic hormones (parathyroid/thyroid hormone, growth hormone, vitamin D and sex steroids) may also modulate these events (Mundy 1996).

Contrary to the assumption that *parathyroid hormone (PTH)* has a catabolic effect on the skeleton, intermittent exposure stimulates osteoblasts and results in increased bone formation in rats (Andreassen, Ejersted *et al.* 1999). It has been shown that low-dose human PTH enhances callus formation by stimulating the early proliferation and differentiation of osteoprogenitor cells, increasing the production of bone matrix proteins and enhancing osteoclastogenesis during callus remodelling. The effect of PTH is likely to be mediated by osteoblastic activity, as PTH receptors are found on osteoblast membranes (Nakajima, Shimoji *et al.* 2002).

Growth hormone is a systemic hormone and its effect on the skeleton is mediated by IGF-1 which promotes bone matrix formation by fully differentiated osteoblasts. Systemic administration in a rat tibial fracture model leads to increased callus formation and enhanced fracture strength (Andreassen and Oxlund 2003).

A link between 24,25dihydroxy *vitamin D* and endochondral bone formation is also supported by the analysis of vitamin D metabolism during fracture healing. The circulating levels of 24,25dihydroxy vitamin D increase during fracture repair as a result of an increase in renal activity (St-Arnaud 1999). This suggests that 24,25dihydroxy vitamin D may be involved in the early phases of the healing process. When the effect of various vitamin D metabolites on the mechanical properties of healed bones was tested *in vivo* (rat), treatment with 1,25 dihydroxyvitamin D alone resulted in poor healing. On the contrary, the strength of healed bones in animals fed 24,25dihydroxy vitamin D in combination with 1,25 dihydroxyvitamin D was equivalent to that measured in a control population fed 25-hydroxyvitamin D (Seo,

Einhorn *et al.* 1997). These suggest an important role of physiological concentrations of 24,25dihydroxy vitamin D in fracture repair.

Sex steroid hormones are also critical regulators of the skeleton (Raisz, Kream *et al.* 2002). Loss of either oestrogens in women or androgens in men is associated with an enhancement of resorption rates in bone without an equivalent increase in bone formation. In women, loss of oestrogens during menopause causes approximately a doubling of the rate of bone loss and increases the risk of developing osteoporosis.

These examples show that although most studies concentrate on investigating autocrine and paracrine signalling in fracture repair, systemic factors probably also play a significant role. The bone morphogenetic proteins and other factors described are also involved in regulation of cartilage growth, bone cell growth and differentiation (see Section 1.2) and are most likely controlled at an earlier stage in tissue development by morphogens. Morphogens are substances that deliver a graded positional signal to developing tissues and result in a particular pattern of tissue development (Campbell and Kaplan 1992). It has been postulated that an understanding of how these genetically programmed systems can be induced and controlled would be a major advance in research on fracture healing (Einhorn 1998). Similarly, all of these studies have addressed bone and cartilage cells, proteins and cytokines. The cells of the immune system and their effect on fracture repair have received very little attention.

1.4 Biological interactions of bone with the immune system

1.4.1 Introduction:

Only recently has the dynamic association between the immune system and the skeletal system become appreciated, with cross regulation of biological events between the two systems. In particular, pathological conditions which lead to excessive bone loss, such as rheumatoid arthritis, periodontal diseases, and some tumour-associated bone abnormalities have been shown to be influenced by cellular components (*e.g.* T lymphocytes) as well as by soluble factors produced by infiltrating lymphocytes (*e.g.* interferon produced by infiltrating lymphocytes) (Teitelbaum and Ross 2003; Walsh and Choi 2003; O'Gradaigh and Compston 2004). The role of T cells in bone biology has been largely accepted since the discovery of the tumour necrosis factor (TNF) family member, known variously as TRANCE (TNF-related activation cytokine) / RANKL (receptor activator of NF-KB ligand) / OPGL (osteoprotegerin ligand) / ODF (osteoclast differentiation factor) which are all the same molecule. TRANCE was initially identified expressed on activated T cells and as a regulator of T cell function (Wong, Rho *et al.* 1997). However, it was subsequently shown that TRANCE is expressed on activated osteoblasts as well and is an essential factor for osteoclastogenesis (Kong, Feige *et al.* 1999; Kim, Odgren *et al.* 2000). Furthermore, immune cells are derived from bone marrow cells and the cytokines expressed by immune cells can regulate bone metabolism (Rho, Takami *et al.* 2004). This work was a combination of a large volume of excellent murine and human *in vivo* and *in vitro* studies and has shown that the skeletal and immune systems are closely related. The

term ‘osteimmunology’ was originally described by Arron and Choi in *Nature* in 2000 to reflect this (Arron and Choi 2000).

1.4.2 Osteoblasts and the immune system:

Osteoblasts have several interactions with the immune system and its cells. They provide a ‘niche’ for haematopoietic stem cells on the endosteal surface of bone, functioning as critical support cells. The osteoblasts involved are on the marrow surface of the endosteum and are early in their commitment to the osteoblastic lineage.

Adhesion of haematopoietic stem cells and osteoblasts appears to be mediated by interaction with adhesion molecules such as N-cadherin (Zhang, Niu *et al.* 2003). It has also been shown that expansion of the osteoblast population in bone by stimulation of the parathyroid hormone receptor on osteoblastic cells increased the number of haematopoietic stem cells in bone marrow (Calvi, Adams *et al.* 2003) while conversely, targeted destruction of osteoblastic cells in mice leads to a decrease in haematopoietic stem cells in bone marrow (Visnjic, Kalajzic *et al.* 2004). This interaction between osteoblasts and haematopoietic stem cells has been demonstrated to act physiologically to inhibit cell division in haematopoietic stem cells while maintaining their capacity for self-renewal (Visnjic, Kalajzic *et al.* 2004). It is the interaction of the tyrosine kinase receptor Tie2 on these stem cells with angiopoietin-1 expressed by osteoblasts that regulates haematopoietic stem cell quiescence in the bone marrow niche, as has been demonstrated elegantly with murine ex-vivo culture models (Arai, Hirao *et al.* 2004).

As well as providing a niche for haematopoietic stem cells, the bone marrow has long been recognized as the site of early lymphocyte development. More recent findings indicate that end-stage and memory lymphocytes also preferentially inhabit the bone marrow (Shapiro-Shelef and Calame 2005). Upon completing differentiation in germinal centres, antibody-producing B cells, or plasma cells migrate from the spleen to the bone marrow, where they are retained by stromal cells, by expression of adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and E-selectin (Tokoyoda, Egawa *et al.* 2004). Plasma cells in serum normally have a half life of at most a few weeks (Ho, Lortan *et al.* 1986). However, plasma cells found in the bone marrow have life spans typically in excess of 90 days suggesting the existence in bone marrow of specialized survival niches (Slifka, Antia *et al.* 1998), thought to be due to the space and microenvironment that the bone marrow can provide. This allows humoral immunity to be maintained for long periods of time, as demonstrated in this nice murine model.

T lymphocyte precursor cells leave the bone marrow and relocate to the thymus for further differentiation. Mature T cells function in the secondary lymphoid organs and at sites of infection. Investigators have recently shown, however, that in the absence of secondary lymphoid tissues the bone marrow can support productive cytotoxic T cell and memory cell generation (Feuerer, Beckhove *et al.* 2003). Bone marrow itself can function as a secondary lymphoid organ during normal immune responses and osteoblasts may also facilitate immune responses (Marriott 2004). They have been shown during bacterial infections of bone in mice to produce of an array of immune regulatory molecules. This pattern of expression promotes the recruitment of leukocytes

to sites of bacterial challenge, initiates antigen-specific activation of infiltrating cells and facilitates the development of cell-mediated host responses to intracellular pathogens of bone tissue. Therefore the osteoblast has been identified as a cell type which was a previously unappreciated component in host responses (Marriott 2004).

Memory T cells form after the expansion and contraction of both the CD4⁺ and CD8⁺ primary response T cells. In normal mice, memory CD8⁺ T cells preferentially reside and homeostatically proliferate in the bone marrow (Becker, Coley *et al.* 2005). The advantage of central memory CD8⁺ T cells relocating to the bone marrow may involve the presence of the crucial survival factor interleukin-15 present in the microenvironment (Mazo, Honczarenko *et al.* 2005).

Immune cells, in turn, regulate osteoblasts. This is mainly *via* the cytokines they produce when activated. Tumour necrosis factor- α inhibits the differentiation of pre-osteoblasts (Gilbert, He *et al.* 2000). Interleukin -1, tumour necrosis factor- α , and interferon- γ inhibit collagen synthesis in osteoblasts (Smith, Gowen *et al.* 1987).

Activated T lymphocytes also produce (unidentified) soluble factors that induce alkaline phosphatase activity in bone marrow stromal cells and elevated expression of mRNA for Runx2 and osteocalcin, factors necessary for osteoblast differentiation. These data indicate that T cell derived factors have the capacity to stimulate the differentiation of bone marrow stromal cells into the osteoblast phenotype (Rifas, Arackal *et al.* 2003).

The above studies represent well conducted and well controlled studies in murine *in vivo* and *ex-vivo* culture experiments, chosen from the vast literature on this topic.

However it is not possible to apply these findings to human pathology until further studies have been completed.

1.4.3 Osteoclasts and the immune system:

It was first recognised that immune cells could influence the activity of osteoclasts in 1972 when factors in supernatant fluid from cultured human peripheral blood leukocytes induced bone resorption (Horton, Raisz *et al.* 1972). Subsequently, a long list of cytokines has been identified which have effects on bone. Stimulators of bone resorption include IL (interleukin)-1, tumour necrosis factor- α (TNF- α), IL-6, IL-11, IL-15, and IL-17. Inhibitors of resorption include IL-4, IL-10, IL-13, IL-18, and interferon- γ . Transforming growth factor- β (TGF- β) and prostaglandins can have either stimulatory or inhibitory effects on resorption, depending on the conditions under which these factors are examined (Martin, Romas *et al.* 1998).

Production of such cytokines by immune cells has been linked to human diseases that involve bone. Perhaps the most extensive studies have been on the role of cytokines in the development of osteolytic lesions observed in rheumatoid arthritis and other inflammatory bone diseases. Although the immunological perspective toward rheumatoid arthritis is typically limited to how it is initiated (*i.e.* by failure of immunological tolerance) and its resultant synovitis (the conventional definition of disease culmination), a hallmark of rheumatoid arthritis is the rapid erosion of periarticular bone, which is often followed by generalised secondary osteoporosis. Osteoclasts are found to be prevalent at the site of focal erosion and are critical for bone

erosion in this disease (Goldring and Gravalles 2000; O'Gradaigh and Compston 2004). In addition to inflammatory bone diseases, altered immune responses or cytokine production may cause other osteolytic diseases. Oestrogen withdrawal after menopause is associated with a rapid and sustained increase in the rate at which bone is lost. This results from an increase in bone resorption that is not met by an equivalent increase in bone formation. Interestingly, activated T cells may cause rapid bone loss under conditions of oestrogen deficiency by enhancing TNF- α production. In a series of well-controlled experiments involving ovariectomy induced bone loss in mice, an animal model for postmenopausal bone disease, it was reported that nude mice did not lose bone mass after ovariectomy, suggesting that T cells are critical for this response (Cenci, Weitzmann *et al.* 2000). Ovariectomy failed to induce bone loss, stimulate bone resorption, or increase mononuclear phagocyte colony-stimulating factor (M-CSF) and TRANCE-dependent osteoclastogenesis in T-cell deficient mice, establishing T cells as essential mediators of the bone-wasting effects of oestrogen deficiency *in vivo*. These findings suggested that the ability of oestrogen to target T cells and suppress their production of TNF- α , is a key mechanism by which oestrogen prevents osteoclastic bone resorption and bone loss.

The role of cytokines in malignancy-related bone disease has also been studied extensively (Guisse and Mundy 1998). Haematological malignancies such as lymphoma, multiple myeloma, and adult T cell leukaemia are associated with increased osteoclast formation and activity, possibly through dysregulation of various cytokines, including IL-1, TNF- α , and IL-6. Unlike bone disease associated with solid tumours, which is

typically mediated by parathyroid hormone related protein, haematological malignancies are often characterized by an uncoupling of resorption from formation and the frequent development of purely lytic bone lesions.

In normal physiological conditions, osteoblasts regulate the formation and activation of osteoclasts. They provide at least two known essential factors for osteoclastogenesis, TRANCE and M-CSF. In addition, stromal cells produce various osteotropic factors that influence osteoblast-induced osteoclastogenesis of bone marrow precursors. These factors can be divided into two groups: those that influence activity of osteoblasts (*e.g.* TNF- α that induces TRANCE expression in osteoblasts), and those that affect osteoclast precursors or osteoclasts themselves. *In vitro*, M-CSF and TRANCE together are sufficient to induce the differentiation of bone marrow precursors, spleen cells, or blood monocytes to become mature osteoclasts (Walsh and Choi 2003). The expression of these factors and their receptors is not limited to bone. However osteoclasts do not appear in soft tissues. The likely reason for this is the presence of co-stimulatory molecules (Kim, Takami *et al.* 2002). A novel cell surface receptor has been identified and is termed osteoclast-associated receptor (OSCAR) and it is preferentially expressed on osteoclasts. It has been shown that in addition to normal TRANCE-RANK signalling, interaction of OSCAR with its putative ligand (OSCAR-L) is important for osteoblast-induced osteoclast differentiation (Kim, Takami *et al.* 2002). Moreover, OSCAR-L expression is most prevalent on osteoblasts. Therefore, the OSCAR receptor/ligand pairing could be characterized as a putative co-stimulation receptor/factor for efficient osteoclast differentiation, and may provide bone-specific

co-stimulation required for the differentiation of osteoclasts in conjunction with the essential factors M-CSF and TRANCE. This signalling combination may provide an explanation of why osteoclasts are found only on bone surfaces *in vivo* (Kim, Kim *et al.* 2005) as there is no OSCAR-OSCAR-L interaction in multinucleated giant cells in other tissues.

It is important to recognise that many of the cytokines that are believed to play a role in regulating bone cells are also produced by non-immune cells such as fibroblasts, as well as immune cells. The discovery of TRANCE as a novel TNF member expressed on activated T cells (Wong, Rho *et al.* 1997) as well as it being a key osteoclast differentiation and survival factor (Fuller, Wong *et al.* 1998; Yasuda, Shima *et al.* 1998) provided evidence of a key link between active immunity and bone homeostasis.

These studies also represent work of the highest quality selected from the literature.

1.4.4 Regulation of bone by TRANCE

Bone homeostasis is maintained *via* a functional balance of two cell types: osteoblasts, which build bone, and osteoclasts, which resorb bone, as described in Section 1.2. In the elucidation of the functions of TRANCE on bone, osteoprotegerin (OPG) was initially identified as a soluble decoy-like receptor, capable of inhibiting osteoclastogenesis *in vitro* (Tsuda, Goto *et al.* 1997). Also, transgenically induced OPG-deficient mice were found to express an *osteoporotic* phenotype and to have an excess of osteoclasts (Bucay, Sarosi *et al.* 1998). Following this the gene identified as encoding the ligand for

OPG was determined to be identical to the gene originally characterised as encoding the activated T cell factor TRANCE (Lacey, Timms *et al.* 1998). TRANCE is capable of inducing osteoclast differentiation, maturation, and activation *in vitro* (Yasuda, Shima *et al.* 1998) and many of the well-known osteotropic factors (as previously discussed, Section 1.4.2), including IL-1, IL-6, and IL-11, are now believed to exert most of their osteoclastogenic activity by inducing TRANCE expression on osteoblasts (Boyle, Simonet *et al.* 2003). It was then established that TRANCE was also a key differentiation factor for osteoclasts *in vivo* as TRANCE deficient mice demonstrated a severely *osteopetrotic* phenotype (Kim, Odgren *et al.* 2000). These mice also exhibit failed tooth eruption, a common defect associated with developmental osteopetrosis, and diversion of haematopoiesis to the spleen and liver because a functional bone marrow cavity fails to form in the absence of osteoclasts.

RANK (receptor activator of nuclear factor kappa-beta) was discovered as the signalling receptor for TRANCE initially through a large-scale analysis of genes expressed in dendritic cells (Anderson, Maraskovsky *et al.* 1997). RANK deficient mice express exactly the same phenotype as TRANCE knockouts and this confirmed the specificity of the TRANCE ligand for osteoclast expressed RANK (Dougall, Glaccum *et al.* 1999). Further research was then carried out into elucidating the signalling mechanisms involved in TRANCE-mediated osteoclastogenesis. RANK signal transduction is mediated by adapter proteins termed TNF receptor-associated factors (TRAFs), of which six are known (TRAF 1-6) (Wong, Josien *et al.* 1998). TRAF6-deficient mice have severe osteopetrosis, implying that the key signals sent through RANK in

osteoclast precursors are mediated by the adapter molecule TRAF6 (Lomaga, Yeh *et al.* 1999). There are several mediators downstream of TRAF6 in TRANCE signalling including the production of reactive oxygen species, but their discussion is out-with the scope of this thesis.

1.4.5 Regulation of immunity by TRANCE

TRANCE is thought to modulate immunity mainly through its action on dendritic cells. Dendritic cells are the most potent antigen presenting cells and are required to initiate T cell-mediated immunity *in vivo* (Steinman, Bonifaz *et al.* 2005). Dendritic cells differentiate from the haematopoietic monocyte/macrophage progenitor cell lineage and are close relatives of osteoclasts (Miyamoto, Ohneda *et al.* 2001). Upon receipt of inflammatory or activating stimuli, dendritic cells home to the T cell areas of lymph nodes to activate antigen-specific T cells. Productive activation relies on numerous dendritic cell-specific factors, including alteration of the chemokine receptor repertoire, up-regulation of co-stimulatory molecules, and cytokine production. TRANCE signalling has been implicated in this dendritic cell function, particularly with regard to prolonging dendritic cell survival, *via* up-regulation of anti-apoptotic proteins (Ouaaz, Arron *et al.* 2002). TRANCE has also been shown to prolong dendritic cell survival *in vivo* as well as enhance cytokine production and formation of T memory cells (Josien, Li *et al.* 2000). TRANCE is also thought to be important in inducing T cell tolerance and one study has demonstrated that TRANCE-mediated signalling is required to prevent the onset of autoimmune disease in a TNF- α -inducible mouse model of diabetes

(Green, Choi *et al.* 2002). TRANCE is also thought to be involved in the regulation of B cell development and function (Yun, Tallquist *et al.* 2001).

1.4.6 TRANCE and disease

Activated T lymphocytes can support osteoclastogenesis *in vitro* and artificial systemic activation of T lymphocytes *in vivo* leads to an osteoporotic phenotype and increased numbers of osteoclasts in mice (Kong, Feige *et al.* 1999). The decreased bone density seen can be prevented by OPG treatment. These studies indicated that activated T cells can disrupt bone homeostasis by modulating TRANCE expression. Also transgenic over-expression of TRANCE in a line which expressed TRANCE specifically in T and B lymphocytes was sufficient to correct partially the osteoporotic phenotype observed in TRANCE-deficient mice (Kim, Odgren *et al.* 2000). Together, these data definitively showed the ability of lymphocytes, by expression of TRANCE, to regulate bone homeostasis *in vivo* and confirmed the interplay between the specific immune system and bone metabolism (Walsh, Kim *et al.* 2006). This is demonstrated in Figure 1.13.

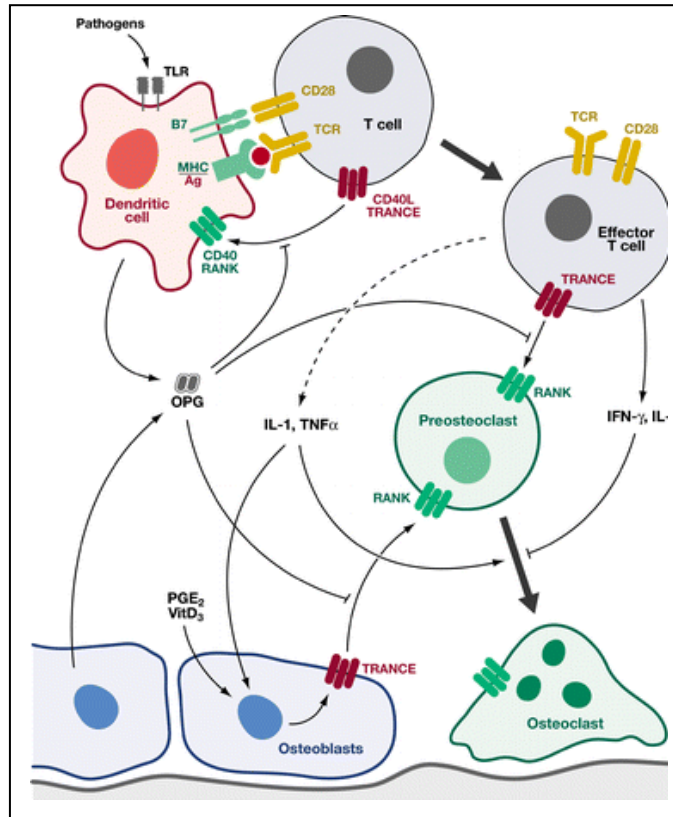


Figure 1.13
A diagram
representing the
cellular
interactions
between specific
immunity and bone
homeostasis.
Modified from Walsh
MC et al, 2006 Annu.
Rev. Immunol. 24:
33-63

In animal models of autoimmune arthritis, TRANCE has been shown to mediate the induction of bone destruction by osteoclasts, but to have no effect on inflammation or cartilage destruction (Pettit, Ji *et al.* 2001). Other investigators have also demonstrated that TRANCE, in combination with M-CSF, can induce transdifferentiation of immature dendritic cells to the osteoclastic lineage and that this is significantly enhanced by rheumatoid arthritis synovial fluid, potentially identifying another mechanism for disease-related bone destruction (Rivollier, Mazzorana *et al.* 2004). $CD4^{+}$ T lymphocytes activated with bacterial antigens are also important in the pathogenesis of periodontitis, specifically with regard to disease-related bone

destruction (Teng, Nguyen *et al.* 2000). In addition to arthritis and periodontal disease, pathological bone loss is observed in patients suffering from other autoimmune diseases (inflammatory bowel disease, diabetes mellitus and lupus erythematosus), chronic viral infections (such as HIV), allergic diseases (such as asthma), and metastatic breast and lung cancers (Walsh, Kim *et al.* 2006). Fortunately, however, most normal T cell immune responses do not elicit aberrant osteoclast differentiation. A further findings was a crucial counter-regulatory mechanism by which activated T cells can inhibit TRANCE induced maturation and activation of osteoclasts (Takayanagi, Ogasawara *et al.* 2000): During the immune response, T cells also secrete cytokines, such as interferon- γ (IFN- γ), IL-4, and TGF- β that inhibit the pro-osteoclastogenic effects of TRANCE (Figure 1.13). The role of IFN- γ , in particular, appears to be crucial in preventing T lymphocyte-mediated osteoclastogenesis (Arron and Choi 2000). It appears that IFN- γ prevents uncontrolled bone loss during inflammatory T-cell responses. It is thought to act *via* degradation of the adapter protein TRAF 6 (see 1.4.3). In this way a productive ‘normal’ immune response is prevented from having an overlapping, deleterious effect on bone in the surrounding environment.

Despite extensive cross-regulation between bone metabolism and the immune system, the mechanisms by which one regulates the other and the biological implications of such interactions, are poorly understood. Further investigation, particularly into the breakdown of normal biological mechanisms which prevent TRANCE induced bone destruction are required.

1.5 The immune system and fracture repair

1.5.1 Introduction and brief overview of immune system:

The previous section (1.4) highlighted the complex interactions between the immune system and bone. The immune system therefore likely has a large role to play when this tissue is injured. This section will review the current knowledge of the role of the immune response in fracture healing. It will become rapidly apparent that there is currently only scanty knowledge available on the contribution of the immune system, particularly the specific immune system in bone healing but that this currently forms an exciting and active forefront of bone biology research.

In simple terms, the immune systems is divided into 2 main arms, the *innate* immune system which is the first line of defence and provides barriers to infection and the *specific* immune system, mainly T and B cells which provide learned responses to foreign antigens. At the cellular level, the innate immune response consists of cells of the myeloid lineage, while specific immune responses are provided by those of the lymphoid lineage. This is illustrated in Figure 1.14:

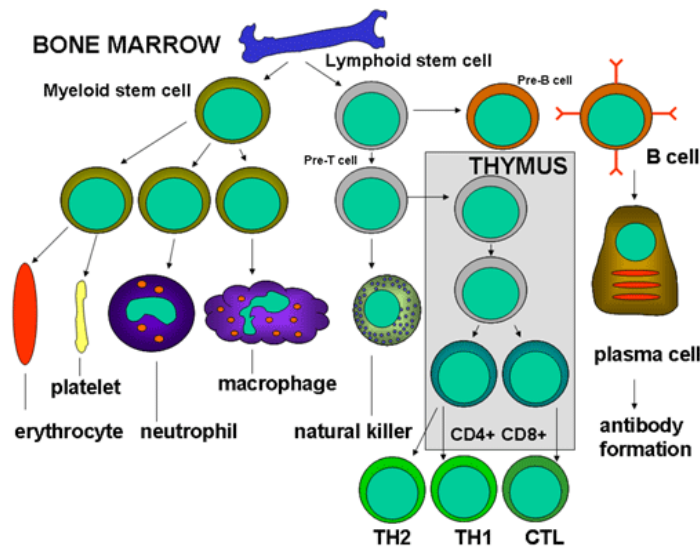


Figure 1.14
Overview of the
immune system.
 Modified from
 pathmicro.med.sc.
 edu

Fracture healing, like all other wound repair responses, is initiated through the induction of an inflammatory response. Fracture trauma involves not only an interruption of skeletal integrity but also a disruption of the normal vascular structures and nutrient flow at the fracture site. This leads to reduced oxygen tension, disruption of the marrow architecture, and results in the infiltration of inflammatory cells, macrophages, and degranulating platelets during formation of a haematoma (Brighton and Hunt 1991; Einhorn 1998). While it is likely that the mechanical stresses, changes in oxygen tension, and loss of vascular nutrients at the fracture site may signal some aspects of healing, the dominant initiators of fracture repair are most likely the numerous cytokines and growth factors released into the fracture site (Bolander 1992). These include the interleukins, IL-1 and IL-6, and tumour necrosis factor α as well as the local growth factors fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), and the bone morphogenetic proteins (BMPs)-2, -3, -4, and -7 among others (see 1.3.8) (Barnes, Kostenuik *et al.* 1999).

However work on the specific cellular components of the immune system involved and their contribution in releasing these signalling molecules remains relatively sparse, as can be seen in the next sections:

1.5.2 The macrophage and fracture repair

The initial stages of *soft* tissue wound healing involve fibrin clot formation and population by haematopoietic cells including peripheral monocytes that differentiate to macrophages. The macrophage plays a pivotal role in wound healing, principally by contributing to wound debridement, and by secreting reparative growth factors (Danon, Kowatch *et al.* 1989). Many of these growth factors regulate mesenchymal stem cell recruitment, proliferation, differentiation, and synthesis of extracellular matrix proteins (Rappolee and Werb 1992). For example, macrophage transforming growth factor (TGF)- β 1 expression regulates fibrosis during cutaneous wound healing (Wu, Yu *et al.* 1997). A similar role for the macrophage in fracture healing has been initially investigated.

Macrophage expression of TGF- β 1 in fracture healing has been shown in several *in vivo* and *in vitro* studies (Moses and Serra 1996; Bostrom and Asnis 1998). The potential contribution of macrophage-derived TGF- β 1 to the osteogenic activity of osteoblastic cells is suggested by observations that a macrophage cell line *in vitro* produces TGF- β 1 and that the addition of anti-TGF- β 1 antibody blocks alkaline phosphatase expression by mesenchymal stem cells. Pro-inflammatory stimulation of macrophages by

lipopolysaccharide (LPS) also reduced BMP-2 expression in the macrophage cell line in this study (Champagne, Takebe *et al.* 2002).

In wound healing the uncommitted macrophage has the potential to differentiate into cells which direct either pro-inflammatory activities (of neighbouring cells) by LPS-induced expression of the predominant pro-inflammatory cytokine, $TNF-\alpha$, or to direct wound healing activities by elaboration of mitogenic growth factors or differentiating cytokines like $TGF-\beta 1$. The factors that control this expression include macrophage chemotactic peptide-1, MCP-1 and possibly other cytokines and chemokines (Champagne, Takebe *et al.* 2002). See Figure 1.15.

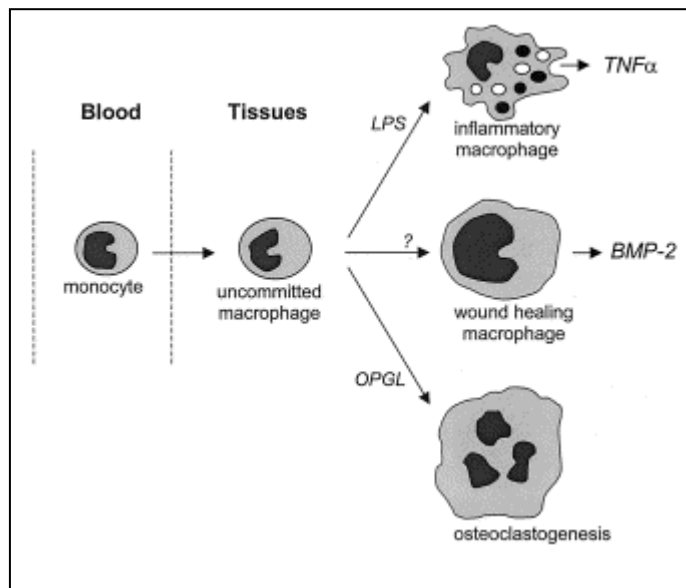


Figure 1.15
Macrophage
phenotypic
development and role
in fracture healing.
Adapted from
Champagne, Takebe *et al.* 2002

It thought likely, therefore, that in fracture repair, under an appropriate wound healing environment (mechanical, physical, biochemical, and physiological) the macrophage differentiates in a way which will allow it to contribute *pro-osteogenic* stimuli. Specifically, these are factors such as bone morphogenetic proteins which are

elaborated to neighbouring uncommitted and undifferentiated osteoblastic precursor cells. It is suggested that this function of macrophages may be essential in bone healing. On the other hand, if the macrophage is induced down the pro-inflammatory pathway (*e.g. via* LPS stimulation from bacteria or other unfavourable conditions) the induction of inflammatory cytokines and fibrosis-promoting growth factors by these stimulated macrophages suggests a prominent negative or osteolytic effect for the *pro-inflammatory* macrophage (Kovacs and DiPietro 1994).

These findings suggest a key role for the macrophage in bone repair, in particular the differentiation pathway which it follows. Macrophages contribute osteoinductive cytokines to the fracture healing environment under the *appropriate* conditions. In particular macrophage elaboration of BMP-2 promotes osteogenesis during aseptic wound healing. Conversely macrophage elaboration of TNF- α would elicit an inflammatory response and fibrosis and perhaps lead to non-union, under unfavourable conditions.

The mechanical conditions have a particular influence on the rate of macrophage infiltration to the fracture site. An *in vivo* study in rats showed that the rate of macrophage immigration to the fracture site was determined by fracture stability, and this again suggests a key role for the macrophage in bone healing (Hankemeier, Grassel *et al.* 2001).

1.5.3 Mast cells and fracture repair

Mast cells are granule rich cells resident in most tissues that have the ability to degranulate when stimulated by injury to release histamine, heparin and proteases. They have been shown to be involved in tissue repair. Specifically tissue proteolysis by the release of enzymes has been shown to be the most important contribution. The most abundant of these enzymes are neutral proteases which are involved in the regulation of tissue repair, cell-matrix and cell-cell interaction and tissue proteolysis. It is thought that they 'pave the way' for new proliferating tissue (Bond and Butler 1987). In bone *in vitro* work has shown that mast cells, through the action of the neutral protease chymase, may alter molecules in the extracellular matrix that are important in osteoblast adhesion, cell spreading, maintenance of cell morphology, and, most likely, cell function (Banovac, Banovac *et al.* 1993). An *in vivo* study of fracture healing concluded that mast cells and their enzymes play a role in the formation of extracellular matrix in callus tissue. In the early stages of healing, mast cell chymase digests loose connective tissue around the blood vessels that could facilitate angiogenesis in the early stages of healing. In the later stages of fracture healing, mast cell chymase, in synergy with other neutral proteases may have a role in the replacement of provisional callus tissue with newly formed bone in the external callus (Banovac, Renfree *et al.* 1995). Others have also suggested that mast cells promote bone resorption, especially during the remodelling phase of callus formed at the fractured site, in a fracture study in rats (Taniguchi 1990). These are the only three studies investigating mast cells in bone and while they were reasonably well conducted they involved only small numbers of

animals, using only immunohistochemical methods and have not been repeated or verified since.

1.5.4 Polymorphonuclear leukocytes and fracture repair

Polymorphonuclear leukocytes (comprising neutrophils, basophils and eosinophils) are part of the aseptic inflammatory response in the first stage of fracture repair, following the extravasation of blood. They form part of the inflammatory response in any injured tissue and are thought to be integral to infection control as tissues repair. However some studies have indicated that their presence may be detrimental and that they may aggravate tissue damage and jeopardise tissue repair as has been shown following myocardial infarction (Romson, Hook *et al.* 1983). However, they *do* play an important scavenger (wound debridement by phagocytosis) and surveillance role, particularly in open wounds (in dealing with infection) but are thought *not* to contribute to tissue regeneration itself (Grogaard, Gerdin *et al.* 1990).

1.5.5 T cells and B cells in fracture repair

The number of studies which have examined the cells of the specific immune system in fracture healing is very limited. It is known that lymphokines, a product of T lymphocytes can stimulate fibroblast migration and collagen synthesis in soft tissue healing (Wahl, Wahl *et al.* 1978). One study investigating the immunogenic properties of demineralised bone matrix described the presence of IL-2 positive lymphocytes in

the granulation tissue of fracture healing (without demineralised bone matrix), simply stating ‘cells with labelled receptors for IL-2 occurred only sparsely’, with no further elaboration or detail of immune cells in the fracture granulation tissue part of this study (Henricson, Hulth *et al.* 1991). They showed a rich induction of T lymphocytes in demineralised bone powder, because of the immunogenic properties of this powder. They suggested a surveillance function of these cells in fracture healing interpreting this as the body’s standard response to injury of any kind to meet a possible immunological challenge. Askalonov found failure of healing of bone tissue in humans to be connected to severe disturbances of cellular immunity (Askalonov 1982) and also carried out *in vivo* work which showed a regulatory role of T cell immunity on osteogenesis (Askalonov, Gordienko *et al.* 1987). These studies were published in the 1980s and the methods used were given in very poor detail.

Andrew *et al.* reviewed the role of inflammatory cells in human fracture healing (Andrew, Andrew *et al.* 1994) and looked at cells of the immune system in human fracture samples taken from healing fractures at variable sites at variable times. Accepted immunohistological techniques were used with approved antibodies and the staining they found appeared specific. They noted that T lymphocytes appeared to be initially selectively recruited into the fracture site at the haematoma stage but become less numerous later and were excluded from areas of cartilage and bone formation. This was deemed ‘selective recruitment’ as they noted that T cells within the haematoma and granulation stage of fracture repair outnumbered B cells at all times, and were not simply present due to haemorrhage from the systemic circulation. In the samples in

which they had been excluded from the cartilage and bone formation, staining could be seen in surrounding fibrous tissue. Santavirta found large number of T cells in non-union samples in humans (Santavirta, Kontinen *et al.* 1992) and with Andrew's findings of T cell exclusion this raises the question of whether T lymphocytes are important in the pathogenesis of failure of bone healing. With regard to the macrophage, Andrew noted large numbers in early wound repair and in granulation tissue but smaller numbers in areas of bone formation. They were not specifically related to areas of debris in a scavenger-type function but seemed to be acting as coordinating cells in a similar fashion as seen in soft tissue healing. They also expressed HLA-DR which implies an antigen presentation function, (a function of macrophages which is vital to a normal immune response to foreign antigens) which, in this situation, was considered to reflect a non-specific immune surveillance function related to tissue injury and repair. Andrew concluded that immune cells control and coordinate fracture healing *via* the cytokines and growth factors they are known to release (see 1.3.8), probably in a similar way as has been proposed for soft tissue wound healing (see 1.5.6 below) (Andrew, Andrew *et al.* 1994). However, he looked at random human fracture samples. No fully controlled, *in vivo* animal study has been conducted to confirm the presence of these cells or suggest their function and importance in fracture healing.

1.5.6 The specific immune system and soft tissue wound healing

It is known that immune cells have an integral function in soft tissue wound healing beyond their role in inflammation and host defence. "*Immune cells are vital to the*

regulation of soft tissue wound-healing through the secretion of signalling molecules, such as cytokines, lymphokines, and growth factors” (Park and Barbul 2004).

Neutrophils arrive first at a soft tissue wound site and their primary role is phagocytosis and wound debridement. Neutrophil dysfunction can lead to increased infection and wound complications and there may be a beneficial role in wound healing for neutrophil growth factors, such as granulocyte/macrophage colony-stimulating factor (GM-CSF) (Canturk, Esen *et al.* 2001).

Macrophages migrate into the wound 48 - 96 hours after injury and become the predominant cell population before fibroblast migration and replication. Macrophages play an integral and critical role in successful wound healing. They participate in and conclude the inflammatory and debridement processes. Their antimicrobial function is carried out by phagocytosis and the generation of reactive radicals, such as nitric oxide, oxygen, and peroxide (Witte and Barbul 1997). The most important contribution is their secretion of cytokines and growth factors. These cytokines act in a paracrine manner to activate and recruit other cells involved in wound healing, such as other macrophages or lymphocytes. Cytokines and growth factors also regulate fibroblast chemotaxis, proliferation, and collagen synthesis, as well as other cells involved in repair, such as endothelial cells (Barbul 1990). Macrophages therefore influence angiogenesis, fibroplasia, and matrix synthesis in soft tissue wound healing. The main cytokines released include TNF- α , TGF- α , TGF- β , IL-1, IL-6, PDGF and IGF-1, very similar to those involved in bone healing, see 1.3.8. The true effects of each of these cytokines *in vivo* are only partially understood in soft tissue wound healing (Park and Barbul 2004).

With regard to T lymphocytes, they have been shown in one study, looking at incisional wounds to the dermis in rats, to migrate into the wound after the appearance of inflammatory cells and macrophages on the fifth day following injury during the proliferative phase, and peak at day 7. However little is known about the role of T lymphocytes in the healing process (Fishel, Barbul *et al.* 1987). Thymectomy studies in rats have suggested an inhibitory role of T-suppressor lymphocytes on soft tissue wound healing (Barbul, Sisto *et al.* 1982). It has also been noted that monoclonal antibody depletion of CD⁸+ T (cytotoxic) cells resulted in increased wound breaking strength and collagen levels in rats as did combined depletion of CD⁴+ (T helper cells) and CD⁸+ cells. Depleting CD⁴+ T cells alone did not make any difference to the wound (Efron, Frankel *et al.* 1990). Martin looked at human soft tissue wounds and showed infiltration of T cells, particularly in the early stages. He concluded that T lymphocytes may play an important regulatory role in wound healing and scar formation (Martin and Muir 1990).

B lymphocytes have been shown to be present in soft tissue wounds in only one study. This looked at pilonidal sinus wounds in humans and was inconclusive as to the effect of these observed B cells and was uncontrolled (Boyce, Jones *et al.* 2000). A better conducted study using monoclonal antibody stains showed no B cells in human wounds (Martin and Muir 1990). Park and Barbul showed there were no activating factors for B lymphocytes present within the wound environment, so B cells are unlikely to play a significant role in the regulation of soft tissue wound healing (Park and Barbul 2004).

1.5.7 The systemic immune response to a fracture

Serious injury including bony fractures predisposes the host to infectious complications and this is thought to be due to a injury-induced suppression of the *specific* immune response (Lederer, Rodrick *et al.* 1999). While the innate immune system appears to demonstrate an increased inflammatory reactivity post-injury, the specific immune response shifts towards a counter-inflammatory phenotype. It is the coexistence of these phenotypic changes which leads to the development of the wide imbalance in immune function frequently observed in seriously injured patients (Harris and Gelfand 1995).

Fracture and the systemic innate immune response:

Fracture or any bodily injury results in systemic release of pro-inflammatory cytokines by cells of the innate immune system including macrophages, fibroblasts and natural killer cells. This is regarded as the acute phase response to injury and it is mediated by enhanced IL-1, IL-6, TNF- α , TGF- β , reactive nitrogen intermediates and prostaglandin E2 production (Ogle, Mao *et al.* 1994). The function of these mediators is to induce chemotaxis of further inflammatory cells, induce the maturation and activation of T- and B-cells, induce pyrogenesis and cause further production of cytokines by yet, unstimulated innate immune cells. These cells are also interdependent in an autocrine and paracrine manner with functions such as phagocytosis dependent on stimulation by the afore mentioned cytokines (Ni Choileain and Redmond 2006). Hyperactivity of this innate immune response and the increased productive capacity for inflammatory mediators by its cells has been implicated in the increased susceptibility to sepsis

following injury as the response becomes detrimental to the host at hyperactive levels giving rise to conditions such as acute respiratory distress syndrome and multi-organ failure syndrome (O'Riordain, Collins *et al.* 1992).

Fracture and the systemic specific immune response

Major impairment of the specific immune system is thought to play an important role in the increased susceptibility to infection following severe injury. Whilst the large majority of fractures have no clinically observable effect on the specific immune system, high-energy trauma such as high speed road traffic accidents which involve long bone or pelvic fractures will show specific immune system sequelae (Ni Choileain and Redmond 2006). T-helper CD⁴⁺ lymphocytes can be divided into two functionally distinct, highly polarized subsets termed Th-1 and Th-2, depending on their pattern of cytokine secretion and related functional activity. Th-1 cells are characterized by their production of IFN- γ and IL-2 while Th-2 cells are characterized by their production of IL-4, IL-5, IL-9 and IL-13. Several other proteins are secreted by both Th-1 and Th-2 cells, including IL-3, TNF- α and granulocyte-macrophage colony-stimulating factor (GM-CSF). Expansion of the Th-1 phenotype is facilitated by exposure to IL-12 while expansion of the Th-2 phenotype is promoted by IL-4 (Trinchieri 1993). In general terms, the Th-1 cytokines activate macrophages and induce pro-inflammatory cellular immunity whilst Th-2 cytokines activate B cells and induce humoral immunity but are also anti-inflammatory (Park and Barbul 2004). It has been demonstrated that the injury-induced change in mitogen-stimulated responses is associated with an increased

production of anti-inflammatory Th-2-type cytokines such as IL-4 with diminished IL-12 and IFN- γ production (Utsunomiya, Kobayashi *et al.* 1997). This preferential expression of the Th-2 phenotype does not occur immediately after the injury but is most prominent at approximately one week, suggesting that it is a developed immune response and it is associated with suppression of Th-1 pro-inflammatory function at this time. This expression probably represents an attempt by the specific immune system to modify the hyperactivated innate immune response at this time, which is often seen in the severely injured patient (see above) (O'Riordain, Collins *et al.* 1992). The low Th-1 type cytokine production and reduced Th-1 type antibody isotype secretion leads to an association between injury-induced suppressed specific immune function and increased risk of developing nosocomial infections (Kelly, O'Suilleabhain *et al.* 1999). This injury-induced immunosuppression can lead to increased susceptibility to subsequent sepsis and multiple organ failure.

Therefore it can be seen that while the very large majority of fractures have no clinically observable affect on the systemic immune response, high energy trauma which almost always involves at least one bony fracture has profound effects on the immune system, some of which can be detrimental and contribute to the demise of the patient.

1.6 Clinical factors which affect both the immune system and fracture repair

1.6.1 Introduction

The previous parts of this introduction have attempted to discuss the cellular and molecular process of fracture repair and the involvement the immune system may have in this process. At a clinical level the relevance of this is highly significant. There are many factors, physiological, pathological and pharmacological which are known to have effects on the immune system in humans. Many of these factors are also known to have effects on fracture repair. Conditions such as diabetes and drugs such as corticosteroids have well documented effects on the immune system. However increasing age, malnutrition and social factors such as alcohol and smoking also have less well known effects on the immune system. The detrimental consequence of these factors in bony healing has been reviewed and discussed below as well as a summary of their effects on the immune system.

1.6.2 Sex and age

Affect of sex and age on fracture healing

There has been no correlation between gender and nonunion or delayed union of fractures, although it is more common amongst males as they have a higher incidence of being involved in high energy accidents. With regards to age, it is well known that children's fractures heal at an increased rate and this is in-part due to a larger subperiosteal haematoma and a thicker periosteum which contribute to a more rapid formation of callus strong enough to render the fracture healed more rapidly than in the

adult (Lindaman 2001). Growth in paediatric bone, which recapitulates fracture healing in many aspects, (Ferguson, Alpern *et al.* 1999) provides an osteogenic environment in children's bones which means that many of the fracture healing mechanisms are already in place at the time of the fracture. Although the difference in fracture healing between children and adults is relatively well understood it is also thought that advancing age in adults also has a significant impact on skeletal repair. Early studies of fracture healing in rats have shown that cartilage and bone formation, and cartilage resorption are delayed in elderly animals (Aho 1966) and there is evidence that accretion of mineral into the callus is reduced in elderly animals (Meyer, Tsahakis *et al.* 2001; Meyer, Meyer *et al.* 2003). Studies into dermal wound healing demonstrate age-related shifts in both macrophage and T cell infiltration into wounds, alterations in chemokine content, and a concurrent decline in wound macrophage phagocytic function, demonstrating a direct link between decline in the immune system with age and wound healing (Swift, Burns *et al.* 2001). A well controlled recent investigation in rats (Lu, Micalau *et al.* 2005) noted that age-related changes in fracture healing included a delay in the onset of the periosteal reaction, delays in cell differentiation, decreased bone formation, delayed angiogenic invasion of cartilage, a protracted period of endochondral ossification, and impaired bone remodelling. They found that this decline in healing capacity continues throughout the life span of the animal. This decline may have resulted from cell-autonomous changes in stem cells or arose from other systemic changes, although this is not yet clear. These systemic changes may include an alteration in the immune system as a result of ageing of the animal, as will be discussed further below. One study suggested the decline may have been due to a decrease in growth hormone production

(Bak and Andreassen 1991), although it is likely to involve interaction of local and systemic systems (Meyer, Desai *et al.* 2006). The clinical literature reveals little evidence about fracture healing in the human elderly being delayed. One well designed prospective study shows an increased risk of clavicular nonunion with advancing age (Robinson, Court-Brown *et al.* 2004). Another, from a retrospective review of a high number of cases, reveals that age is predictive of fracture union after internal fixation of intracapsular femoral neck fractures (Parker 1994).

The literature does suggest an effect of age on fracture repair and this may well be linked to the decline in immune function.

Affect of age on the immune system

In the immunological literature there is a general consensus, supported by experimental data and clinical observations that the function of the immune system declines with age (Wick and Grubeck-Loebenstein 1997). Thus, on a worldwide basis, infections are still the main cause of death in the elderly. Changes in T lymphocyte populations underlie much of the age-related decline in the protective immune response. Aging leads to the replacement of virgin T cells by memory T cells and to the accumulation of cells with signal transduction defects (Miller 1996). While immune reactivity against foreign antigens drops significantly in older age, giving rise to a higher frequency of infections, autoreactivity paradoxically rises. This increased autoreactivity appears to be due to a decreased potential for down-regulation of the immune reaction by a shift from Th1 to Th2 cells with autoreactive potential (Globerson, Tomer *et al.* 1993) as has been demonstrated in experiments with aged mice. The alterations in both the innate and

specific immune system are known collectively as *immunosenescence* (Hakim and Gress 2007). Chronic involution of the thymus gland is thought to be one of the major contributing factors to loss of immune function with increasing age and this has been demonstrated in mice and humans (Gruver, Hudson *et al.* 2007). The topic of immunosenescence has received a large amount of attention in the field of immunology and a full review of the current literature is not in the remit of this thesis. However, a change in the function and shifts in the sub-populations of T cells related to thymic changes seems to be the overall consensus in age related immune changes (Wick and Grubeck-Loebenstein 1997). This may have profound effects on fracture repair, particularly if T cells have an active role in this process.

1.6.3 Diabetes

Diabetes mellitus and fracture repair

There is a strong association between diabetes and impaired bone healing. It is often associated with reduced bone mass and delayed fracture healing in humans and clinical studies have demonstrated significant incidence of delayed union, non-union, and pseudarthrosis and a doubling of the time to fracture healing in diabetic patients compared to non-diabetic patients (Cozen 1972; Levin, Boisseau *et al.* 1976; Loder 1988).

There have been several animal trials examining the effects of diabetes. Diabetic rats can be induced by two means; chemical induction using streptozotocin directly poisons the pancreatic islets eliminating insulin production (Rees and Alcolado 2005); spontaneously diabetic BB/OK rats have autoimmune induced disease (Follak, Kloting

et al. 2004). A good study using streptozotocin induced diabetes showed that after two weeks of healing, fracture callus from the untreated diabetic animals had a 29% decrease in tensile strength and a 50% decrease in stiffness compared with the controls (Macey, Kana *et al.* 1989). Treatment of the diabetic animals with insulin restored the tensile strength and stiffness of the callus to a value that was not statistically different from that of the controls. They also showed that between the fourth and eleventh days of healing, there was a 50 - 55% decrease in the collagen content of the callus of the untreated diabetic animals compared with the controls. In addition, on the fourth day of healing, DNA content, an indicator of cellularity of the callus, was decreased by 40% in the untreated diabetic group.

In several other animal models, diabetes has led to reduced biomechanical properties of the healing fracture, reduced cellular proliferation in the early callus and reduced collagen synthesis and content compared to non-diabetic control animals (Herbsman, Powers *et al.* 1968; Funk, Hale *et al.* 2000; Beam, Russell Parsons *et al.* 2002). All these studies have shown that the most important factor is physiological glycaemic control which will return fracture healing back to that seen in the control animal.

Poor glycaemic control in association with bone healing leads to reduced collagen production (type II), reduced cellular proliferation and osteoblast activity and results in inadequate mineralisation, insufficient biomechanical stability and delayed healing (Gooch, Hale *et al.* 2000).

It is thought that some of the consequences of diabetes on fracture repair are related to inhibition of growth factors although the underlying mechanism is largely unknown.

Tyndall described diabetes as affecting the early phase of fracture healing by inhibiting

cell proliferation through decreasing expression of platelet derived growth factor (Tyndall, Beam *et al.* 2003). Gandhi showed that levels of PDGF, IGF-1, VEGF and TGF- β were all significantly reduced in diabetic animals; this group also showed that local delivery of platelet rich plasma restored normal cell proliferation and chondrogenesis in the early stages and partially improved mechanical strength in the later stages of fracture repair (Gandhi, Doumas *et al.* 2006). The same group demonstrated that direct delivery of insulin to the fracture site in diabetic animals returned fracture healing to normal, suggesting a direct effect of insulin on repair (Gandhi, Beam *et al.* 2005).

Although there is no study looking at fracture healing in patients with good and poor blood glucose control, it would seem from the above work that good blood sugar control in fracture patients will minimize the potential complications of delayed fracture healing, wound complications, and development of Charcot arthropathy (Bibbo, Lin *et al.* 2001).

Diabetes mellitus and the immune system

Both patients with insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) have an impaired cell mediated (or specific) immune response as has been demonstrated in human and animal studies (Delespesse, Duchateau *et al.* 1974; Plouffe, Silva *et al.* 1978). The abnormalities in the immune system have been considered a major factor contributing to infections in diabetic patients (Fox 1984). Cell-mediated immunity is mediated by T lymphocytes and monocytes / macrophages and is particularly crucial in host defence against intracellular

pathogens such as mycobacteria, fungi and pseudomonas. Prolonged depression of cell-mediated immunity could explain the increased incidence and severity of pulmonary tuberculosis and the increased prevalence of all infections in diabetic patients (Edsall, Collins *et al.* 1970). T cell proliferation is thought to be under the control of the cytokine IL-2 and the subsequent expression of the IL-2 receptor (Smith 1988). It is thought that defective expression of the IL-2 receptor in T cells is the mechanism of a low lymphocyte proliferative response in diabetic patients which has been demonstrated in T cells from human diabetic patients (Chang and Shaio 1995). Culture studies showed that this was not due to the hyperglycaemia alone but the presence of 'advanced glycosylation end-products', well known to be up-regulated in the cytosolic environment in diabetic patients, were shown to affect the expressivity of this receptor (Giardino, Edelstein *et al.* 1994).

While diabetes most likely has affects on all cell types, its well documented effects on the immune system may well have a bearing on its affects on fracture healing.

1.6.4 Non-steroidal anti-inflammatory drugs

NSAIDs and fracture repair

Non-steroidal anti-inflammatory drugs (NSAIDs) specifically inhibit cyclooxygenase (COX) activity and are widely used as anti-arthritis, post-surgical analgesics, and for the relief of acute musculoskeletal pain. They include aspirin, ibuprofen, COX-2 inhibitors and naproxen. These inhibit prostaglandin synthesis which accounts for their side effects of which the worst is gastric ulceration and haemorrhage. There is much conflicting evidence in the literature as to their effects on fracture repair. Well

conducted animal experiments with mice where the COX 2 gene is knocked out show a dramatic decrease in repair of long bone fractures (Simon, Manigrasso *et al.* 2002). They also showed that fracture healing was impaired in rats treated with COX-2-selective NSAIDs (celecoxib and rofecoxib). Their histological observations suggested that COX-2 was required for normal endochondral ossification during fracture healing but was not required for embryonic skeletal development: Knock-out animals deficient in COX 2 showed normal skeletons. Others studies have shown a harmless effect of COX inhibitors (Gerstenfeld, Thiede *et al.* 2003) but this may relate to dosing and rapid first-pass metabolism in the livers of male rats in this study.

Clinically it is important to know whether NSAIDs inhibit fracture repair as they are often prescribed for post-operative analgesia. There is very good evidence for their use in preventing heterotopic bone formation (Fransen and Neal 2004). In a well constructed randomised controlled trial looking at the role of indomethacin in preventing heterotopic ossification following the surgical treatment of acetabular fractures, the authors subsequently evaluated the incidence of long-bone non-union in fractures that had occurred in the same injury as the acetabular fracture. There was a significant increase in the rate of non-union in the patients receiving indomethacin compared to those who received a single dose of prophylactic radiotherapy to the hip or no treatment (Burd, Hughes *et al.* 2003). A retrospective study in patients with femoral fractures found there was a marked association between nonunion and the use of NSAIDs after injury (Giannoudis, MacDonald *et al.* 2000). Delayed healing was noted in patients who took NSAIDs and whose fractures had united.

Investigations of the molecular link between prostaglandins have shown that prostaglandin E(2) can control expression of both BMP-2 and BMP-7 (bone morphogenetic proteins) (Paralkar, Grasser *et al.* 2002; Arikawa, Omura *et al.* 2004). It appears that NSAIDs exhibit inhibitory effects on bone formation in humans. It is indeterminate as yet as to the exact nature of the effect on fracture healing.

NSAIDs and the immune system

It is accepted that NSAIDs exhibit anti-inflammatory effects through the inhibition of prostaglandin production, as noted above, by the inhibition of COX activity and it is thought that this produces down-regulation of the innate cellular immunity response (Vane 1971). However the effects on the specific immune system have only been minimally investigated. Tanaka has shown that indomethacin enhanced specific cell-mediated responses such as lymphocyte proliferation and cytotoxic activity in a dose-dependent manner (Tanaka, Tanaka *et al.* 1998) in culture studies. This was thought to be due to the ability of NSAIDs to up-regulate IL-2 production and down-regulate IL-6 affecting the production of T-cell subsets (Tsuboi, Tanaka *et al.* 1995). It has been shown that these effects are independent of prostaglandin inhibition but are rather a direct effect on specific immune cells (Tanaka, Tanaka *et al.* 1998).

1.6.5 Corticosteroids

Steroids and fracture repair

Corticosteroid treatment is, for some patients, an essential part of treating their health problems and making daily life tolerable. Such diseases include widespread conditions

such as asthma, chronic obstructive airways disease, inflammatory bowel disease and tolerance of transplanted organs. Corticosteroids are immunosuppressive and anti-inflammatory and consequently have serious side effects, particularly if used at moderate to high doses for prolonged periods. It is well understood that prolonged systemic administration of corticosteroids causes osteoporosis and increased risk of fracture due to their inhibitory effects on the production of IGF-1 and TGF- β (Aaron, Francis *et al.* 1989). However few studies have been conducted to investigate specifically the effect on fracture repair. Waters *et al* found that prolonged chronic systemic administration in a non-critical sized defect (defects small enough to heal spontaneously) long bone rabbit model led to an 85% nonunion rate compared to 18% in the control group (Waters, Gamradt *et al.* 2000). Aslan investigated the effects of short term prednisilone on rats demonstrated no inhibitory effects (Aslan, Simsek *et al.* 2005) and this was confirmed in an earlier study investigating short term administration of methylprednisilone in rats (Hogevold, Groggaard *et al.* 1992). These animal studies therefore suggest that long term steroid therapy will be detrimental to fracture repair but that short term treatment may not be deleterious.

Steroids and the immune system

Corticosteroids profoundly affect the inflammatory and immune response by way of vasoconstriction, decreased chemotaxis, and down-regulation of macrophages and T and B cells (Claman 1975). Intracytoplasmic corticosteroid-specific receptors appear to be an important common pathway for steroid-induced changes (Cupps and Fauci 1982). Circulating mononuclear cells also redistribute out of the intravascular compartment

following treatment with corticosteroids, accounting further for the immunosuppression seen (Cupps and Fauci 1982). Administered steroids also can have lytic effects on activated lymphocytes (Akdis, Blesken *et al.* 1997). While it is known that corticosteroids have an overall immunosuppressive action on both arms of the immune system, the immuno-regulatory effects of corticosteroids on lymphocyte immune responses are complex and a full discussion is out-with the remit of this thesis.

1.6.6 Alcohol

Alcohol and fracture repair

Chakkalakal reported that chronic consumption of excessive alcohol eventually results in an osteopenic skeleton and increased risk for osteoporosis. Alcoholics experience not only increased incidence of fractures from falls, but also delays in fracture healing compared with non-alcoholics. Alcohol-induced osteopenia results mainly from decreased bone formation rather than increased bone resorption (Chakkalakal 2005). Human, animal and cell culture studies of the effects of alcohol on bone strongly suggest alcohol has a dose-dependent toxic effect on osteoblast activity. In fracture healing, the effect of alcohol is to suppress synthesis of an ossifiable matrix, possibly due to inhibition of cell proliferation and maldifferentiation of mesenchymal cells in the repair tissue. This results in the deficient bone repair observed in animal studies, characterized by repair tissue of lower stiffness, strength and mineral content (Chakkalakal, Novak *et al.* 2005). Of note inhibition of fracture healing in animal models by alcohol can be reversed by the use of IL-1 and TNF antagonists (Perrien,

Wahl *et al.* 2004) suggesting the deleterious effects may be mediated *via* the immune system or its products.

Alcohol and the immune system

The depression of the immune system by alcohol is well described in the immunological literature (Messingham, Faunce *et al.* 2002). Individuals who chronically abuse alcohol exhibit neutropaenia, impaired lymphocyte function, reduced natural killer (NK) cell activity, and altered humoral immunity (Grossman, MendeHall *et al.* 1988). These same defects are also observed in animal models of acute ethanol exposure (Szabo 1998). At a molecular level the production of cytokines by T cells is depressed and lymphocyte subset distribution is altered (Jayasinghe, Gianutsos *et al.* 1992). It is thought that these alterations result from apoptosis-driven loss of T and B cells from the thymus, spleen, and lymph nodes (Slukvin and Jerrells 1995). Cells of the innate immune system (monocyte lineage, mainly macrophage) are even more sensitive to the immuno-modulatory effects of ethanol, even at low doses (Bautista, D'Souza *et al.* 1991). Here the antigen presenting function of macrophages is disrupted and instead they take on a more inflammatory phenotype producing pro-inflammatory cytokines such as IL-1, IL-6, IL-8, IL-10, and TNF- α , and these are implicated in the pathogenesis of alcoholic liver disease (Messingham, Faunce *et al.* 2002). The dysregulation of immunity with alcohol is again a complex area. However it is clear to see again how alcohol may have an affect on fracture healing through its effects on immunity.

1.6.7 Smoking

Smoking and fracture repair

Smoking has been shown to adversely affect bone mineral density, lumbar disc disease, the rate of hip fractures, and the dynamics of bone and wound healing (Porter and Hanley 2001). Smoking is an undisputable high risk factor for atherosclerosis not only of the cardiac vessels but also the large and medium arteries of the limbs. Multiple studies have shown the adverse affects of tobacco use on fracture repair: A low-energy injury has a 4.1-fold risk of causing a closed tibial shaft fracture in smokers, compared with non-smokers. Smokers have a significantly longer mean time to clinical union and a higher incidence of delayed union of closed tibial fractures (Kyro, Usenius *et al.* 1993). A prospective multi-centre trial of 268 compound tibial fractures showed that smokers were 37% more likely to develop nonunion and twice as likely to develop infection. Ex-smokers are also at increased risk of non-union and osteomyelitis (Castillo, Bosse *et al.* 2005). As well as delayed and non-union, smokers also show an increased rate of flap (plastic surgery procedure to soft tissue requiring good vascularity) failure in open tibial fracture with rates up to 20% (Adams, Keating *et al.* 2001). Relative risk of hip fracture among smokers is between 1.5- 2.0 and this is worsened by an insufficient dietary intake of vitamin E and C (Melhus, Michaelsson *et al.* 1999). Smoking is also linked to a higher rate of nonunion and poorer results after surgery to fuse the ankle and spine (Perlman and Thordarson 1999).

There are several hypotheses for the mode of action in smoking. Reduced blood supply, high levels of reactive oxygen intermediates, low concentrations of antioxidant vitamins and nicotinic effects on arteriole endothelial receptors have all been postulated. Nicotine

at high doses is also directly toxic to proliferating osteoblasts although low dose nicotine may be stimulatory (Gullihorn, Karpman *et al.* 2005). In one of the few animal studies carried out, tobacco extract (not containing nicotine) significantly reduced the mechanical strength of healing femoral fractures in rats while nicotine alone did not affect mechanical properties (Skott, Andreassen *et al.* 2006). Smoking is associated with other factors including low socioeconomic status, poor nutrition, general ill-health and other lifestyle factors making it hard to determine the precise risk analysis of the habit in fracture healing

Smoking and the immune system

Cigarette smoke has been shown to have multiple effects on the immune system. Nicotine has been shown to be immunosuppressive (Sopori and Kozak 1998) causing a lack of responsiveness in T cells in culture. Smoking in human studies has been shown to alter lymphocyte populations, increasing numbers of T cells and changing the ratios of subsets of T cells (Tollerud, Clark *et al.* 1989). Smoking and its effects on immunity receives much attention for conditions such as tuberculosis and lung cancer and again these are complex and wide ranging. The effects on fracture healing have received little attention as described above. The finding that nicotine has a negative effect on T cells and may have even a stimulatory effect on fracture healing (Presented ORS 2007, Betchtold JE *et al.*, Minneapolis) is important.

In summary many common clinical factors affect the immune system and impair fracture healing. The understanding of the interaction between the immune system and fracture repair is incomplete and further elucidation could be of great clinical benefit. This forms the basis of the experiments to be described in this thesis.

Section two: Hypothesis and aims

1.7 Hypothesis

The hypothesis to be evaluated in this thesis was:

“The Specific Immune System Modulates the Process of Fracture Repair”

1.8 Aims of study

The importance of the cells of the immune system in bone biology has been outlined in Section one. There is a dearth of information in the role of the specific immune system in fracture healing. In order to address this and evaluate the hypothesis above, the aims of this thesis were as follows:

1. The establishment of a clinically relevant, reproducible and reliable small animal model of fracture repair which does not involve potentially immunogenic internal fixation.

2. To evaluate the time sequence of fracture healing in the wild type Balb/c mouse using this model; To compare this with a genetic 'knock-out' mouse lacking a functioning specific immune system, specifically the Balb/c SCID (severe combined immuno deficient).
3. To quantify and compare fracture healing in the two strains of animal using the following techniques: histology; histomorphometry; radiographic assessment; bone mineral density determination; mechanical assessment.
4. To establish a method of reconstitution of SCID animals, to 'give back' the specific immune system; Determination of success of reconstitution by the technique of ELISA; To compare bony healing using the fracture model in these animals, compared again to wild type and SCID strains using the outcomes detailed in aim 3.
5. Evaluation of the temporal expression of mononuclear leukocytes (the key cells thought to be involved in wound healing – T cells, B cells, and macrophages) locally at the fracture site temporally over the course of fracture repair, using the fracture model detailed in aim 1.
6. Evaluation of the temporal systemic expression of mononuclear leukocytes in murine blood following fracture, using the fracture model detailed in aim 1.

Chapter 2

General Methods: Fracture model and Fracture Repair Assessment

2.1 Introduction:

In this chapter the animal model used will be discussed. The overall plan was to validate a previously used closed tibial murine fracture procedure which could then be applied to these studies. This was used in wild type mice to monitor fracture repair with comparative studies involving selectively immunodeficient mouse strains. It was also used to assess the immune cells involved in normal murine bone repair in immunocompetent mice at both the local and systemic levels. The development and validation of the model and the method detail including specimen harvest, processing, storage and retrieval will be described in this chapter.

The main outcome measures of fracture repair recorded were:

1. Histological / histomorphometric analysis of fracture site.
2. Radiographic assessment of fracture repair.
3. Calculation of tibial bone mineral density.
4. Mechanical testing of healing fractures.
5. Body weight.
6. Statistical testing was applied to all of the above results.

2.2 Animal model

2.2.1 Need for an animal model

The use of animal models to study fracture healing is designed to answer questions that relate to the most efficient and effective way to treat fractures in people. Much of the information on fracture healing in humans has been inferred from animal models and has been supported by human clinical and epidemiologic studies (Nunamaker 1998).

The parameters that have been reported to affect the rate of fracture repair include (Neyt, Buckwalter *et al.* 1998):

1) Age	4) External environment
2) Sex	5) Type of fracture
3) Race	6) Type of stabilisation.

These factors need to be controlled in any experiment investigating fracture repair. It would be very difficult to control all of these in a human study. The use of experimental animals, however, allows sufficient control of all these parameters.

It has been noted for many years that there are only very few differences in normal fracture repair between human patients and animals (Yamagishi and Yoshimura 1955). This original study also noted that callus formation in animals, as in man, was closely related to biomechanical factors related to the fixation method.

Numerous methods have been devised to induce fractures in rodents in order to study fracture healing. These include the use of digital pressure (Hyldebrandt, Damholt *et al.* 1974), breaking the bone using a pneumatic punch press (Jackson, Reed *et al.* 1970) or cutting the bone with a handsaw (Yamagishi and Yoshimura 1955). These techniques

have been shown either to lack reproducibility and/or to not resemble a natural fracture and therefore gave a distorted picture of fracture repair. Nunamaker has reviewed the more modern fracture and osteotomy models used to investigate fracture repair and reported that reliable models can be created along the phylogenetic scale from rodents right up to primates (Nunamaker 1998). All have their particular advantages and disadvantages and he recommended circumspection when relating results to the response in humans. The commonly used validated model of fracture repair in mice is that of Bonnarens and Einhorn (Bonnarens and Einhorn 1984) whereby a standardised weight is dropped from a height with a blunt guillotine connected to create a fracture of the femur which is stabilised with an intramedullary pin. This was deemed unsuitable for this study due to the internal fixation used and the animal model used in this thesis is discussed below.

2.2.2 Animals used

Mice were chosen for use in this research project for the following reasons:

1. Easy to procure and maintain
2. Inexpensive
3. Easy to handle
4. Validated methods for creating reproducible fractures and assessing fracture healing
5. Readily available transgenic strains.

Limitations of mice when used as a fracture model:

1. More primitive bone structure when compared to humans
2. No Haversian remodelling occurs in murine fracture repair (Nunamaker 1998).

2.2.3 Animal husbandry

2.2.3.1 Environment and diet

Wild type animals were housed in groups of six in standard cages with wood shaving bedding. Immunodeficient animals were also housed in groups of six but their cages were individually ventilated with clean air and all bedding and feed was sterilised prior to use. For all animals temperature was maintained at 15 to 20 degrees Celsius with cycles of 12 hours of light and dark every 24 hours. All procedures and husbandry were carried out during hours of light. Animals were delivered one week before creation of the fracture and handled daily to allow acclimatisation to handlers and environment.

2.2.3.2 The animals

Balb/c wild type and balb/c severe combined immuno-deficient (SCID) mice were used. Skeletal maturity was reached in each strain used in this study at 12 weeks. Therefore the age range of all the animals used was 12 – 14 weeks at delivery to the facility. All animals were male to control for the effects of sex hormones on fracture repair. All were of a similar weight at the commencement of the experiment ($\pm$ 1 gram). The SCID balb/c mouse is a natural mutant which lacks functioning T and B cells. It does not manufacture mature T and B cells but natural killer and macrophage cell function is normal, as is circulating complement. They carry a natural mutation that results in failure to generate functional immunoglobulins and T cell receptors. However all other components of their immune system are intact. They are used widely in immunological and transplant studies and provided an ideal animal to investigate the absence of the specific immune system.

2.2.3.3 Choice of time points for euthanasia

Animals were euthanased by carbon dioxide inhalation. The time points chosen to examine the fracture callus were based on previous work looking at fracture healing in mice which showed that the key stages in fracture repair in this animal were (Bourque, Gross *et al.* 1992):

Stage 1: (Days 0 – 2) Inflammatory response stage: Formation of haematoma with influx of inflammatory cells.

Stage2: (Days 2 – 5) Periosteal fibrous callus stage: Expansion of periosteum, appearance of fibroblast-like mesenchymal cells and formation of fibrous bridging callus.

Stage 3: (Days 5 – 9) Chondrogenic stage: Production of cartilaginous callus.

Stage 4: (Days 9 – 21): Endochondral stage: Replacement of cartilage callus by new trabecular bone through the process of endochondral ossification, with almost complete replacement of cartilaginous callus by bone at day 21.

The time points therefore used to assess histology and immunohistochemistry in this animal were days 1, 3, 7, 14 and 21 with a minimum of 4 animals (see power calculation later in chapter) at each time point for each strain. Radiology was assessed at days 7, 14 and 21. Mechanical assessment and bone mineral density measurements were done at day 21 when fracture repair will be almost complete in the wild type mouse. A minimum of 6 animals in each strain were used for these measures (see power calculation Section 2.11.5.1).

2.2.4 The fracture model:

2.2.4.1 Development and validation:

A closed model of fracture repair was required as the inflammatory/immune response in fracture healing was under investigation. The use of an osteotomy which would involve a surgical wound and the use of internal fixation which would involve a foreign body was to be avoided, to prevent confounding results from immune responses to these insults. The widely established and accepted technique described by Bonnarens (Bonnarens and Einhorn 1984) could therefore not be used as this involves an intramedullary pin. The literature was searched for a reproducible, reliable closed tibial fracture in mice. The fracture technique used in the present work was developed based on the model described by Bourque *et al.* (Bourque, Gross *et al.* 1992) of Dalhousie University, Halifax, Nova Scotia. This technique has been used and cited by many other authors (Ashurst 1994; Ferguson, Miclau *et al.* 1998; Nunamaker 1998; Le, Miclau *et al.* 2001; Thompson, Miclau *et al.* 2002; Lu, Miclau *et al.* 2005). All described Bourque's model which is a tibial diaphyseal fracture created by three point bending using a custom made apparatus (Figure 2.1A and B) and Professor Brian Hall of this group kindly supplied me the apparatus which Bourque originally used.



Figure 2.1 A (Top). A custom designed apparatus which can be operated by one person. The dimensions are 30cm long and 12 cm wide at the base and 7 cm high.
Figure 2.1 B (Bottom). The tibia is placed across the fulcrum at the point marked by the arrow. The 2 braces on the upper part of the apparatus swing down with the lever handle and break the tibia over the fulcrum.

Bourque *et al.* described results from 100 mice tibiae in which fractures were created. This was controlled with 'a sham' in which the same number of mice underwent the same procedure except that no fracture was produced. The experimental mouse was anaesthetised, had its right hind leg shaved and was then placed on an adjustable pillow support adjacent to the fracture apparatus. The tibia was palpated and, to ensure no fracture of the fibula was produced, it was placed in the gap behind the fulcrum of the apparatus. The anterior aspect of the leg therefore faced away from the operator of the apparatus (see Figure 2.2). Minimal force was then applied to the lever until the fracture was both felt and heard. This could be easily completed by one person. This created a closed tibial fracture, leaving the fibula intact. Internal limb stabilisation therefore resulted from the intact fibula and collateral soft tissue support. The mice were then allowed to recover from anaesthetic and within the first hour the post-fracture mice were observed moving around the cages. Comparison of the mice that had undergone fracture with unfractured controls showed no observable differences in recovery time from anaesthesia. They were observed bearing weight on the fractured limb within 24 hours post-fracture. Dietary and sleeping habits appeared identical to those of the control mice. Radiographic studies showed that the fracture configuration produced was sharp and complete and provided excellent alignment during the early stages of fracture repair. This minimised discomfort and allowed the animal to use the limb early in fracture repair. This model required no surgical wound or manual manipulation at the fracture site and its closed nature negates the risk of sepsis, an advantage when studying the immune system in fracture repair. Bourque described no occurrences of infections, malnutrition or unexpected local injuries. The degree of bony displacement and soft

tissue damage were minimal, predictable, and controllable when compared to other previously described models of fracture repair (Jackson, Reed *et al.* 1970; Hyldebrandt, Damholt *et al.* 1974).

After full review of all models available for which the facility was available we found that Bourque's closed tibial fracture model was appropriate because:

- 1) it provides the smallest reasonable long bone site at which reproducible closed fractures may be produced in mice,
- 2) it is the site most likely to provide tissue samples of sufficient size and quality for subsequent analysis,
- 3) it is a site of anatomical and clinical relevance with regard to the bone repair mechanisms under investigation,
- 4) it is a site which, in this particular species, is likely to provide the sufficient collateral support during repair without the need for internal fixation,
- 5) it addressed the need for a closed fracture technique to prevent fracture repair sites becoming compromised due to introduction of low grade contaminants or infection, likely to elicit an immune response.

Direct discussion with Professor Brian Hall was carried out to ascertain the fine detail of the model and to ensure that no undue pain, suffering or distress had occurred to the animals with this model. This was confirmed. As noted previously, to ensure reproducibility as closely as possible to this study, they kindly supplied the same apparatus to produce the fracture which they had used.

To validate this model for use in our unit initial studies were carried out on 30 cadaveric 3-6 month old balb/c mice, kindly donated by the University of Edinburgh Small Animal Research Facility. Cadaver mice underwent the fracture procedure as described above in Bourque's model. See Figure 2.2.



Figure 2.2. Validation study on mouse cadaver. A support is in place to hold the mouse at the correct level for placement of a hind leg in the apparatus. A gaseous anaesthetic delivery apparatus had also been set up, which is the set-up used in live mice undergoing the procedure anaesthetised at all times. The tibia was palpated and placed on the fulcrum, ensuring the fibula was in the gap. The lever was then depressed with the minimum of force until the fracture is felt and heard.

The cadavers of ten mice had the soft tissues dissected off the fractured tibia to reveal a short oblique fracture of the tibia and an intact fibula, with minimal crushing to the surrounding soft tissues. See Figure 2.3.

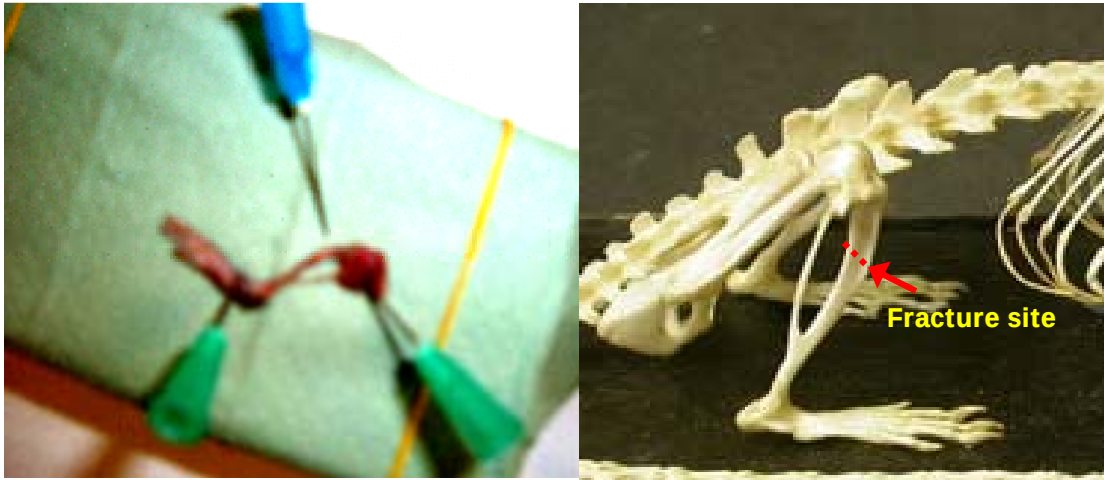


Figure 2.3 Dissected cadaveric tibia, which had undergone fracture using the method described by Bourque. The blue arrow point to the position of the fracture on the tibia The pictorial representation shows the site of the fracture more clearly

A further ten cadaveric mice underwent the fracture procedure. These were assessed clinically by moving the limb in a similar way to how normal movement would occur in the live animal while palpating the fracture site. This revealed some movement at the fracture site which was greater than the diameter of the tibia. Also, radiographic studies were carried out after the fracture and again after placing the limb at the extremes of joint movement – these revealed an increase in displacement at the fracture site following movement of the limb. Following discussion with the Named Veterinary Surgeon, the Named Animal Welfare Care Officer (NAWCO) and the Home Office inspector it was decided that further external stabilisation in the form of a plaster of Paris cast should be used to prevent excess movement at the fracture site by decreasing

joint movement to prevent undue distress to the live animals. This has been described in the literature (Williams 1988) and there was experience of this within the laboratory. The fracture was carried out in a further ten cadaveric animals in which a plaster of Paris cast was applied to the lower limb. Clinical assessment did not reveal any evidence of movement at the fracture site. Similarly radiographic assessment now showed no gross displacement at the fracture site. See Figure 2.4. These findings were confirmed by an independent observer. This modification to Bourque's model was therefore adopted and used as the fracture model for this study.

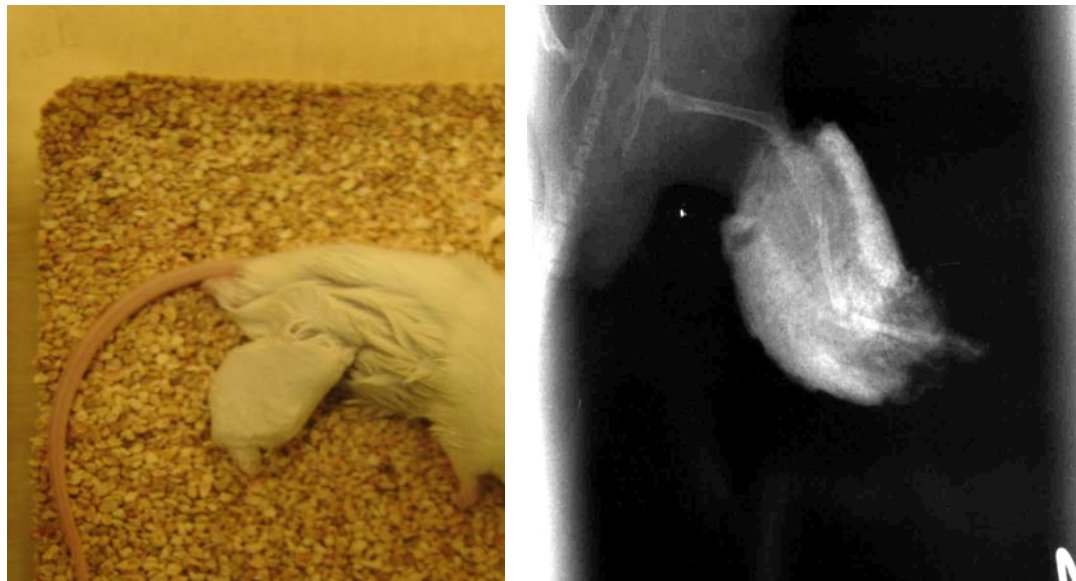


Figure 2.4 Picture showing application of plaster of Paris cast to lower limb of mouse (left). Radiograph showing fracture to tibia stabilised externally with plaster of Paris cast (right).

2.2.4.2 *In vivo* fracture creation and stabilisation

Induction and maintenance of general anaesthesia

Prior to anaesthesia the animal was weighed and the mass recorded. Anaesthesia was induced by gas inhalation using Isoflurane at a concentration of 5% (in 10 litres of oxygen) to induce anaesthesia in a single mouse, and was used for maintenance of anaesthesia at a dose of 1-2%. A satisfactory level of anaesthesia was confirmed by loss of corneal reflex and withdrawal response and by monitoring of the pattern and depth of respiration.

Fracture Technique

When full anaesthesia was obtained the mouse was transferred to the fracture apparatus as shown in Figure 2.2. The apparatus was placed on a heated mat to maintain the animal's body temperature. Under anaesthesia, analgesia was administered in the form of subcutaneous Buprenorphine at a dose related to the animal's mass (0.05mg/kg). The mouse was also administered 1-2 mls of subcutaneous warm saline, to aid maintenance of body temperature and replace fluid lost during fracture. The right hind leg was shaved. The mouse was placed on an adjustable foam support adjacent to the fracture apparatus.

The tibia was palpated and to ensure no fracture of the fibula occurred it was placed in the gap behind the fulcrum of the apparatus. The anterior aspect of the leg was therefore placed so that it faced away from the operator of the apparatus. Minimal force was then applied to the lever until the fracture was both felt and heard. See Figure 2.1. The mouse was then placed on the heated mat and whilst still maintaining anaesthesia a cast

was applied to the fractured limb. Padding with soft wool was initially applied ensuring plenty of padding over the front of the ankle. The cast was then applied in strips until a full cast of the limb from toes to knee was constructed. A confirmatory radiograph was taken – see Section 2.6.1 and Figure 2.4. The mouse was then placed back in a heated recovery cage in the lateral recumbency position and allowed to recover from anaesthesia and was monitored constantly until fully conscious (see below). To ensure satisfactory recovery of the animal, longer oral fluid sipper tubes were used, tissues lined the floor of the cage to protect the cast and food was mulched with water and placed on the floor of the cage. A further weight was taken to ascertain the mass of the applied cast, as soon as the cast appeared dry. All animals were noted to move comfortably around the cage at 1 hour post-procedure but were subject to the strict monitoring procedure detailed below.

Monitoring and Post-Procedure Care

Monitoring of the animal was carried out at regular intervals: 15 minutes post – procedure and then hourly for 6 hours and then daily or as required. This included

- (1) pain assessment – posture, behaviour, fur (piloerection), mobility, eating, drinking. Administration of analgesics – injectable Buprenorphine subcutaneously – was carried out as necessary depending (minimum three post-operative doses) on this assessment,
- (2) bodyweight (daily) (with subtraction of the weight of the cast),
- (3) assessment of body temperature - warmth supplied as required,
- (4) weight bearing status of the limb.

Monitoring was recorded on a mouse monitoring chart – See Appendix 1 – with a scoring system for the variables listed above. Any concerns of animal welfare resulted in seeking of advice from the NAWCO. In consultation with the NAWCO Schedule 1 euthanasia was carried out if necessary.

2.3 Specimen preparation

2.3.1 Specimen harvest

The animals were killed using carbon dioxide inhalation and death was ensured by dislocation of the cervical vertebrae. Radiographs of limbs requiring radiographic assessment were taken at this time. They were taken in the same way as radiographs taken with the animals alive but under anaesthesia (see 2.7.1). The lower limb was then removed from the animal by transection through the lower femur and the tibia was freed from surrounding skin and most of the surrounding soft tissue except the tissue immediately surrounding the fracture site. Tibiae that were to be used for mechanical testing and measurement of bone mineral density had these procedures carried out. The limbs for histology and immunohistochemistry were processed in the following way.

2.3.2 Specimen preparation for histological analysis

As it was of primary interest to investigate the morphology of the healing fractures, it was decided that samples should be fixed in formalin, decalcified and embedded in paraffin to provide sections with optimum morphology. The possible effects of this method of specimen preparation will now be considered.

2.3.2.1 Fixation

In this study, the preservation of tissue architecture and cell detail was essential for histology and immunohistochemistry. Thus 4% formaldehyde buffered with Phosphate Buffered Saline (PBS) was chosen for fixation. Following harvest the samples were immediately immersed in 4% formaldehyde in PBS at pH 7.2-7.4 and left for 48 hours. A period of 24 to 48 hours is accepted as sufficient for fixation of most tissues after Helander showed the quantity of fixed tissue plateaus after this period (Helander 1994). Formalin fixation causes protein cross-linking. While this has no effect on histological appearance of the specimen, it has implications for immunohistochemical techniques and this will be discussed further, later in this chapter.

2.3.2.2 Decalcification

Bone requires decalcification prior to mounting to permit sectioning in paraffin. Removal of calcium and resulting dissolution of hydroxyapatite can be achieved by strong acids or chelating agents. Acids such as nitric and hydrochloric have the advantage of achieving rapid decalcification but at the cost of hydrolysis of nucleic acids and denaturising enzymes (Kiernan 1999). This can significantly hinder subsequent assays and immunohistochemical techniques (Athanasou, Quinn *et al.* 1987). For this reason, specimens were decalcified in disodium ethylenediamine tetraacetic acid (EDTA) at pH 7, which acts as a chelating agent and is known to be a gentle decalcifying agent, preserving most of the protein during decalcification. It has been shown that treatment of mouse hind legs with EDTA resulted in an efficient decalcification and good preservation of morphological details, with preservation of

lymphoid cell surface antigens, particularly relevant to this study (Athanasou, Quinn *et al.* 1987).

Complete decalcification was verified by radiographic analysis of specimens using a table-top x-ray unit (Faxitron, USA). All samples were completely decalcified after 3 weeks at 37°C with weekly changes of EDTA.

2.3.2.3 Paraffin embedding

After complete decalcification and further trimming, the specimens were processed in an automated processor to allow embedding in paraffin. Samples were taken through 70% (three hours), 90% (one hour) and 100% (three changes, two hours for each step) of alcohol and three changes of paraffin wax at 60 °C (1 hour for each step) The specimens were then embedded in paraffin wax blocks and great care was taken to orientate the specimens horizontally. This was to permit longitudinal cross sections of the tibiae to be cut. The longitudinal plane has been assumed to be optimal as the tissue heterogeneity proximal and distal to the fracture site can be observed (Kon, Cho *et al.* 2001).

2.3.2.4 Sectioning

5µm sections were cut on a standard microtome (Shandon, Thermo Fisher Scientific TM, Waltham, MA) and mounted on “Superfrost plus” slides (BDH biosciences TM, Poole UK) after floating on deionised water at 40°C to remove ridges. Slides were stood vertically to dry and then baked at 37°C for 12 hours to optimize section adherence.

Slides were then stored in a dry, dark environment at room temperature until use in histological or immunohistochemical procedures.

2.4 Histological analysis with haematoxylin and eosin staining

2.4.1 Morphology:

In order to study the general morphology of the healing fractures three slides from each specimen (four specimens (*i.e.* four animals at each time point) at days one, three, seven, 14 and 21 post-fracture) were stained with haematoxylin and eosin (H&E). See Appendix 2 for staining protocol. Sections were taken 50µm apart to control for variability throughout the specimen. The sections were then viewed under light microscopy at magnification from 40x to 400x, and a general assessment of morphology made. H&E staining is the most commonly used histological staining technique. It has the ability to demonstrate clearly a number of different tissue structures from many different sites and is relatively simple to use. The haematoxylin component stains the cell nuclei blue-black and shows good intra-nuclear detail. The eosin stains the cell cytoplasm and most connective tissue fibres in varying shades and intensities of purple, pink and red. In general this combination of staining gives excellent detail of fracture healing morphology. Tissue types such as cartilage and bone formation are easily identified and chondrocytes, in particular can be easily seen. This is demonstrated by the example pictures below, Figure 2.5 and in the results in Section 4.2.

2.4.2 Tissue quantification:

Formal quantification of tissue types present at the fracture site was performed by photographing images of the fracture callus at a magnification of 100x using the microscope camera (Nikon DXM 1200) and image grabber software. A 10 x 10 grid was placed over the image on the computer screen (Adobe Photoshop Elements 3.0 TM). This applied 100 cross points to a field of view each corresponding to an area of 0.03mm². See Figure 2.5 below. The fracture site was defined as beginning where the periosteum became elevated to where it returned to cortical contact beyond the fracture. The fracture site was examined systematically and the number of cross points that overlaid each different tissue type was counted. Tissue types were recorded as:

- Newly formed bone
- Cartilage
- Undifferentiated tissue

and the relative proportions of each were calculated. Results were expressed as the number of points for each tissue type at the fracture site over the total number of points mapped (100) and the results were expressed as a percentage. The results from the three sections were averaged to provide a mean score for each specimen (Brownlow and Simpson 2000).

2.4.3 Limitations:

The tissue sections did not show the entire callus but reflected the largest longitudinal sections taken across it. As callus is not a uniform tissue, these differences were reduced by taking three sections per specimen at 50 μm apart and four specimens (animals) per time point as shown in the graphs in results Section 4.3 (Bourque, Gross *et al.* 1992; Brownlow and Simpson 2000; Saijo, Kitazawa *et al.* 2003).

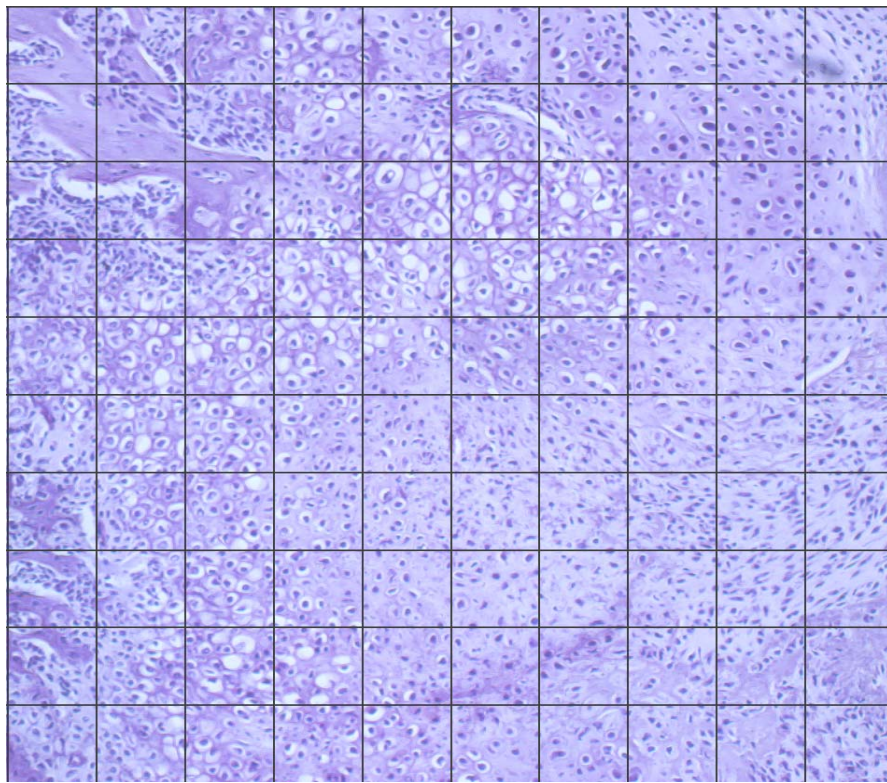


Figure 2.5 Tissue quantification method. 100 points on the grid, each corresponding to 0.03 mm^2 . For description of the tissue types seen, see results Section 3.3, histology quantification, H&E staining. Magnification x100.

2.5 Histomorphometric analysis

To quantify the formation of cartilage and new bone more formally, a histomorphometry technique was employed. Three sections from each specimen (four specimens (*i.e.* four animals at each time point) at days seven, 14 and 21 post-fracture) were stained with safranin-O (this stains cartilage a bright orange by demonstrating proteoglycans) and Goldner's modified mason's trichrome (which stains mineralised bone a bright green by demonstrating collagen). See Appendix 3 for staining protocol. Three sections for each stain were taken 50 μm apart to control for variability throughout the specimen. The sections were viewed under light microscopy at magnification 200x. From each section, two images were taken randomly throughout the callus area. With four animals at each time point, this gave a total of 24 images per time point per strain. This therefore gave a uniform distribution of the tissue present and was equal to the minimal nominal value (six images per callus as suggested by Gerstenfeld – there were six per callus in this study) required to reach statistically valid measurements representative of the whole callus tissue (Gerstenfeld, Wronski *et al.* 2005). The images were digitised using the microscope camera (Nikon DXM 1200) and image grabber software and transferred into the histomorphometry software package (Bioquant-USA). This was calibrated for 200x magnification to allow real measurements of area in μm^2 . The number of pixels comprising each tissue component was used to estimate the area and this was determined by selecting the pixels using the lasso tool. Cartilage size was determined by selecting pixels stained by safranin-O. Size of newly formed mineralised bone was determined by selecting pixels stained green after trichrome staining and then deselecting those in cortical bone (*i.e.* the fractured

ends of bone occasionally present in the callus images). The standard total field area of each image was known and was always the same and therefore the proportion of the callus comprising cartilage and bone was determined and expressed as a percentage of the total area for each time point of seven, 14 and 21 days post fracture (Thompson, Miclau *et al.* 2002; Gerstenfeld, Wronski *et al.* 2005; Lu, Miclau *et al.* 2005).

2.6 Immunohistochemistry

2.6.1 Introduction

Immunohistochemistry (IHC) or immunocytochemistry is the combination of immunology and microscopy. Biological macromolecules such as proteins, carbohydrates, lipids, nucleic acids and synthetic polypeptides are capable of evoking antibody production, which can be correlated with cell structure using immunohistochemistry. This technique relies on the very specific binding of an antibody to the antigen of a tissue. This reaction is then localised by attaching a microscopically dense marker. If the antibody is specific, immunohistochemistry can be performed on any biological specimen provided the antigen remains intact and able to bind with antibody. The specimen preparation technique exposes the antigen for immunolabelling by the antibody (Beesley 1993).

2.6.2 Direct and indirect immunolabelling:

The two most common methods of immunohistochemistry are the direct and indirect techniques. In the direct method a labelled primary antibody reacts directly with the antigen in the tissue. This method only uses one antibody and is quickly completed.

However, the technique provides little signal amplification and is therefore rarely used on paraffin sections. See Figure 2.6.

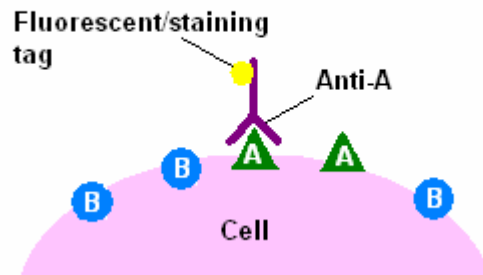


Figure 2.6 Diagram representing direct immunolabelling

In the indirect method an unlabelled primary antibody is amplified by a labelled secondary antibody which is directed against the immunoglobulin of the animals species in which the primary antibody has been raised. This method is more sensitive than the direct method as several antibodies can react with different antigenic sites on the primary antibody (Beesley 1993). The indirect technique was the method used within this thesis and is the method of choice. See Figure 2.7.

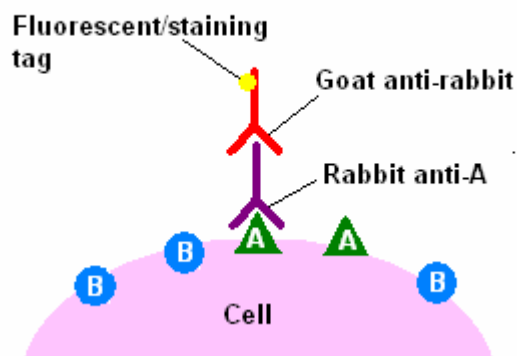


Figure 2.7 Diagram representing indirect immunolabelling

2.6.3 The antibody

The choice of antibody is critical to immunohistochemistry. A good antibody must be highly specific, possess a high titre (amount of antibody activity) and high affinity.

Many monoclonal and polyclonal antibodies are available commercially. These are usually fully characterised for use in an experimental situation.

2.6.3.1 Polyclonal antibody

Polyclonal antibodies contain a mixture of high affinity antibodies, each active against different epitopes on the antigen. Polyclonal antibodies are produced by injecting an antigen into a host animal. The serum is then periodically collected and tested for antibody. A well known problem with polyclonal antibodies in immunohistochemistry is that they bind to non-specific sites, which can produce some misleading results. This non-specific binding can be reduced by the use of antigen affinity purification, where the polyclonal sera is run down a column to which antigen has been bound. The polyclonal antibodies that have bound to the antigen can then be retrieved. Polyclonal antibodies are regularly used as a secondary or tertiary reagent when indirect immunohistochemistry is used, as they allow considerable signal amplification whilst maintaining specificity through the monoclonal primary antibody.

2.6.3.2 Monoclonal antibody

Monoclonal antibodies are specific to one epitope and therefore reduce the risk of non-specific binding. The activity of a monoclonal antibody is susceptible to fixation of the antigen because the specificity of monoclonal antibodies to one epitope means that if

this epitope is destroyed, all reactivity is lost. A weak fixative such as paraformaldehyde or formalin, as was used in the experiments in this thesis, is usually necessary. Monoclonal antibodies are largely produced by immunising mice. The plasma cells are removed from the host animal and fused with a malignant myeloma to form hybridoma cells. These hybridoma cells are isolated and cultured *in vitro* or *in vivo* in the peritoneal cavity of a suitable host to produce unlimited monoclonal antibodies raised against a specific epitope (Beesley 1993).

2.6.4 Immunolabelling techniques

There are several methods available in immunohistochemistry to demonstrate antigens in tissue sections. The aim is to achieve reproducible and consistent demonstration of specific antigens with a minimal amount of background staining. The secondary antibody is usually labelled with a marker such as a fluorescent dye, an enzyme or colloidal gold. Frequently the secondary antibody is labelled with an enzyme such as a biotinylated secondary antibody. This utilises the indirect avidin-biotin complex (ABC) technique. This relies upon detection of a biotinylated secondary antibody by an avidin-biotin-horse radish peroxidase complex. This, in turn, is identified by utilisation of the reaction between the colourless chromogen diaminobenzidine (DAB) and peroxidase which results in production of a visible, insoluble brown product at the site of the reaction. The improved sensitivity of this technique compared with other IHC methods was shown by Hsu *et al.* (Hsu, Raine *et al.* 1981). This technique was most commonly used in the IHC experiments in this thesis. Due to the use of this enzyme, endogenous peroxidase activity has to be blocked with 3% hydrogen peroxide. Similarly the use of a

fluorescent marker required a fluorescence microscope for visualisation of the stain (Boenisch 2001).

2.6.5 Reducing non-specific background labelling

Background staining and non-specific labelling can occur due to the attachment of non-specific binding of the antibodies or chromagen to the section. The use of a monoclonal antibody is preferred as polyclonal antibodies are likely to produce an increased background. Incorrect fixation or enzyme pre-treatment can cause alteration of the structure of proteins, which may result in non-specific labelling by the antibody. This can be reduced by using a gentle fixative such as 4% formalin used in this study, and avoiding or minimising antigen retrieval techniques.

Much non-specific background labelling can be reduced by the addition of reagents to buffers or their application to the section prior to application of the primary antibody. The following reagents, which are available to reduce non-specific background labelling, were used in this study:

1. Blocking agents, such as serum, contain a high concentration of proteins which saturate the binding sites that cause the non-specific binding. The host from which the serum is derived is usually the same as the host of the secondary antibody. For example, if the secondary antibody is goat anti-mouse, the most appropriate blocking serum would be goat serum. Blocking serum needs to be carefully matched to the primary and secondary antibody to prevent cross-reactivities. A serum-free protein block is now available. This is preferable to serum as it can be used with any primary and secondary antibodies and eliminates cross-reactivities. Blocking serum is applied to the tissue

before the primary antibody. Dako™ (Dako UK Ltd, Ely) protein block was used in this study.

2. Some tissues may bind avidin, biotinylated horseradish peroxidase or other biotin/avidin detection system components without prior addition of a biotinylated antibody. This binding may be due to endogenous biotin or biotin-binding proteins, lectins, or nonspecific binding substances present in the section. To prevent a high non-specific background when a biotinylated secondary antibody is used, pre-treatment of the tissue with avidin is required. This is followed by pre-treatment with biotin (to block the remaining biotin binding sites on the avidin) and again this was used in this study when a ABC-DAB chromagen detection system was employed (Boenisch 2001).

3. Reagents can be added to the rinse buffers as an additional blocking procedure. Tween 20 is a surfactant reagent that reduces electrostatic attachment of molecules to a section (Boenisch 2001). Tween-20 (Sigma-Aldrich™, UK) was added to the rinse buffers in this study.

2.6.6 Antigen retrieval

During fixation with formalin, cross-linking bonds within proteins can occur which may mask the antigenic sites causing false negative staining. There are several methods available that can retrieve antigens, such as proteolytic enzymes, microwave treatment or boiling. It is important that antigen retrieval is only carried out if necessary, as otherwise it may cause false antigenic-sites (Beesley 1993). During this study Proteinase K (Dako™) pre-treatment was used when necessary; each primary antibody required several trials to obtain optimum staining with positive and negative controls.

The duration that Proteinase K was applied was determined from this, ensuring no unspecific staining in the positive control. Other methods of antigen retrieval such as boiling or microwaving have limited use in bone as the tissue does not tend to remain on the slide.

2.6.7 Controls for immunostaining:

In immunohistochemistry it is possible to produce either false positive or false negative results. It is therefore essential that adequate controls are chosen for each stain to minimise this. Positive controls test the reactivity of the antigen, antibody and substrate marker used. The efficacy of the antibody should be confirmed by immunolabelling with a known antigen positive tissue. In this study, cells of the immune system were under investigation and therefore spleen from the same species was used as a positive control to confirm the efficacy of each primary antibody.

Negative controls assess the non-specificity of the immunolabelling technique. There are several methods by which this can be carried out. A tissue can be used where it is known that the target antigen is absent. More common methods are to omit the primary antibody or use an unrelated antibody. During this study the primary antibody was omitted to act as negative controls.

2.6.8 Immunohistochemistry protocol

Appendix 4 details the general protocol used. The modifications to this protocol specific to each antibody used as well as the antibody concentrations, incubation times and times for protein blockade are detailed in Appendix 4 for each specific antibody used.

2.6.9 Quantification of cells identified by immunohistochemistry

For quantifying the immunostaining, the sections were analyzed under a Nikon research microscope (Nikon DXM 1200). A validated method of quantifying immunostaining results in fracture repair was adopted (Fox, Leek *et al.* 1995; Li, White *et al.* 2002). Under 100x magnification, the area containing the maximum number of positively labelled cells was chosen in five defined regions near the fracture site, namely, the fracture gap and two sub-regions distal and proximal to the fracture gap on both sides of the fractured cortices. Three random images from each selected area were then taken under high magnification (x400). All the images of each specimen were captured via a digital camera and saved as JPEG files. The images were opened in an image software package (Image J TM - freely available from rsbweb.nih.gov/ij) and the number of positively labelled cells in each image was counted. The mean numbers of positively labelled cells per high-power field (for each antibody used at each time point) was then calculated and data was expressed as mean +/- standard error.

2.7 Radiographic analysis

2.7.1 Radiological technique

At zero (post-fracture, with the same anaesthetic), seven and 14 days after fracture all animals were anaesthetised (see Section 2.2.4.2 for anaesthetic protocol) and placed on a table top X-ray unit (Faxitron X-ray Corporation, IL, USA) and radiographed using an exposure of 60kV output for 0.6sec and a focal distance of 72cm. At day 21 radiographs were taken after Schedule 1 euthanasia of the animals. Radiographs were taken in both antero-posterior (AP) and lateral planes and the planes were controlled in each animal using a specifically designed jig (which also controlled for magnification). The animal was positioned centrally using laser crosshairs for guidance. The radiographs produced from this were scanned using a high resolution scanner (UMAX TM powerlook 2100XL, 500dpi) specially designed to scan radiographs. Because each radiograph had been taken with a steel number (all of the same density and completely radio-opaque) to label the images they were standardised by setting the grey-level over each steel number to white, within the image analysis software (Chhabra, Zijerdi *et al.* 2005). Digital images were then saved in TIFF format and analysed using Image JTM image analysis software.

2.7.2 Callus area

The callus area was measured using pixilation: the marquee tool was used to delineate the callus circumferentially in the 2-dimensional plane. The number of pixels contained by the selected area was then recorded (the area on the lateral view was recorded as the fibula image overlay the tibia on the AP view which would have confounded the

results) and gave a result in squared inches (as the scanner scanned in dpi) (Chhabra, Zijerdi *et al.* 2005). This is the method described by Brownlow and Simpson (2000).

2.7.3 Callus index

Callus index is the maximum transverse width of the callus divided by the width of the bone at the fracture site. It was determined using the image software (both medial-lateral (AP radiograph) and antero-posterior (lateral radiograph)), measurements again taken using pixilation (Sarisozen, Durak *et al.* 2002). This method corrects for any initial differences in bone size between animals. The AP and lateral ratios were measured separately.

2.7.4 Relative bone mineral content

The changes in pixel density across the fracture gap were compared using Image J™ software. Changes in pixel density correspond with changes in mineralised bone tissue. In the early period of repair the fracture gap is filled with relatively less dense inflammatory tissue and thus the pixel density is low. As repair progresses, hard callus is formed resulting in a rise of pixel density. The mean pixel density of two areas in the fracture gap is compared to two adjacent areas of uninjured bone. Initially there is less bone at the fracture gap than in the adjacent areas. However with healing, callus is laid down and the pixel density at the fracture becomes relatively greater than in the uninjured area adjacent to it. The mean density value within the fracture gap is subtracted from the mean density value in the normal bone and these values represent ‘relative bone mineral content’. The intra and inter-observer variability of pixel density analysis has been shown to be highly reproducible (Murnaghan, McIlmurray *et al.* 2005).

2.8 Calculation of tibial bone mineral density

To ascertain the bone mineral density of whole tibial bone specimens from mice, the following measurements were made (Aro, Wippermann *et al.* 1989). Any residual soft tissues were stripped from the bone before assessment.

2.8.1 Volume

Volume was calculated using Archimedes' principle.

The tibiae were stripped of all remaining soft tissue and the fibulae removed. The tibiae were then weighed (mass A).

A bijou was filled with water and sealed underwater to ensure the absence of air-bubbles. The filled bijou was then dried externally and weighed (mass B).

The tibia was then placed in the filled bijou which was once again sealed underwater to ensure the absence of air. This was then weighed again after externally drying (mass C).

From the principle that 1 ml of water weighs 1g, the volume of the tibia was calculated from the following formula:

Mass of water in bijou displaced by bone = $B - (C - A)$

Mass of displaced water (g) = volume of bone (ml)

2.8.2 Ash weight

Wet mass (Mw): Weight A (grams)

Dry mass (Md): Each tibia was placed in an individual, pre-weighed and pre-dried crucible (weight D) and dehydrated at 105°C for 48 hours and the crucible weighed (weight E) (grams).

Dry Weight = E - D

Ash mass (Ma): The same crucible as above was then placed in a muffle furnace at a temperature of 600°C for 24 hours. The crucible plus the ash was then weighed (weight F) (grams).

Ash weight of each bone = F – D

2.8.3 Bone mineral density

Bone mineral density (BMD) is calculated:

$$\text{BMD (g/ml)} = \text{Ma (g)} / \text{Volume of bone (ml)}$$

Other derivations from above:

Water content is difference between wet and dry masses: $M_w - M_d$

Organic content is given by difference between dry mass and ash mass: $M_d - M_a$

Mineral content is given by final mass after ashing: M_a

All of these are expressed as fractions of the wet mass M_w .

This method of bone mineral density assessment was previously used within the research group and is modified from Li and Aspden (1997).

2.9 Mechanical testing

2.9.1 Introduction to biomechanical testing

A key outcome in fracture healing is the restoration of the bone's mechanical properties, in particular stiffness and strength. Stiffness is a measure of the load required to deform the bone and strength is a measure of the load required to break bone. Biomechanical testing of bone is typically in tension, compression or torsion. Stress by definition is the force per unit area and can be compressive, tensile or shear. Applied force that shortens a material is compressive, force that stretches is tensile and applied force that causes one area of material to move relative to another is known as shear. Strain is the percentage change in length or deformation. The relationship between the loads (force) applied to a structure and its resulting deformation can be plotted graphically to produce the load-deformation curve. This curve has two parts, the elastic and plastic deformation regions. The slope of the elastic region reflects the stiffness of the material being tested and is linear (Hooke's law): if the load is removed within this linear region, the structure will return to its original shape, like a spring. In the plastic region of the curve there will be permanent deformity after removal of the load and this region of the curve is non-linear. The point of yield is that at which the curve becomes non-linear and this signifies where permanent structural changes occur within the material being tested, as it goes from the elastic to the plastic region of the curve. The highest point on the curve refers to the ultimate tensile stress of the material and is the maximum stress it can withstand before failing. The extrinsic properties of a material can be obtained from a load-deformation curve and these relate to both the innate properties of the material and its size. The intrinsic properties of a material are independent of size or dimensions and

are constant for each material. These can be calculated from a stress strain curve, see Figure 2.8. This is inferred from the load deformation curve by taking into account the cross sectional area to calculate stress from load and the percentage deformation to give strain (see later in this section for calculation in bone). Young's modulus of elasticity is the linear slope of the elastic region of the stress-strain curve and represents the intrinsic stiffness of the material *i.e.* a measure of the stress required to deform the material.

Young's modulus is a constant for each material (Turner and Burr 1993).

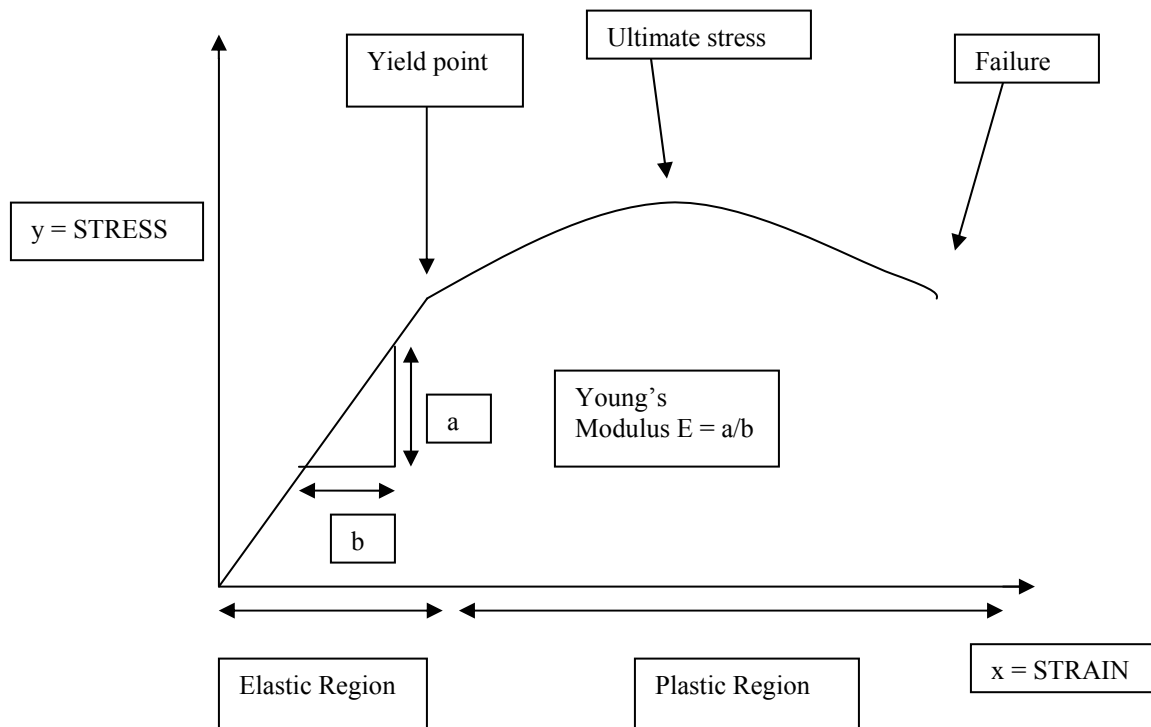


Figure 2.8 Stress – strain curve for a typical material

2.9.2 Methods of biomechanical testing

The healing fractures in this thesis were tested by four point bending as described below. Bending is a practical and well accepted technique for obtaining biomechanical data from small animal bones or healing fractures (Turner and Burr 1993). Tensile testing does provide accurate assessment of biochemical properties of both cortical and cancellous bone (Turner and Burr 1993). However, the technicalities of mounting specimens for tensile testing mean that this requires relatively large or well machined specimens and it is therefore rarely used for testing rodent bone. Previous workers have tested healing fractures in torsion (Northmore-Ball, Wood *et al.* 1980; Wang, Lewallen *et al.* 1994; Bouxsein, Turek *et al.* 2001). This provides information about the behaviour of the sample in shear. However, the specimen requires to be circular in cross section so that a constant centre of rotation exists at which shear is zero. With a non-circular specimen the polar moment of inertia changes during the test rendering the results inaccurate (Turner and Burr 1993). The shape of the tibia and the irregular shape of the healed fracture callus rendered the specimens from this work unsuitable for testing in torsion for this reason. Testing in compression can provide useful information on the mechanical properties of cancellous bone in particular. It has the additional advantage of reproducing a more physiological model with, for example, vertebra normally being under compression *in vivo*. However, uniform compression must be used and an even distribution of stress needs to be applied with precise alignment at the point of contact between the bone and loading plates. Reproducible perfect alignment of the murine tibia in the axial plane is impossible without cutting the ends of the bone off to create a flat plane. This carries the risk of damaging the fracture site itself in such a

small specimen and therefore it was thought that the mouse tibia should not be tested under compression in the axial plane.

Bending tests are applicable to small specimens and reproduce an element of the normal physiological environment under which long bone fractures heal. Bending a bone results in tension on one side and compression on the other with failure usually occurring on the tension side as bone is weaker in tension than compression (Reilly and Burstein 1975). Bending can be achieved using three or four contact points. Three point bending is simple to perform but results in significant shear at the mid-loading point, which in these experiments would be the site of healing *i.e.* the callus (Wilson and Carlsson 1991). This problem is compounded as small specimens result in a low span to depth ratio as biological specimens are of a predetermined length and depth. A span to depth ratio of greater than 16 is recommended to exclude shear but this cannot be achieved with rodent tibia or femur. Four point bending is slightly more technically challenging as it relies on equal contact by all four loading points, which is elementary on standardized samples but can be more difficult with irregular bone. However, the use of four points of loading results in total bending with minimal shear between the two mid-loading points. In this study the intrinsic properties without shear of a specific region of the bone (*i.e.* the healing fracture) were required and therefore four point bending was thought most appropriate. To ensure equal contact at all four loading points, a pivot is usually incorporated into the design of the loading contact to ensure constant contact throughout. Consequently, it was decided to design an apparatus to test specimens in four point bending to failure.

2.9.3 Conditions for testing

The mechanical properties of bone have been shown to vary according to the conditions that the bone is stored in and tested in as well as the method of testing. Particularly important are hydration, temperature, preservation and type of jig stabilisation. For these reasons the results obtained are most likely only relevant to that study and inter-study comparison of data is unlikely to be accurate (Schriefer, Robling *et al.* 2005).

Also real time controls are essential to make the data obtained interpretable and therefore mechanical testing of the contralateral limb was carried out in this study.

Some mechanical tests require the bone ends being fixed in blocks of cement to grip the specimen. As four point bending was the chosen method of testing in this study, immobilisation of the bone ends was not essential as the specimen was immobilised by the four contacts once the bone had settled into a stable position and the contacts start to apply pressure on the bone from two opposing directions.

Fixation in formalin, frozen storage from -20 to -70 and a change in the level of hydration and temperature have been shown to produce slight changes in the biomechanical properties in bone (Turner and Burr 1993). As the mechanical testing apparatus and animals were housed in the same site, no fixation or freezing was carried out. The samples were kept moist in warm saline at 37⁰C until the point of testing. All tests were carried out a controlled room temperature of 20⁰C. Also, all mechanical tests were carried out within 1 hour from animal death.

2.9.4 Preparation of samples for testing

Immediately after death both the fractured and unfractured tibiae were dissected free of the fibulae and all soft tissue with disarticulation of the tibio-femoral and tibio-talar joints. The samples immersed in warm (37°C) 0.9% normal saline (NaCl). They were taken immediately to the materials testing area for four point bending. The fracture calluses were measured in the antero-posterior and medio-lateral planes using a precision calliper measuring to 0.1mm. The mean of three readings was taken for each orientation for each sample. Samples were re-immersed in the warmed saline for a further five minutes, then removed and immediately tested to failure. On average the total time from removal from the warm saline to end of the experiment was 90 seconds.

2.9.5 Testing apparatus and specification

All tibiae were tested to failure by four point bending using a Zwick/Roell Z005 materials testing machine (Series 4500, Instron Corp. Canton, Mass, USA).

The four point loading jig shown in Figure 2.9 was designed by a musculoskeletal engineer Dr A McLean, University of Edinburgh and produced specifically for this work by the University of Edinburgh Engineering Workshop, Kings Buildings, Edinburgh.

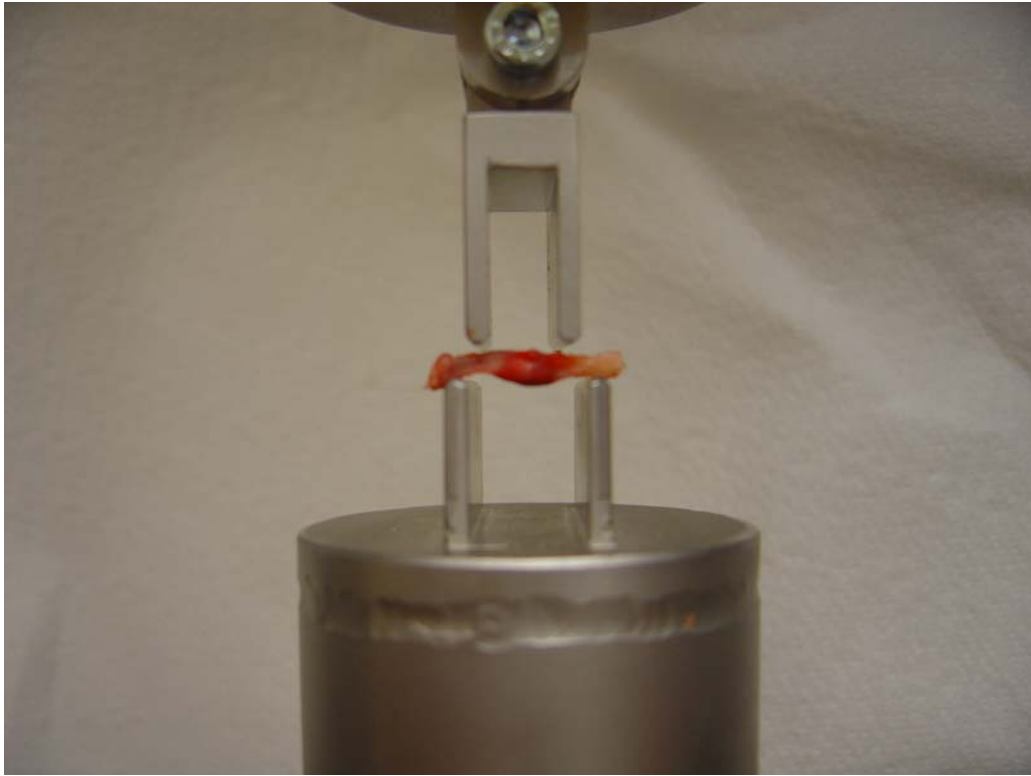


Figure 2.9 Four point bending apparatus used to test tibia until failure through fracture callus.

The upper loading assembly was designed to incorporate a pivot to ensure symmetrical loading by the upper two contacts. The upper loading span was 8mm and the lower, static span was 12mm. Contact points were rounded to minimise notching of the bone after application of load.

All samples were tested in the same orientation with the tibiae resting on their lateral surface and force being applied from the medial aspect, with the healing fracture between the two upper contacts. The tibiae were found to be stable in the testing

apparatus in this orientation after a trial of various different orientations with cadaveric mouse tibial samples.

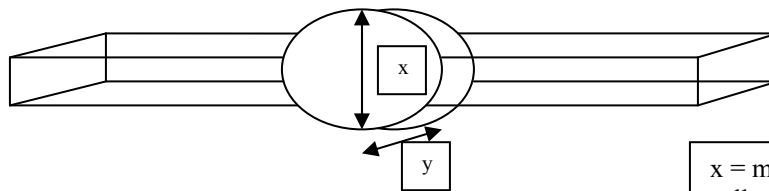
Testing specifications were as follows:

- Load cell 0.5KN
- Rate of deformation 2mm/minute
- Temperature of specimen 37⁰C
- Room temperature 22⁰C
- Room humidity 50%.

2.9.6 Expression of results

Data derived from the materials testing machine was expressed as load (N) / deformation (mm) in the form of a load / deformation graph (TestXpert TM). Load at yield and failure were taken directly from the load / deformation graph with yield defined as the point where the load / deformation curve became non-linear. Load at failure was defined as the maximum load applied.

Other workers have directly measured the cross sectional area of the fracture plane using image analysis and pixilation (Cao, Mori *et al.* 2002). This highly accurate technique was not available and the cross sectional area was therefore estimated from biplanar (antero-posterior (which is the horizontal width when the bone was sitting on the bending jig) and medio-lateral (which is the vertical height when the bone was sitting on the bending jig)) measurements – see diagram below. The fracture plane was considered to be an ellipse (Gerstenfeld, Wronski *et al.* 2005).



The area of an ellipse is:

$$\text{Width (y)} \times \pi \times \frac{\text{Height (x)}}{4}$$

x = medio-lateral measurement of callus *i.e.* vertical height of callus as bone was sitting on bending jig

y = antero-posterior measurement of callus *i.e.* horizontal width of callus as bone was sitting on bending jig

The cross sectional moment of inertia (I) is a measure of material distribution around a neutral axis. In order to calculate stress, I must be known.

Cross sectional moment of inertia for an ellipse (I):

$$I = \frac{1}{4} \times \pi \times (\text{vertical radius}) \times (\text{horizontal radius})^3$$

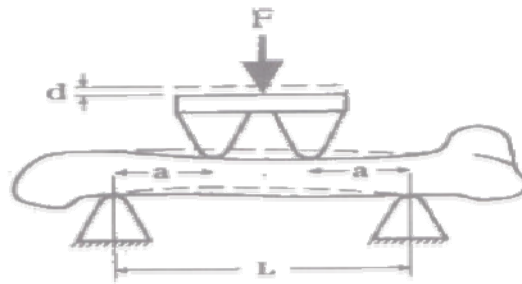
Where
vertical radius = x/2

and

horizontal radius = y/2

where vertical radius is half the vertical height as described above and horizontal radius is half the horizontal width as described above

Figure 2.10 allows interpretation of the following formulae:



(KEY: F=force, d=displacement, L=span, a=distance from outer to inner contact)

Figure 2.10 Diagram of four point bending model

Stress can therefore be calculated from:

$$\text{Bending Stress } (\sigma_b) = \frac{(F \times a \times c)}{2I}$$

(where c=distance from the centre of the mass or half the vertical height (or medio-lateral measurement) of the callus).

Strain describes the deformation of an object under load and is expressed as change in length / original length. For bone which undergoes relatively small degrees of strain, this is usually expressed as μstrain . Strain can be measured directly using a strain gauge but in this study was derived from load/deformation data as follows:

$$\text{Strain } (\epsilon) = \frac{(6 \times \text{vertical radius} \times \text{displacement})}{a(3L - 4a)}$$

Young's Modulus (E) was calculated from the data as follows:

$$E = (\text{Force } F / \text{Displacement}) \times (a^2 / 12I) \times (3L - 4a).$$

The above were all calculated at the point of yield due to the increased inaccuracy of the deformation data derived beyond this point (Burstein, Currey *et al.* 1972).

Units

Load (or force)	(F)	Newton (N)	
Moment of inertia	(I)	(mm ⁴)	
Stress	(σ)	megapascals (MPa)	(1 Pascal = 1 Newton/m ²)
Strain	(ε)	μstrain	
Young's Modulus	(E)	megapascals (MPa)	

All measurements of distance expressed in mm.

2.10 Body weight

All animals were weighed daily to an accuracy of 0.01 grams.

2.11 Statistical treatment of experimental data

2.11.1 Introduction

Raw data were first organised into a spreadsheet using the computer programme ‘Microsoft Excel TM’. The first column contained a list of identification numbers of the individual experimental animals. The second column listed the independent variable ‘Experiment’ in which the subgroups *i.e.* specific experimental animal (*e.g.* SCID fractured limb; wild type control limb) were designated. In the subsequent columns to the right of this, each dependent variable (*e.g.* bone mineral density, Young’s modulus) was recorded. When the Excel spreadsheet was completed with all of the morphological and physiological results it was imported into a statistics computer programme (‘Statistica version 6.0’ — Statsoft Inc, 2300 East 14th Street, Tulsa, O.K. 71404, U.S.A.). This programme is an extensive package for statistical analysis and graph plotting.

2.11.2 Rejection of outliers

It was necessary to identify and reject outliers within the raw data. This was accomplished by plotting half-normal probability plots in which the selected variable was plotted in a scatterplot against the values calculated as those ‘expected from the normal distribution with the same mean and variance’ for each column of data. The half-normal probability plot was constructed in the same way as the standard normal probability plot (see below), except that only the positive half of the normal curve was considered. Consequently, only positive normal values were plotted on the Y-axis. On each plot it was possible to draw an ellipse representing the 95% confidence intervals

for the distribution. Points lying outside this ellipse were considered to be outliers and rejected from the study. The resulting spreadsheet was termed ‘weeded data’. See Figure 2.11

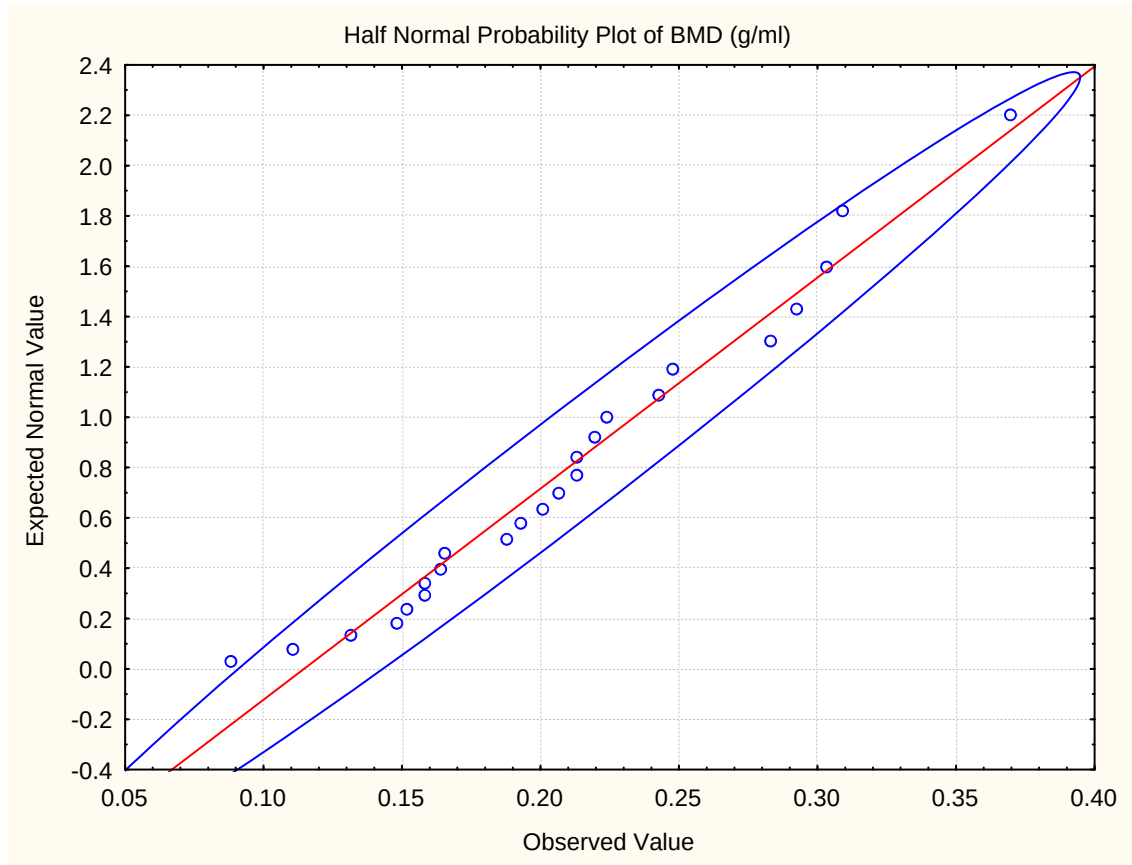


Figure 2.11. An example of a half normal probability plot for bone mineral density in the SCID compared to the wild type mice (see chapter 4). One point lies outside the 95% ellipse and is therefore regarded as an outlier.

2.11.3 Determination of normality

After rejecting the outliers, the columns of data were then re-plotted as normal probability plots to determine whether or not the data fitted a normal distribution.

The standard normal probability plot was constructed as follows. First, the values were rank ordered. From these ranks, Z values (*i.e.* standardised values of the normal distribution with the same mean and variance as the data) were computed based on the assumption that the data came from a normal distribution. These Z values were plotted on the y-axis in the plot and if the observed values (plotted on the x-axis) were normally distributed all values should have fallen onto a straight line in the plot. If the values were not normally distributed, they would have deviated from the line. The fit of the computed line to the scatter of the raw data was tested by the programme using the Shapiro–Wilk W test. If the W statistic so produced was significant (*i.e.* resulting in P value <0.05), then the hypothesis that the respective distribution was normal had to be rejected. The Shapiro-Wilk W test is the preferred test of normality because of its good power properties as compared to a wide range of alternative tests (Shapiro, Chen *et al.* 1968). At this point, for ease of recognition, each of the columns in the spreadsheet of dependent variable data that were not normally distributed were converted to a red font for easy identification. See Figures 2.12 and 2.13.

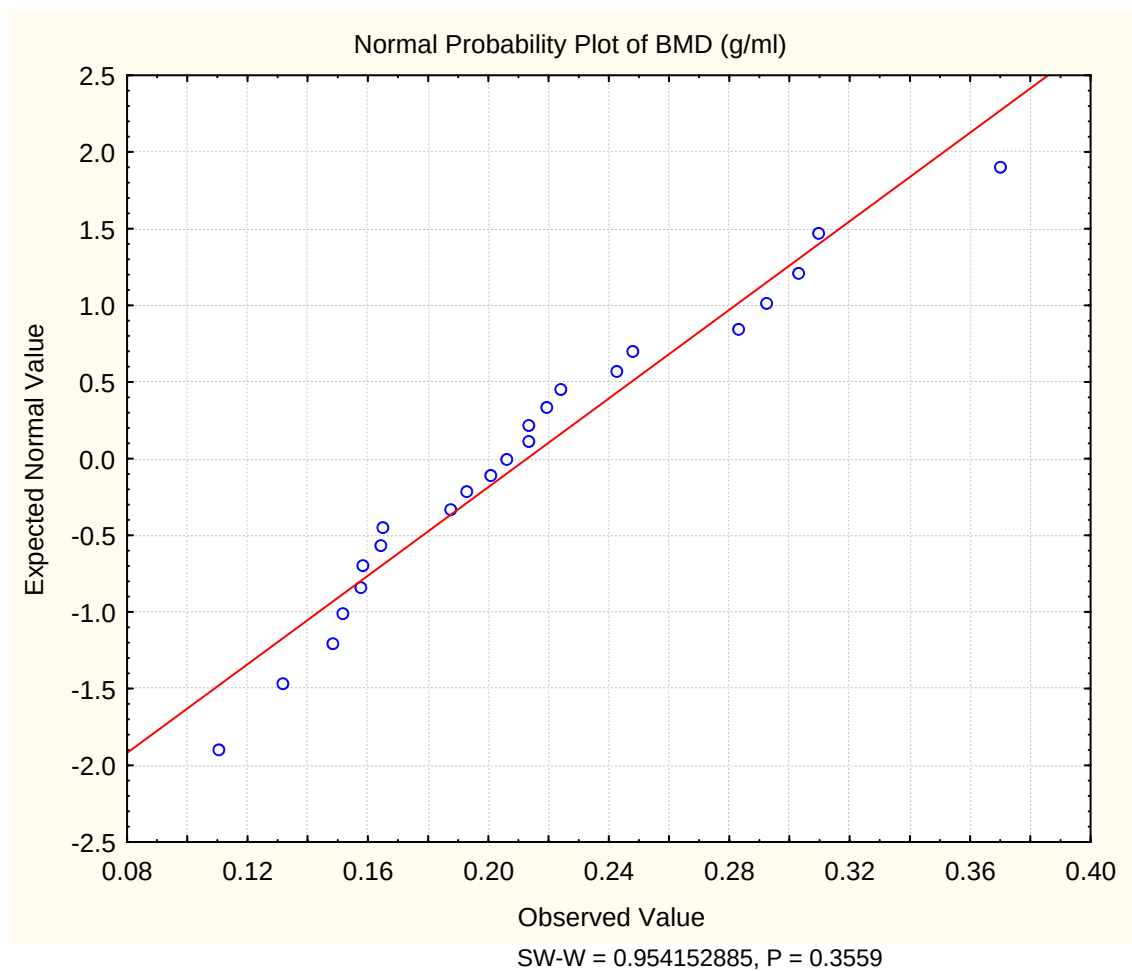


Figure 2.12. Example 1 of normal probability plot, for bone mineral density in the SCID compared to the wild type mice (see chapter 4). The W statistic is not significant; therefore the hypothesis that the distribution is normal can be accepted.

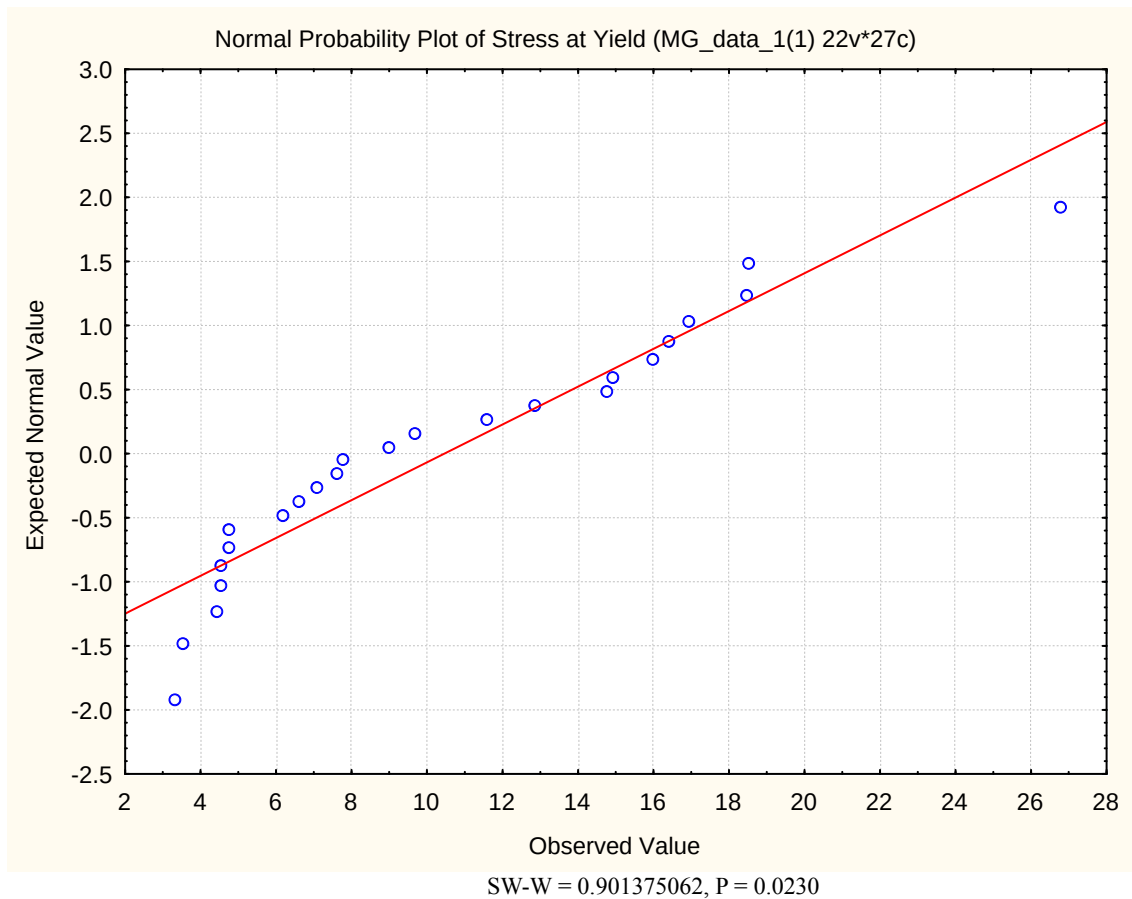


Figure 2.13. Example 2 of normal probability plot, for stress at yield in the SCID compared to the wild type mice (see chapter 4). The W statistic is significant ($P < 0.05$); therefore the hypothesis that the distribution is normal must be rejected.

2.11.4 Application of statistical tests

The next two stages in statistical testing were directed at identifying the presence of differences (variants of the F test) and identifying where these differences lay (variants of Student's t test). Different algorithms had to be adopted for normally and non-parametrically distributed data.

For normally distributed data, the *F* test was applied in the form of one way ANOVA (analysis of variance). The independent variable with its subgroups (*e.g.* SCID fractured limb) was the ‘grouping’ or ‘factor’ in the ANOVA calculation and the dependent variables were the columns of weeded data. The ANOVA test identifies differences due to ‘between-groups variation’ whilst neutralising differences due to ‘within-groups variation’ From the ANOVA test ‘p’ values for statistical differences were identified. To find where these differences lay it was necessary to perform *post hoc* tests which tested the null hypothesis by comparing the means of these independent groups of data. The Student’s *t* test compares the means of two independent samples. This test asks the question: ‘What is the probability that the two samples (represented by their means and variances) were drawn from the same population?’ The Student’s *t*-test, is not however appropriate where ANOVA discloses multiple differences (or not as may be the case) and this is likely in experiments such as those encountered here. If repeated *t* tests were performed on a single cohort of data, there would be an increased likelihood of a Type I error in the analysis. A Type I error is the acquisition of a ‘false positive’. To reduce this occurrence when, within the independent variable there are more than two subgroups (represented in Statistica by ‘codes’), the more conservative Scheffé test is used in preference to the *t*-test. It is a variant of the *t* test specifically for analysis of variance where multiple groups are being considered (*e.g.* when comparing fractured limbs to unfractured limbs in SCIDs and wild types groups – four independent variables).

On the rare occasions when data were not parametrically (normally) distributed, statistical analysis based upon the ranking of data had to be used. There is a variety of

tests equivalent to the more sensitive parametric tests used above. The Kruskal–Wallis test is a non-parametric alternative to one-way (between-groups) ANOVA. It was used to compare three or more samples, and it tests the null hypothesis that the different samples in the comparison were drawn from the same distribution or from distributions with the same median. Thus, the interpretation of the Kruskal–Wallis test is similar to that of the parametric one-way ANOVA, except that it is based on ranks rather than means.

In the case of non-parametrically distributed data for testing where the differences lay, the Mann–Whitney U test was used. The Mann–Whitney U test assumes that the variable under consideration has been measured on at least an ordinal (rank order) scale. The interpretation of the test is essentially identical to the interpretation of the result of a t-test for independent samples, except that the U test is computed from rank sums rather than means. The U test is the most powerful (*i.e.* sensitive) nonparametric alternative to the t-test for independent samples.

2.11.5 Statistical power

2.11.5.1 Initial power calculation: evaluation of sample sizes required

The Act of Parliament governing the experimental use of animals states that a minimum number of animals consistent with a statistically significant result shall be used (Home Office (HMSO) 1986). Using the results of previous similar studies (Murray AW: MD thesis, University of Edinburgh, 2007) conducted within the research group, power studies were conducted to estimate the appropriate sample size required. The ‘Statistica’ software (see below) contains a module into which previous results and their standard

deviations may be entered to produce a graph of sample size against power. Statistical power is defined as: ‘The probability of rejecting a false statistical null hypothesis’. An error of this type is termed a Type II error and is the failure to reject a false null hypothesis, effectively a false negative. The probability of detecting a Type II error is defined as:

$$P(\text{Type II}) = \beta$$

The power of a test is therefore $1-\beta$: ideally it should be 1.0 but an experimental value of 0.9 is considered excellent and therefore the desirable power to be achieved was 0.9 or better. In the previous studies used to calculate power, Young’s modulus was recorded in a tibial fracture healing model in rat in groups of five animals. Three experiments were carried out which gave mean values for Young’s modulus of: Normal control: 220MPa, Ovariectomised: 150MPa, Ovx + Simvastatin: 40 MPa. The population standard deviation was 63.3 MPa. With a probability limit of $p < 0.05$ ($\alpha = 0.05$) and a ‘power goal’ of 0.9, the following relationship was obtained:

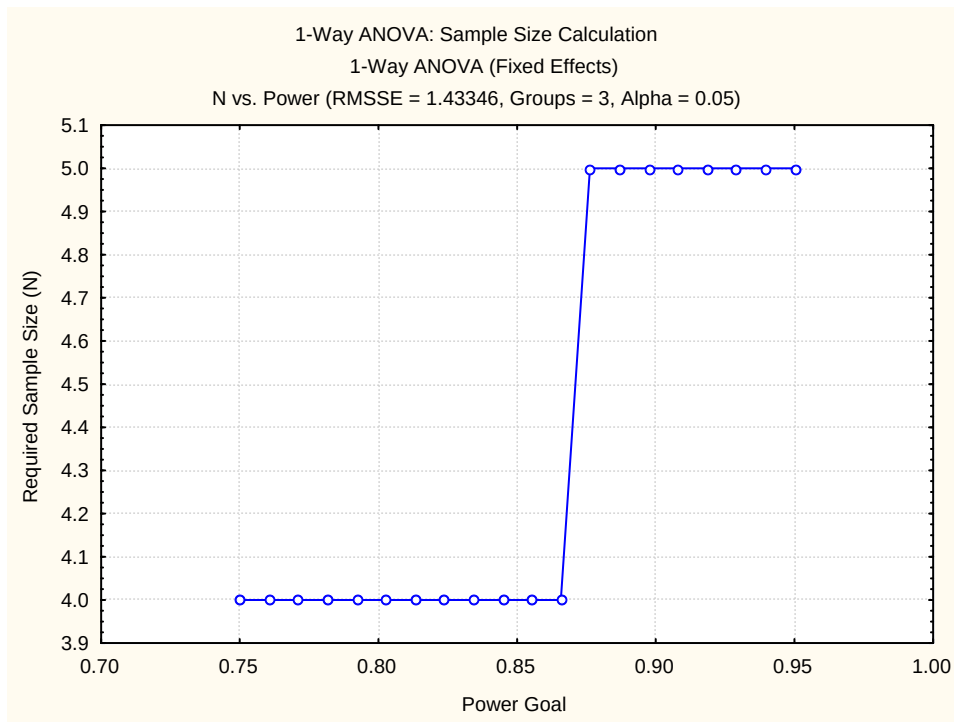


Figure 2.14 Sample size versus power based on previous data assessing fracture healing in rat

The graph indicates that the desired power (0.90) for this particular variable could be obtained with a group of five animals. To see the effect of increasing the sample size the same data were plotted with group size on the x-axis and power on the y-axis. This gave the following relationship:

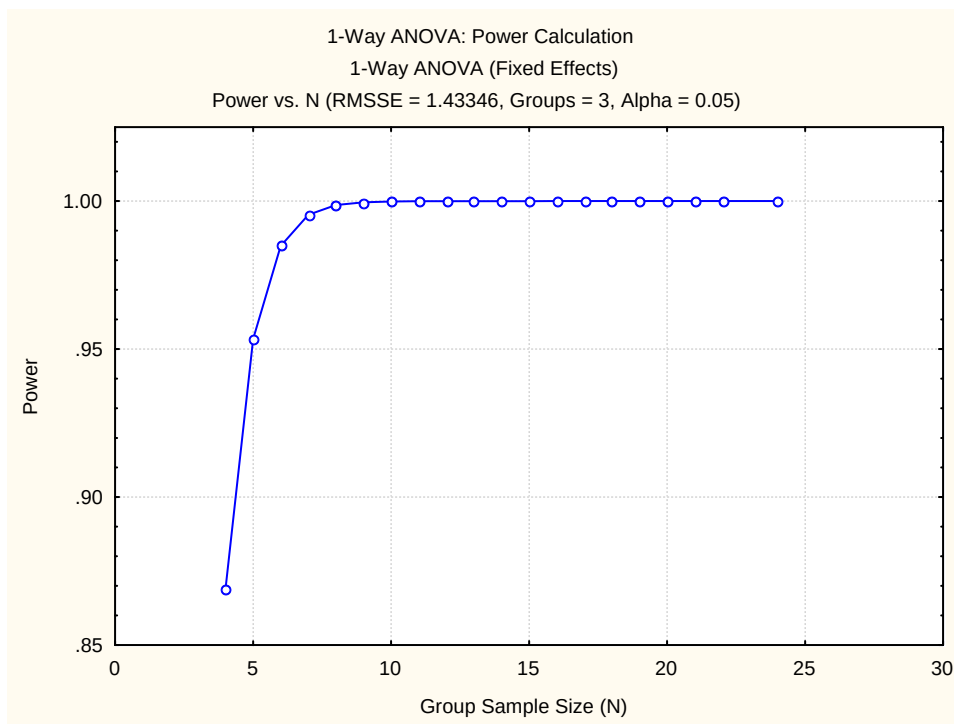


Figure 2.15 Power versus N based on previous data assessing fracture healing in rat

It can be seen that a large change in power takes place between sample sizes of five and ten. Sample size calculations based on similar data for different variables from previous work produced similar results.

Therefore the sample sizes for this study were chosen to achieve power of greater than 0.9. Sample sizes of 6 animals per group were chosen for mechanical, radiographic and bone mineral density assessment. This would allow for unexpected diminution by death, illness or experimental failure of one animal per group and still maintain power at above 0.9. These experiments only required one group of animals per experiment. In the case of histological, histomorphometric and immunohistochemical studies, animals groups were required at five different time points (namely days 1, 3, 7, 14 and 21 post fracture) per experiment. In order to keep the use of animals to a minimum and fulfil Home

Office requirements a group size of four animals was chosen for each of these time points. As can be seen from the graph above this still maintains power at above 0.8, which remains completely acceptable and is often the standard in the scientific literature.

2.11.5.2 Evaluation of power following completion of experiments:

In any study where statistics have been used it is important to consider the statistical power of the tests that have been used. As well as predicting the useful size of samples before undertaking experiments, the accuracy of the analysis that was used must be assessed at the end. 'Statistica' contains a comprehensive '*Power Analysis*' module which allows these tests to be carried out. Using this it was possible to test the power of each of the tests which had been performed for each of the dependent variables. It was then possible to compare these results retrospectively with the computations for sample size which had been made at the start of the project.

2.11.6 Experimental application of the tests

The tests described above, variously in combination, were used to investigate a number of different outcomes of fracture repair in different groups of mice as well as investigate the cellular specific immune response both locally and systemically. These investigations are recorded in Chapters 3 to 8. Taken together, this body of experimental evidence may be used also to evaluate the tests themselves and the animal model. The conclusions drawn from these aspects of the study are recorded in Chapter 9.

Chapter 3

**Results: The expression of mononuclear
leukocytes in murine fracture repair**

3.1 Introduction

The aim of this part of the study was to establish the quantity of the common cells of the immune system at the fracture site and change in their expression over the time course of fracture repair in mouse. The numbers of T and B cells (lymphocytes) were established in the wild type Balb/c mouse. To further ascertain the role of monocyte-macrophages, from that described in the literature (1.5.2), they were also quantified. Macrophages and lymphocytes are known collectively as mononuclear leukocytes. Both play important roles in cellular and humoral immunity. The specific and innate systems communicate mainly through the antigen presenting function of macrophages and their release of cytokines which modulate T and B cell function. These cells are able to exit and re-enter the circulation whilst retaining their function. The remaining cellular component of the immune system consists of granulocytes, the other cells of the innate immune system, an example of which is the neutrophil whose main function is phagocytosis of bacteria. Granulocytes are in general less complex and are thought to have a less significant effect on wound healing (Ogawa, Porter *et al.* 1983).

3.2 Study design

The fracture model described in chapter 2 was employed for this part of the study. Four animals (of each strain, wild type and SCID) at 1, 3, 7, 14 and 21 days following fracture underwent schedule 1 euthanasia. This was the minimum number of animals required from the power calculation (see 2.11.5.1). The tibial fracture specimens were prepared, decalcified and mounted following paraffin embedding as described in Section 2.3. Sections were taken 50 μ m apart to control for variability throughout the

fracture specimen. Immunohistochemistry, as discussed in Section 2.6 was used to identify the different immune cells within the fracture callus. These were quantified as described in Section 2.6.9.

3.3 Immunohistochemistry on control tissue for CD3 (T cells), B220 (B cells) and F4/80 (monocytes-macrophages)

T cells: Immunohistochemistry for T cells (rabbit anti mouse CD3, see Appendix 4) was carried out on positive control tissue (mouse spleen), Figure 3.1. This revealed intense green fluorescent staining in the normal distribution of T cells in spleen, which is abundance within the white pulp area, surrounding the central arteriole.

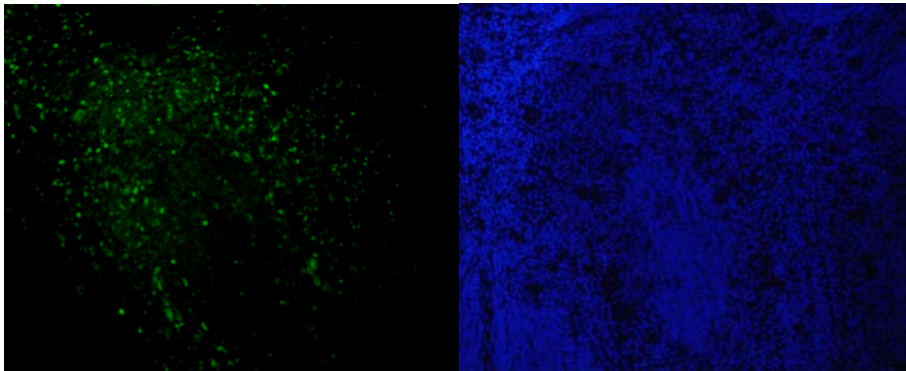
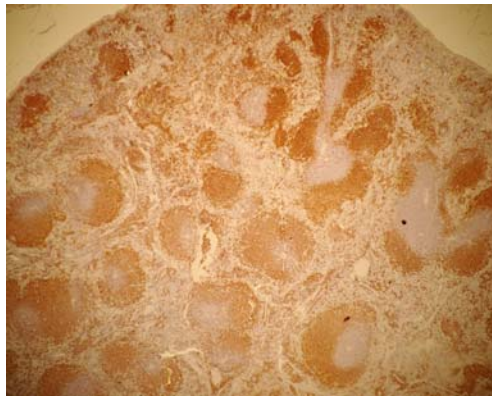


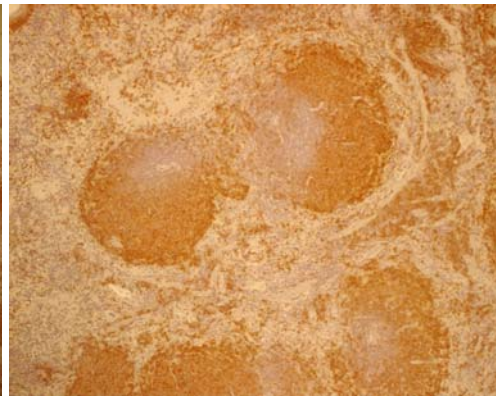
Figure 3.1 Positive control for T cell staining in spleen (left) with corresponding nuclear (DAPI) counter-stain (right). Magnification x200

The negative control tissue with the primary antibody omitted in the spleen and fracture samples revealed negligible labelling for the anti CD3 antibody.

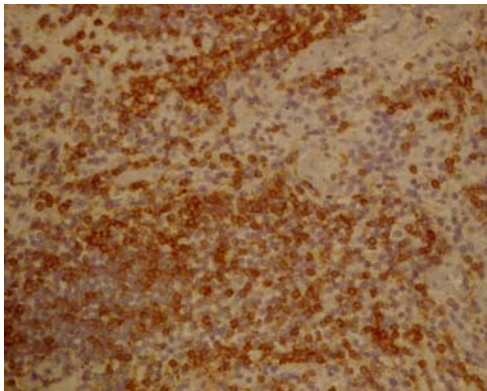
B cells: Immunohistochemistry for B cells (rat anti mouse CD45/B220, see Appendix 4) was carried out on positive control tissue (mouse spleen of wild type animals) and revealed intense brown staining in the normal distribution of B cells in spleen, again in the white pulp, surrounding the central arteriole (the white pulp of the spleen is formed of a mass of T and B cells) see Figure 3.2.



x40



x100

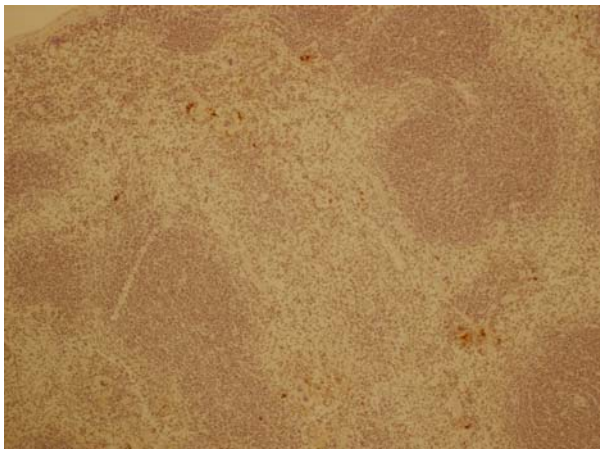


X200

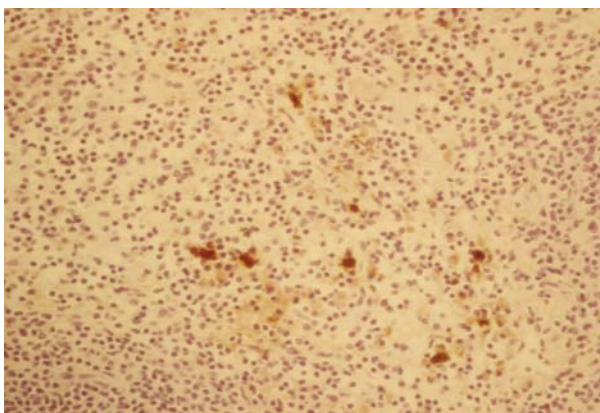
Figure 3.2 Positive control for B cell staining in mouse spleen using rat anti mouse B220. Counterstain: Haematoxylin. Magnification shown below each picture

With the primary antibody omitted (negative control) in spleen or fracture samples there was no staining for B220 detected.

Monocytes-macrophages: Immunohistochemistry for monocytes-macrophages (rat anti mouse to F4/80, see Appendix 4) was carried out on positive control tissue (mouse spleen) and revealed positive brown staining in the normal distribution for macrophages in spleen, in the marginal zones. See Figure 3.3.



x100



X200

Figure 3.3 Positive control for monocyte-macrophage staining in mouse spleen using rat anti mouse F4/80. Counterstain: Haematoxylin. Magnification shown below each picture

With the primary antibody omitted (negative control) in spleen and fracture tissue there was no staining for F4/80 detected.

3.4 Immunohistochemistry results in wild type fracture samples – descriptive.

T cells:

At day one post fracture in the wild type there were multiple CD3 positive cells present within the fracture gap as a result of the fracture haematoma – see Figure 3.4

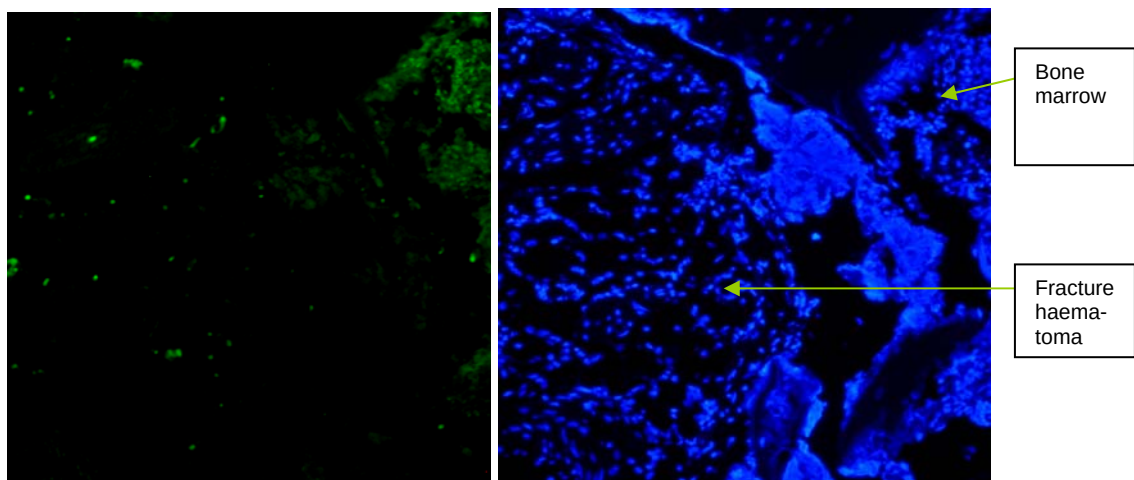


Figure 3.4 CD3 positive staining for T cells in fracture haematoma at day 1 post fracture in mouse (left) with corresponding nuclear (DAPI) counter-stain (right). Magnification x200.

There was also CD3 positive staining abundant within the raised periosteum adjacent to the fracture site both proximal and distal to the fracture – see Figure 3.5.

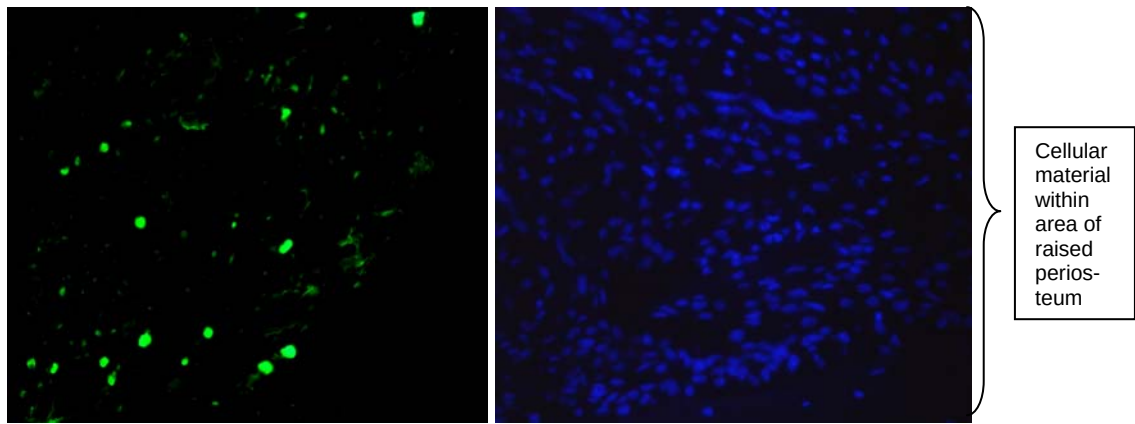


Figure 3.5 CD3 positive staining (left) within the raised periosteum at day 1 post fracture in mouse with corresponding nuclear (DAPI) counter-stain (right). This image shows the typical image used for cellular quantification with this antibody. Magnification x400

B cells:

As with the T cells, there was abundant B220 positive staining within the fracture haematoma at day one post fracture in wild type animals. However, less B220 staining was evident in the periosteal region. See Figure 3.6.

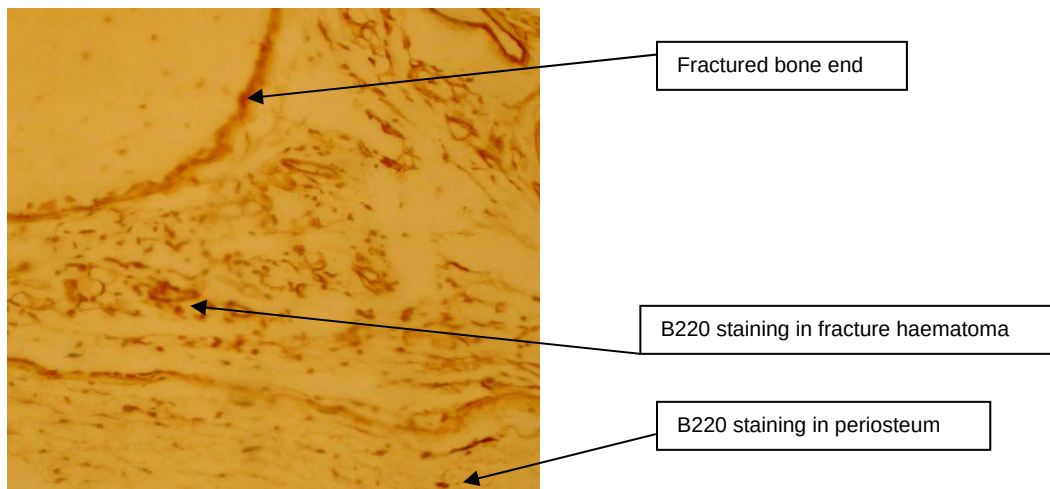


Figure 3.6 B220 staining at fracture site in wild type mouse. Magnification x200

Monocytes-macrophages

At the initial stages of fracture repair there was also multiple F4/80 positive cells in the fracture gap and in the raised periosteal region, in a similar distribution to the T cells, see Figure 3.7



Figure 3.7 Areas of high density F/480 positive cells in the fracture gap of day 3 fracture sections in wild type mouse. Magnification x400.

As fracture repair progressed within the wild type sections there was still positive CD3 staining for T cells at day three both within the fracture gap and in the tissues surrounding both the proximal and distal fractured bone end. This included periosteum, muscle and the undifferentiated tissue forming the beginning of fracture callus. Although the mean number of cells was less than at day one, the T cells did appear to be part of the organising fracture tissue. There remained high numbers of T cells in the raised periosteal area of the fracture at day three. See Figure 3.8. There was still significant B220 staining for B cells in the wild type sections at day three, but mostly in the fracture gap and the numbers of cells had decreased on quantification. See Table 3.1. There was much less B220 staining in the periosteum, with only a few scattered cells seen in this area. F4/80 staining for monocyte-macrophages was still visible in

both the gap and periosteum at the fracture site at day three following fracture. F4/80 staining often appeared near to vessels, giving it a characteristic ‘perivascular’ location – see Figure 3.9.

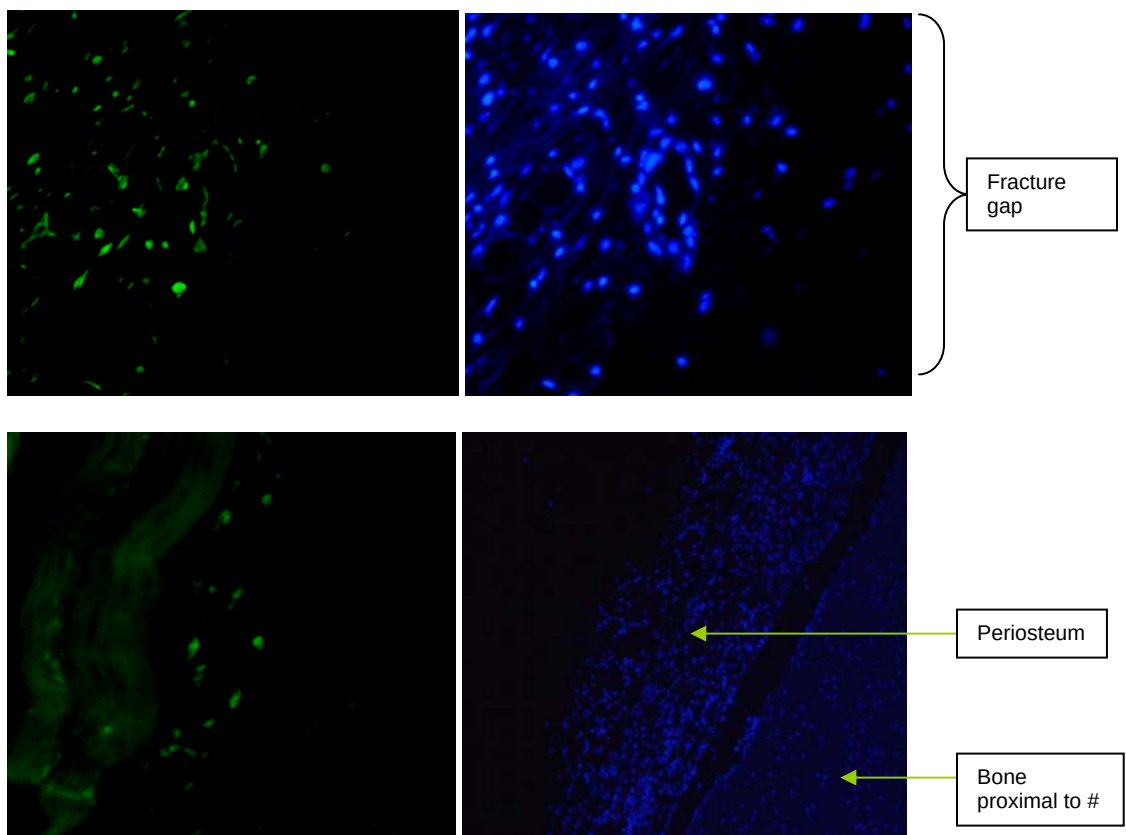


Figure 3.8 CD3 positive staining in mouse fracture sections at day 3. These were high power field images typical of those used for quantification. The above images shows typical staining within the fracture gap region and the below images show typical staining at the periosteal region, just proximal to the fracture. CD3 staining is shown on the left while corresponding DAPI counterstain is shown on the right. Maginification x400

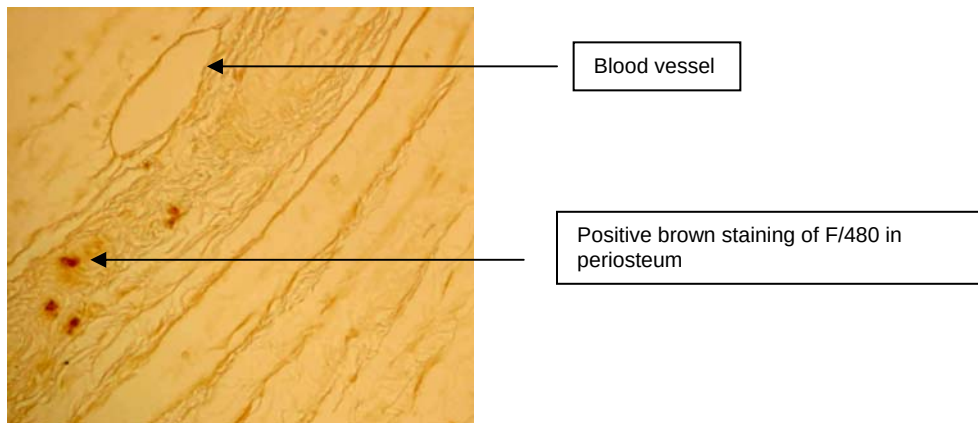


Figure 3.9 F/480 positive cells in the periosteal area of day 3 fracture sections in wild type mouse. Magnification x400.

By day seven the fracture site had become organised into areas of periosteal callus formation and showed differentiation of the tissues within the fracture gap (see Chapter 4 for description of histological sequence of fracture healing in this model). The number of T cells as shown by CD3 staining had decreased but occasional staining was still seen in the periosteal and gap regions, in association with the regenerating tissue. B cell staining with B220 was not evident in any sections at this time point, except for occasional positive cells noted in the bone marrow and within the vessels of the samples (which acted as internal controls, ensuring adequacy of staining). Similar to T cells, the density of F4/80 staining for monocytes-macrophages continued to decrease. There were a few detected in the region of the fracture gap and more seen in the raised periosteum, again in association with the regenerate tissue. F4/80 positive cells were noted *adjacent* to areas of early ossification and cartilage formation in a similar manner to the CD3 positive cells.

By day 14 the fracture repair site was now becoming clearly organised into periosteal cartilaginous callus which had areas of ossification and abundant cartilaginous callus formation within the gap. There was no CD3 positive staining within these areas of regeneration. Positive staining could still be found within the periosteum as was seen at day seven, see Figure 3.10.

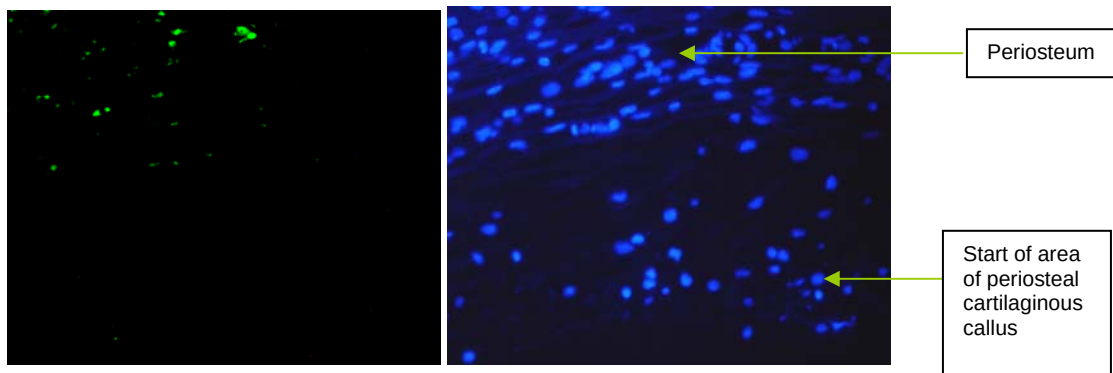


Figure 3.10 CD3 positive staining continues within the periosteum in day 14 post fracture section of wild type mouse (left). DAPI (nuclear) counterstain (right). No CD3 staining was seen in areas of regenerative tissue. Magnification x400.

There continued to be no positive B220 staining for B cells within the fractured area.

The numbers of monocytes-macrophages detected by F4/80 staining also continued to decrease. They were in a similar distribution to day seven in that they were associated with areas of regeneration and with vessels. It is important to note that the F4/80 antibody does not bind to the murine osteoclast .

By day 21 the fracture gap was now largely filled with cartilaginous callus with areas of endochondral ossification progressing across this to form woven bone. The occasional CD3 positive cell was noted in existing bone marrow and in areas of new marrow

formation. However T cells associated with bony regeneration were very sparse at this time point with only the occasional periosteal cell seen in some of the sections. Similarly, there was only occasional perivascular staining noted for monocytes-macrophages with F4/80, near to areas of new bone formation.

3.5 Immunohistochemistry results in wild type fracture samples – quantitative.

The following table and graph summarises the results of immunohistochemical quantification of T cells, B cells and monocytes-macrophages in the wild type mouse. Appendix 10 details an intra- and inter-observer reliability study related to this data.

	Mean number of positive cells per high power field +/- standard error				
	Day 1	Day 3	Day 7	Day 14	Day 21
CD3 (T cells)	20.6 +/- 1.3	17.1 +/- 2.2	7.2 +/- 0.69	5.13 +/- 0.97	1.1 +/- 0.21
B220 (B cells)	14.8 +/- 1.2	10.9 +/- 1.3	0	0	0
F4/80 (monocytes / macrophages)	24.2 +/- 1.4	21.9 +/- 1.0	9.2 +/- 0.63	5.1 +/- 0.51	3.4 +/- 0.44

Table 3.1 Immunohistochemistry results in wild type mouse: Quantification data showing numbers of T cells, B cells and monocyte-macrophages over the time course of fracture repair in wild type animals.

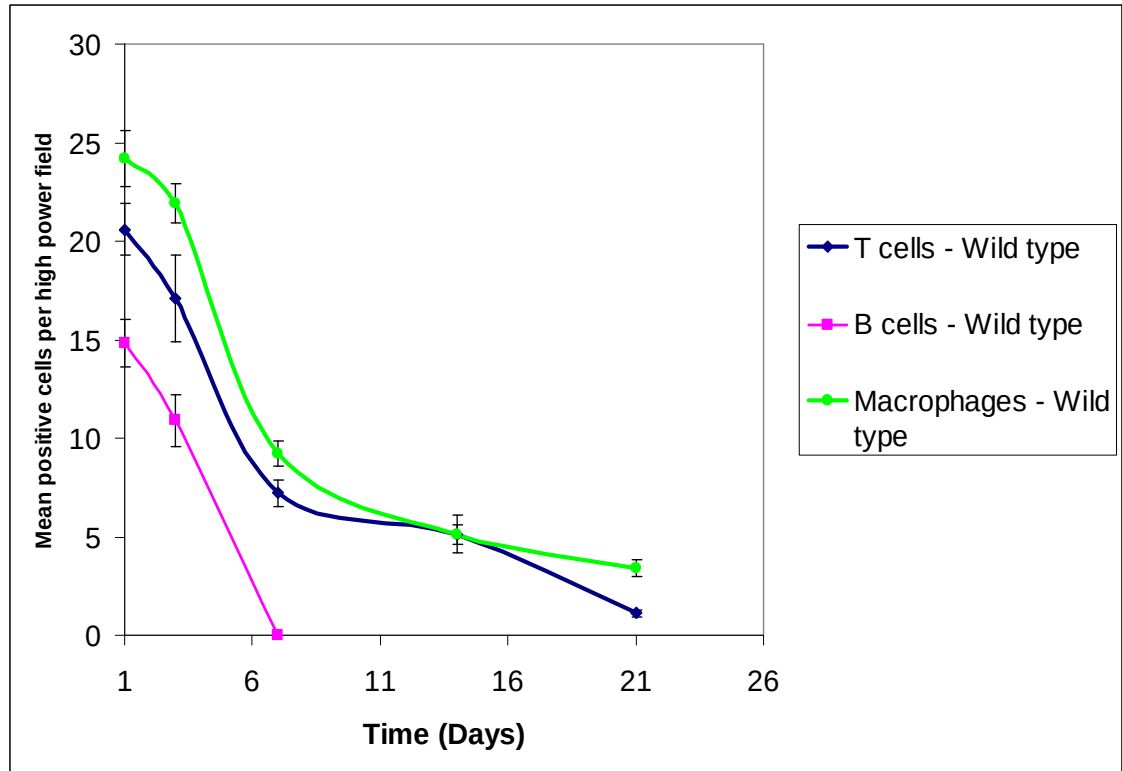


Figure 3.11 Graph demonstrating the expression of mononuclear leukocytes (T cells, B cells and monocyte-macrophages) as a function of time during fracture repair in the wild type mouse

Chapter 4

**Results: Comparison of fracture healing
between Wild Type and SCID mice**

4.1 Introduction

Having shown that cells of the specific immune system are present during the process of fracture repair in Chapter 3, it was decided to evaluate the following hypothesis:

“Lack of the specific immune system will cause perturbation of fracture repair in vivo”

In order to investigate this a ‘severe combined immuno deficient’ or SCID mouse was used with application of the fracture model and assessment methods as detailed in chapter 2 with comparison to wild type controls. The immunodeficient Balb/c SCID mutant mouse lacks functioning T and B cells. It is a naturally occurring mutant that is commercially available. The natural mutation prevents the V(D)J recombination in B and T lymphocytes, resulting in failure to generate functional immunoglobulins and T cell receptors. They therefore lack adaptive (or specific) immunity but have intact innate immunity on an identical BALB/c genetic background (Adarichev, Vermes *et al.* 2005). Therefore natural killer and macrophage function is normal, as is circulating complement. For this reason wild type BALB/c controls, as were used in chapter three, were used. The SCID mice were matched for age, sex (all males) and weight to the controls.

The results comparing fracture repair between the two strains are presented in three sections. Section one deals with descriptive and quantitative histology and histomorphometry. Section two compares organic measures of fracture repair with radiographic assessment and bone mineral density measurements. Section three compares biomechanical properties using four point bending mechanical testing. Also included is *post hoc* power assessment results, validating the numbers of animals used in the studies.

Section one: Descriptive and quantitative histology and histomorphometry

4.2 Histology observations: H& E staining: Descriptive comparison of morphology of fracture healing between wild type and SCID

Histological assessment was performed on animals euthanased at time points 1, 3, 7, 14 and 21 days following fracture, with comparison between SCID and wild types.

4.2.1 Day 1 post fracture:

SCID:

The appearances were not discernibly different at day 1 post fracture in the SCID animals compared to the wild type, including magnifications up to 400x.

Wild type:

The fracture gap contained mainly haematoma and a few undifferentiated cells. The influx of polymorphonuclear leukocytes and monocytes into the fracture site could be seen. There was also undifferentiated tissue in close association with the surrounding soft tissues and muscles. See Figure 4.1.

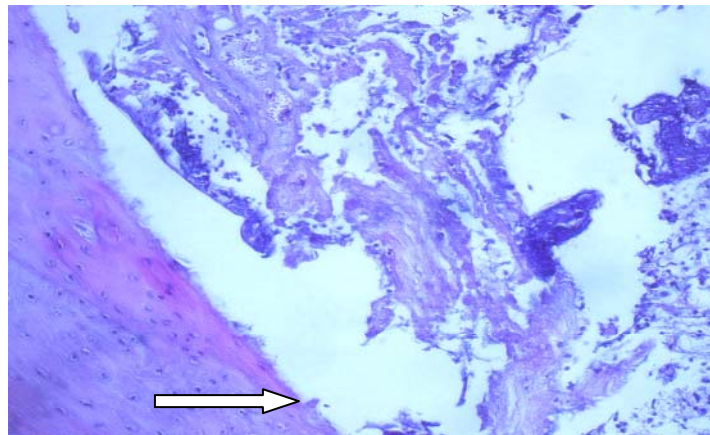


Figure 4.1 Day 1 post fracture (wild type) H& E stain. Haematoma seen. Fractured bone end (arrow). (200x).

4.2.2 Day 3 post fracture:

SCID:

There were more undifferentiated cells (fibroblast-like mesenchymal cells) compared to the wild type. The periosteum was more active and merged with the periosteal callus. Cellular activity was most evident in the cambial layer of the periosteum which was expanded and periosteal callus (cartilaginous) was being formed. The first bridge across the fracture site was becoming evident at this stage formed from a fibrous fracture callus. See Figure 4.2.

Wild type:

Wild type animals showed some undifferentiated tissue filling the fracture gap but less periosteal reaction in comparison to the SCID. There was no evidence of bridging callus at this time point. See Figure 4.3.

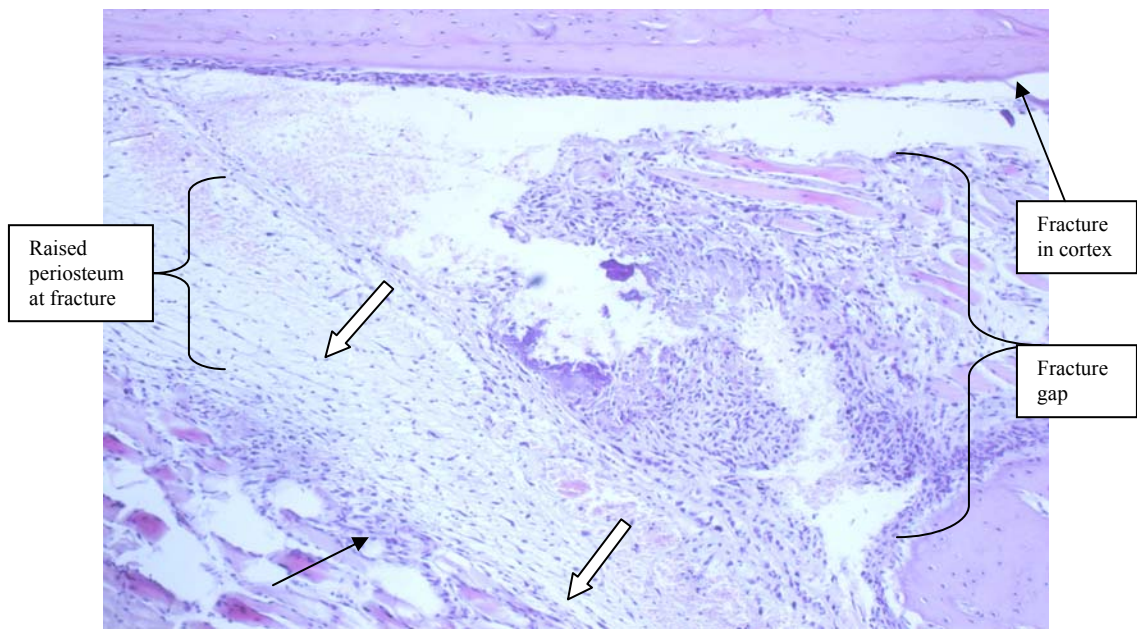


Figure 4.2 Day 3 post fracture, (SCID) H&E stain. Active cambial layer of periosteum (open arrows). Fibroblast-like mesenchymal cells (closed arrow) (100x).

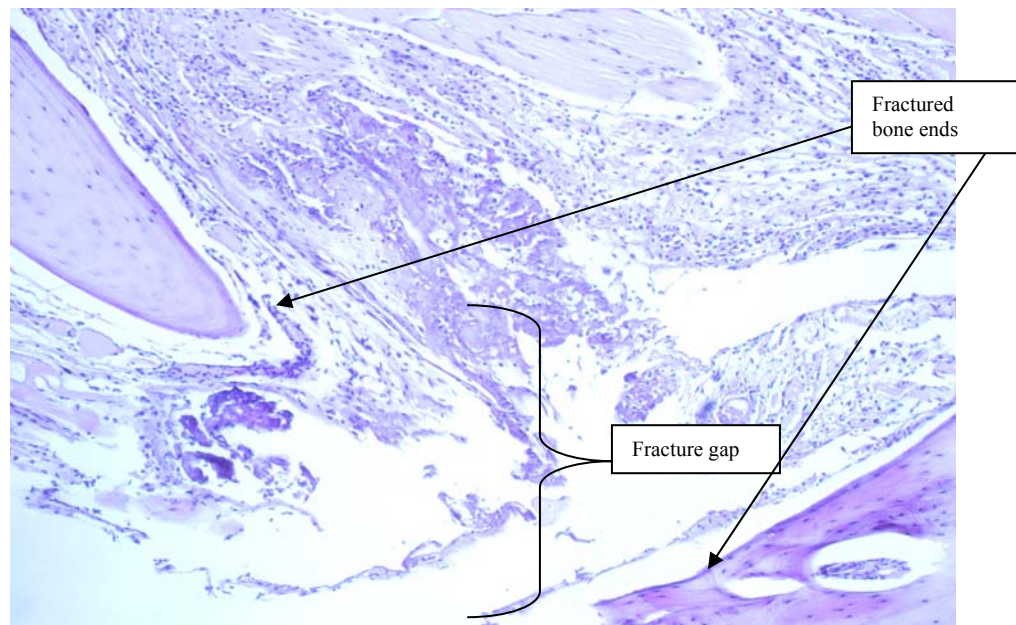


Figure 4.3 Day 3 post fracture, (wild type) H&E stain. Undifferentiated tissue only. (100x)

4.2.3 Day 7 post fracture:

SCID:

At day 7 there was a clear histological difference between the groups. The production of cartilaginous callus was evident in many areas in the SCID animals. The fibroblast like mesenchymal cells had undergone 'chondrification'. There was also evidence of new bone formation along the surfaces of the original cortices with ossification of the periosteal cartilaginous callus. There were also the beginnings of endochondral ossification within the cartilage of the external callus. The callus now fully bridged the fracture gap. See Figure 4.4.

Wild type:

Wild type animals showed an abundance of undifferentiated tissue (fibroblast-like mesenchymal cells) filling the fracture gap at this stage but little evidence of cartilage formation, in comparison to the SCID. Periosteal callus formation was now evident. See Figure 4.5.

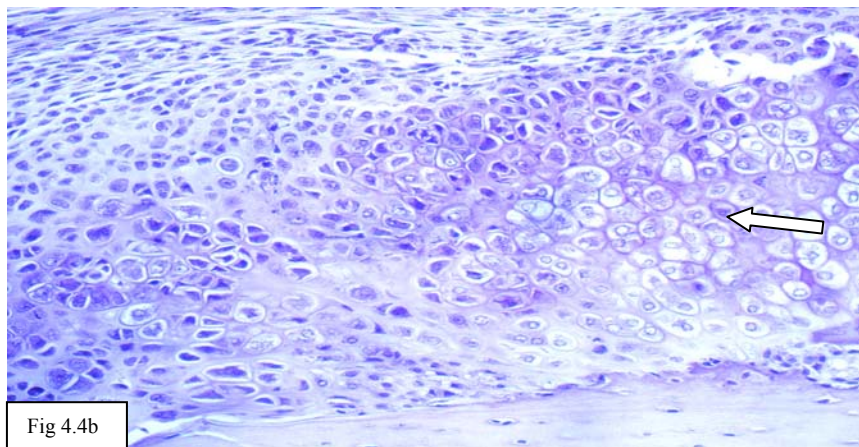
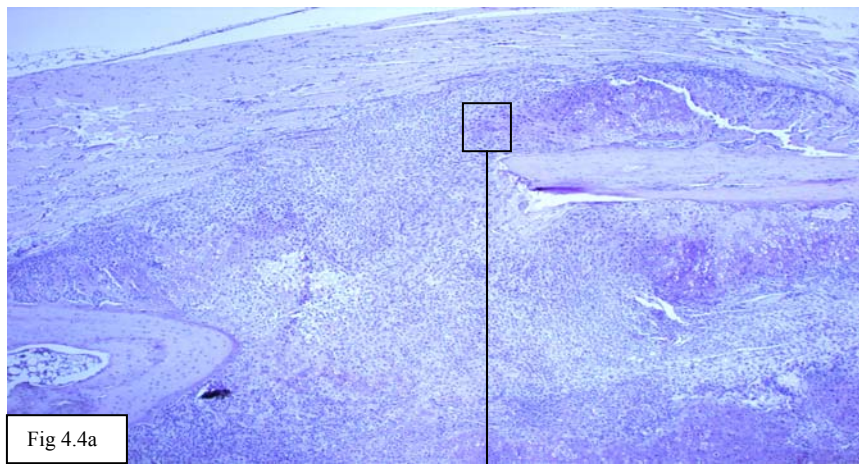


Figure 4.4

Day 7 post fracture, (SCID) H&E stain. 40x (a) and 200x (b). Chondrocytes producing cartilage (open arrow)

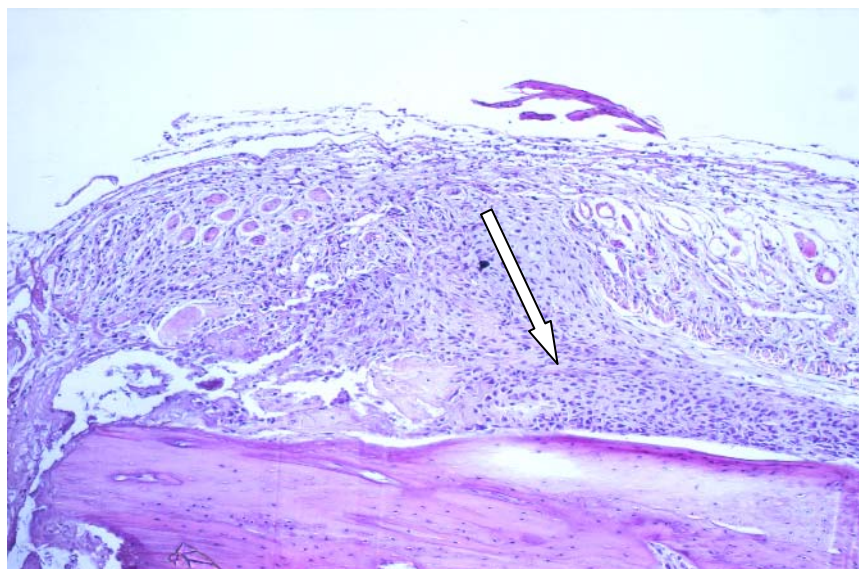


Figure 4.5 Day 7 post fracture, (wild type) H&E stain. Periosteal callus formation (open arrow). (100x).

4.2.4 Day 14 post fracture:

SCID:

The majority of the tissue in the fracture gap had been converted to cartilage. Abundant endochondral ossification replaced the cartilaginous callus with new trabecular bone in many areas, giving a 'bony callus'. Morphologically fracture repair was proceeding to completion. See Figure 4.6.

Wild type:

Cartilaginous callus production was now becoming abundant in the wild type, with the proportion of undifferentiated tissue decreasing. There were only a very few areas of endochondral ossification seen. The callus now fully bridged the fracture gap in the wild types. See Figure 4.7.

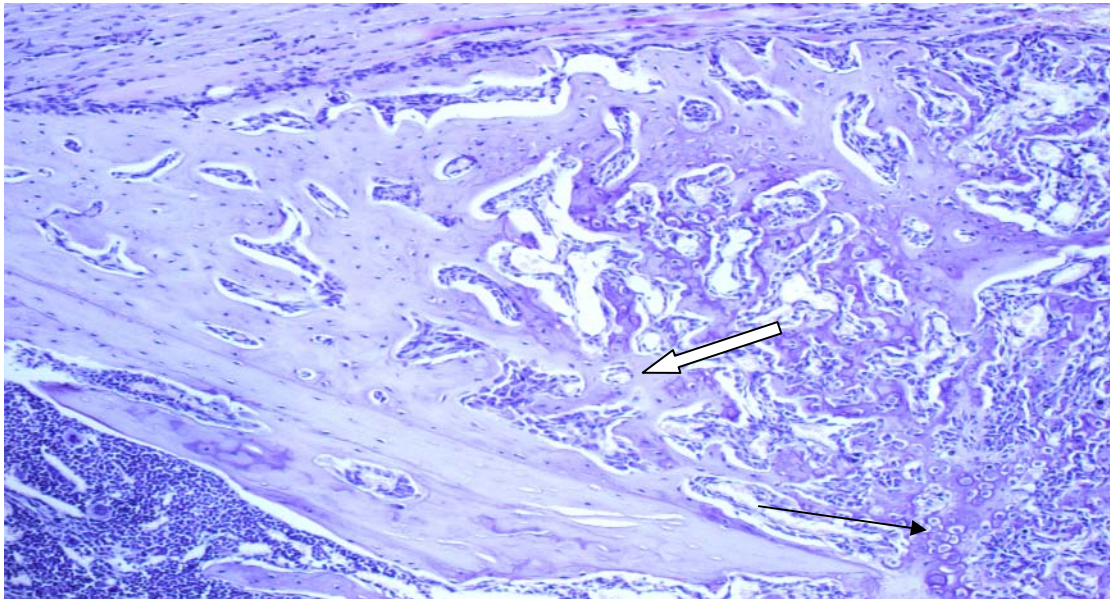


Figure 4.6 Day 14 post fracture, (SCID) H&E stain. Endochondral ossification replacing cartilage (closed arrow) with new trabecular bone (open arrow) (100x).

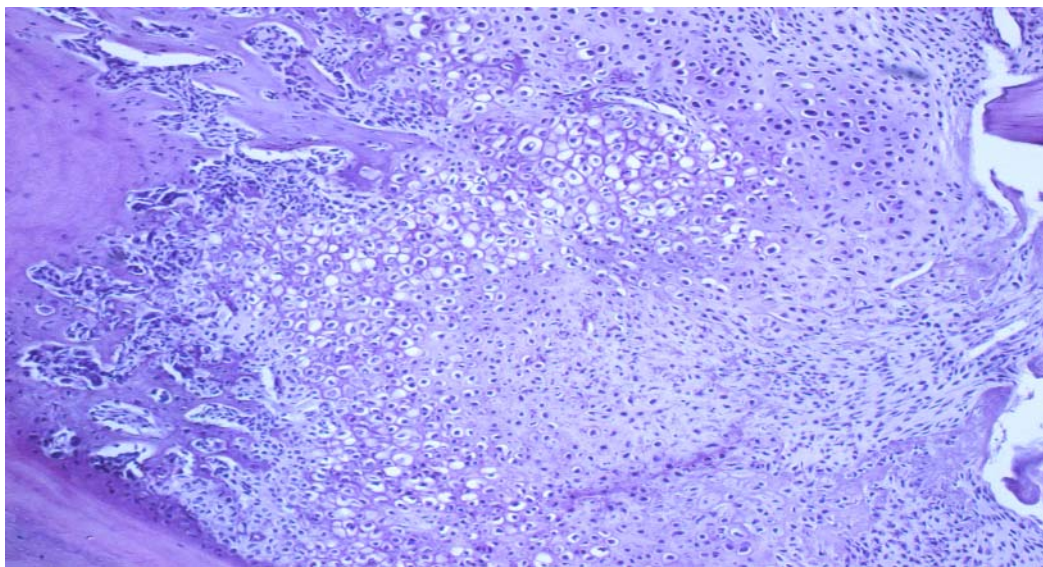


Figure 4.7 Day 14 post fracture, (wild type) H&E stain. 'Chondrification' of fibroblast-like mesenchymal cells and abundant cartilaginous callous production (100x).

4.2.5 Day 21 post fracture:

SCID:

Most of the fracture callus had undergone endochondral ossification. Newly formed woven bone was the predominant tissue with the rest of the areas showing ongoing endochondral ossification. Bone marrow could be seen infiltrating into the bone trabecules. The woven bone was showing evidence of remodelling and the callus size had actually started to decrease on most sections. See Figure 4.8.

Wild type:

The fracture gap was largely filled with cartilaginous callus and the areas of endochondral ossification were increasing. The time lag behind the SCID animals was noted but otherwise the characteristics of fracture repair were showing similar appearances. See Figure 4.9.

Notes on histology:

- 1) Of note in this model there was no intramembranous bone formation in that all bone deposition followed a cartilage intermediate *i.e.* endochondral ossification.
- 2) The histological descriptions given were consistent across all four animals in each group and the next section summarises the quantification of this histology.

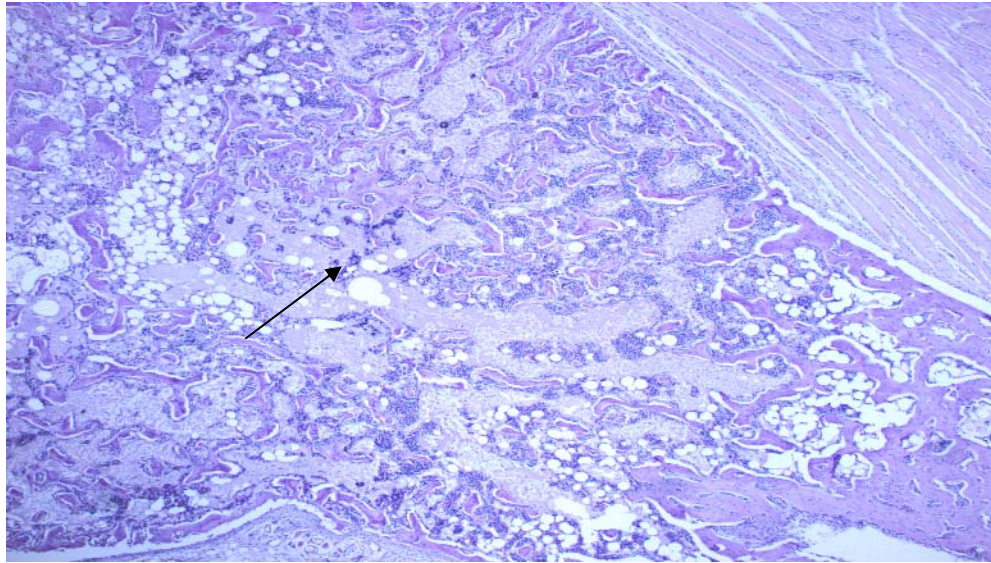


Figure 4.8 Day 21 post fracture, (SCID) H&E stain. Fracture site now mainly composed of new trabecular bone, with evidence of bone marrow infiltration (arrow). (40x).

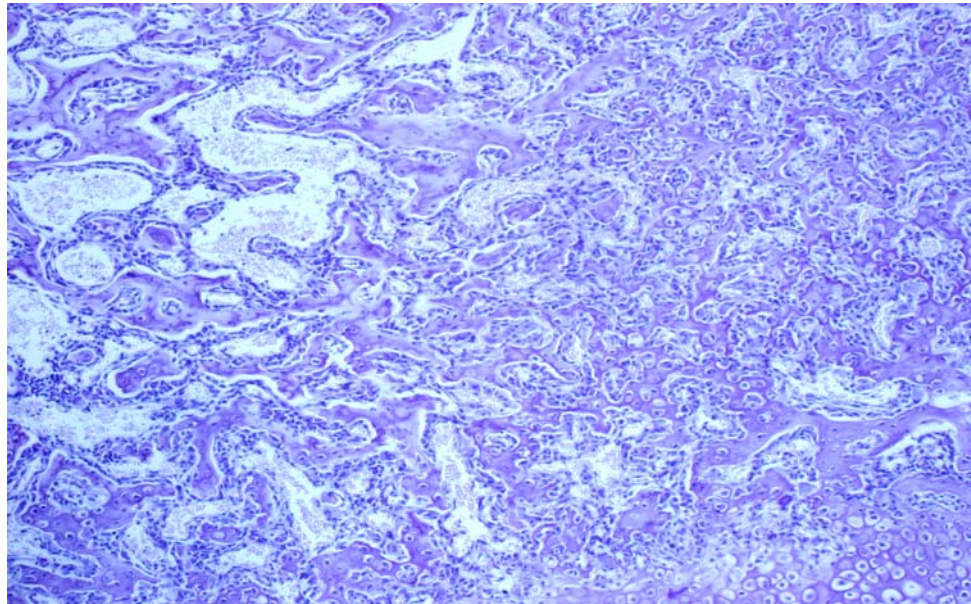


Figure 4.9 Day 21 post fracture, (wild type) H&E stain. Endochondral ossification replacing cartilage with woven bone (100x).

4.3 Quantification of tissue types: results of H&E staining

As mentioned in the morphology observation results, fracture callus less than seven days showed predominantly undifferentiated tissue in both wild type and SCID animals. Also because the fractures had not yet united in either group, tissue processing often resulted in the fractured bone ends becoming disrupted, which again prevented formal quantification of tissue present. Tissue types were therefore quantified at days 7, 14 and 21 post fracture as described in Section 2.4.2.

In each graph the x-axis shows the number of days post fracture at which the specimen was taken.

The y-axis shows the percentage area of tissue in the fracture callus

Each bar represents the mean of data from three sections from each of four animals (each section being mapped three times), each for the wild-type and SCID at each time period.

4.3.1 Amount of cartilage formation (Figure 4.10)

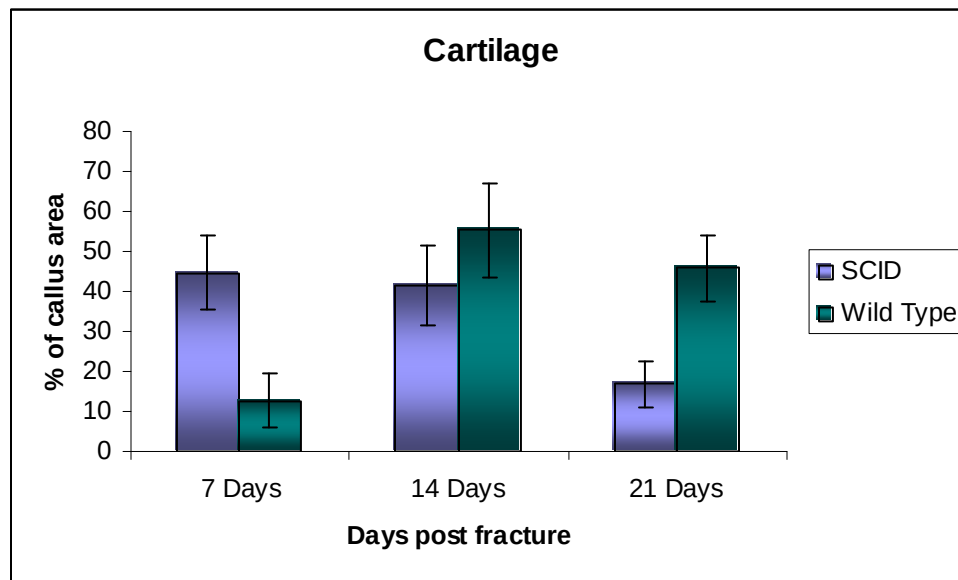


Figure 4.10 Cartilage formation during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 1 standard deviation).

At day 7 post-fracture there was significantly more cartilage formed in the SCID specimens compared to the wild types.

Statistics: Data normally distributed: Shapiro-Wilk $W = 0.917586883$, $P = 0.0592$

Statistical test used: 1 way ANOVA revealed a difference in the percentage of cartilage at day 7 between wild type and SCID animals, $F=98.0$. *Post hoc* Scheffé test showed a significant difference with $P < 0.005$.

At day 14 post-fracture there was no significant difference in the amount of cartilage between the two strains. However, it can be seen from the graph that the temporal peak in cartilage formation in the wild type animal occurred at this time (about a week later

than the SCIDs), while the amount of cartilage in the SCIDs had started to decrease, as endochondral ossification was underway. This was seen in the histology observations, Section 4.2.

At day 21 post-fracture there was significantly more cartilage formed in the wild type animal compared to the SCID, as fracture healing in the latter was mostly in the bone forming phase.

Statistics: Data not normally distributed: Shapiro-Wilk W: 0.896177193, P = 0.0211

Statistical test used: Kruskal-Wallis ANOVA by ranks revealed a difference in the percentage of cartilage at day 21 between wild type and SCID animals. Mann-Whitney U test showed a significant difference ($Z=4.06$) with $P < 0.005$.

4.3.2 Amount of new bone formation (Figure 4.11)

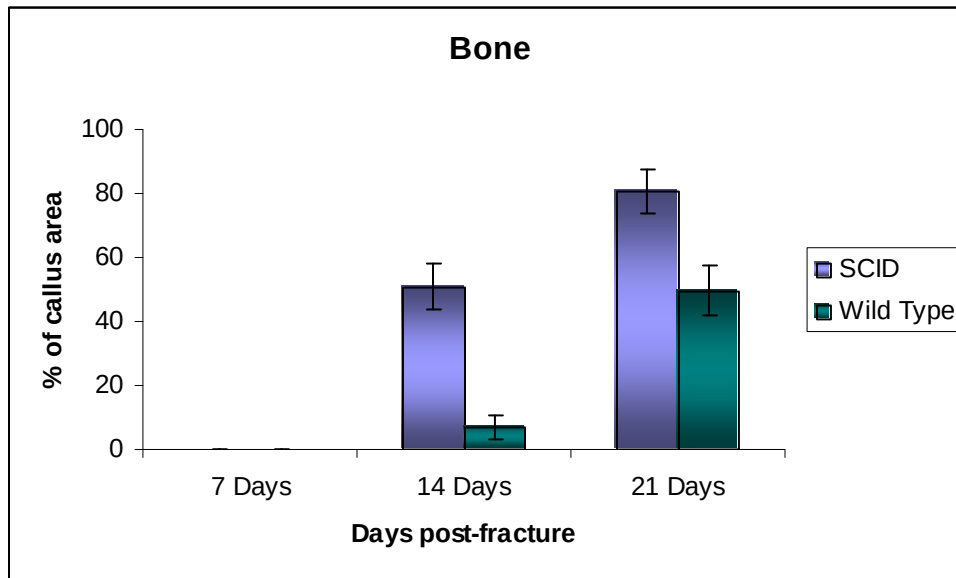


Figure 4.11 Bone formation, during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 1 standard deviation).

At day seven there was no evidence of new bone formation in either strain. At days 14 and 21 there was significantly more new endochondral bone formation in the SCID strain compared to the wild type.

Statistics, Day 14: Data not normally distributed: Shapiro-Wilk W: 0.896177193, P = 0.0211. Statistical test used: Kruskal-Wallis ANOVA by ranks revealed a difference in the percentage of bone at day 14 between wild type and SCID animals. Mann-Whitney U test showed a significant difference (Z=4.07) with P< 0.005.

Statistics, Day 21: Data not normally distributed: Shapiro-Wilk W: 0.891171314, P = 0.0167. Statistical test used: Kruskal-Wallis ANOVA by ranks revealed a difference in the percentage of bone at day 21 between wild type and SCID animals. Mann-Whitney U test showed a significant difference (Z=4.06) with P< 0.005.

4.3.3 Amount of undifferentiated tissue (Figure 4.12)

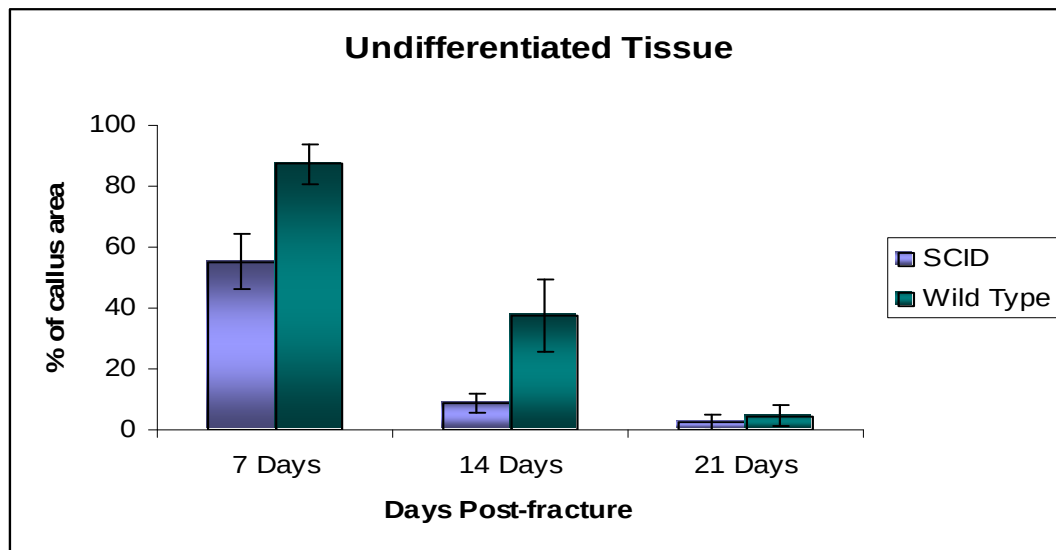


Figure 4.12 Amount of undifferentiated tissue, during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 1 standard deviation).

The amount of undifferentiated tissue decreased much faster in the SCID animals compared to the wild types, with statistical significance at days 7 and 14.

Statistics, Day 7: Data not normally distributed: Shapiro-Wilk W: 0.887227856, $P = 0.0139$. Statistical test used: Kruskal-Wallis ANOVA by ranks revealed a difference in the percentage of undifferentiated tissue at day 7 between wild type and SCID animals. Mann-Whitney U test showed a significant difference ($Z=4.06$) with $P < 0.005$.

Statistics, Day 14: Data not normally distributed: Shapiro-Wilk W: 0.868373649, $P = 0.0049$. Statistical test used: Kruskal-Wallis ANOVA by ranks revealed a difference in the percentage of undifferentiated tissue at day 14 between wild type and SCID animals. Mann-Whitney U test showed a significant difference ($Z=4.16$) with $P < 0.005$.

4.3.4 Summary: Histological findings

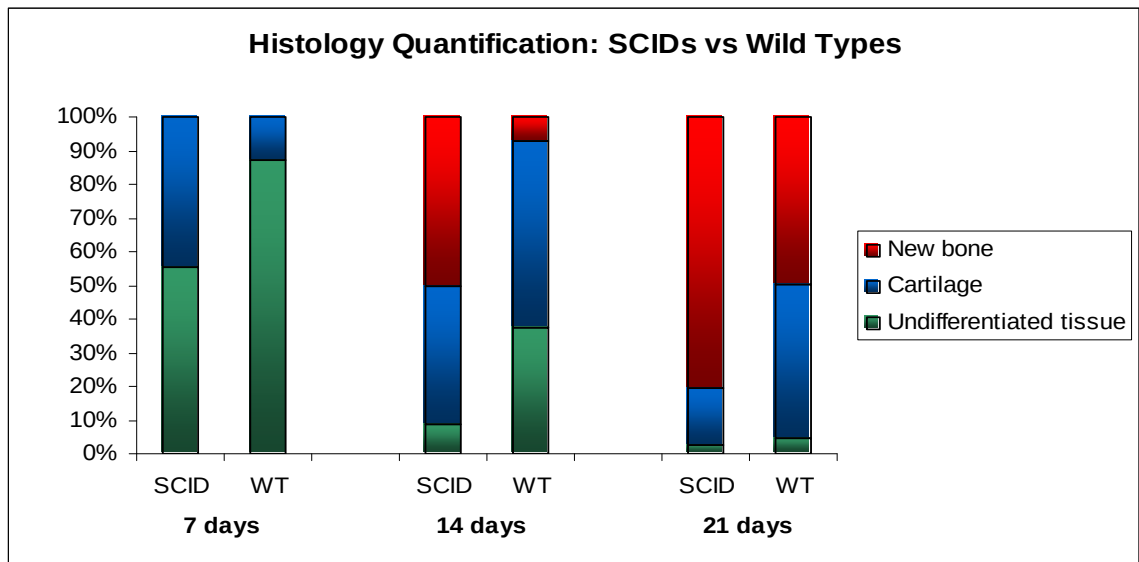


Figure 4.13 Summary graph of histology results during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

The histological analysis, both quantitatively and qualitatively demonstrated the following:

- 1) The formation of cartilage in the SCID animals took place earlier compared to the wild types.
- 2) The amount of cartilage formation in the wild type did increase to similar levels as the SCID strain, but at a later time point.
- 3) The amount of new bone formed in the SCID strain was greater at all stages examined in this study.
- 4) The process of fracture healing followed the same histological steps, but at a slower pace in the wild types compared to the SCIDs.

- 5) The amount of undifferentiated tissue decreased to a very low level at day 14 in the SCID strain, again suggesting more rapid healing.

Overall the SCID animals had less undifferentiated tissue at the earlier time points and increased bone formation at the later time points compared to the controls suggesting more rapid fracture healing.

4.4 Histomorphometric results

Formal histomorphometry methods (see Section 2.5) were used to assess the amounts of cartilage and new mineralised bone formed more accurately and to compare this between the two strains.

4.4.1 Safranin-O histomorphometry

Safranin-O staining was used to assess cartilage formation, again at days seven, 14 and 21 post fracture. The distal epiphysis of the fractured limb was used as a control to check for the efficacy of safranin-O staining, as can be seen in Figure 4.14.

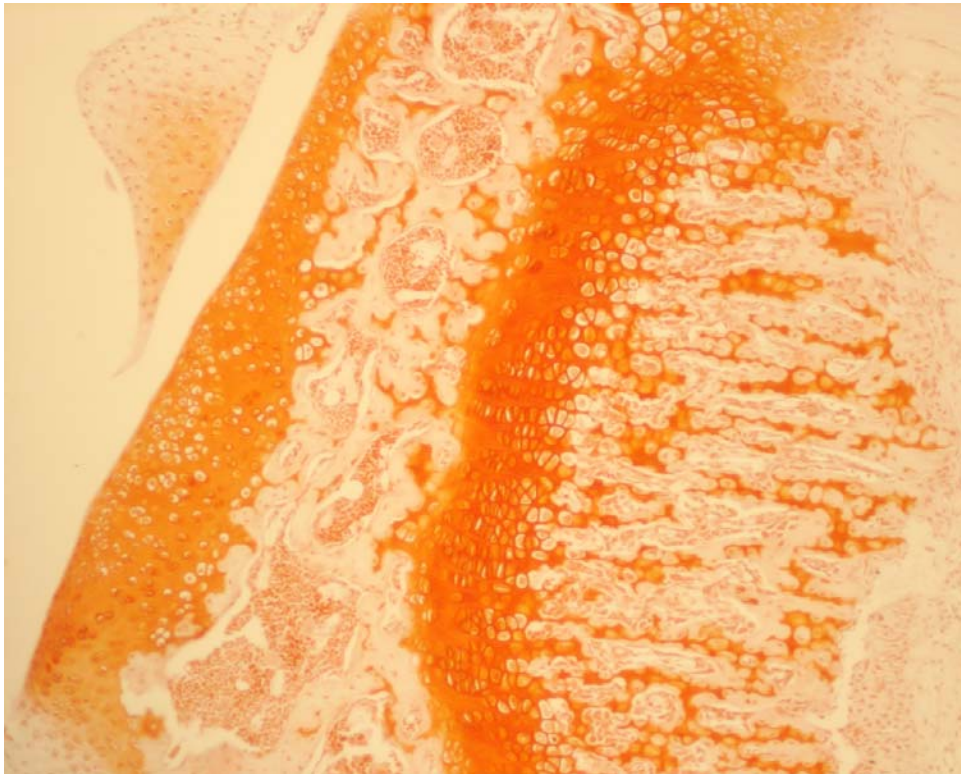


Figure 4.14 Safranin-O staining in the distal epiphysis of the wild type mouse showing bright orange staining of cartilage formation (Magnification x 100)

The following figure shows the typical staining obtained with Safranin-O at day 7 in the SCID animal



Figure 4.15 Safranin-O staining at day 7 in SCID animal (Magnification x40)

Images were taken at x200 magnification for the purpose of histomorphometry:

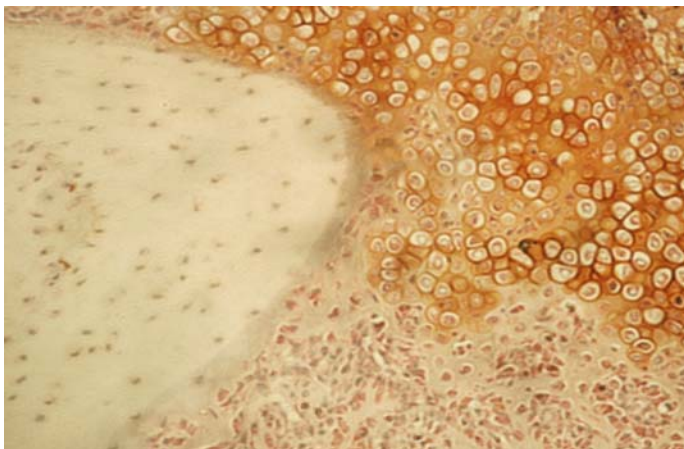


Figure 4.16
x200 magnification
image of Safranin-O
staining at day 7 in
SCID strain. For
histomorphometry
method see section
2.5

The results are displayed in Table 4.1 and the amount of cartilage is expressed as a percentage of the total callus tissue in the image field.

Days post-fracture	7	14	21
% Cartilage Wild type (Mean $\pm$ 1 SD)	7.0 +/-1.5	60.9 +/-2.5	39.7 +/-2.2
% Cartilage SCID (Mean $\pm$ 1 SD)	60.6 +/-3.7	43.4 +/- 2.9	9.6 +/-1.9
P value	P=0.021 (Non parametric; Mann- Whitney U test)	P=0.0001 (Parametric; ANOVA F=82.2, <i>post</i> <i>hoc</i> Scheffé test)	P=0.021 (Non parametric; Mann-Whitney U test)

Table 4.1 Results of Safranin-O staining histomorphometry during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

The Safranin-O staining revealed a remarkable difference at day seven post fracture, with abundant formation of cartilage in the SCID strain compared to the controls, as can be seen from the table. The sections from the wild type animals showed only small pockets of cartilage formation at day seven. This confirms the findings from Section 4.3.1: the peak of cartilage formation occurred approximately one week later in the wild types compared to the SCIDs. At day 21 there was very little cartilage remaining in the SCID images and the amount of cartilage had began to decrease in the wild types. These were largely in keeping with the quantification results from the H&E histology.

4.4.2 Goldner's modified Mason's trichrome histomorphometry

Mason's Trichrome staining was used to assess the formation of new mineralised bone at day 14 and 21 post-fracture.

Figure 4.17 shows the typical staining obtained with Mason's Trichrome at day 14 in the SCID animal.

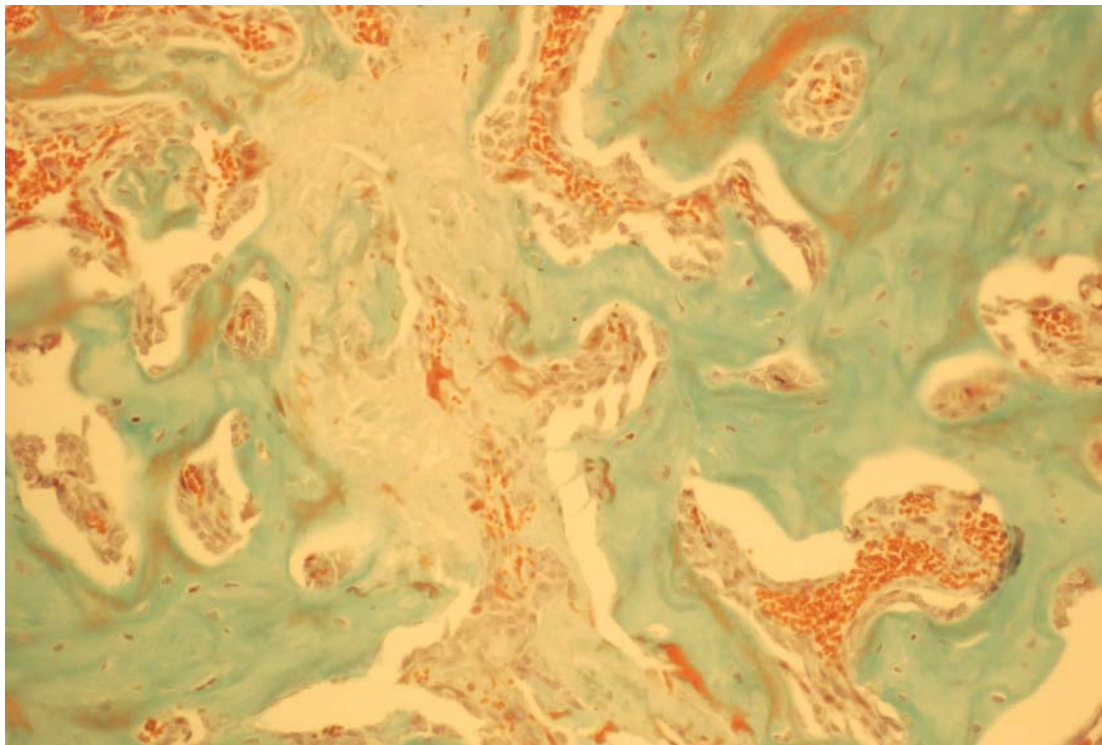


Figure 4.17 Mason's Trichrome staining at day 14 in SCID animal (Magnification x200)

The results are displayed in Table 4.2 and the amount of new mineralised bone formed is expressed as a percentage of the total callus tissue in the image field.

Days post-fracture	14	21
% Bone Wild type (Mean $\pm$ 1 SD)	15.0 +/-1.5	53.2 +/-3.2
% Bone SCID (Mean $\pm$ 1 SD)	53.5 +/- 4.3	79.2 +/-2.8
P value	P=0.021 (Non parametric; Mann-Whitney U test)	P=0.021 (Non parametric; Mann-Whitney U test)

Table 4.2 Results of Mason's Trichrome staining histomorphometry during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

The Mason's Trichrome staining revealed a remarkable increase in bone formation at day 14 in the SCID animals and only minimal bone formation in the wild types (Table 4.2). At day 21 new endochondral bone fills the majority of the callus tissue in SCIDs. In the wild types, bone formation is now well underway and occupies approximately half of the callus volume.

These findings again corroborate the quantification results from the H&E histology, Section 4.3.1.

The accurate assessment of the amount of cartilage and new bone by histomorphometry has confirmed that the SCID animal progressed more rapidly through the process of fracture repair.

Section two: Radiographic assessment and bone mineral density measurements

4.5 Radiographic Assessment

The healing fractures of both strains were assessed radiographically as detailed in Section 2.6. Radiographs were taken at days 7, 14 and 21 post fracture in six animals in each strain. The following radiographs show examples from each time point:

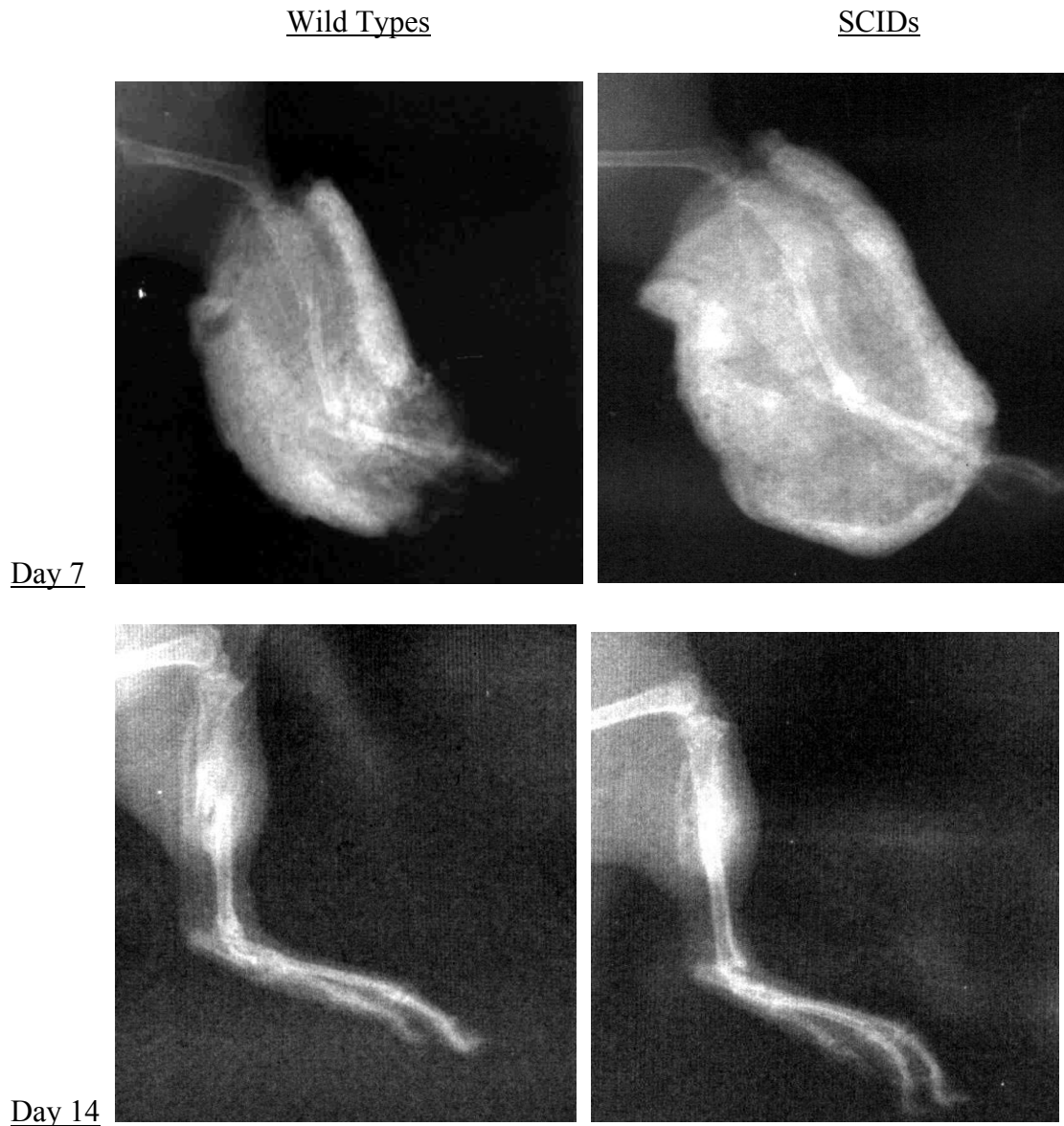


Figure 4.18 Example radiographs taken at days 7 and 14: lateral views

4.5.1 Callus area results

Callus area was measured by pixilation as described in 2.7.2. The mean results from the six animals and standard deviations are displayed in the Table 4.4

Days post-fracture	7	14	21
Callus area Wild type (Mean $\pm$ 1 SD) (inch ²)	0.00717 +/- 0.0019	0.014 +/- 0.0017	0.022 +/- 0.0030
Callus area SCID (Mean $\pm$ 1 SD) (inch ²)	0.01083 +/- 0.0017	0.02767 +/- 0.0028	0.02483 +/- 0.0031
P value	P=0.006 (Parametric; ANOVA F=11.9, <i>post hoc</i> Scheffé test)	P=0.004 (Non parametric; Mann- Whitney U test)	P=0.138 (Parametric; ANOVA F=2.5, non-sig)

Table 4.3 Results of callus area measurement during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

These results are also shown in the graph in Figure 4.19, which demonstrates the trend in change of callus area size, comparing the 2 strains

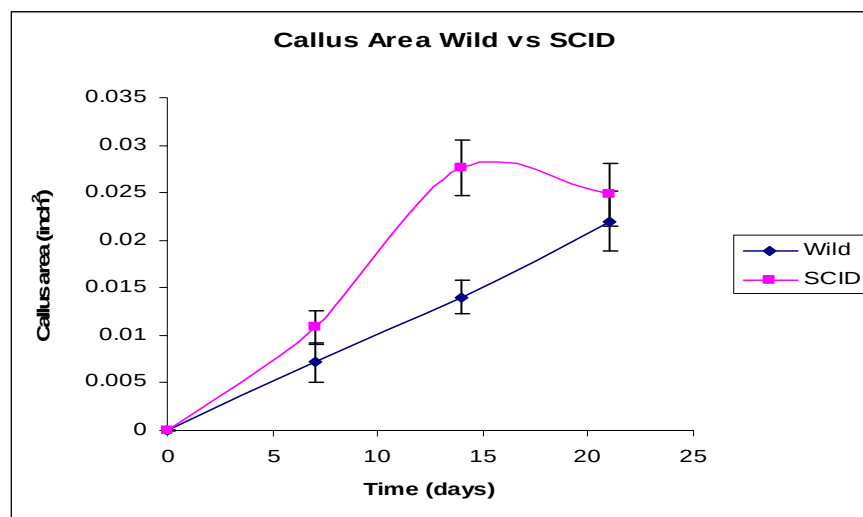


Figure 4.19 Change in callus area with time, during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 95% confidence intervals).

4.5.2 Callus index results

Callus index in both orthogonal planes (antero-posterior and lateral: at 90 degrees to each other) was measured as described in Section 2.7.3. The mean results from the six animals and standard deviations are displayed in the tables and graphs below

Days post-fracture	7	14	21
Callus index AP Wild type (Mean $\pm$ 1 SD)	1.45 +/-0.08	1.5 +/-0.17	2.86 +/-0.47
Callus index AP SCID (Mean $\pm$ 1 SD)	1.95 +/-0.09	2.79 +/- 0.32	3.05 +/-0.17
P value	P=0.00001 (Parametric; ANOVA F=106, <i>post hoc</i> Scheffé test)	P=0.00005 (Parametric; ANOVA F=76.5, <i>post hoc</i> Scheffé test)	P=0.38 (Parametric; ANOVA F=0.84, non-sig)

Table 4.4 Results of antero-posterior callus index measurement during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

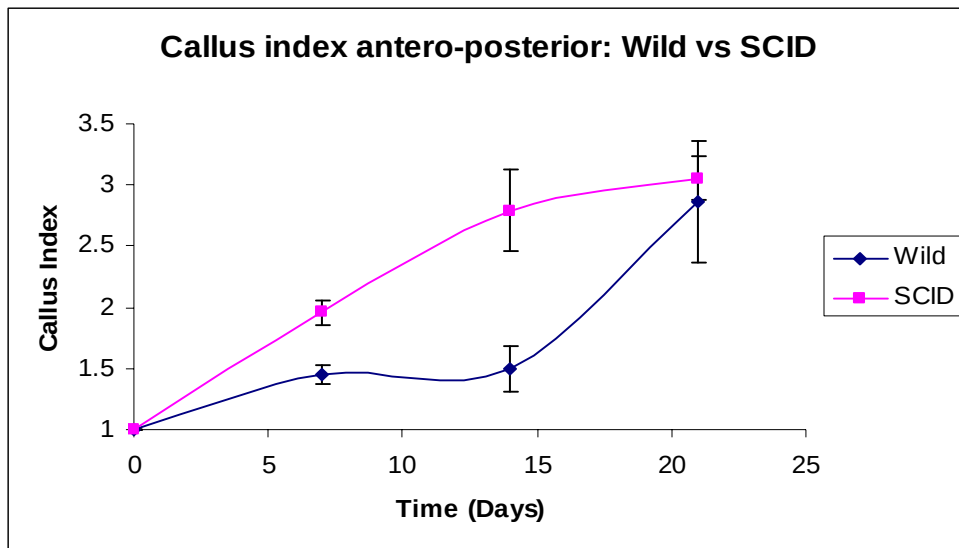


Figure 4.20 Change in antero-posterior callus index with time during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 95% confidence intervals). Callus index is a ratio: No units.

Days post-fracture	7	14	21
Callus index med-lat Wild type (Mean $\pm$ 1 SD)	1.39 +/-0.11	1.67 +/-0.11	2.49 +/-0.31
Callus index med-lat SCID (Mean $\pm$ 1 SD)	2.04 +/-0.20	2.76 +/- 0.22	2.12 +/-0.39
P value	P=0.00004 (Parametric; ANOVA F=47.2, <i>post hoc</i> Scheffé test)	P=0.004 (Non parametric; Mann- Whitney U test)	P=0.13 (Non parametric; M-W U Z = 1.52, non-sig)

Table 4.5 Results of medial-lateral callus index measurement during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

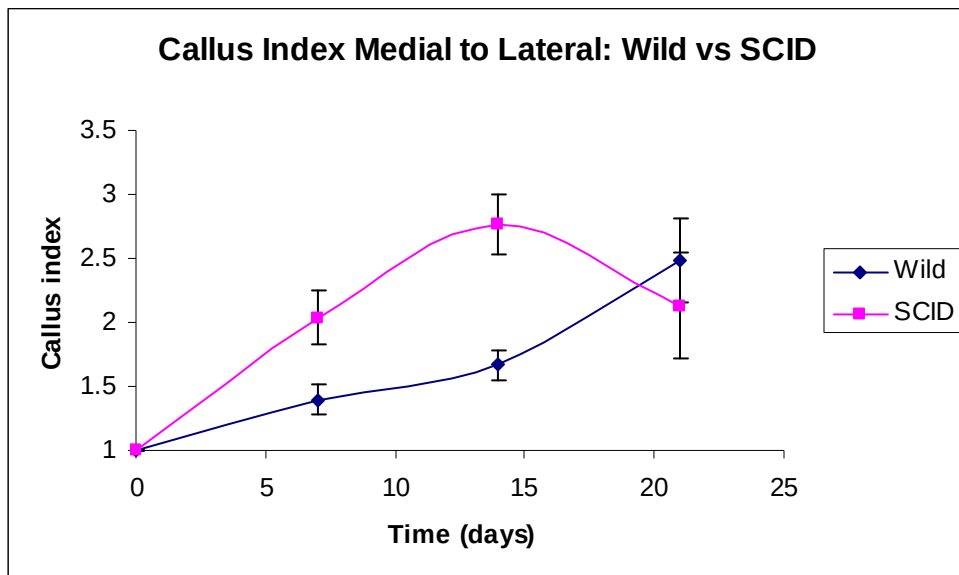


Figure 4.21 Change in medial-lateral callus index with time, during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 95% confidence intervals). Callus index is a ratio: No units.

4.5.3 Relative bone mineral content results

Relative Bone Mineral Content was measured as described in Section 2.7.4. The mean results from the six animals and standard deviations are displayed in the table and graph below.

Days post-fracture	7	14	21
RBMC Wild type (Mean $\pm$ 1 SD)	-961.5 +/-72.5	545.7 +/-53.9	888.7 +/-57.7
RBMC SCID (Mean $\pm$ 1 SD)	-356.7 +/-77.6	1406.8 +/- 191.6	1996.2 +/-101.9
P value	P=0.004 (Non parametric: M-W U Z = 2.88)	P=0.004 (Non parametric: M-W U Z = 2.88)	P=0.004 (Non parametric: M-W U Z = 2.88)

Table 4.6 Results of Relative Bone Mineral Content measurement during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

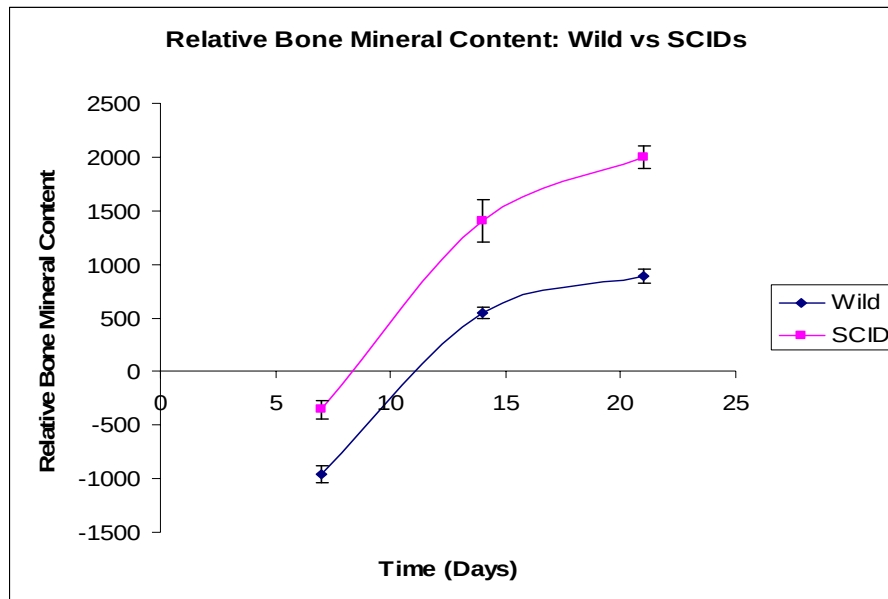


Figure 4.22 Change in Relative Bone Mineral Content (of callus relative to normal bone) with time, during fracture repair in wild type mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 95% confidence intervals). RBMC is a ratio: No units.

4.5.4 Summary: Radiographic assessment

Radiographic analysis of fracture healing in the two strains revealed a significant increase in the amount of callus as measured by callus area and callus index at days 7 and 14 post fracture in the SCID animals, although the difference in these parameters was no longer significant at day 21. This is indicative of the SCID strain having progressed further along the healing pathway and supports earlier findings in this chapter. Callus density which is an indication of maturity of the regenerate was calculated *via* relative bone mineral content as described by Li *et al.* (Murnaghan, McIlmurray *et al.* 2005). This was significantly higher in the SCID strain compared to wild types at all time points.

It is important to note that both callus area and index and relative bone mineral content calculations correct for the normal bone dimensions and density thereby controlling for these between the strains.

4.6 Bone mineral density assessment

The bone mineral density of the whole tibia of both strains was assessed at day 21 post fracture as detailed in Section 2.8 for six animals in each strain. The contralateral, unfractured tibia was also assessed for bone mineral density measurements, to assess for differences between the strains in normal bone. The results obtained were bone mineral density measured in grams per millilitre. Results were also obtained for water content and organic content and these were expressed as a function of the original bone volume, to account for differences in size.

4.6.1 Bone mineral density results

The following table and graph show the results for bone mineral density

	Bone mineral density (g/ml) Mean $\pm$ 1 SD
Wild Type – fractured	0.154 +/- 0.05
SCID – fractured limb	0.289 +/- 0.05
Wild Type – unfractured limb	0.188 +/- 0.06
SCID – unfractured	0.199 +/- 0.03

Table 4.7 Bone mineral density results. Values are shown for both the fractured and unfractured limb in both strains

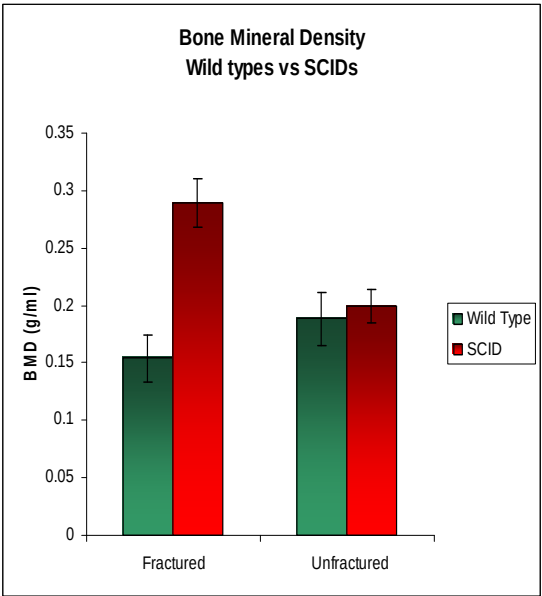


Figure 4.23 Graphical representation of bone mineral density results. Error bars are standard error of the mean

Statistics for BMD: Data normally distributed; Shapiro-Wilk W: 0.954152885, P = 0.3559. Statistical test used: 1 way ANOVA revealed a difference in the bone mineral density between the fractured limbs of wild type and SCID animals, F=7.32 with *post hoc* Scheffé test showing P=0.005. There was no significant difference between the unfractured limb, P=0.98.

4.6.2 Water content results

	Water content (g/ml) Mean $\pm$ 1 SD
Wild Type – fractured limb	0.689 +/- 0.11
SCID – fractured	0.685 +/- 0.10
Wild Type – unfractured limb	0.664 +/- 0.27
SCID – unfractured	0.832 +/- 0.42

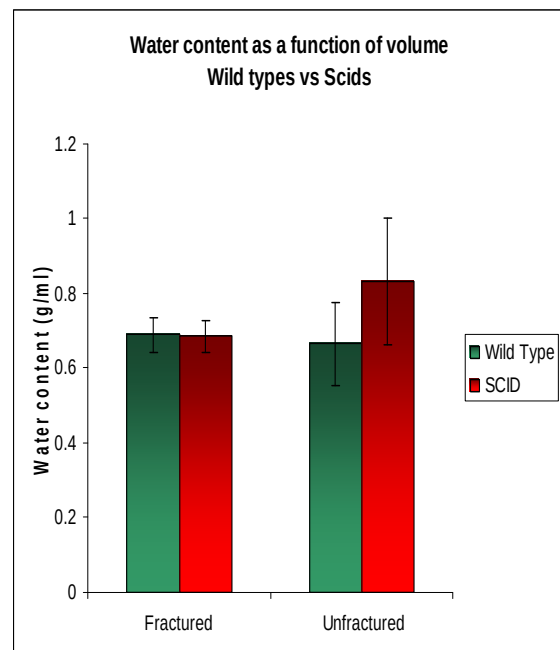


Table 4.8 Water content results. Values are shown for both the fractured and unfractured limb in both strains

Figure 4.24 Graphical representation of water content results. Error bars are standard error of the mean

Statistics for water content results: Data normally distributed; Shapiro-Wilk W: 0.955118092, P = 0.3722. Statistical test used: 1 way ANOVA revealed no significant difference in the water content between the fractured limbs and unfractured limbs of wild type and SCID animals, F=1.99, P=0.15.

4.6.3 Organic content results

	Organic content (g/ml) Mean $\pm$ 1 SD
Wild Type – fractured limb	0.251 +/- 0.07
SCID – fractured	0.392 +/- 0.05
Wild Type – unfractured limb	0.347 +/- 0.10
SCID – unfractured	0.392 +/- 0.14

Figure 4.25 Graphical representation of organic content results. Error bars are standard error of the mean

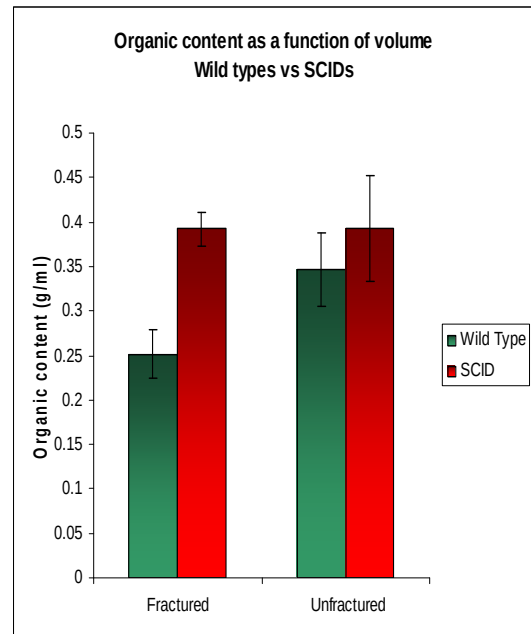


Table 4.9 Organic content results. Values are shown for both the fractured and unfractured limb in both strains

Statistics for organic results: Data normally distributed: Shapiro-Wilk W: 0.971277494, P = 0.7196. Statistical test used: Although the fractured limb of the wild type animal had a lower organic content than the SCID animal, 1 way ANOVA did not reach statistical significance for the difference between the fractured limbs or of the unfractured limbs of wild type and SCID animals, F=2.68, P=0.07. While this is a highly sensitive statistical method for looking for differences across the four groups, a simple independent by groups t test between the fractured limb wild type and SCID (*i.e.* two groups) shows a significant difference, t=4.3, P=0.0016. This shows that it was important to display the results graphically so as obvious differences were not missed by sensitive statistical testing.

4.6.4 Bone mineral density assessment summary

This data shows that the SCID strain healing fractured tibia at day 21 had a higher bone mineral density and higher organic content compared to the fractured bone of wild types. The water content of fractured tibiae was not significantly different between the two strains. These parameters in unfractured bone showed no significant differences. This data reveals that the SCID strain fractured bone had a significantly increased amount of mineral and organic material. Importantly there was no baseline difference in mineral or organic content in the bone of these two strains (in the unfractured limb), as this would confound the results in fractured bone.

Section three: Biomechanical properties

4.7 Mechanical testing results

The mechanical properties of the fractured tibiae were determined at day 21 post fracture in six SCID animals and six wild type animals as described in Section 2.9. The contralateral (left), unfractured tibiae also underwent mechanical testing to ascertain if there were any differences in the normal bone between the two strains. The results were processed to determine the intrinsic properties of the healing fracture or normal bone. The values were recorded at the point of yield (where the load-deformation curve becomes non-linear).

4.7.1 Typical load-deformation curve

The typical Load-Deformation graph obtained from the mechanical testing is shown in Figure 4.26.

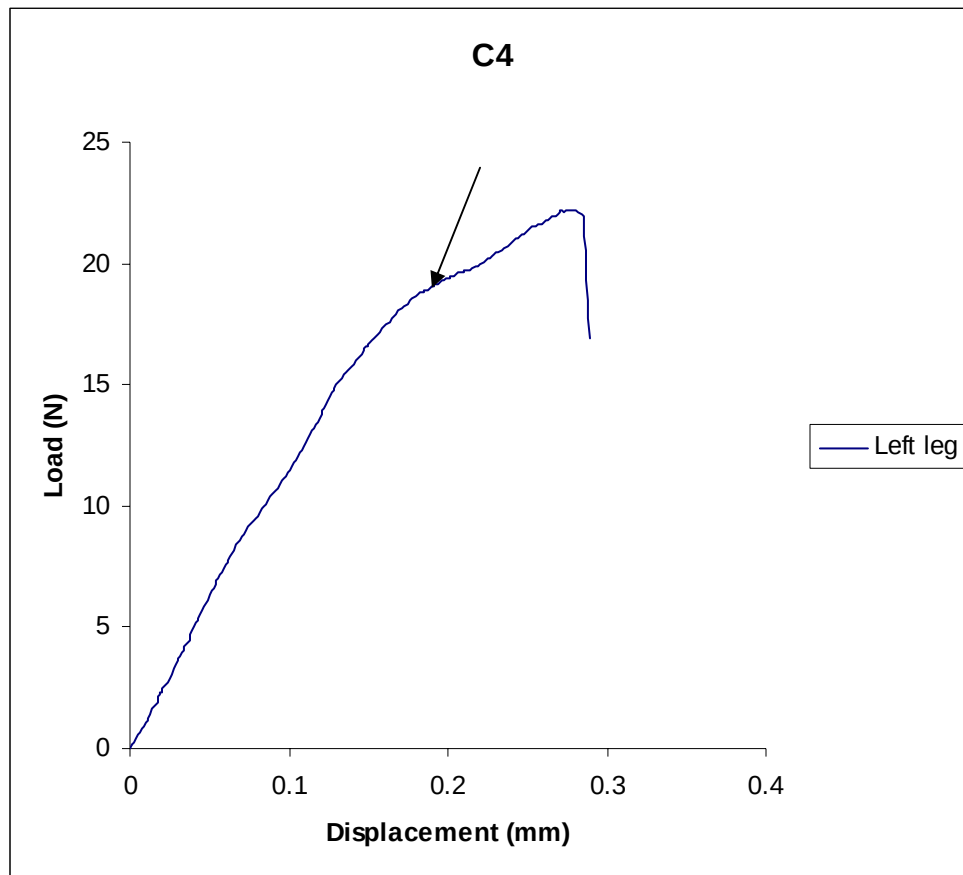


Figure 4.26 Typical Load-Deformation line graph obtained from materials testing machine with 4 point bending of murine tibia. The arrow depicts the point of yield (where the curve becomes non-linear and plastic deformation begins). The values of load and displacement were recorded at this point.

Values were read from the graph displayed above at the point of yield. This gave the displacement in millimetres and load in Newtons. If the load at yield is divided by the displacement at yield a value for stiffness is given (N/mm). However these results are not independent of the size of the callus as the dimensions of the callus are not factored into them – the stiffness calculated above refers to the *extrinsic* properties of the particular bone sampled. Therefore the results were processed with the data for the dimensions of the bone as described in Section 2.9.6 to give the *intrinsic* properties of

the bone sampled. It was thought this would give more consistent and meaningful results. The dimensions of the bone were recorded as detailed in Section 2.9.4. These are applied to the formulae as detailed in 2.9.6 to give the intrinsic properties of the experimental bone.

4.7.2 Results for fractured bone (right tibiae)

Mechanical testing comparing fractured bone between the wild type and SCID strains are displayed in the Table 4.11.

	Cross sectional moment of inertia $I \text{ (mm}^4\text{)}$	Stress at point of yield $\sigma \text{ (MPa)}$	Strain at point of yield $\epsilon \text{ (}\mu\text{strains)}$	Young's Modulus $E \text{ (MPa)}$
Wild type	14.53	4.19	202961	22.15
Mean $\pm$ 1 SD	+/-5.53	+/-0.61	+/-57700	+/-7.33
SCID	7.28	7.07	129054	60.44
Mean $\pm$ 1 SD	+/-2.57	+/-1.46	+/-38499	+/-24.29
P value	P=0.015 (Non parametric; Mann-Whitney U test)	P=0.004 (Non parametric; Mann-Whitney U test)	P=0.04 (Parametric; ANOVA $F=16.9$, <i>post hoc</i> Scheffé test)	P=0.015 (Non parametric; Mann-Whitney U test)

Table 4.10 Intrinsic properties of fractured bone at day 21, comparing wild type and SCID strains

The cross sectional moment of inertia, which is a function of the size of the callus (proportional to (horizontal radius)³) is smaller in the SCID strain. This is statistically significant, reflecting a difference in the distribution of material in the SCIDs. The area of the callus ellipse in the SCID fractured limb was only slightly less than the wild type (SCID mean ellipse area 10.5mm², Wild type mean ellipse area 10.7 mm²) with no statistically significant difference [P=0.54, Mann-Whitney U test]. This is in keeping with the findings from the radiographic studies of callus area and index (Section 4.5). This showed that the callus size and index had peaked and were starting to decrease in the SCID strain at day 21 post-fracture, with no significant difference in radiographic area at day 21 between the two strains, see Table 4.5.

The stress at yield of the fractured limb which is an indication of the intrinsic *strength* of the bone was significantly higher in the SCID strain compared to the wild type, see Figure 4.27.

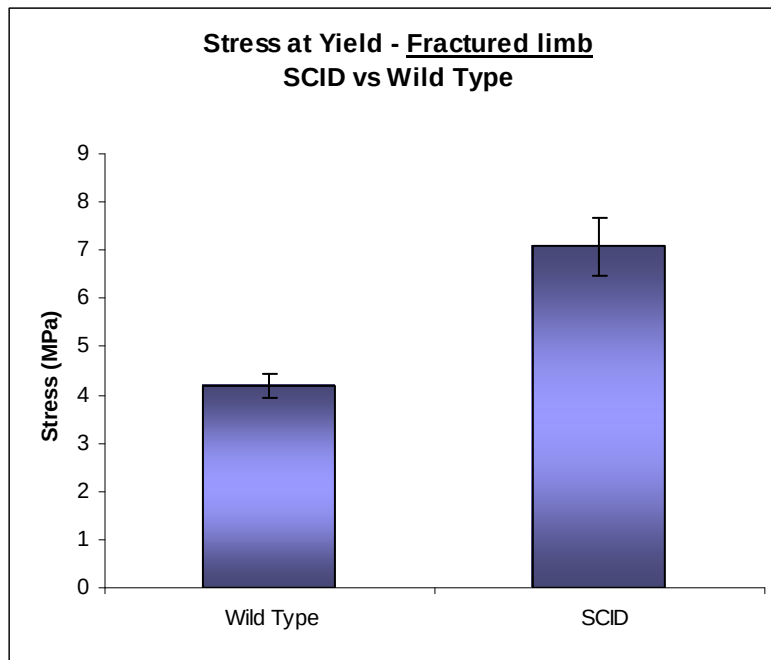


Figure 4.27
Histogram representing stress at yield, comparing fractured bone in wild type to SCID strain. Error bars shown are standard error of the mean.

The strain at the point of yield, which is the intrinsic *deformation* of the bone at this point, was significantly less in the SCID strain compared to the wild type, see Figure 4.28.

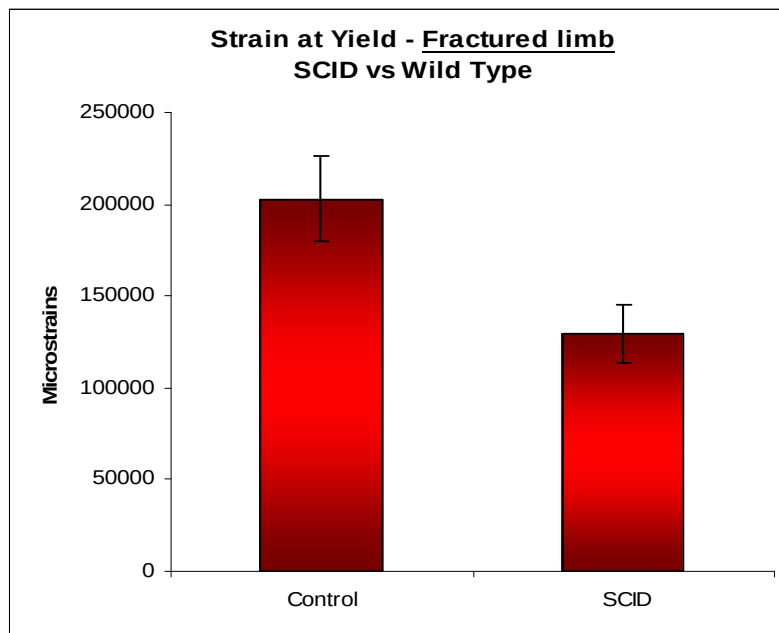
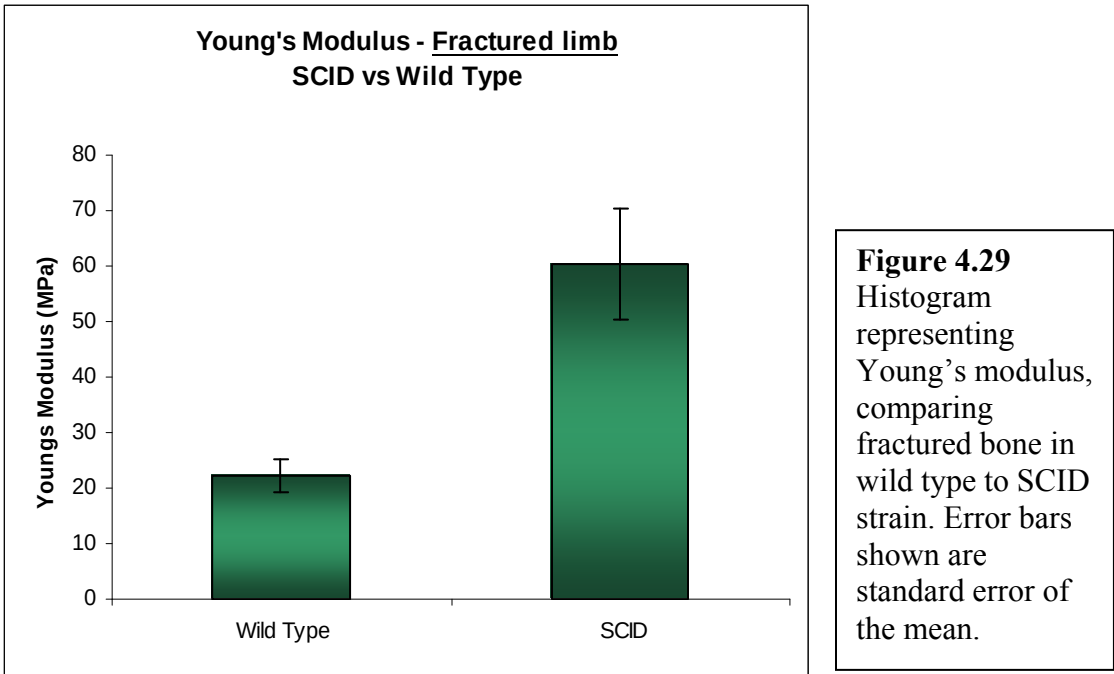


Figure 4.28
Histogram representing strain at yield, comparing fractured bone in wild type to SCID strain. Error bars shown are standard error of the mean.

The Young's modulus, an indication of the intrinsic *stiffness* of the healing bone, was significantly higher in the fractured tibia of the SCIDs, see Figure 4.29.



4.7.3 Results for unfractured bone (left tibiae)

Mechanical testing comparing unfractured bone between the wild type and SCID strains are displayed in the table below

	Cross sectional moment of inertia $I \text{ (mm}^4\text{)}$	Stress at point of yield $\sigma \text{ (MPa)}$	Strain at point of yield $\epsilon \text{ (}\mu\text{strains)}$	Young's Modulus $E \text{ (MPa)}$
Wild type	1.634	14.84	58049	339.4
Mean $\pm$ 1 SD	+/-0.67	+/-3.61	+/-38400	+/-155.6
SCID	1.03	15.74	62642	246.2
Mean $\pm$ 1 SD	+/-0.54	+/-6.57	+/-17061	+/-60.65
P value	P=0.26 (Non parametric ; M-W U Z = 1.12)	P=0.58 (Non parametric; M-W U Z = 0.56)	P=0.99 (Parametric; ANOVA, <i>post</i> <i>hoc</i> Scheffé test)	P=0.15 (Non parametric ; M-W U Z = 1.44)

Table 4.11 Intrinsic properties of unfractured bone at day 21, comparing wild type and SCID strains

In the contralateral, unfractured tibiae, there were no significant differences in stress at yield, strain at yield or Young's modulus; see Figures 4.30, 4.31 and 4.32.

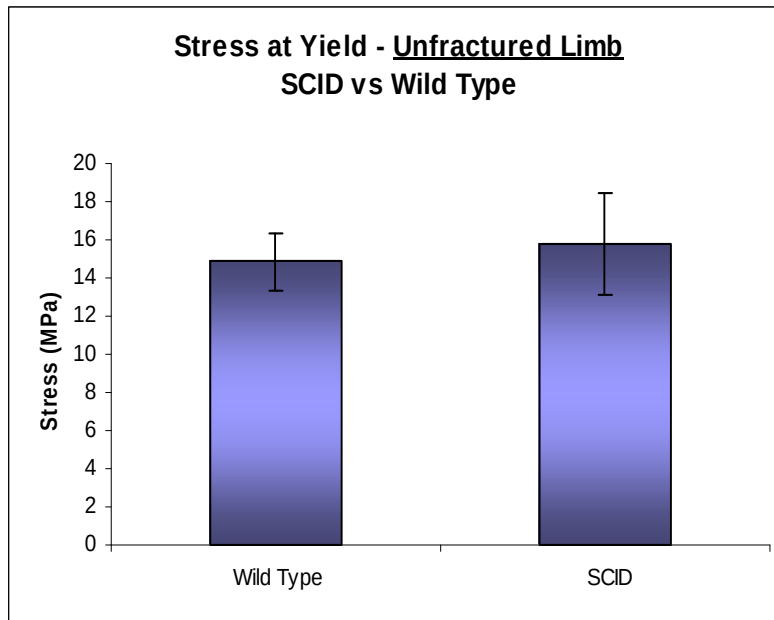


Figure 4.30
Histogram representing stress at yield, comparing unfractured bone in wild type to SCID strain. Error bars shown are standard error of the mean

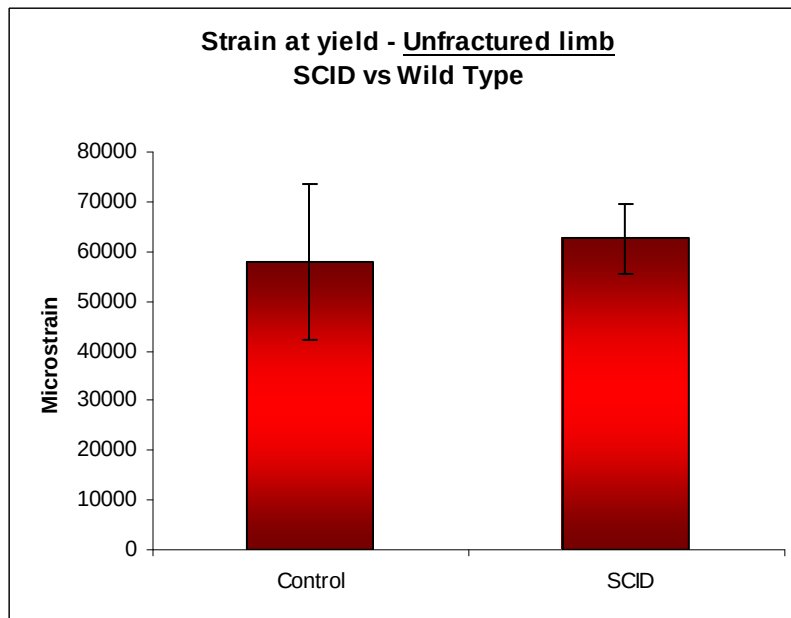


Figure 4.31
Histogram representing strain at yield, comparing unfractured bone in wild type to SCID strain. Error bars shown are standard error of the mean

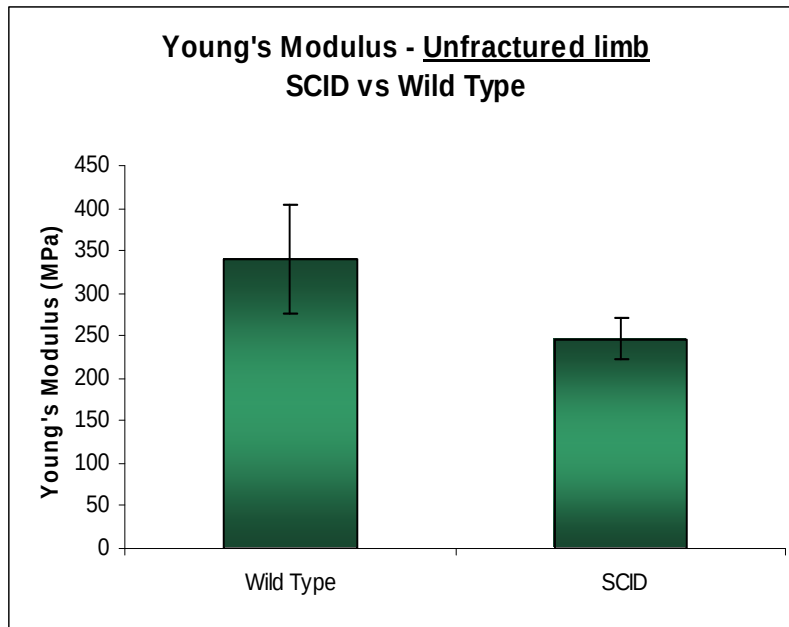


Figure 4.32
Histogram representing Young's modulus, comparing unfractured bone in wild type to SCID strain. Error bars shown are standard error of the mean

In the unfractured bone no differences were found in the intrinsic mechanical properties between the SCID strain and wild types.

4.7.4 Comparison of intrinsic mechanical properties of fractured and unfractured bone

It can be seen from the results in Sections 4.7.2 and 4.7.3 that the intrinsic properties at three weeks of the unfractured bone were quite different to those of the fractured bone. This would be expected as the mechanical testing was carried out at post-fracture day 21, when the fractured bone has entered the remodelling phase but is still a long way from complete repair. The unfractured bone has higher stress at yield values and lower strain at yield values and is therefore acting as a stronger but more brittle material, as would be expected. The stress at yield findings for both animal groups is displayed graphically below. See Figure 4.33.

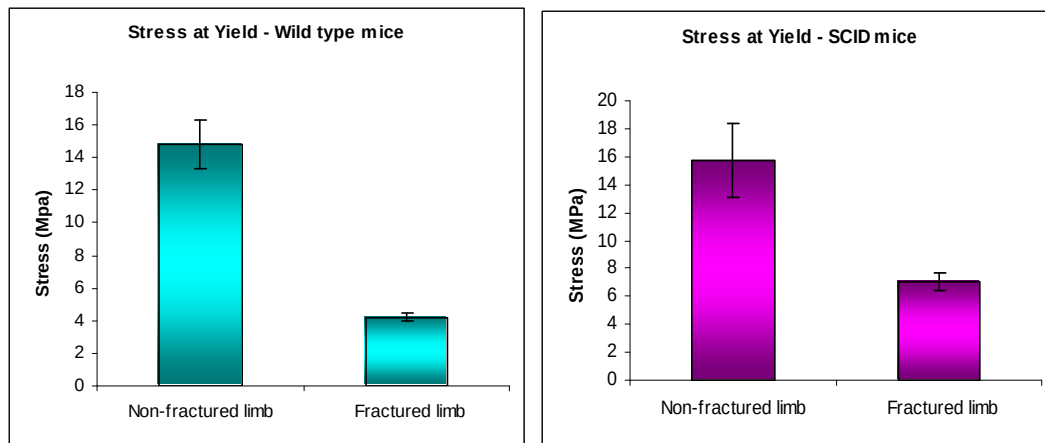


Figure 4.33 Stress at yield in both control and SCID strains, comparing unfractured limb to fractured limb. Error bars shown are standard error of the mean.

The unfractured bone also had a significantly higher Young's modulus compared to the fractured bone and therefore was intrinsically stiffer than the healing, fractured bone.

The Young's modulus findings for both strains of animal is displayed graphically below; Figure 4.34.

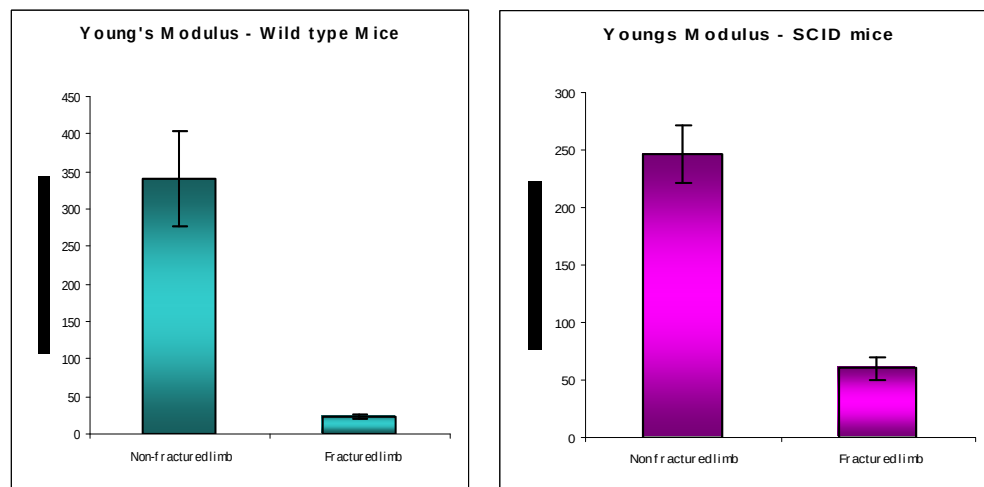


Figure 4.34 Young's modulus in both control and SCID strains, comparing unfractured limb to fractured limb. Error bars shown are standard error of the mean.

It can be seen that the SCID fractured limb results approach the values of the unfractured limb more than the wild type which again reveals that the SCID fractured bone is showing increased strength and stiffness. Figure 4.35 shows the relative values of the Young's modulus for each experimental group.

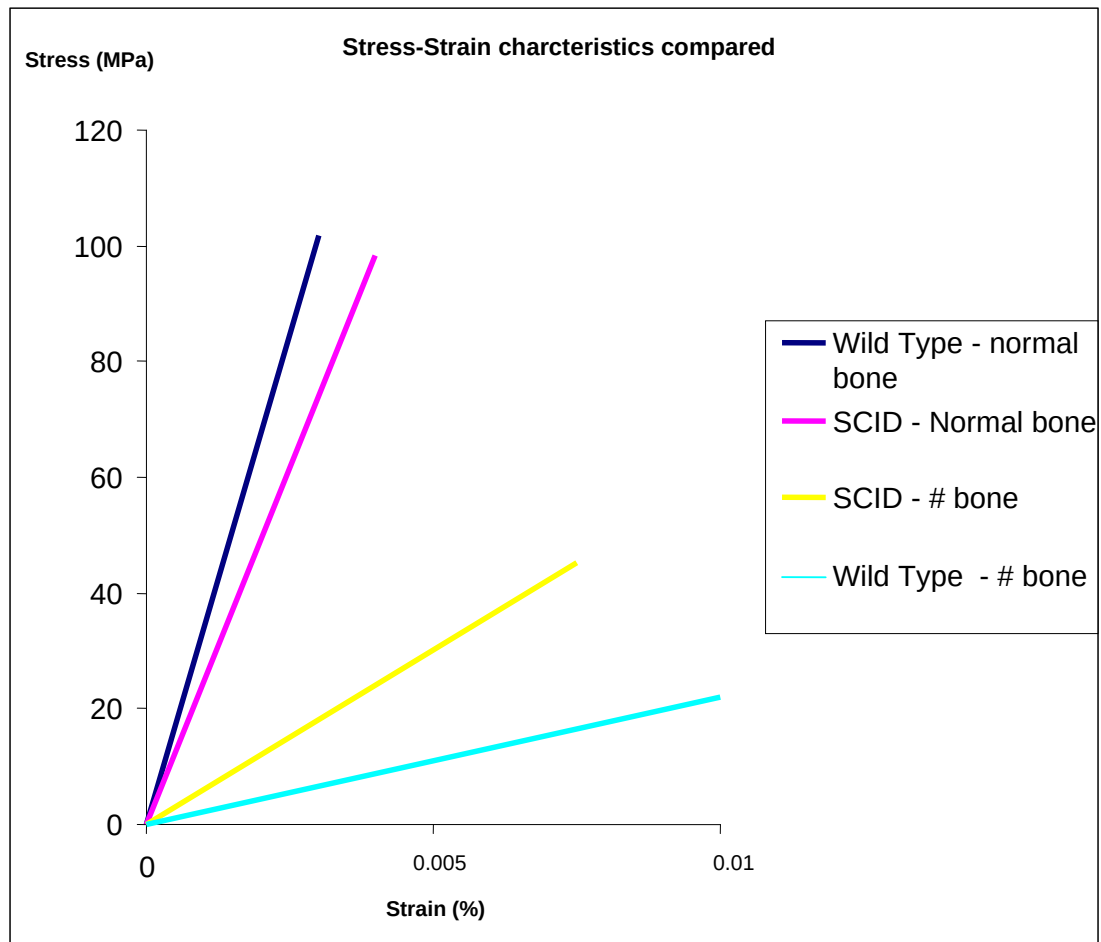


Figure 4.35 Graph which compares the stress-strain characteristics of each experimental group

4.7.4 Mechanical testing summary

Mechanical measurements have revealed an enhancement of fracture healing in the SCID mouse strain compared to the control strain, as stress at yield and Young's modulus were significantly higher in SCID mice than controls at day 21 post fracture, and were closer to the parameters of normal bone compared to the wild type fractured bone. There was no difference in the mechanical properties of unfractured bone between the strains.

4.8 *Post hoc* power assessment

As described in Section 2.11.5.2 the power of the studies completed was calculated *post hoc* based on the results obtained. The table below gives examples of these calculations:

	Bone Mineral Density	Young's Modulus	Stress at Yield	RBMC at day 7	AP index at day 14	Cartilage at day 7	New bone at day 21
Number of Groups	4.0	4.0	4.0	2.0	2.0	2.0	2.0
Group Sample Size (N)	6.0	6.0	6.0	6.0	6.0	12.0	12.0
RMSSE	0.82	0.97	0.92	1.32	1.27	1.25	1.29
Noncentrality Parameter (Delta)	12.2	17.0	15.3	10.5	9.7	18.7	20.1
Type I Error Rate (Alpha)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Effect Df	3.00	3.00	3.00	1.00	1.00	1.00	1.00
Error Df	20.0	20.0	20.0	10.0	10.0	22.0	22.0
Critical Value of F	3.09	3.09	3.09	4.96	4.96	4.30	4.30
Power	0.8	0.9	0.9	0.8	0.8	0.9	0.9

Table 4.12 *Post hoc* power analysis for several parameters measured in chapter 4.

As can be seen in the above table, all the parameters measured met the power requirement of 0.8 or above, and this was consistent throughout the studies in this thesis.

4.9 Body weight results

All animals were weighed daily. Mass did not fluctuate more than + / - 1.0g for any animal. The NAWCO remained satisfied with their condition throughout all the experiments.

Chapter 5

Lymphoid reconstitution of SCID mice with allogenic bone marrow: method, assessment and results

5.1 Introduction

5.1.1 Rationale

It has been demonstrated in chapter four that an animal model which lacks the specific immune system underwent accelerated fracture repair. This supported the hypothesis stated in 4.1, “*Lack of the specific immune system will cause perturbation of fracture repair in vivo*”. To ensure this is directly due to a lack of T and B cells, it is important to exclude the following two causes for the acceleration seen in the SCID animal:

- 1) An unrelated genetic aberration in the SCID mutant animal that directly affects and regulates fracture repair. This has not been defined or previously documented.
- 2) Intrinsic enhancement of the innate immune system in the SCID animal causing up-regulation of macrophage or natural killer cell function. This may be responsible for driving the enhancement of bone healing seen.

Enhancement of soft tissue wound healing has been previously documented in nude mice, which also lack a functioning specific immune system (Barbul, Shawe *et al.* 1989). In these experiments, which used skin wounds, the authors reconstituted the nude mice with functioning T and B cells and demonstrated a return of healing parameters to control levels.

To progress the work for this thesis further it was decided to carry out a similar experiment in SCID mice with reconstitution of their specific immune system and to assess the effect on fracture repair. The aim of this was to exclude causes 1 and 2 above as reasons for the enhanced fracture repair seen in chapter four.

The hypothesis for the next set of experiments was:

“Reconstitution of the specific immune system in SCID animals will return fracture healing to the same as controls”

The method and assessment of reconstitution of the specific immune system in SCID mice will be discussed in this chapter and the results will be demonstrated. The results of assessment of fracture repair in these animals compared to wild type controls and unreconstituted SCID controls will be shown in chapter six.

5.1.2 Background of SCID reconstitution

An autosomal recessive mutation in mice causing severe combined immunodeficiency (SCID) was first reported in 1983. This disease was already recognised in humans in whom the differentiation of both T and B lymphocytes was severely impaired. Affected infants are highly susceptible to recurring infections of viruses, fungi and bacteria and invariably die within two years of birth. Prior to the murine mutation, SCID had only been observed in animals in Arabian foals. The SCID mouse represented a new model to investigate how lymphoid differentiation may be impaired in SCID and regulated normally (Bosma, Custer *et al.* 1983). Investigation of these SCID mice revealed a few non-functional T cells in thymus and spleen. However these cells seemed highly disposed to neoplasia as thymic T-cell lymphomas were observed in 41 of 269 mice. No pre-B or B cells could be identified and cells of the myeloid lineage appeared normal (Custer, Bosma *et al.* 1985).

Before the discovery of SCID mice, allogenic mouse chimeras (mouse which receives and incorporates immunologically and genetically different tissue) had been developed to study complex immune cell interactions *in vivo*. Chimeric mice were originally

produced by combining the eight-cell stage embryos of two animals and allowing them to develop in the uterus of a pseudopregnant female (Mintz and Silvers 1967). A later technique used adult mice that had been irradiated lethally and repopulated with allogeneic bone marrow cells (von Boehmer, Hudson *et al.* 1975). One disadvantage of this method was that host haematopoietic and stromal cells were, on many occasions, damaged by the irradiation, often confounding the results.

The SCID mice thereafter became the ideal hosts for chimerism using allogeneic bone marrow cells without prior irradiation, as these animals lacked both T and B lymphocytes capable of reacting against donor alloantigens (Cowing and Gilmore 1992). Reconstitution of SCID mice was first reported in 1985 (Custer, Bosma *et al.* 1985) and immunologically functional allogeneic chimerism in adult SCID recipients was reported in 1988 (Zinkernagel, Ruedi *et al.* 1988). These mice and the technique of reconstitution have subsequently become widely used in studies of bone marrow transplant and immunological research. Human lymphocytes have been shown in many studies to transfer successively and function normally in response to antigens in SCID mice, showing the same functionality they would in the donor host (Martino, Anastasi *et al.* 1993). Furthermore, these SCID chimeras do not develop graft-versus-host disease, (where the engrafted cells attack the host tissue) as no clinical features of this were found in the study by Cowing and Gilmore (1992).

It was suggested initially by some authors that low dose irradiation (400 Rad) before engraftment of SCID mice should be carried out to permitted full lymphoid reconstitution by syngeneic stem cells (Fulop and Phillips 1986). However, it was subsequently demonstrated by the same authors that the SCID defect also diminishes

the repair of radiation-induced DNA damage in non-immunocompetent cells such as fibroblasts (Fulop and Phillips 1990). Thus, even low dose irradiation could be detrimental to non-lymphoid cells in the SCID host which would almost certainly have an effect on fracture healing, and it was preferable in this study to employ a method for reconstituting unirradiated SCID mice. The method described by Zinkernagel was therefore adopted (Zinkernagel, Ruedi *et al.* 1988).

5.2 Method of reconstitution

5.2.1 Study design

Six Balb/c SCID mice were reconstituted. A further six Balb/c SCID mice and six wild type Balb/c animals were the two control groups. All were male and had a similar weight (+/- 1 gram) at the start of the experiment. The animals were received into the facility at one week old, as reconstitution of lymphoid cells has been shown to be more successful in immature animals (Leibson, Hunter-Laszlo *et al.* 1986). Immunodeficient animals were housed in groups of six and their cages were individually ventilated with clean air and all bedding and feed was sterilised. The temperature was maintained at 15 to 20 degrees Celsius. There were cycles of 12 hours of light and dark every 24 hours. All procedures and husbandry were carried out during hours of light. Animals had one week in the facility to allow acclimatisation and had daily handling before commencement of the experiment.

The study plan was to reconstitute the six SCID mice and assess reconstitution (see 5.3 below) in these mice 10 weeks following reconstitution, when it is thought to be complete (Zinkernagel, Ruedi *et al.* 1988). Reconstitution was compared to wild type

and SCID mice, as controls. The aim was to run the fracture model on these 6 reconstituted mice at 10 weeks post reconstitution (when the animals would be 12 weeks old, the age at which the animals were fractured in chapter 4). Six SCID and six wild type mice also underwent fracture as control groups. Fracture assessment with radiographs, mechanical testing and bone mineral density was carried out on all 18 animals at 21 days post fracture. To keep the use of animals to a minimum, only these assessment measures would be carried out in the first instance. In the light of the results further animals could be reconstituted to allow histological and histomorphometric measurements to be undertaken if necessary. The controls in this study also allowed confirmation of the findings in chapter 4, which compared fracture repair between SCID mice and wild types.

5.2.2 Bone marrow harvest and reconstitution

One wild type Balb/c mouse underwent schedule 1 euthanasia. The femora and tibiae were removed under sterile conditions and placed in sterile phosphate buffered saline (PBS). These were taken immediately to a sterile tissue culture hood. They were placed in a flat sterile dish in a small amount of PBS. The muscle and all other soft tissues were stripped from the bone. Both ends were cut off the bone to expose the marrow cavity. 1 ml of 10% Dulbecco's fetal calf complete media (see Appendix 5) was used to flush through the marrow cavity of each of the 4 bones. The marrow was collected in a sterile universal container. The complete media was used to maintain cellular viability at a maximum throughout the reconstitution process. A 10 μ l sample was then taken for cellular counting and diluted 1:10. A 10 μ l sample of this diluted sample was placed in a

haemocytometer to count cell numbers and therefore calculate the total number of cells that had been harvested. Trytan blue was then added to the sample in the haemocytometer to evaluate cell death and determine the number of live cells attained. Cellular death was less than 1%. 35×10^6 cells were obtained. This allowed reconstitution of more than 5×10^6 cells into each animal. The universal container was then spun in a centrifuge at $300 \times g$ for three minutes to produce a pellet of cells. This was taken immediately to the animal facility. The pellet of cells was reconstituted in normal saline at 37°C to give six samples of 0.5mls of normal saline each containing 5×10^6 bone marrow cells. Each of the six SCID animals were then immobilised by scruffing, had their abdomens cleaned with alcohol and the bone marrow was injected into the peritoneal cavity. Sterility of the bone marrow reconstitute was maintained throughout collection and transfer. The animals were kept in individually ventilated cages for the next 10 weeks. They were monitored as detailed in Chapter 2 and additionally were monitored for signs of graft versus host disease, such as rash, dry mucosal membranes, jaundice and breathing difficulties. At 10 weeks post reconstitution they were assessed for the presence of a functioning specific immune system, as detailed in the next section.

5.3 Assessment of reconstitution

5.3.1 Introduction

The aim of this part of the experiment was to assess if a functioning specific immune system had been obtained in the reconstituted animals. It is known that cellular reconstitution takes place, not only in the recipient's bone marrow cavity, but also in

peripheral lymphoid organs, such as spleen, lymph nodes and the peritoneal cavity (Cowing and Gilmore 1992). However a method of assessment not requiring harvest of tissue was needed as the live animal was needed for the fracture part of the experiment. It is known that the collaborative action of both T and B cells is required for an antibody response. Wild type mice have a baseline level of the immunoglobulin IgG which is produced by B cells when they are activated by CD4⁺ T cells. Therefore the mechanisms that regulate production and homeostasis of serum IgG are under the control of a functioning specific immune system and measuring the level of this is an adequate method to assess reconstitution (Waldschmidt, Panoskaltsis-Mortari *et al.* 2002). There was local experience of this method of assessing reconstitution in SCID mice in the Department of Immunology, University of Edinburgh (Professor Sarah Howie) and advice and assistance with the protocol was kindly given.

5.3.2 Measurement of serum total IgG

Enzyme linked immunosorbent assay (ELISA) is a well established technique for measuring the serum concentration of immunoglobulins. The use of this technique allowed reconstitution to be assessed from a small venous blood sample taken from the live mouse.

5.3.2.1 Collection of serum sample

A blood sample was obtained from the tail vein of each of the 18 mice. Each animal was given a short anaesthetic (as described in Section 2.2.4.2). They were placed on a warming mat. Approximately 1cm of the end of the tail was cut off using a sterile

scalpel (Hoff 2000). The blood drops were collected into an EDTA (ethylenediaminetetraacetic acid) lined container which prevented coagulation of the sample. Approximately 0.1mls of blood was obtained from each animal. The samples were centrifuged at 1500 x g for 15 minutes to separate the plasma from the cellular component of the blood sample. The plasma was pipetted off and used for ELISA determination of serum total IgG.

5.3.2.2 ELISA technique

The principle of an ELISA is to use an enzyme to detect the binding of antigen (Ag) to antibody (Ab). The enzyme converts a colourless substrate (chromogen) to a coloured product, indicating the presence of Ag:Ab binding. An ELISA can be used to detect either the presence of Ags or Abs in a sample, depending on how the test is designed. The seminal work on ELISA was carried out by Engvall and Perlman (1971) and the first described quantitative ELISA was on immunoglobulin G. One of the most useful immunoassays is the two antibody “sandwich” ELISA. This assay determines the antigen concentration in unknown samples. This ELISA is fast and accurate, and if a purified antigen standard is available, the assay can determine the absolute amount of antigen in an unknown sample. The sandwich ELISA requires two antibodies that bind to epitopes (specific macromolecule on the antigen that the antibody binds to) that do not overlap on the antigen. This can be accomplished with either two monoclonal antibodies that recognise discrete sites or one batch of affinity-purified polyclonal antibodies. This ‘sandwich’ technique was employed in the present study (Jeddi, Keusch *et al.* 1997).

To utilise this assay, a “capture” antibody, polyclonal rabbit anti-mouse immunoglobulin G was bound to the bottom of an ELISA microtitre well plate, the solid phase. The samples, (the “antigen”) were then added and allowed to complex with the bound antibody. Unbound products were then removed by washing. A labelled second antibody, the “detection” antibody, polyclonal rabbit anti-mouse immunoglobulin IgG/HRP was allowed to bind to the antigen, thus completing the ‘sandwich’. The assay was then quantitated by measuring the amount of labelled second antibody bound to the matrix, using ortho-phenylenediamine (OPD), a colourimetric substrate. OPD was converted by the horse radish peroxidase (HRP) on the detection antibody to an orange colour. The optical density was determined by a microplate reader set to the specific wavelength for the substrate used. The optical density of serial dilutions of the mouse serum samples in duplicate was determined. Purified mouse IgG of a known concentration was similarly serially diluted to allow the determination of a standard curve, and optical density readings were recorded from this standard for each assay. The concentration of IgG in the serum samples was determined from this standard curve. Each microwell plate had duplicate negative controls (‘blanks’) which had no primary or secondary antibody. The optical density reading from the blanks was subtracted from the optical density results from the rest of the plate. Each plate also had two randomly placed duplicate ‘quality controls’. These were known concentrations of purified mouse IgG which were used to check the accuracy of determination of IgG concentration from the standard curve. The technique used for IgG determination in this study was that described by Reboussou *et al.* (Reboussou, Wijdenes *et al.* 1998) and the protocol was that as laid out in Appendix 6.

Major advantages of this technique are that the antigen does not need to be purified prior to use, and that these assays are very specific. However, one disadvantage is that not all antibodies can be used. Monoclonal antibody combinations must be qualified as “matched pairs”, so that they can recognise separate epitopes on the antigen and not hinder each other’s binding. This was eliminated in this study by using polyclonal antibodies. Competitive ELISA assays can be used when only non-purified primary antibodies are available. This works on the principle of ‘competitive binding’ between the antigen under investigation and a conjugated immunogen to an unlabelled primary antibody. In this case, the more immunogen in the sample or standard, the lower the amount of conjugated immunogen is bound. Substrate is used to develop colour change by the conjugate. This is measured as for a double sandwich ELISA, but it is important to recognise that the curve of the standard curve will be in an opposite direction – *i.e.* more colour equates to a lower concentration (Fleming and Pen 1988).

In this study affinity-purified polyclonal antibodies against mouse total IgG were commercially available and therefore the double antibody sandwich technique was used. See Figures 5.1 and 5.2.

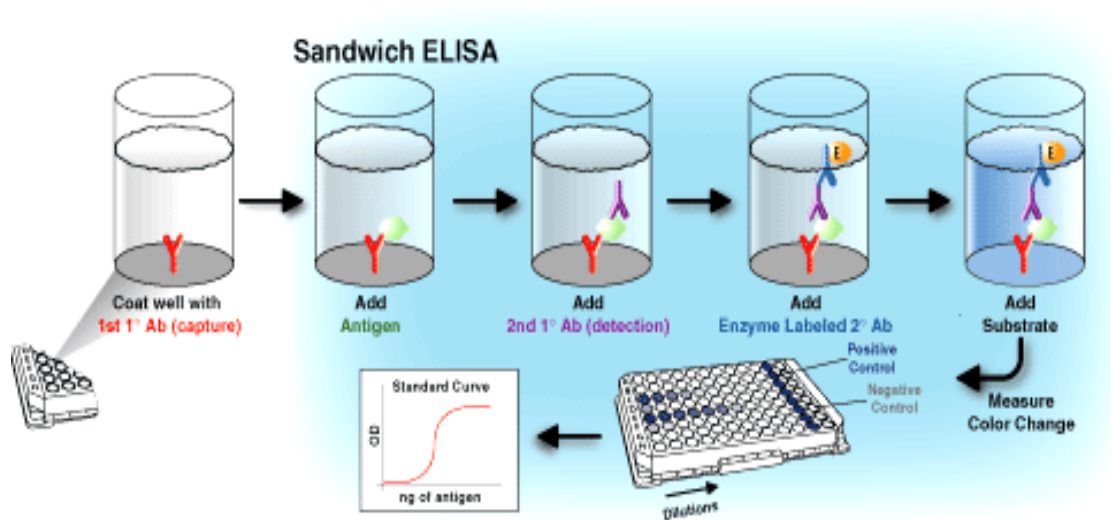


Figure 5.1 Schematic of double antibody sandwich ELISA technique.



Figure 5.2 A microtitre plate (left) and the optical density measuring equipment (right).

Each assay contained known, serially diluted purified mouse IgG concentrations, ‘blanks’ as negative controls, ‘quality controls’ (see text 4.3.2.2) as positive controls, as well as the serially diluted serum samples under investigation. Each plate had samples from one reconstituted SCID mouse, one wild type mouse and one SCID mouse.

5.3.3 Results of reconstitution in SCID mice

Initial adjustments were made to the dilutions used of the purified mouse IgG and of the serum samples to achieve an appropriate standard curve and readable optical densities.

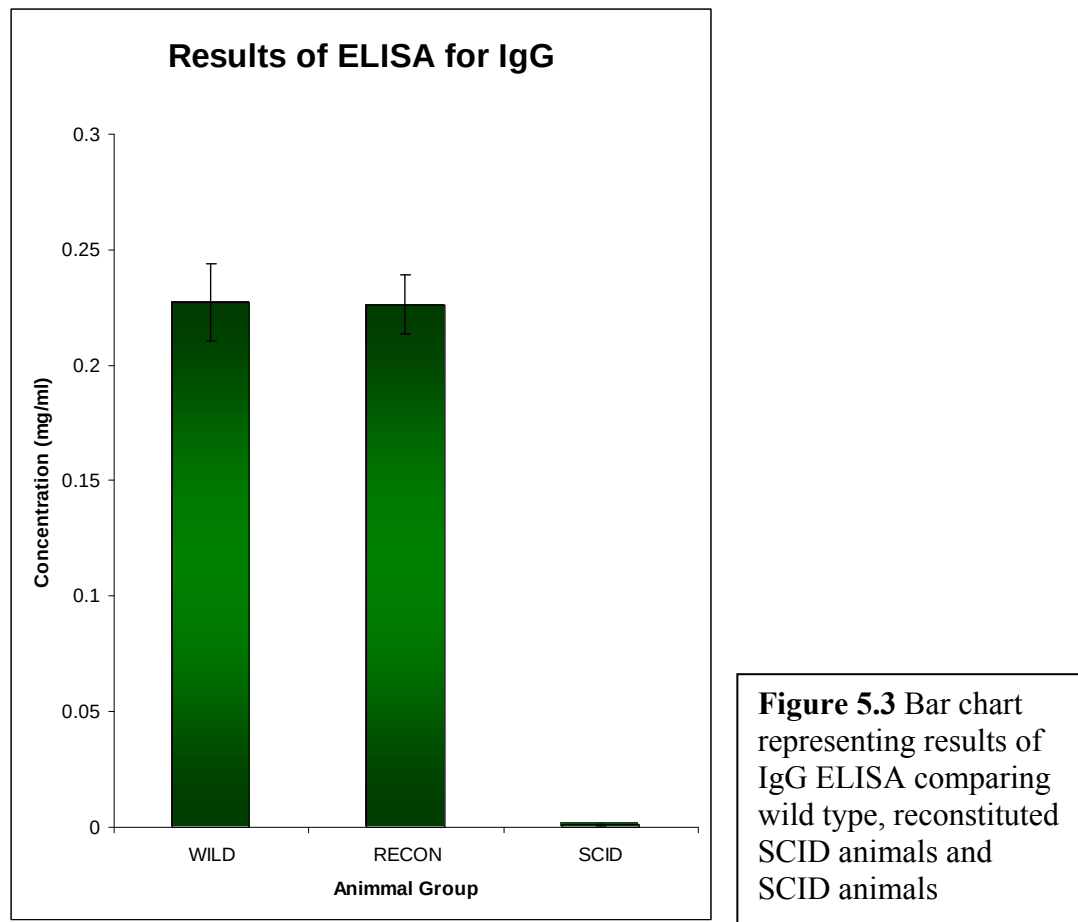
It was determined that dilutions of 1:5000 (200ng/ml) for the purified IgG with doubling of dilutions in the wells below this, was most appropriate. Starting dilutions for the serum sample were determined at 1:100, and were doubled serially below this. This gave the widest range of readable optical densities. Each assay determined IgG concentration in one reconstituted SCID mouse, one wild type mouse and one SCID mouse and after determination of the appropriate dilutions, six assays were run which determined concentrations of IgG for each of the 18 animals in the study.

See Appendix 7 for a typical ELISA plate plan, optical density results read out, formation of standard curve and sample concentration determination to include error determination from quality controls.

The total IgG concentration was determined in all 18 animals and the results are presented in Table 5.1 and Figure 5.3.

		CONCENTRATION of IgG (mg/ml)		
		WILD	RECON	SCID
ANIMAL	1	0.166	0.227	0.00113
	2	0.207	0.188	0.0009
	3	0.259	0.22	0.00089
	4	0.203	0.209	0.00078
	5	0.261	0.28	0.00094
	6	0.266	0.234	0.00085
Mean Conc (mg/ml)		0.227	0.226333	0.000915
Standard deviation		0.040983	0.03082	0.000118

Table 5.1 Results of reconstitution of SCID animals as measured by total IgG concentration. Results of controls, wild types and SCIDs shown. RECON: Reconstituted animals



These results indicate that each of the SCID reconstituted animals had IgG concentrations similar to wild type animals. There were negligible concentrations of IgG in untreated SCID control animals. Statistical analysis was carried out as detailed in Section 2.10. Half normality plots revealed no outlying data points. Normality plots with Shapiro Wilk W test revealed the data was not normally distributed: SW-W = 0.778487589, $P = 0.0008$. The Kruskal-Wallis test revealed unsurprisingly that there were differences between the sample distributions:

Kruskal-Wallis test: $H = 11.38012$, $P = .0034$. Mann Whitney U test was then completed to test for where the differences lay and showed that there no significant difference

between the wild type and reconstituted SCID IgG result ($P=0.87$). However, there were significant differences between the wild types and untreated SCID animals and reconstituted SCID mice and untreated SCID mice ($P<0.05$). The average error as determined from quality controls was less than 1% at 0.46% across all the assays carried out.

5.4 Summary

Successful reconstitution was demonstrated in the SCID mice. The IgG concentrations obtained were closely comparable to previously published murine IgG concentrations (Jeddi, Keusch *et al.* 1997; Waldschmidt, Panoskaltsis-Mortari *et al.* 2002). Although it could not be concluded that T and B cell function was restored to *normal*, the mechanisms that produce and regulate these immunoglobulins come from both these groups of cells and require collaborative effort. Thus there seemed to be functional recovery of the B and T cells in the reconstituted SCID mouse.

The next step in assessing the hypothesis:

“Reconstitution of the specific immune system in SCID animals will return fracture healing to the same as controls”

was to carry out fracture healing assessment in these reconstituted SCID mice, again with wild type and untreated SCID mice as controls. The methods of fracture assessment described in chapter 2 were employed and the results of this are the subject of the next chapter.

Chapter 6

A comparison of fracture healing between wild type mice, SCID reconstituted mice and SCID mice using radiographic, mechanical and bone mineral density outcomes.

6.1 Introduction

Six reconstituted SCID mice were subjected to the fracture model described in chapter two with concurrent control groups consisting of six wild type mice and six untreated SCID mice. The results comparing fracture repair between the two strains at 21 days post fracture are presented in two sections. Section one compares organic measures of fracture repair with radiographic assessment and bone mineral density measurements. Section two compares biomechanical properties using four point bending mechanical testing. The methods as described in chapter two were again used.

Section one: Radiographic assessment and bone mineral density measurements

6.2 Radiographic results

The healing fractures of all three animal groups were assessed radiographically as detailed in Section 2.6. Radiographs were taken at days 7, 14 and 21 post fracture in six animals in each strain.

6.2.1 Callus area results

Callus area was measured by pixilation as described in 2.7.2. The results are displayed graphically below:

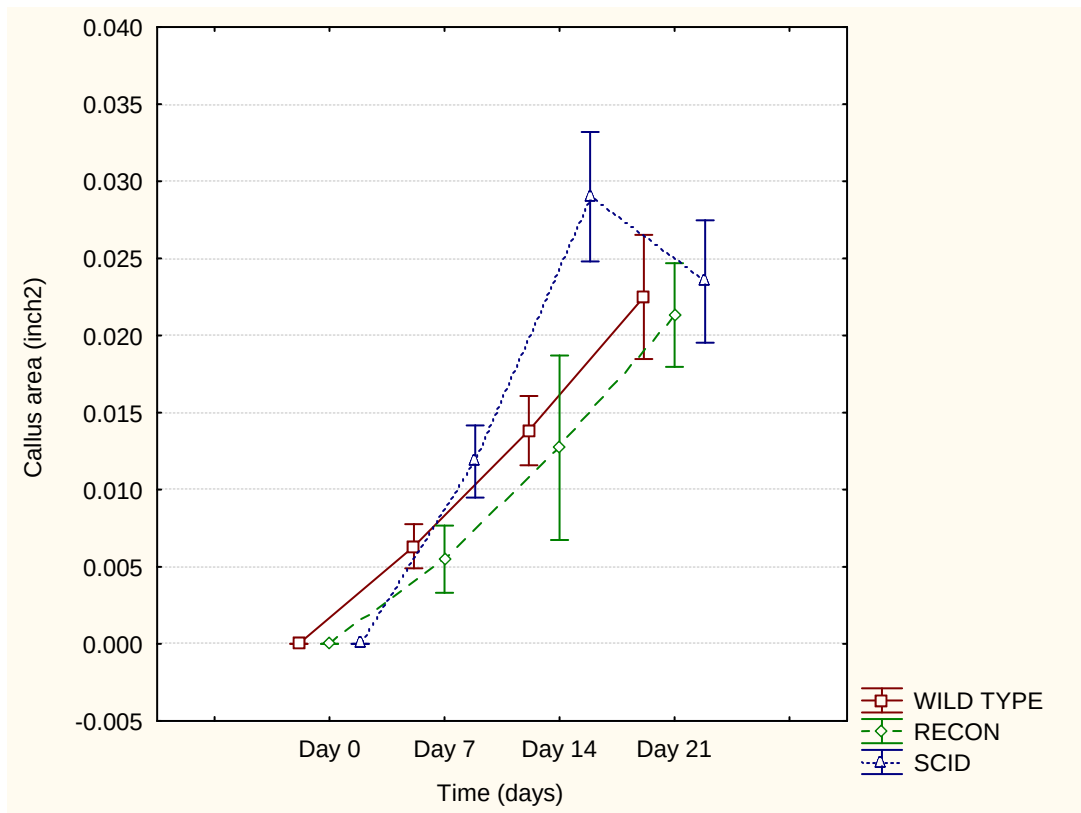


Figure 6.1 Change in callus area with time, during fracture repair in wild type mouse compared to RECON mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 95% confidence intervals).

The mean results from the 3 different groups of animals and standard deviations are displayed in the table below

Days post-fracture	7	14	21
Callus area Wild type Mean $\pm$ 1 SD (inch ²)	0.0063 +/- 0.0013	0.0138 +/- 0.0021	0.0225 +/- 0.0038
Callus area RECON Mean $\pm$ 1 SD (inch ²)	0.0055 +/- 0.00207	0.0127 +/- 0.0057	0.0213 +/- 0.0032
Callus area SCID Mean $\pm$ 1 SD (inch ²)	0.011.8 +/- 0.0022	0.029 +/- 0.0040	0.0235 +/- 0.0038

Table 6.1 Results of callus area measurement during fracture repair in wild type mouse compared to RECON mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

Statistical analysis (as described in Section 2.11) revealed significant differences in the callus area at day 7 between the wild type and SCID, $P=0.0007$ (Scheffé test) and between the RECON and SCID, $P=0.0002$ (Scheffé test) but no differences between the wild type and RECON, $P=0.76$.

At day 14 there were significant differences in callus area between wild type and SCID, $P=0.004$ (Mann-Whitney U test (MW-U)) and between RECON and SCID, $P=0.004$ (MW-U) but again not between the wild type and RECON ($P=0.69$)

At day 21 there were no statistical differences found between any of the groups, $P=0.81$, (1 way ANOVA).

Hence the SCID callus volume increased at a faster rate than either the wild type or RECON and actually started to decrease in size at day 21 which resulted in there being no differences between the groups at day 21. These findings are in keeping with that in Section 4.5.1 and show that the RECON animal is behaving in the same manner as the wild type.

6.2.2 Callus index results

Callus index in both orthogonal planes was measured as described in Section 2.7.3. The mean results from the three groups of animals are displayed in the Tables 6.2 and 6.3 and Figures 6.2 and 6.3.

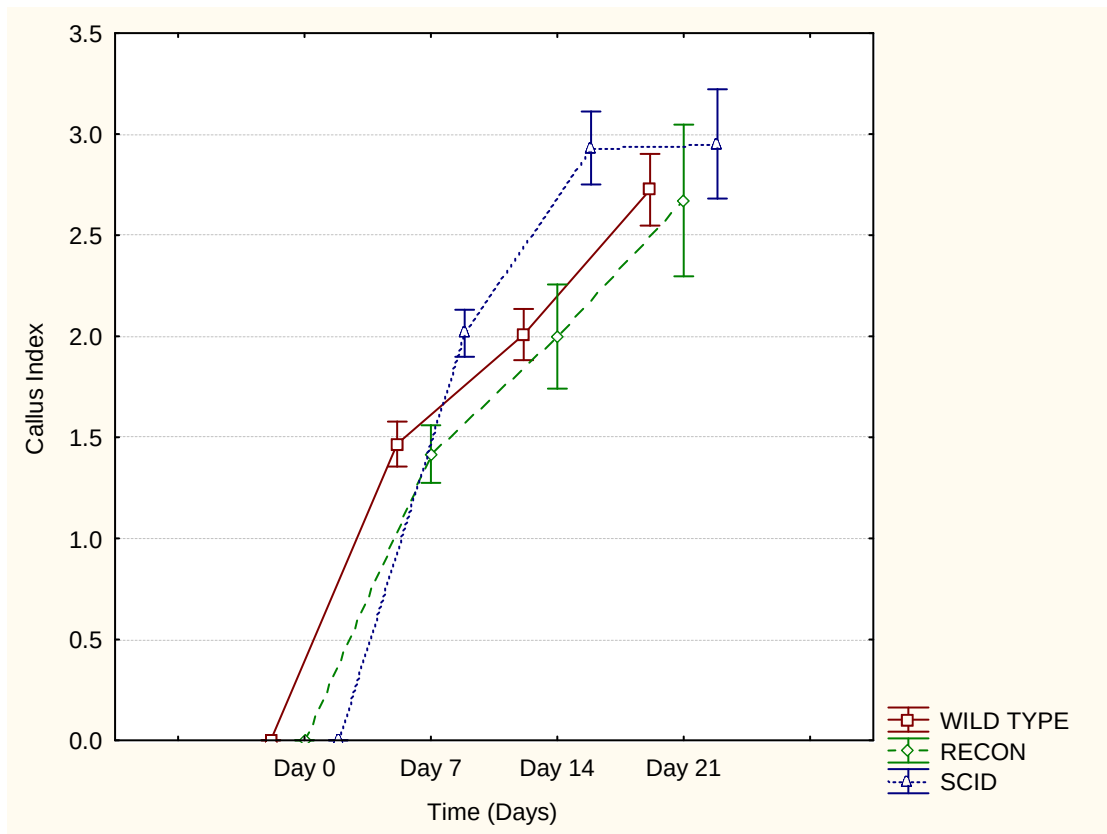


Figure 6.2 Change in antero-posterior callus index with time, during fracture repair in wild type mouse compared to RECON mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 95% confidence intervals). Callus index is a ratio: No units.

Days post-fracture	7	14	21
Callus index AP Wild type. Mean $\pm$ 1 SD.	1.47 +/-0.11	2.01 +/-0.12	2.73 +/-0.17
Callus index AP RECON. Mean $\pm$ 1 SD.	1.41 +/-0.14	1.99 +/-0.25	2.67 +/-0.36
Callus index AP SCID. Mean $\pm$ 1 SD.	2.02 +/- 0.11	2.93 +/-0.17	2.95 +/-0.26

Table 6.2 Results of antero-posterior callus index measurement during fracture repair in wild type mouse compared to RECON mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

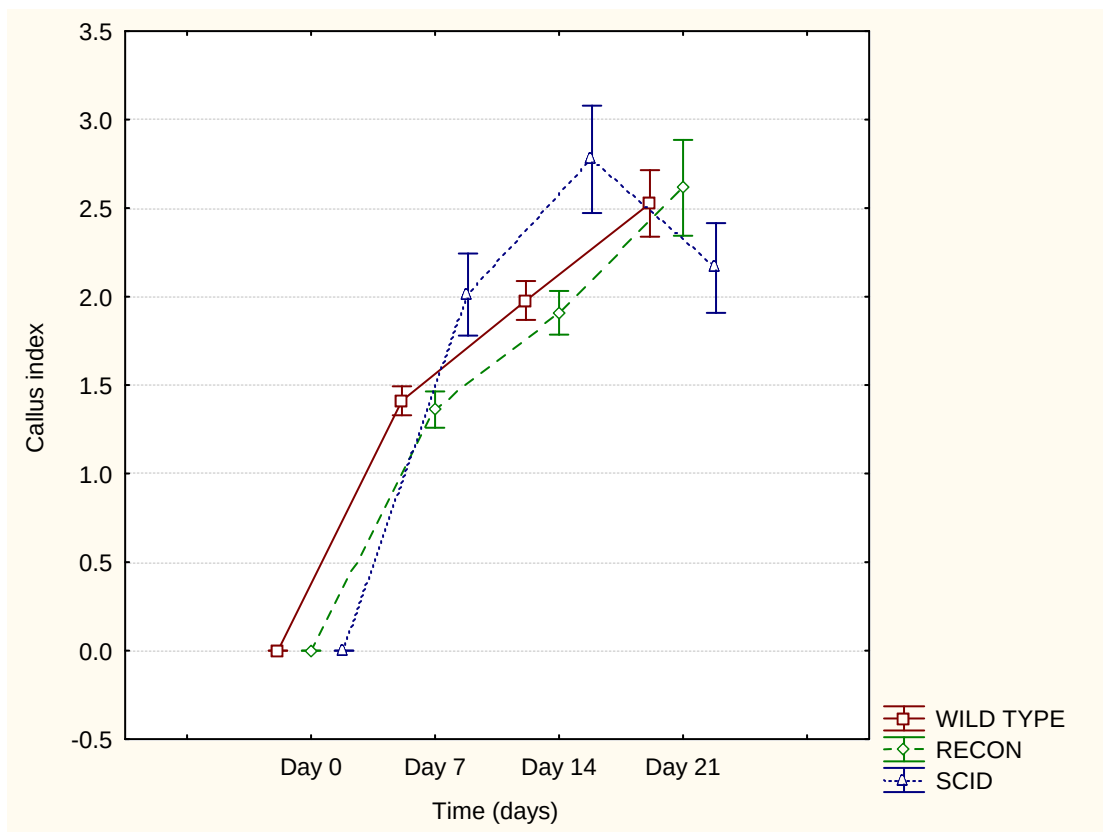


Figure 6.3 Change in medial-lateral callus index with time, during fracture repair in wild type mouse compared to RECON mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 95% confidence intervals). Callus index is a ratio: No units.

Days post-fracture	7	14	21
Callus index ML Wild type. Mean ± 1 SD.	1.41 +/-0.078	1.98 +/-0.10	2.53 +/-0.17
Callus index ML RECON. Mean ± 1 SD.	1.36 +/-0.098	1.91 +/-0.12	2.62 +/- 0.26
Callus index ML SCID. Mean ± 1 SD.	2.01 +/- 0.22	2.78 +/-0.29	2.16 +/-0.24

Table 6.3 Results of medial-lateral callus index measurement during fracture repair in wild type mouse compared to RECON mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

Statistical analysis of callus index revealed similar results to that of area.

At day 7 there were significant differences in both AP and ML between wild and SCID (AP: $P=0.00002$, (Scheffé test), ML: $P=0.004$, (MW-U)) and between RECON and SCID (AP: $P=0.00005$ (Scheffé test), ML: $P=0.004$, (MW-U)) but not between wild and RECON (AP: $P=0.77$, ML: $P=0.33$).

At day 14 there were again significant differences in AP and ML callus index between wild type and SCID (AP: $P=0.00007$ (Scheffé test), ML: $P=0.004$, (MW-U)) and between RECON and SCID (AP: $P=0.00006$ (Scheffé test), ML: $P=0.004$, (MW-U)) but not between the wild type and RECON (AP: $P=0.99$, ML: $P=0.87$).

At day 21 there were no statistical difference in the AP callus index between any of the groups ($P=0.21$, 1 way ANOVA). Interestingly, at day 21 the medial-lateral callus index was significantly *lower* in the SCID compared to wild type ($P=0.04$ (Scheffé test)) and RECON ($P=0.01$ (Scheffé test)).

The callus index results were similar to the results for callus area, showing that the amount of SCID callus increased to a peak and had started to decrease at day 21. The callus index in the wild types still continued to rise up to day 21 and this supports the findings in Section 4.5.2. The reconstituted SCID mice again show similar results to the wild types showing no significant differences to the wild types.

6.2.3 Relative bone mineral content results

Relative Bone Mineral Content (RBMC) was measured as described in Section 2.7.3.

The mean results from the 3 groups of animals and standard deviations are displayed in Table 6.4 and Figure 6.4.

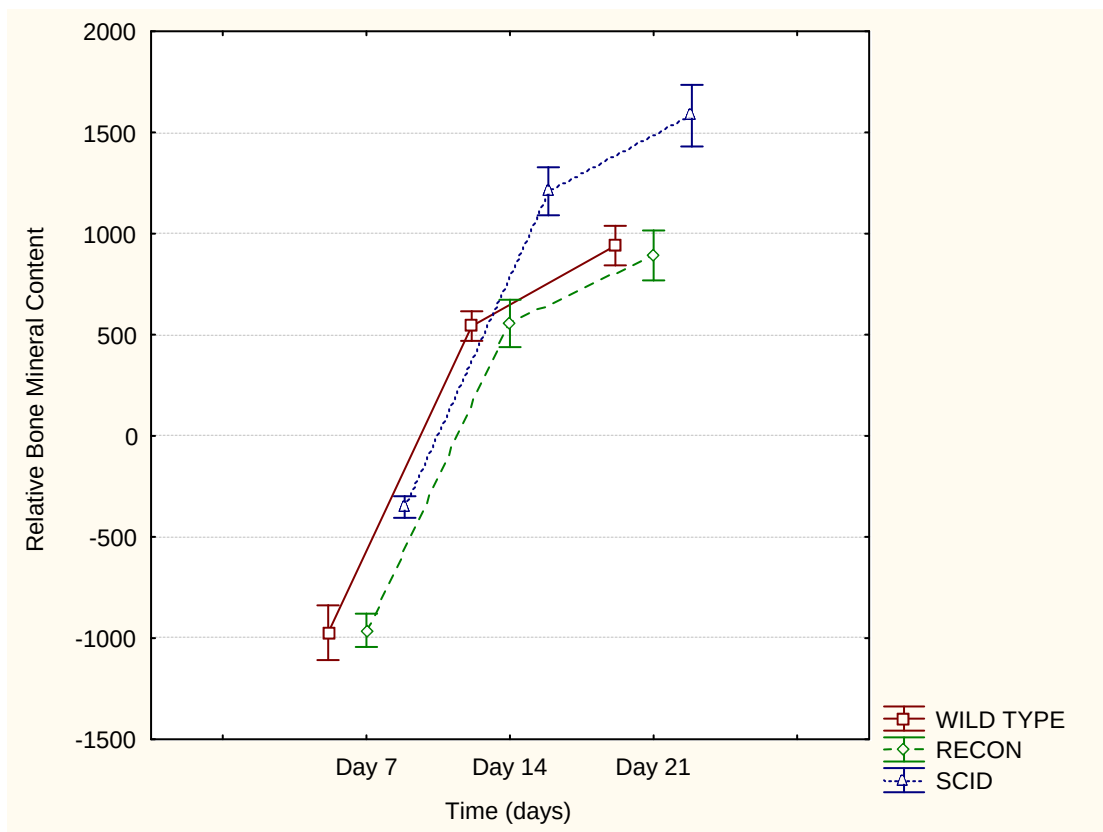


Figure 6.4 Change in Relative Bone Mineral Content with time during fracture repair in wild type mouse compared to RECON mouse compared to SCID mouse at 7, 14 and 21 days following fracture (error bars shown are 95% confidence intervals). RBMC is a ratio: No units.

Days post-fracture	7	14	21
RBMC Wild type.	-973	543	940
Mean $\pm$ 1 SD.	$\pm$ 129	$\pm$ 69.4	$\pm$ 93
RBMC RECON.	-877	555	892
Mean $\pm$ 1 SD.	$\pm$ 117	$\pm$ 111	$\pm$ 118
RBMC SCID.	-361	1209	1583
Mean $\pm$ 1 SD.	$\pm$ 80.4	$\pm$ 113	$\pm$ 145

Table 6.4 Relative Bone Mineral Content measurement during fracture repair in wild type mouse compared to RECON mouse compared to SCID mouse at 7, 14 and 21 days following fracture.

The RBMC was significantly higher at days 7, 14 and 21 in the SCID compared to both the wild type and RECON ($P=0.004$ (MW-U)). There were no significant differences between the wild types and RECON at any time point (Day 7: $P=0.63$ (MW-U); Day 14: $P=0.87$ (MW-U); Day 21: $P=0.64$ (MW-U)).

6.2.4 Radiographic assessment summary:

Radiographic analysis of fracture healing comparing the three groups of animals revealed a significant increase in the amount of callus as measured by callus area and callus index at days 7 and 14 post fracture in the SCID animals compared to both wild type and RECON, although the difference in these parameters was no longer significant at day 21, except for medio-lateral callus index which was significantly *less* in the SCIDs.

Callus density which is an indication of maturity of the regenerate was calculated via relative bone mineral content as described by Li *et al.* (Murnaghan, McIlmurray *et al.* 2005). This was significantly higher in the SCID strain compared to wild types and RECON at all time points, indicating faster mineralisation.

These results indicate that the RECON animals behaved in a similar manner to the wild type animals, having the same callus volume and density at the respective time points. It is important to note that index and relative bone mineral content calculations correct for the normal bone dimensions and adjacent bone density respectively, thereby controlling for these factors between the strains.

6.3 Bone mineral density assessment

The bone mineral density (BMD) of the whole tibia of each of the three animal groups was assessed at day 21 post fracture as detailed in Section 2.8 for six animals in each strain. The contralateral, unfractured tibia was also assessed for BMD measurements, to assess for differences between the strains in normal bone. The results attained were bone mineral density measured in grams per millilitre. Results were also attained for water content and organic content and these were expressed as a function of the original bone volume, to account for differences in size.

The statistical analysis of the results is presented in Appendix 8.

6.3.1 Bone mineral density results

The results for bone mineral density are presented in Figure 6.5.

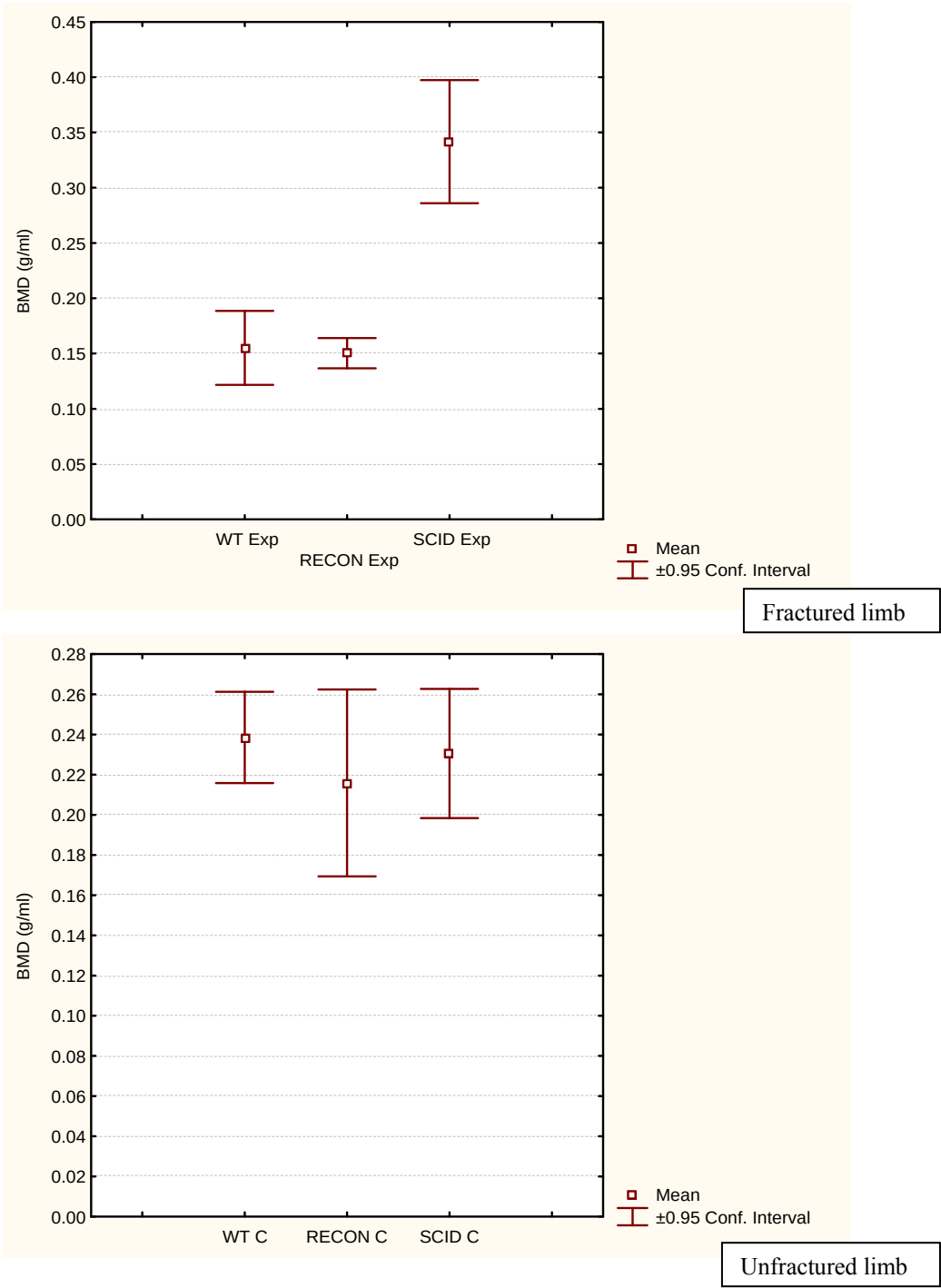


Figure 6.5 Bone mineral density (BMD) (g/ml) comparing wild type to RECON to SCID in both fractured (Exp) and unfractured (C) tibiae

6.3.2 Water content results

The results for water content are presented in Figure 6.6.

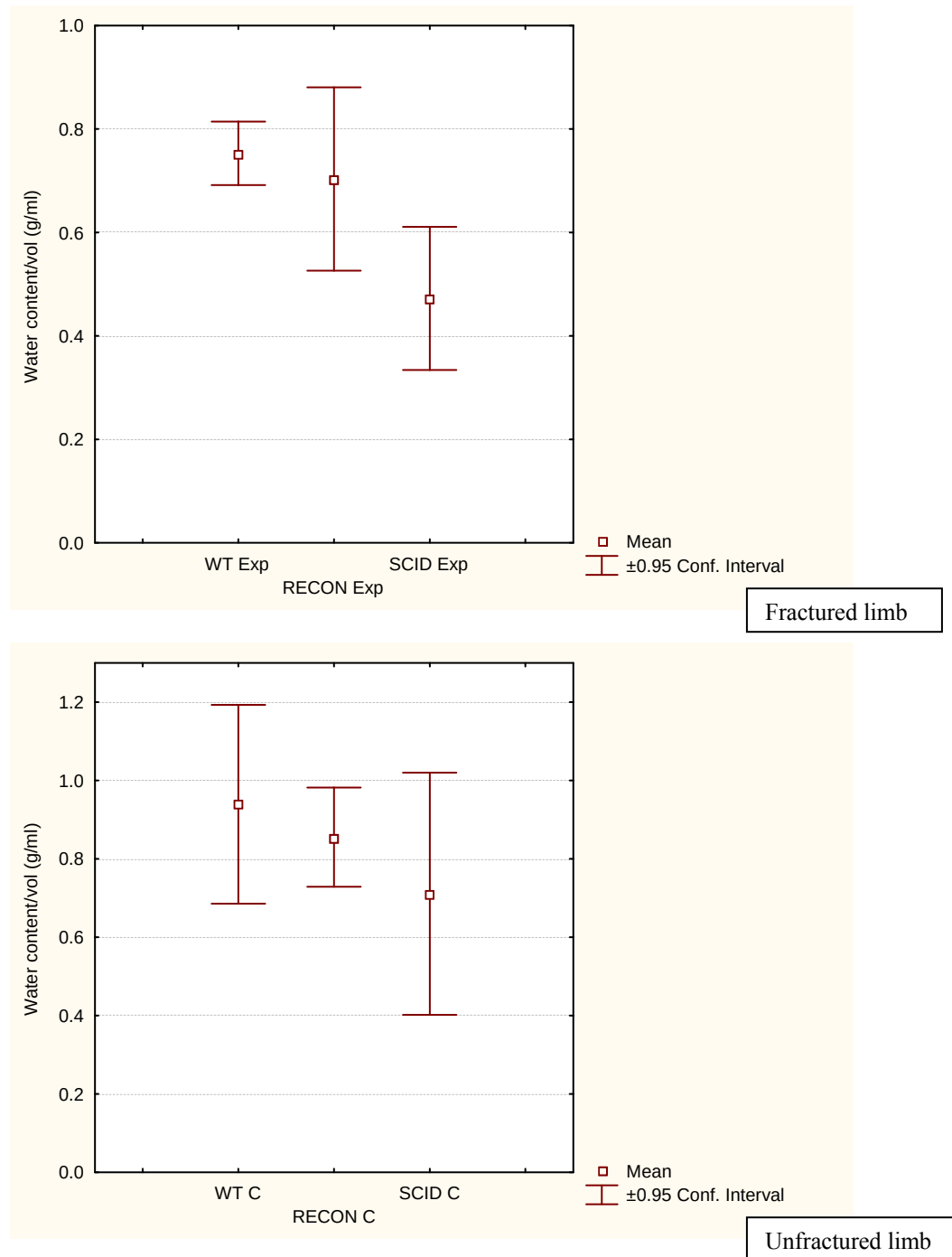


Figure 6.6 Water content (g/ml) comparing wild type to RECON to SCID in both fractured (Exp) and unfractured (C) tibiae

6.3.3 Organic content results

The results for organic content are presented in Figure 6.6.

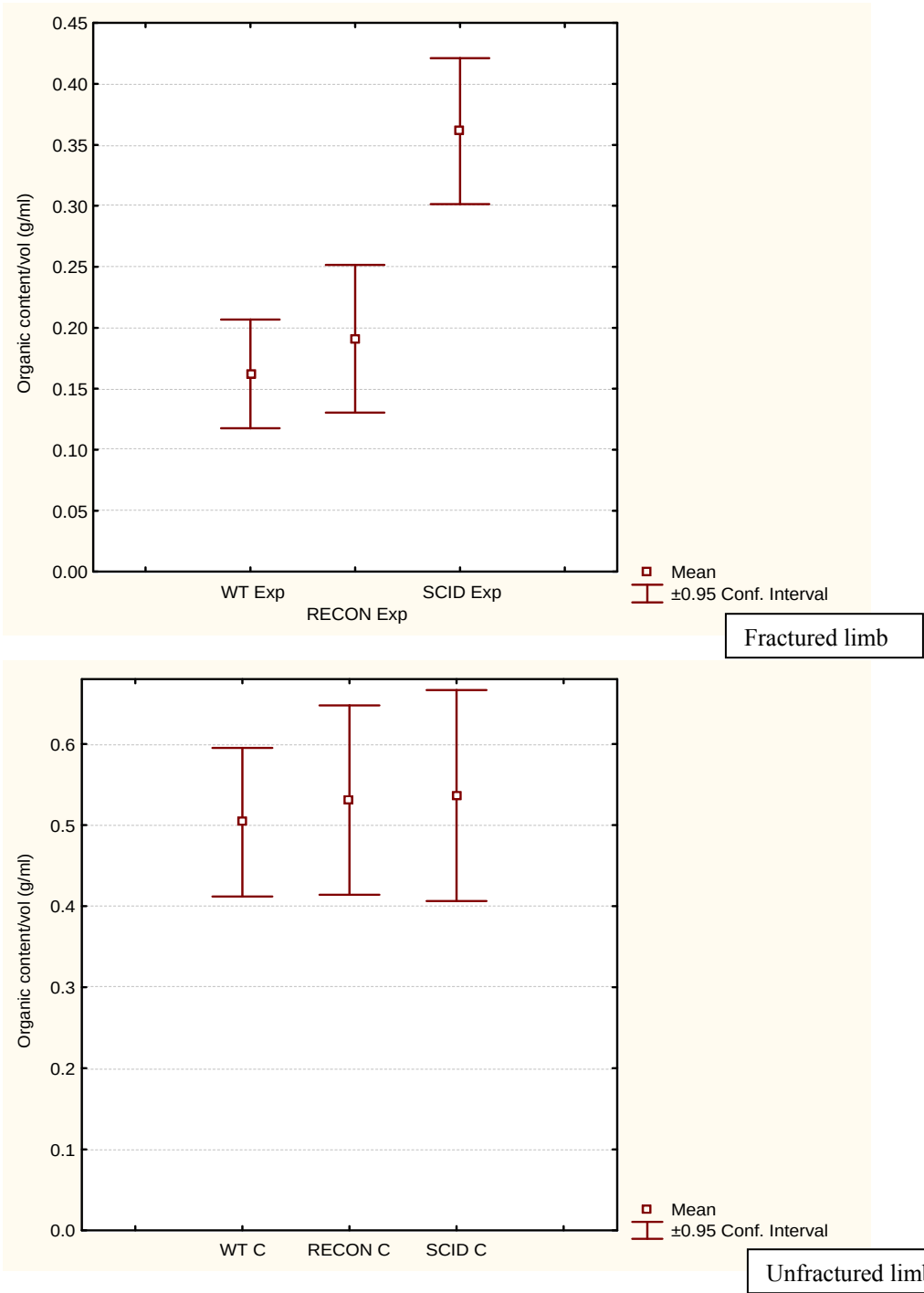


Figure 6.7 Organic content (g/ml) comparing wild type to RECON to SCID in both fractured (Exp) and unfractured (C) tibiae

6.3.4 Bone mineral density assessment summary

The bone mineral density (BMD) results revealed that the fractured limb in the SCID mice had a significantly higher BMD when compared to both the wild type and RECON mice groups, which did not significantly differ from each other (see statistical analysis, Appendix 8). The BMD in the control, unfractured limb showed no significant differences across the three groups. This confirms the results in 4.6.1 and shows the RECON mice group had a similar outcome to the wild type group.

The results for organic content are in keeping with this and demonstrated a higher organic content in the fractured SCID group compared to both the wild type and RECON mice groups, although the difference between SCID and RECON did not quite reach statistical significance ($P=0.053$) but did between SCID and wild type ($P=0.015$). There were no significant differences in the organic content of the unfractured limbs across the three groups.

There were no significant differences found in water content between fractured limbs of all three groups or between unfractured limbs of all three groups.

The BMD and organic content results corroborate the results in Section 4.6 and show that the RECON mice group to be behaving in a similar manner to wild type, with regard to these measurements.

Section two: Biomechanical properties

6.4 Mechanical Testing Results

6.4.1 Introduction

The mechanical properties of the fractured tibiae were determined at day 21 post fracture in six animals in each of the three groups as described in Section 2.9. The contralateral (left), unfractured tibiae also underwent mechanical testing to ascertain if there were any differences in the normal bone between the three groups. The values gained were processed as described in Section 2.9.7 to determine the intrinsic properties of the healing fracture or normal bone. As previously discussed, the values shown are at the point of yield (where the load-deformation curve becomes non-linear).

6.4.2 Results of mechanical testing

The results of mechanical testing in the three strains are presented in the Table 6.5.

Independent variable	Stress at Yield Means	Stress at Yield Std.Dev.	µStrain at Yield Means	µStrain at Yield Std.Dev.	Youngs Means	Youngs Std.Dev.
WT Exp	4.44409	0.615853	203362.5	42442.38	21.0829	5.1076
RECON Exp	4.06395	0.592104	196289.3	44745.54	21.4516	5.0587
SCID Exp	7.21571	1.002827	105847.9	13050.72	68.5404	9.1921
WT C	15.71989	3.736579	44455.7	12249.95	338.1325	43.9321
RECON C	13.05533	2.545526	40629.5	11799.21	330.8397	55.9551
SCID C	13.90439	0.965036	47344.9	16902.04	331.6687	43.7843

Table 6.5 Intrinsic properties of fractured and unfractured bone at day 21, comparing wild type, RECON and SCID strains

6.4.2.1 Results for stress at yield

The results for stress at yield in fractured bone are presented in Figure 6.8 and in unfractured bone in Figure 6.9.

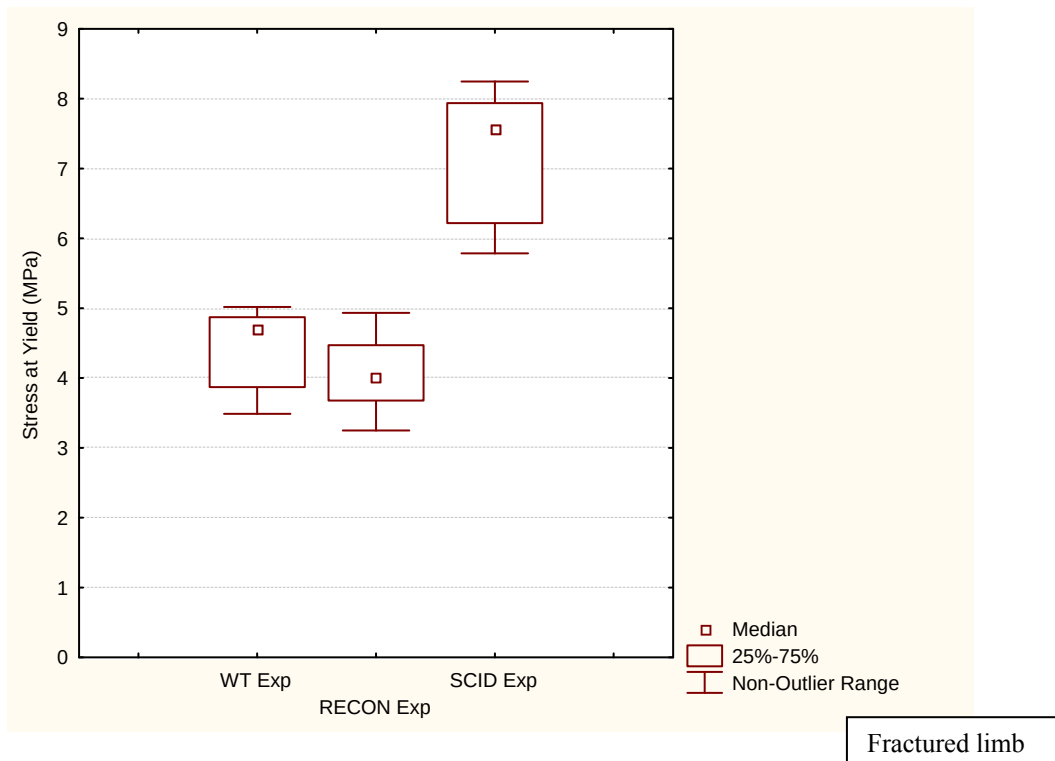


Figure 6.8 Box plot representing stress at yield in fractured bone comparing wild type, RECON and SCID strains

The SCID group had significantly higher stress at yield (which is an indication of the intrinsic *strength* of the bone) than both the RECON and wild type groups, $P=0.004$ for both (MW-U) while there was no significant difference between wild type and RECON groups.

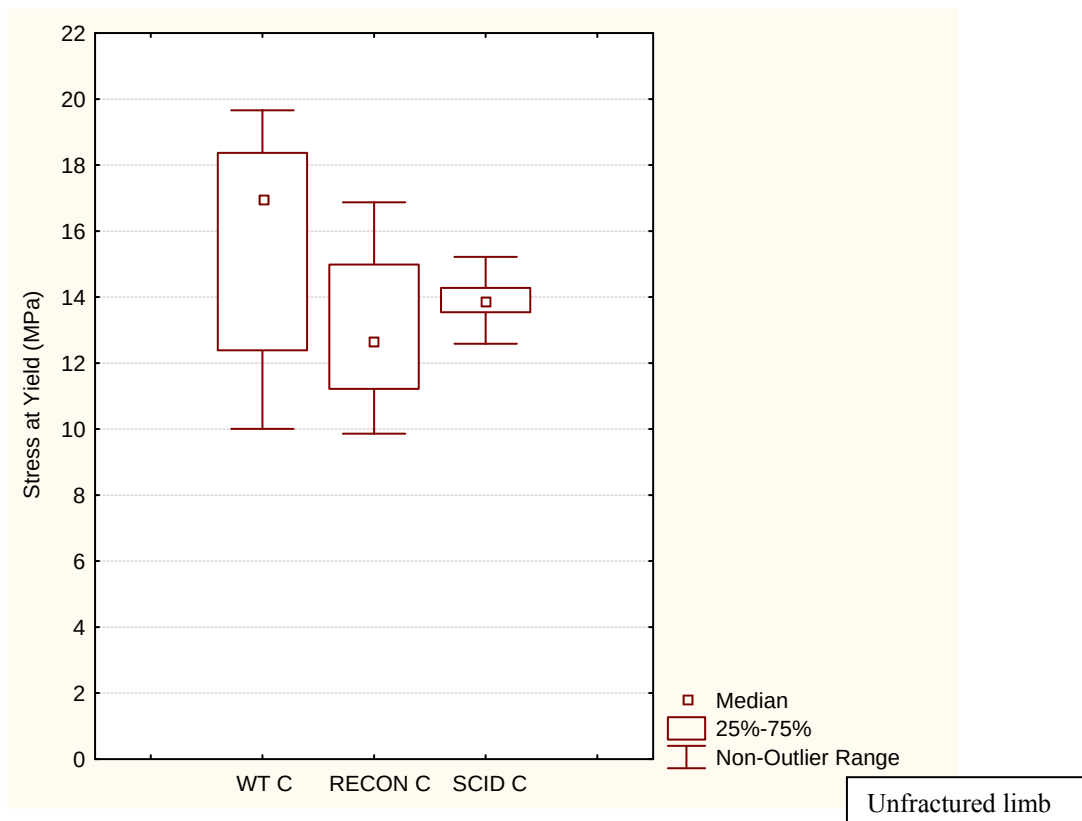


Figure 6.9 Box plot representing stress at yield in unfractured bone comparing wild type, RECON and SCID strains

There were no significant differences in stress at yield in the unfractured bone of the three groups of mice.

6.4.2.2 Results for strain at yield

The results for strain at yield in fractured bone are presented in Figure 6.10 and in unfractured bone in Figure 6.11.

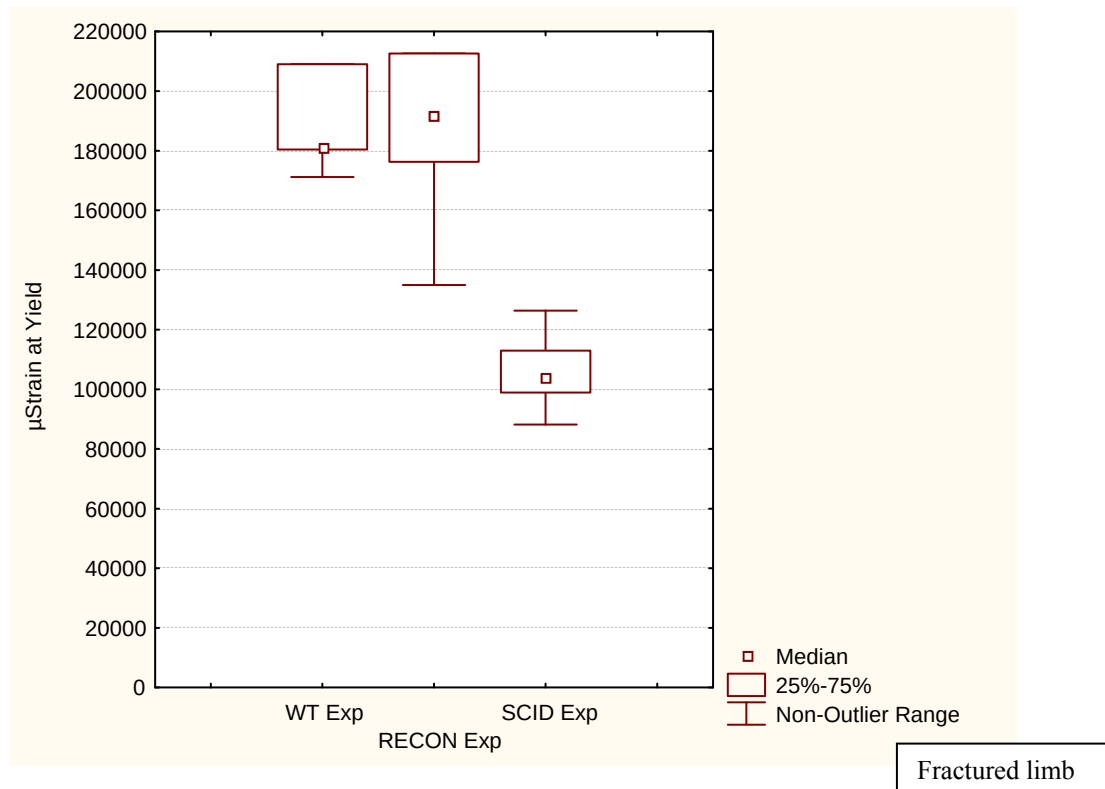


Figure 6.10 Box plot representing strain at yield in fractured bone comparing wild type, RECON and SCID strains

The strain at the point of yield in fractured bone (which is the intrinsic *deformation* of the bone at this point) was significantly less in the SCID group compared to the wild type and RECON groups, $P=0.006$ (SCID v WT) and $P=0.004$ (SCID v RECON) (MW-U). There was no significant differences between the wild type and RECON groups, $P=0.34$.

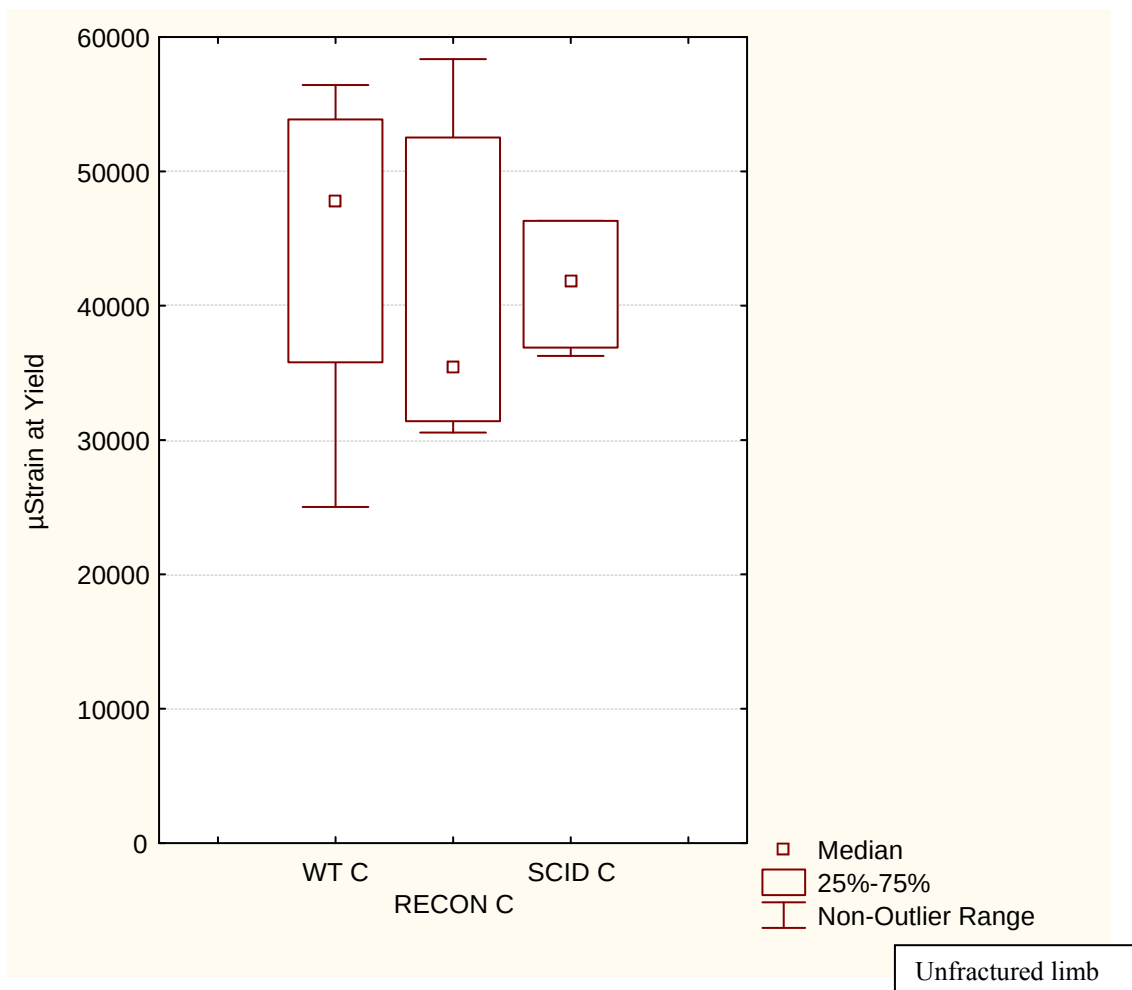


Figure 6.11 Box plot representing strain at yield in unfractured bone comparing wild type, RECON and SCID strains

There were no significant differences in strain in the unfractured bone of the three groups of mice.

6.4.2.3 Results for Young's modulus

The results for Young's modulus in fractured bone are presented in Figure 6.12 and in unfractured bone in Figure 6.13.

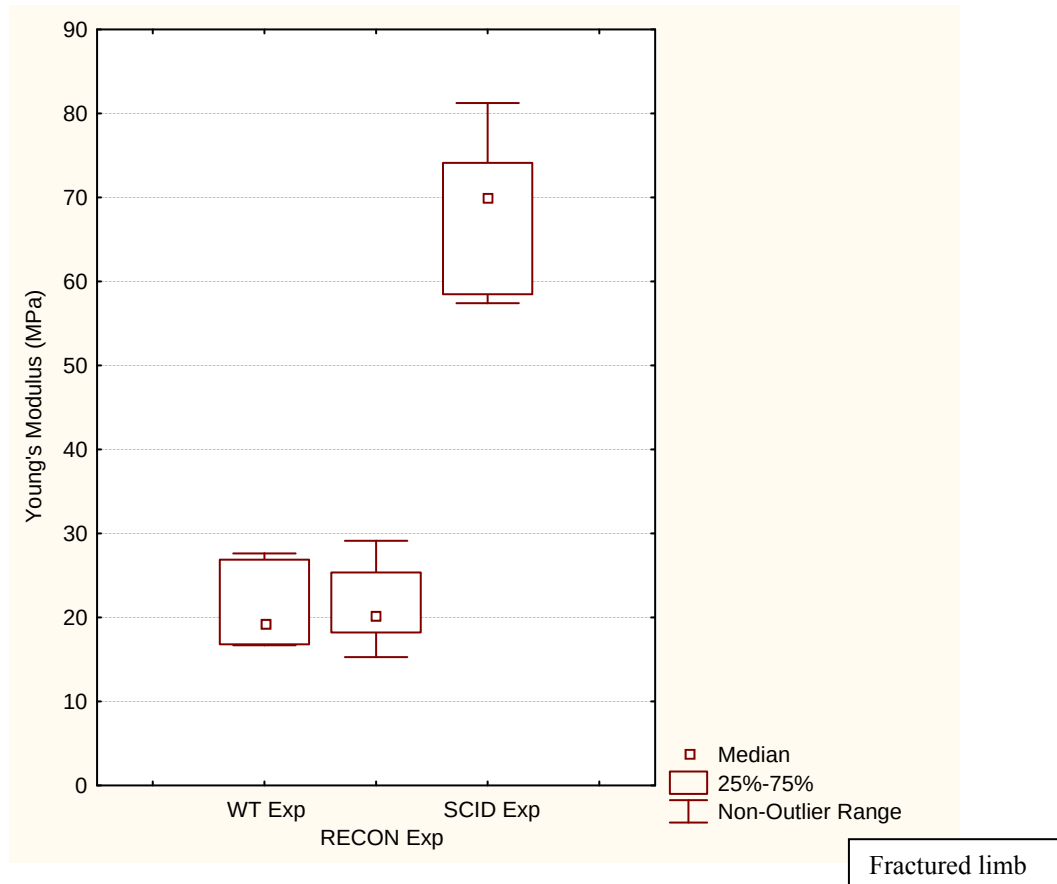


Figure 6.12 Box plot representing Young's modulus in fractured bone comparing wild type, RECON and SCID strains

The Young's modulus (which is an indication of the intrinsic *stiffness* of the bone) was significantly higher in the SCID group compared to the wild type and RECON groups, $P=0.004$ for both (MW-U) while there was no significant differences between the wild type and RECON groups $P=0.74$.

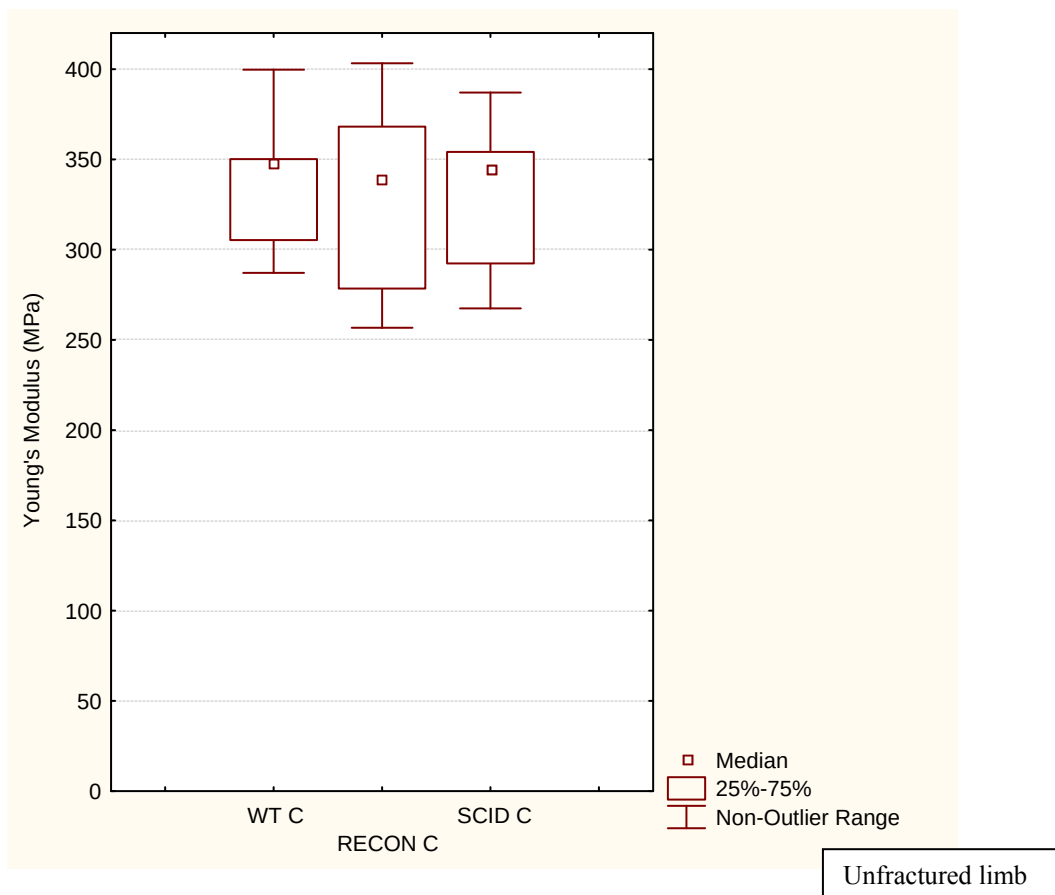


Figure 6.13 Box plot representing Young's modulus in unfractured bone comparing wild type, RECON and SCID strains

There were no significant differences in Young's modulus in unfractured bone between the three strains.

6.4.3 Mechanical testing summary

These results revealed a mechanical enhancement of fracture healing in the SCID group of mice compared to the wild type and reconstituted SCID mice groups, with stress at yield and Young's modulus significantly higher in SCID mice at day 21 post fracture. The SCID mice values were closer to the values of these parameters in normal bone compared to wild type and reconstituted SCID mice fractured bone, as is demonstrated by Figure 6.14. There was no difference in the mechanical properties of unfractured bone in the three groups of mice. The mechanical results confirm findings from Section 4.7 as well as showing the RECON strain to have behaved in a similar manner to the wild type. Figure 6.14 shows the *relative* values of the Young's modulus for all three groups of mice and demonstrates that the SCID fractured bone is showing increased stiffness when compared to RECON and wild type fractured bone.

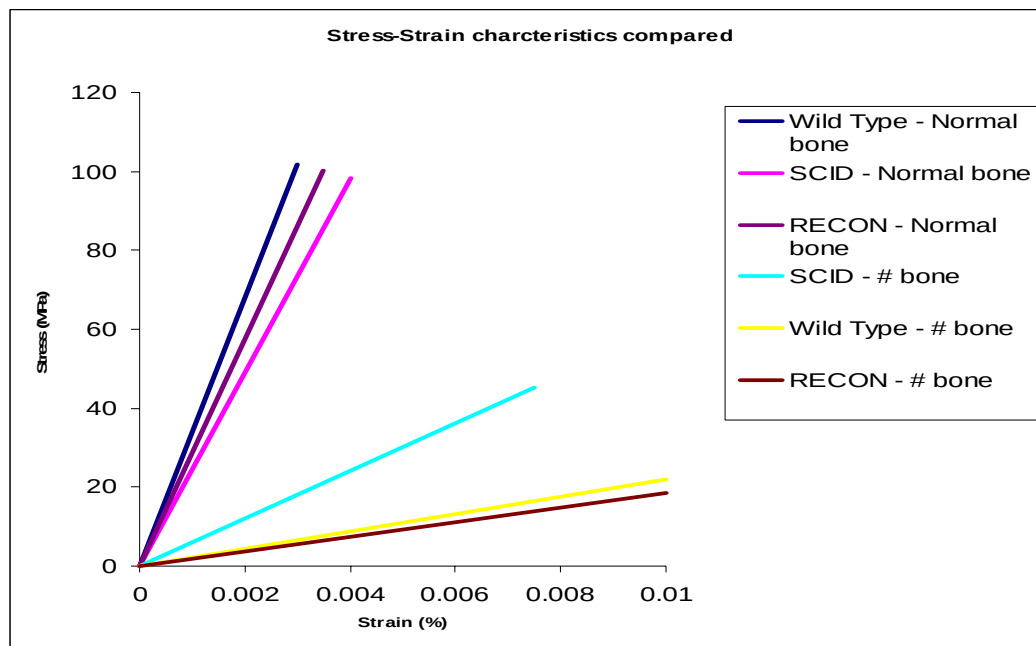


Figure 6.14 Graph which compares the stress-strain characteristics of the SCID and RECON and wild type experimental groups

Chapter 7

**Results: The expression of mononuclear
leukocytes in severe combined immuno-
deficient murine fracture repair**

7.1 Introduction

Chapter six has demonstrated that reconstitution of SCID mice as demonstrated by the ability to produce antibody, resulted in loss of the enhancement of fracture repair seen in SCID mice. The results in chapter six also demonstrated confirmation of the results in chapter four, confirming the enhanced bony repair in SCID mice compared to wild type mice and repeating and confirming the viability of the methods described in chapter two.

This work confirms the hypothesis in Section 5.1.1 stating

“Reconstitution of the specific immune system in SCID animals will return fracture healing to the same as controls”

While this has shown that a lack of the specific immune system is the most likely cause of enhanced fracture healing in SCID mice, it is important to visualise and quantify mononuclear leukocytes present in the callus tissue in this mouse strain. The results of this are the subject of this chapter.

Mononuclear leukocytes were quantified in the SCID animal throughout the time course of fracture repair, to determine if there were different expression patterns compared to wild type considering that the T and B cells are non-functioning and to look for differences in monocyte-macrophage expression. Immunohistochemistry as described in Section 2.6 was used to identify cells at the fracture site. The results obtained for wild type mice are described in chapter three and will be referred to here.

7.2 Study design

The fracture model described in chapter two was employed for this part of the study. Four SCID mice at 1, 3, 7, 14 and 21 days following fracture underwent schedule 1 euthanasia. The tibial fracture specimens were prepared, decalcified and mounted following paraffin embedding as described in Section 2.3. Sections were taken 50 μ m apart to control for variability throughout the fracture specimen. Immunohistochemistry was then used to identify the different immune cells within the fracture callus.

7.3 Immunohistochemistry results in SCID mice

The results in SCID mice will be compared to that in wild type mice, which were presented in Chapter 3. The positive and negative controls as presented in chapter 3 apply to this section of the results, see Figures 3.2 and 3.3.

In the SCID sections at day one there were similar numbers of CD3 positive cells seen in the fracture haematoma as in the wild type. There were however very few cells detected in and around the periosteum in SCID (see Figure 7.1) and the overall mean number of CD3 positive cells quantified was less overall. See Table 7.1.

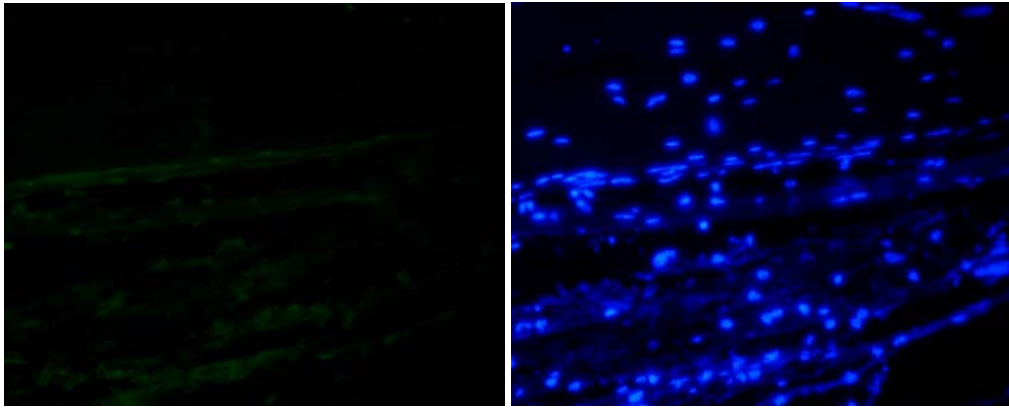


Figure 7.1 CD3 staining was not detected within the periosteum at day 1 post fracture in SCID (left). Corresponding nuclear (DAPI) counter-stain (right). Magnification x400

In the SCID sections there was no B220 staining for B cells detected at *any* time point.

There were multiple F4/80 positive cells in the fracture gap with infiltration into the periosteal region in both the wild type and SCID sections at day 1 post fracture, as shown in Figure 3.7.

Table 7.1 summarises the immunohistochemistry results at day 1 following fracture comparing the wild type mice to the SCID mice:

	Mean number of positive cells per high power field +/- standard error		P value
	Wild Type	SCID	
CD3 for T cells	20.6 +/- 1.3	14.9 +/- 1.2	P=0.19
B220 for B cells	14.8 +/- 1.2	0	P=0.00004
F4/80 for monocytes- macrophages	24.2 +/- 1.4	24.9 +/- 2.2	P=0.99

Table 7.1 Immunohistochemistry results at **day 1** post fracture

Statistics: All outliers were rejected and all the data was normally distributed by the Shapiro–Wilk W test. A one way ANOVA was applied with a *post hoc* Scheffé test to give the P values as displayed above.

At day three post fracture the fracture site in the SCID mice was rapidly organising into periosteal and fracture gap callus (see Section 4.2). By this stage there was now no CD3 staining evident within the fractured areas of the SCID sections. Occasional positive cells were seen in the bone marrow and within vessels. F4/80 staining for monocytes-macrophages was still visible in both the gap and periosteum of both wild type and SCID sections (see Figure 3.9), although the density of staining was less so in the SCIDs.

Table 7.2 summarises the immunohistochemistry results at day 3 following fracture comparing the wild type mice to the SCID mice.

	Mean number of positive cells per high power field +/- standard error		P value
	Wild Type	SCID	
CD3 for T cells	17.1 +/- 2.2	0	P=0.00011
B220 for B cells	10.9 +/- 1.3	0	P=0.00001
F4/80 for monocytes- macrophages	21.9 +/- 1.0	13.7 +/- 1.1	P=0.007

Table 7.2 Immunohistochemistry results at **day 3** post fracture

Statistics: All outliers were rejected and all the data was normally distributed by the Shapiro–Wilk W test. A one way ANOVA was applied with a *post hoc* Scheffé test to give the P values as displayed above.

By day seven in the SCID sections (which were clearly showing more rapid fracture repair by this stage, see Section 4.2) no CD3 staining for T cells could be seen in any area of cartilage formation within the external callus of the fracture gap or within ossification areas which were beginning to appear in the periosteal callus. Occasional positive cells were noted in other areas *e.g.* bone marrow which ensured an adequate staining procedure technically. There continued to be no staining for B cells with B220. The density of F4/80 staining continued to decrease. There were very few detected in the region of the fracture gap in either SCID or wild type mice. In SCID mice the positive cells appeared to be adjacent to areas of early ossification or cartilage formation rather than within them and this was more evident in the SCID sections as these had more distinct areas of ossification and cartilage formation – see Figure 7.2.

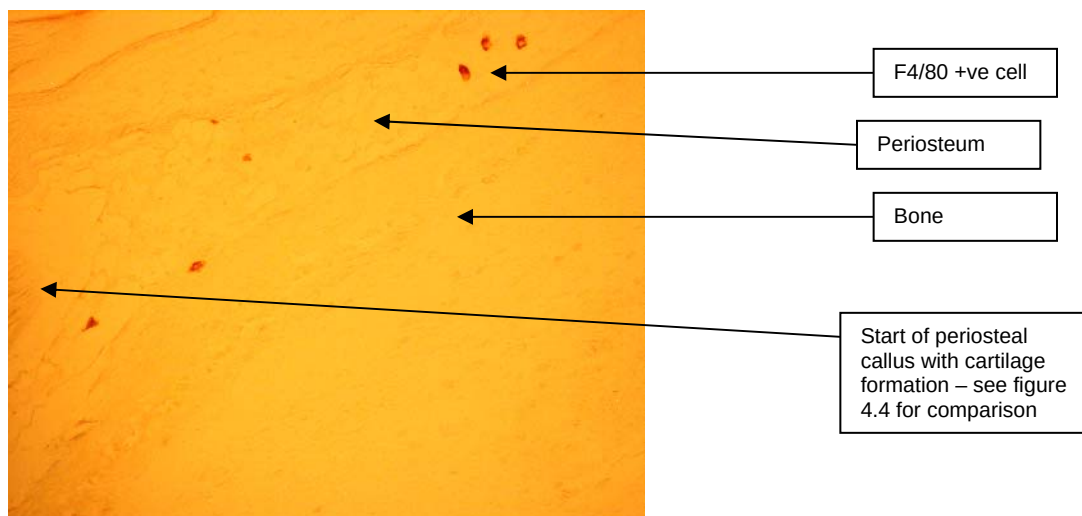


Figure 7.2 F4/80 staining in SCID section at day 7 post fracture. Magnification x200.

Table 7.3 summarises the immunohistochemistry results at day 7 following fracture comparing the wild type mice to the SCID mice.

	Mean number of positive cells per high power field +/- standard error		P value
	Wild Type	SCID	
CD3 for T cells	7.2 +/- 0.69	0	P=0.00002
B220 for B cells	0	0	
F4/80 for monocytes- macrophages	9.2 +/- 0.63	6.7 +/- 0.91	P=0.098

Table 7.3 Immunohistochemistry results at **day 7** post fracture

Statistics: All outliers were rejected and all the data was normally distributed by the Shapiro–Wilk W test. A one way ANOVA was applied with a *post hoc* Scheffé test to give the P values as displayed above.

At the latter stages of fracture repair in the SCID sections (days 14 and 21, where there was abundant cartilaginous callus formation and increasing areas of endochondral ossification and woven bone formation) there continued to be no CD3 positive staining within the areas of regeneration. There continued to be no positive B220 staining for B cells. With regard to staining for monocytes-macrophages with F4/80 there was only occasional perivascular staining noted near to areas of new bone formation in both strains. See Figure 7.3.

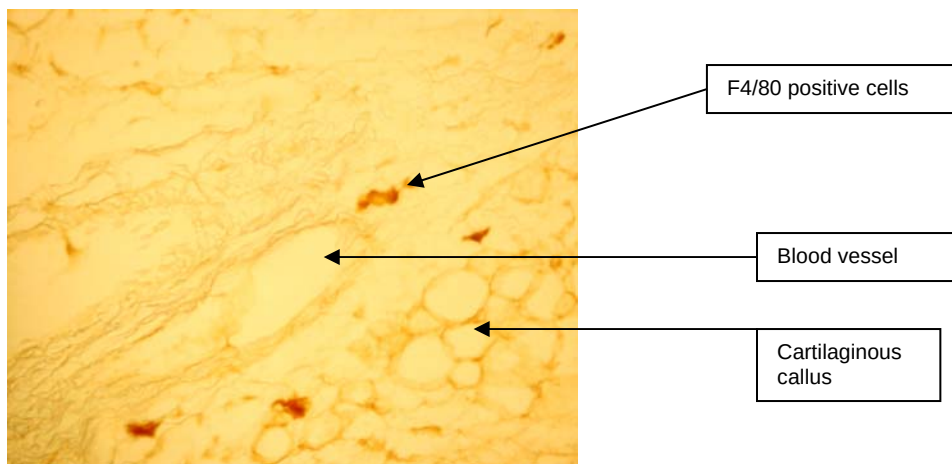


Figure 7.3 F4/80 staining in day 14 post fracture section from SCID mouse. Macrophages appear to be associated with a vessel and an area of cartilaginous callus about to undergo endochondral ossification. Magnification x400.

Tables 7.3 and 7.4 summarise the immunohistochemistry results at day 14 and day 21 following fracture comparing the wild type mice to the SCID mice.

	Mean number of positive cells per high power field +/- standard error		P value
	Wild Type	SCID	
CD3 for T cells	5.13 +/- 0.97	0	P=0.00001
B220 for B cells	0	0	
F4/80 for monocytes-macrophages	5.1 +/- 0.51	2.80 +/- 0.60	P=0.14

Table 7.4 Immunohistochemistry results at **day 14** post fracture

Statistics: All outliers were rejected and all the data was normally distributed by the Shapiro–Wilk W test. A one way ANOVA was applied with a *post hoc* Scheffé test to give the P values as displayed above.

	Mean number of positive cells per high power field +/- standard error		P value
	Wild Type	SCID	
CD3 for T cells	1.1 +/- 0.21	0	P=0.12
B220 for B cells	0	0	
F4/80 for monocytes- macrophages	3.4 +/- 0.44	2.3 +/- 0.33	P=0.15

Table 7.5 Immunohistochemistry results at **day 21** post fracture

Statistics: All outliers were rejected and all the data was normally distributed by the Shapiro–Wilk W test. A one way ANOVA was applied with a *post hoc* Scheffé test to give the P values as displayed above.

7.4 Temporal expression of immune cells at the fracture site following fracture in wild type mice and SCID mice – summary

See Figure 7.4 on next page

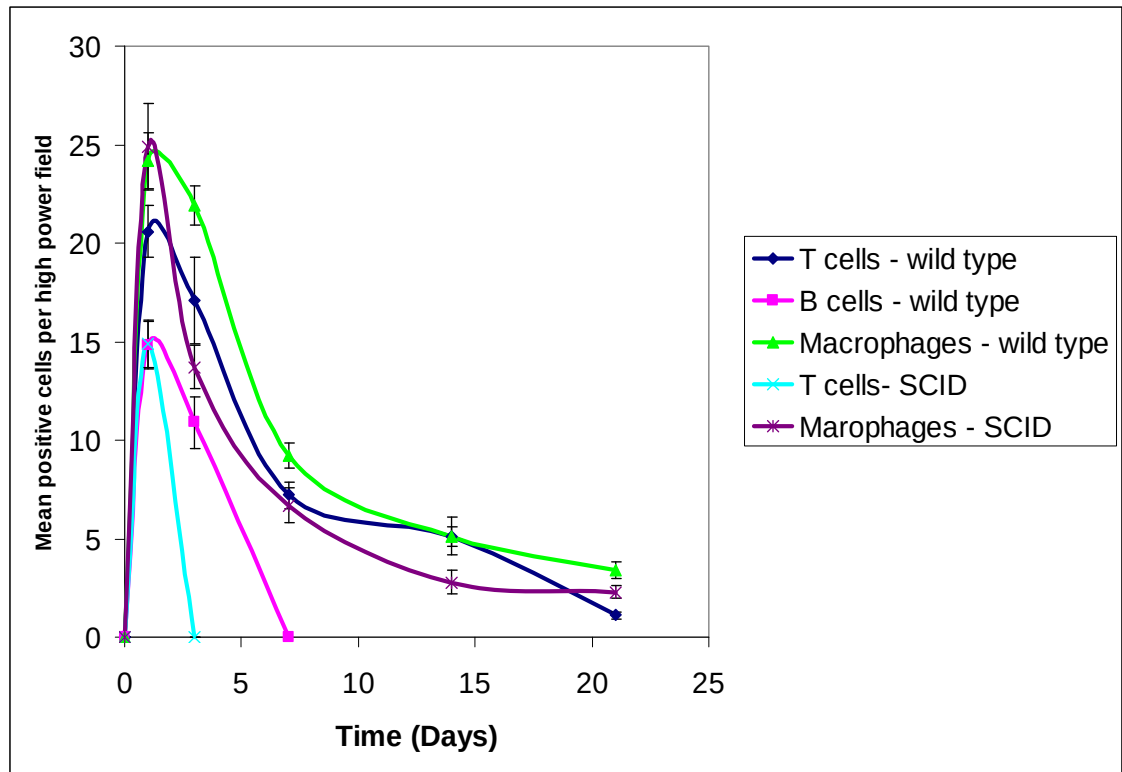


Figure 7.4 Graph demonstrating the expression of mononuclear leukocytes (T cells, B cells and monocyte-macrophages) as a function of time during fracture repair in the wild type mouse and the SCID mouse.

Figure 7.4 compares the presence of mononuclear leukocytes at the fracture site between the wild type and SCID mice. The T cells, which are non-functioning in the SCID, dropped to undetectable levels very quickly. The number of monocytes-macrophages in the SCID closely followed the pattern of the wild type, although the numbers were at slightly lower level throughout. No B cells were detectable in SCID mice.

While these findings are in themselves highly significant and will be discussed in Chapter 9, it was important to assess the *systemic* response with regard to these immune cells when a fracture occurs. This is the subject of the next chapter.

Chapter 8

**Results: Assessment of temporal systemic
expression of mononuclear leukocytes in wild
type and SCID mice**

8.1 Introduction

In order to investigate the systemic immune response to a closed fracture, mononuclear leukocytes were quantified in the blood of both wild type and SCID mice, over the time course of fracture repair. This was not only to gain information on the systemic response itself but also to determine if the immune response locally at the fracture site was related to the systemic response, or if indeed the immune cells present at the fracture site, particularly in the early stages, were simply related to fracture haemorrhage. The technique of fluorescence-activated cell sorting with quantification of the cells by flow cytometry was used to identify cells present in blood and will now be discussed in this chapter.

8.2 Study design

The fracture model described in chapter two was employed for this part of the study. Four animals (of each strain, wild type and SCID) at 1, 3, 7, 14 and 21 days following fracture underwent schedule 1 euthanasia. A 1 ml blood sample by cardiac stab was obtained from each animal immediately following confirmation of death. This was added to a container pre-filled with 10mls of phosphate buffered saline and 10U/ml of heparin to prevent coagulation of the sample and then processed for flow cytometry.

8.3 Fluorescence-activated cell sorting (FACS) & flow cytometry

8.3.1 Introduction

A fluorescence-activated cell sorter can rapidly separate cells in a suspension on the basis of size and the colour of their fluorescence. A cell suspension containing cells labelled with a fluorescent dye is directed into a thin stream so that all the cells pass in single file. The dye is coupled to a monoclonal antibody that binds to those cells containing the antigen for which the antibody is specific. This stream emerges from a nozzle vibrating at some 40,000 cycles per second which breaks the stream into 40,000 discrete droplets each second. Some of these droplets may contain a cell. A laser beam is directed at the stream just before it breaks up into droplets. As each labelled cell passes through the beam, its resulting fluorescence is detected by a photocell. If the signals from the two detectors meet either of the criteria set for fluorescence and size, an electrical charge (+ or -) is given to the stream. The droplets retain this charge as they pass between a pair of charged metal plates. Positively-charged drops are attracted to the negatively-charged plate and *vice versa* and separated off into separate containers. Uncharged droplets (those that contain no cell or a cell that fails to meet the desired criteria of fluorescence and size) pass straight into a third container and are later discarded. This apparatus can sort as many as 300,000 cells per minute. The cells are not damaged by sorting.

Flow cytometry refers to quantification of the cells in each sample. All light signals detected by the photocell in the FACS machine, whether from fluorescently labelled cells or from the beam scattered by unlabelled cells, are transferred to a computer and transformed into digital signals. These signals are displayed in channels along a

histogram graph. The typical output is a single parameter graph of intensity versus the number of cells, see Figure 8.1.

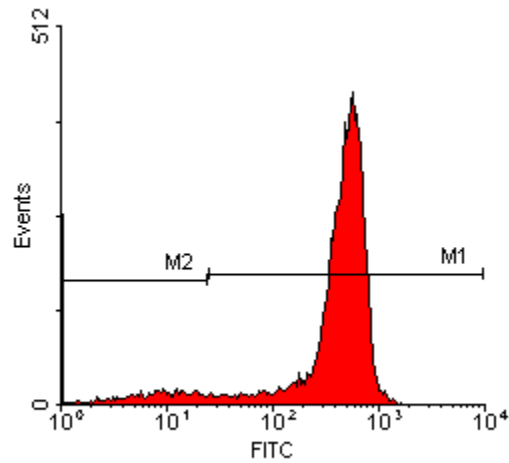


Figure 8.1 Single parameter histograms display the relative fluorescence (x-axis) plotted against the number of events (cells) (y-axis).

More complex two-dimensional scatter plots are required, when one wishes to compare multiple parameters that are collected at the same time. In these diagrams, one parameter is plotted against another in an X versus Y axis display, often referred to as forward scatter and side scatter, see Figure 8.2.

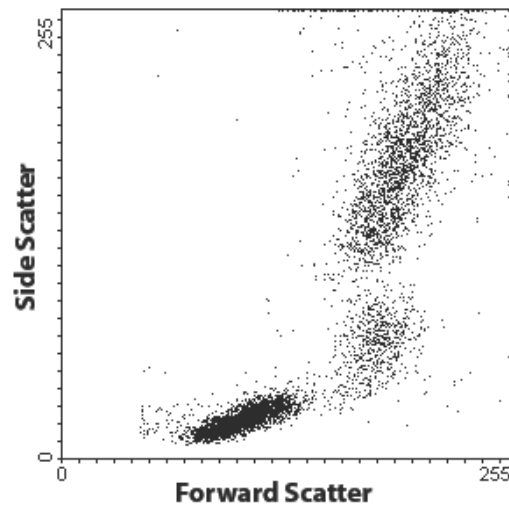


Figure 8.2 Two dimensional histogram, displaying a typical scatter plot produced when flow cytometry is used to quantify two subsets of cell populations

Such scatter plots display the relationship between each of the two parameters.

Individual subsets or cell populations within a sample are resolved based on the combined intensity levels of each of the two parameters. This allows simultaneous quantification of two subsets of cell populations *e.g.* T cells and B cells. When comparing cell populations, subcellular debris or unimportant cell populations can be eliminated from the data by gating that uses flow cytometry software to select a population within the graph that needs to be plotted. In this study gating was used to select the true population of leukocytes by using a cell-lineage specific gate with an antibody to CD45 labelled with fluorescein isothiocyanate (FITC). This gave volumetric absolute counts for all immune cells present within each sample, and discarded all non-immune cells. The gated cells were then displayed in a new graph using additional parameters (for example, antibodies to T cells and antibodies to B cells, see below) to give measurable data. The software was able to produce the results for

each antibody expressed as a percentage of gated cells (Brando, Barnett *et al.* 2000; Bergeron, Nicholson *et al.* 2002; Janossy, Jani *et al.* 2002) .

8.3.2 Method

In this study cells of the immune system were analyzed phenotypically using a FACS scan flow cytometer (Becton Dickinson, San Jose, CA). The blood samples were collected as described in Section 8.2. For each sample obtained, two analyses were carried out:

1. Fifty microlitre aliquots of blood were incubated for 20 minutes with three antibodies: Thy-1 PE (phycoerythrin)-conjugated rat anti-mouse and B220 APC (allophycocyanin)-conjugated rat anti mouse and CD45 FITC (fluorescein isothiocyanate)-conjugated rat anti mouse. This gave the subpopulations of T (Thy-1) and B cells (B220) after gating with CD45 to exclude all other cellular material (*i.e.* non-immune cells). All of the reagents were from BD Biosciences PharMingen, San Diego, CA.
2. Fifty microlitre aliquots of blood were incubated for 20 minutes with two antibodies: CD11b PE (phycoerythrin)-conjugated rat anti-mouse and CD45 FITC (fluorescein isothiocyanate)-conjugated rat anti mouse (all BD Biosciences PharMingen, San Diego, CA). This gave the subpopulations of macrophages/monocytes (CD11b) after gating with CD45 to exclude all other cellular material *i.e.* non-immune cells.

To remove erythrocyte contamination, blood samples were incubated for 10 minutes in lysis buffer (BD Biosciences PharMingen) and centrifuged at 1,000 rpm for eight minutes. The pellets were washed in phosphate buffered saline, pH =7.4, centrifuged

at 1,000 rpm for eight minutes and fixed in 300 ml of cold Cytofixt buffer (BD Biosciences PharMingen). The analysis was performed using a flow cytometer and data were analyzed with a Macintosh G3 workstation, which contains graphics software packages used for data acquisition and analysis. CD45 gating was used to give volumetric absolute counts for all immune cells present within each sample, allowing the total leukocyte count to be obtained. The numbers of T cells (Thy-1), B cells (B220) and monocytes-macrophages (CD11b) was then expressed as a percentage of this for each sample.

Of note, the Thy-1 antibodies and CD11b antibodies here give similar populations of T cells and monocyte-macrophages respectively as CD3 and F4/80 antibodies used in the immunohistochemistry experiments but are antibodies more amenable to flow cytometry.

8.4 Quantification of immune cells in fracture healing in the systemic circulation – results

Examples of the typical scatter plots obtained from the flow cytometry are shown in Appendix 9. The systemic expression of the total leukocyte count and of each of the cells described as determined by FACS are shown as a function of time in Section 8.5. The normal (i.e pre-fracture) peripheral blood mononuclear leukocyte composition obtained by FACS from both wild type and SCID mice will first be described. The results from the experimental tissue obtained at each time point will then be described.

8.4.1 Normal peripheral blood leukocyte composition before fracture

Blood samples were obtained from unfractured Wild type balb/c mice and SCID Balb/c mice, again following schedule 1 euthanasia and cardiac stab, to give baseline counts for each strain.

Figure 8.3 shows the findings in wild type mice.

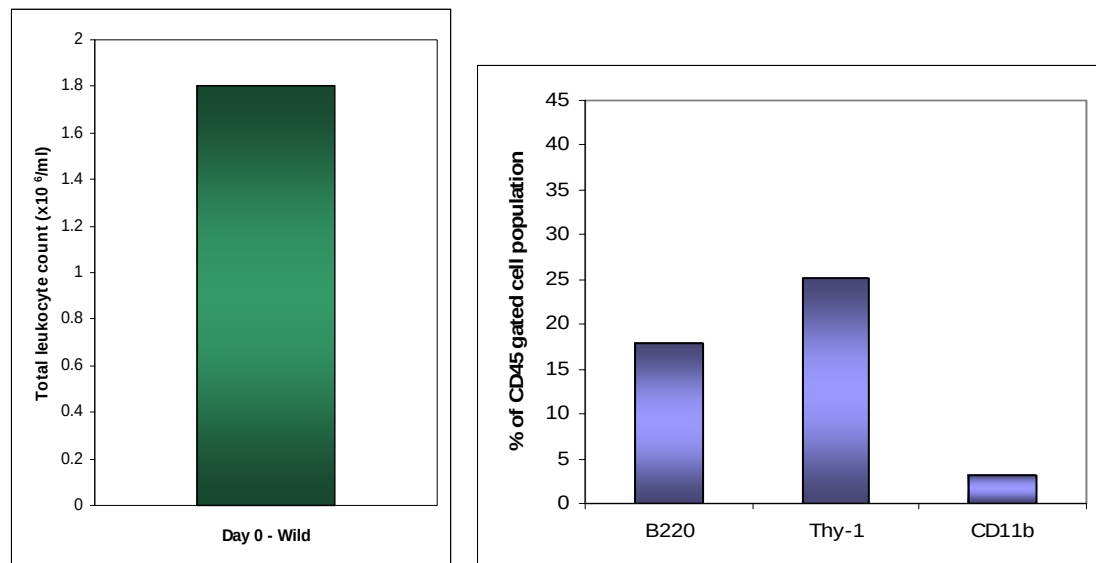


Figure 8.3 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) prior to fracture in wild type animals.

These findings were in keeping with previously published blood parameters in murine blood from cardiac sampling (Doeing, Borowicz *et al.* 2003).

Figure 8.4 shows the finding in SCID mice.

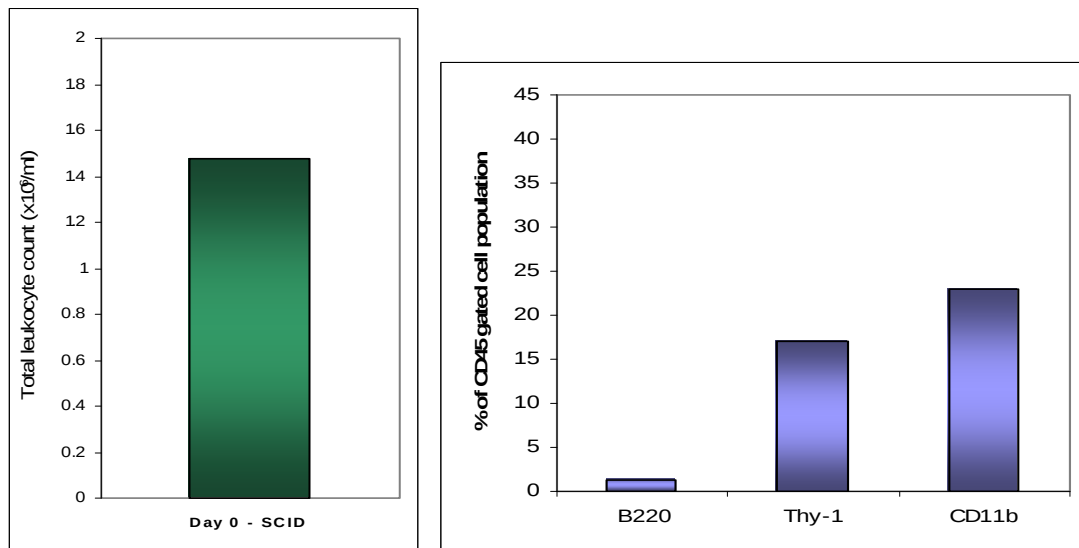


Figure 8.4 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) prior to fracture in SCID animals.

The above results demonstrate that the SCID animals had a lower baseline leukocyte count. The differential of the mononuclear leukocytes revealed that there was a higher number of circulating macrophages compared to the wild type both in percentage and total number. The percentage of B cells was negligible, and accounting for the accepted error with FACS analysis (1 to 1.5%, (Carter, Newport *et al.* 1983)) is probably insignificant. Thy-1 analysis for circulating T cells revealed a level of 17% (compared to 25% in the wilds). All of these findings are in keeping with previous studies (Custer, Bosma *et al.* 1985) and it is known that these T cells are non-functional.

8.4.2 FACS, day 1:

The FACS analysis results for the circulating immune cells at day 1 post fracture are given in Figures 8.5 and 8.6:

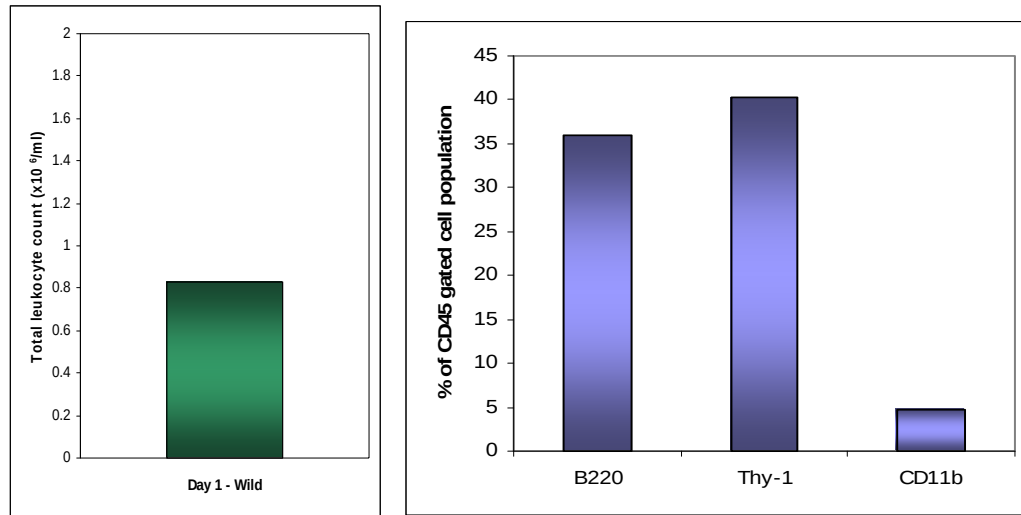


Figure 8.5 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) at day 1 post fracture in **wild type** animals.

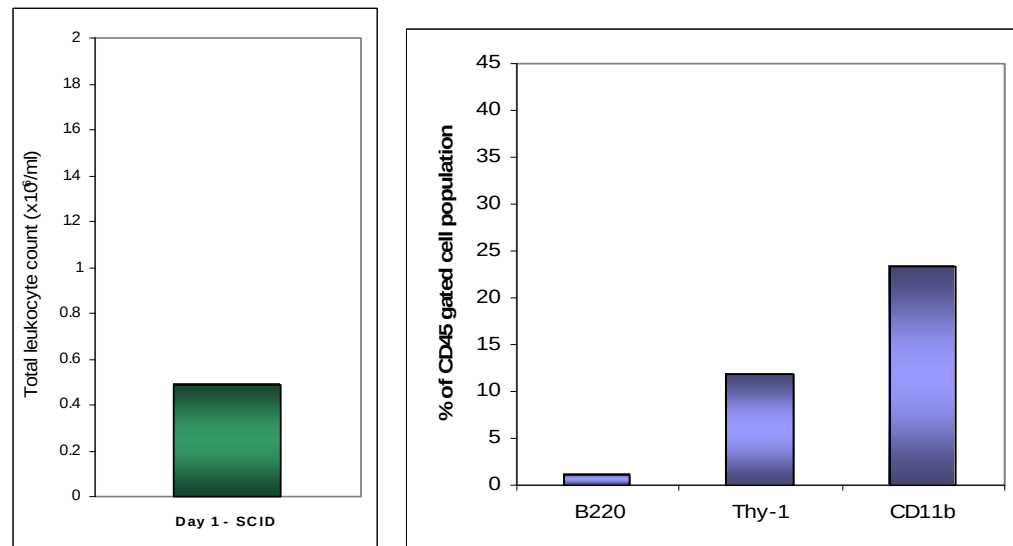


Figure 8.6 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) at day 1 post fracture in **SCID** animals.

8.4.3 FACS, day 3

The FACS analysis results for the circulating immune cells at day 3 post fracture are given in Figure 8.7 and 8.8:

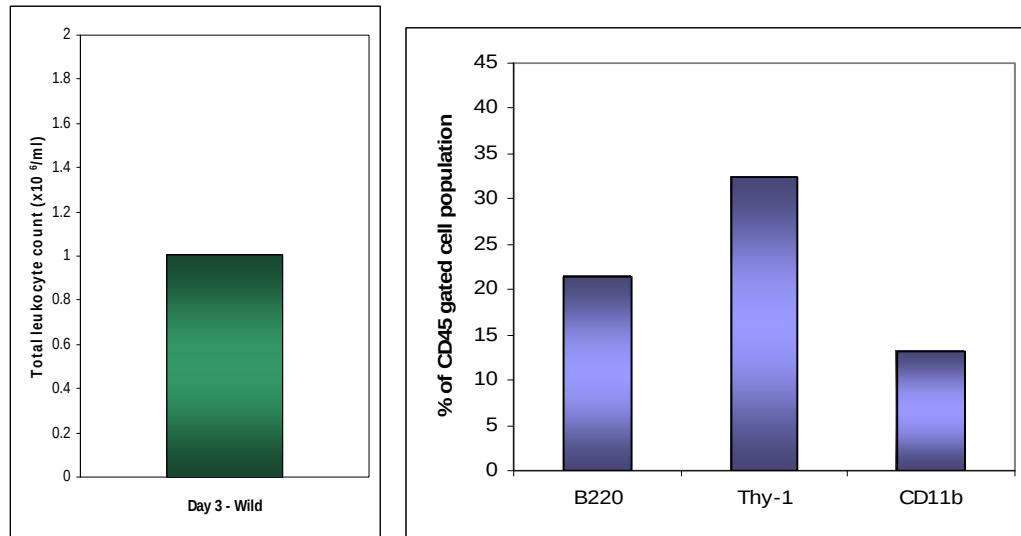


Figure 8.7 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) at day 3 post fracture in **wild type** animals.

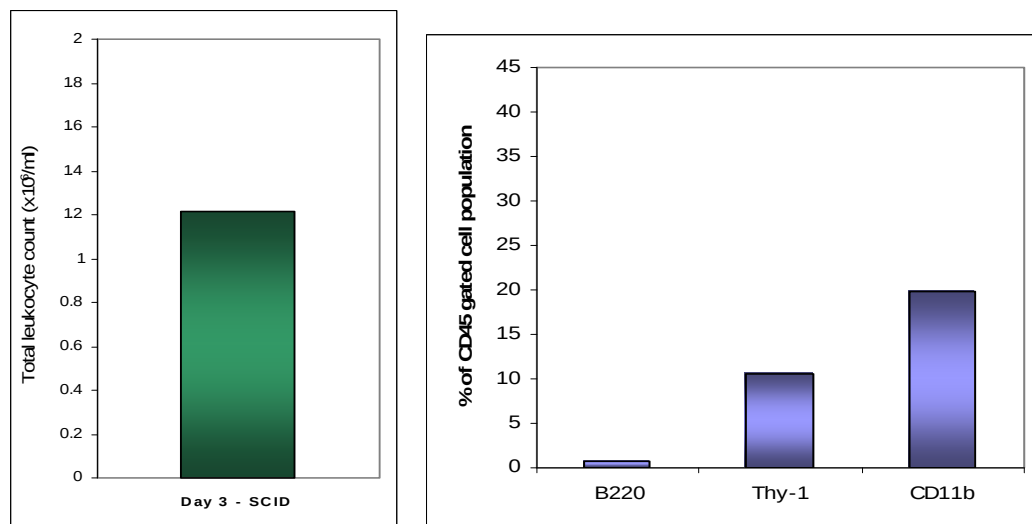


Figure 8.8 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) at day 3 post fracture in **SCID** animals.

8.4.4 FACS, day 7:

The FACS analysis results for the circulating immune cells at day 7 post fracture are given in Figure 8.9 and 8.10:

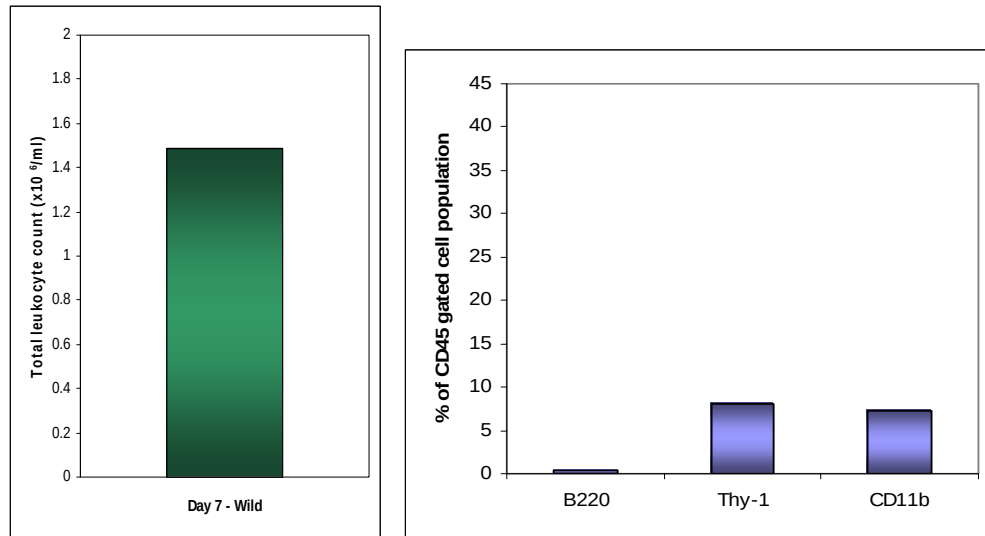


Figure 8.9 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) at day 7 post fracture in wild type animals.

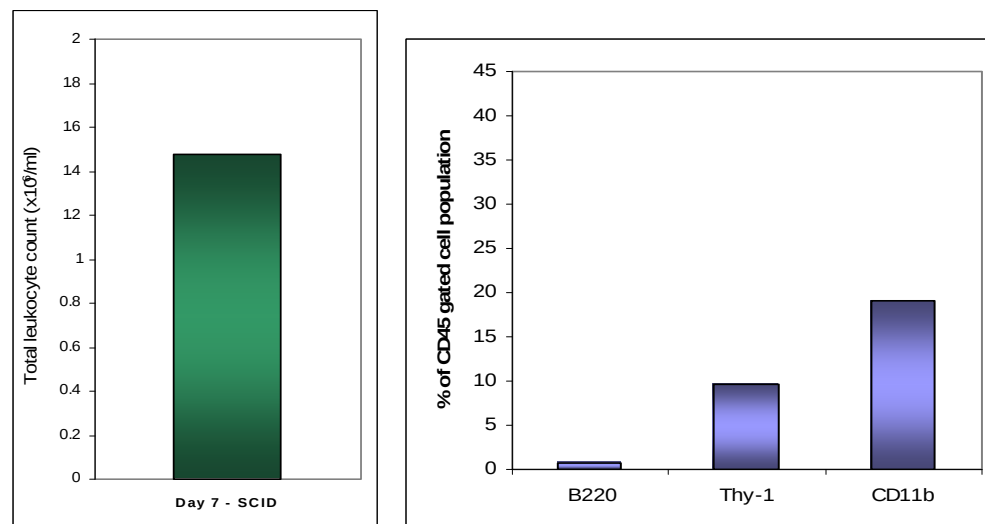


Figure 8.10 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) at day 7 post fracture in SCID animals.

8.4.5 FACS, day 14

The FACS analysis results for the circulating immune cells at day 14 post fracture are given in Figures 8.11 and 8.12

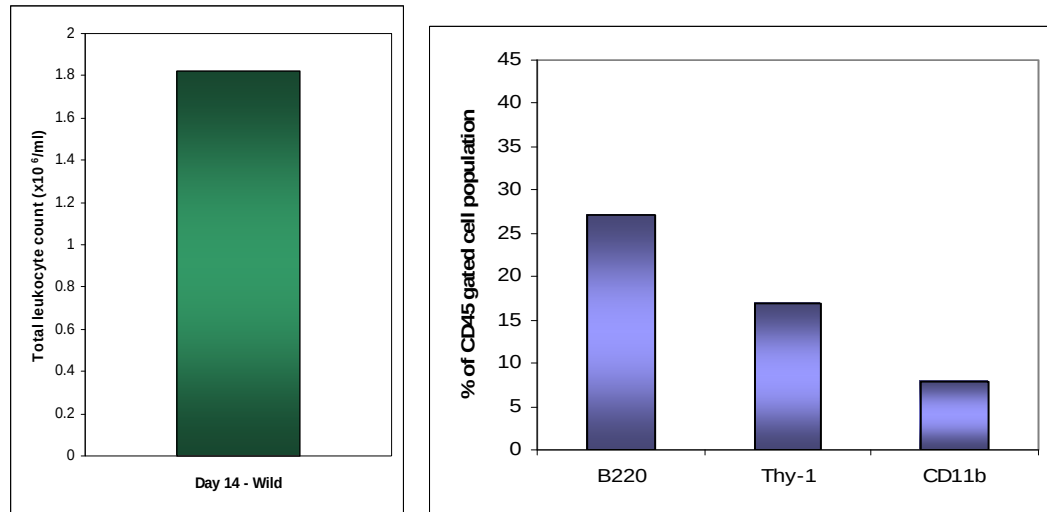


Figure 8.11 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) at day 14 post fracture in **wild type** animals.

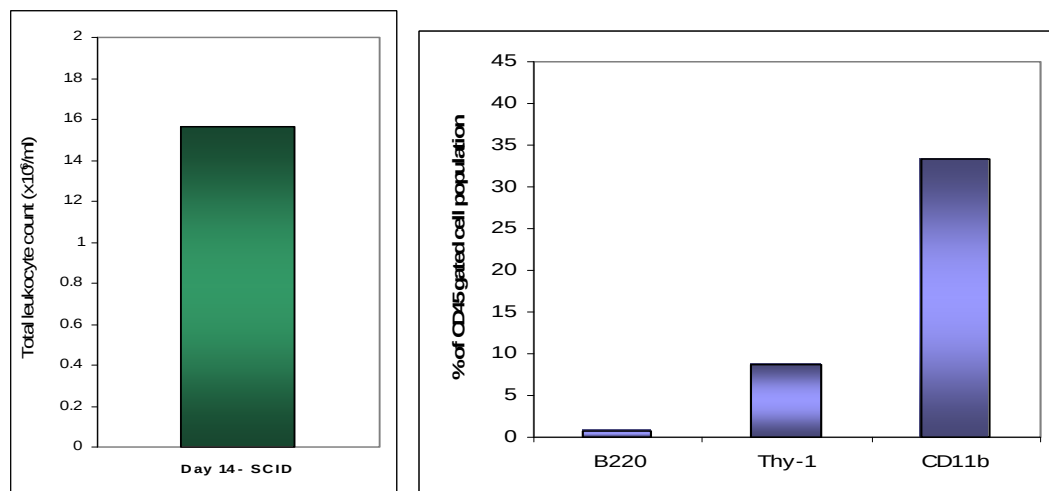


Figure 8.12 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) at day 14 post fracture in **SCID** animals.

8.4.6 FACS, day 21

The FACS analysis results for the circulating immune cells at day 21 post fracture are given in Figure 8.13 and 8.14

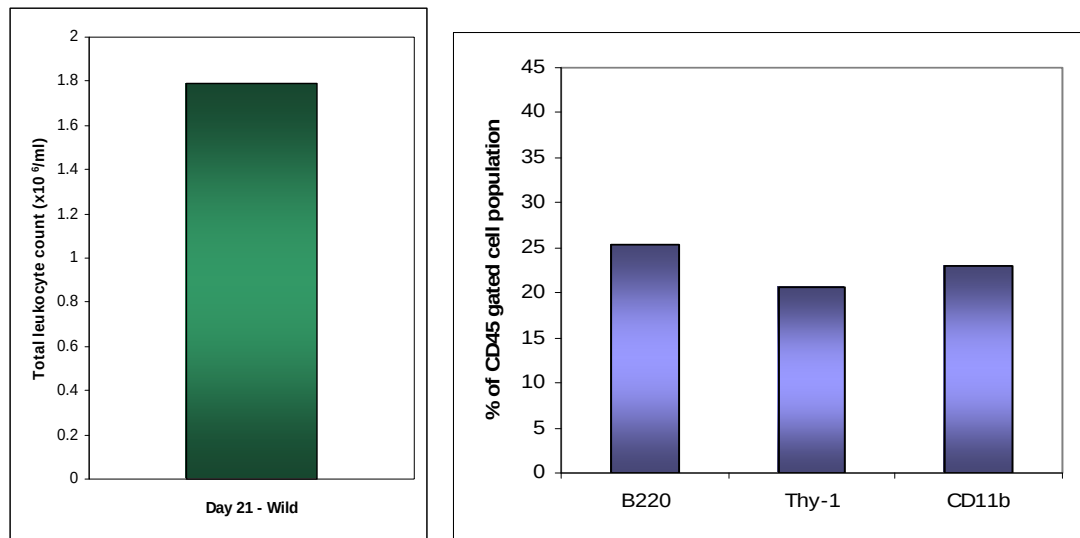


Figure 8.13 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) at day 21 post fracture in **wild type** animals.

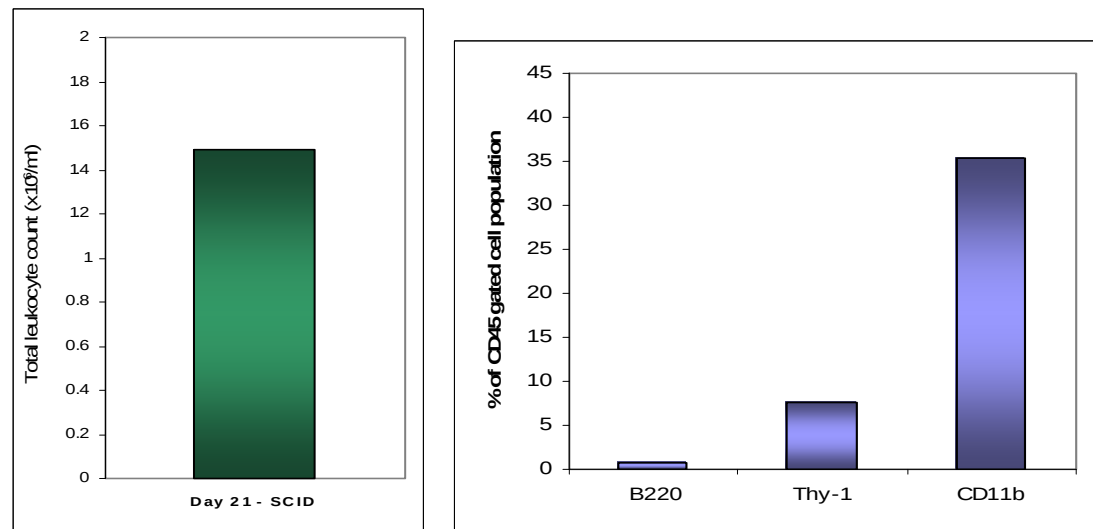


Figure 8.14 Histograms representing total leukocyte count and percentage of B cells (B220), T cells (Thy-1) and monocytes-macrophages (CD11b) at day 21 post fracture in **SCID** animals.

8.5 Temporal expression of immune cells in blood following fracture

8.5.1 Wild type:

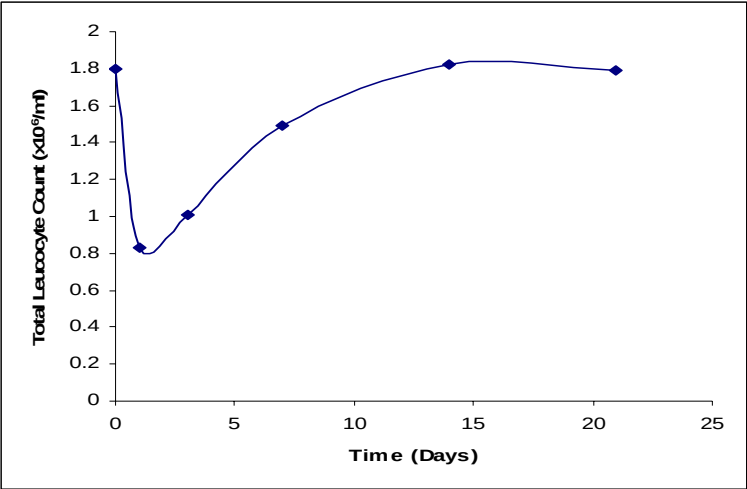


Figure 8.15 Total leukocyte count expressed as a function of time following fracture in wild type animals

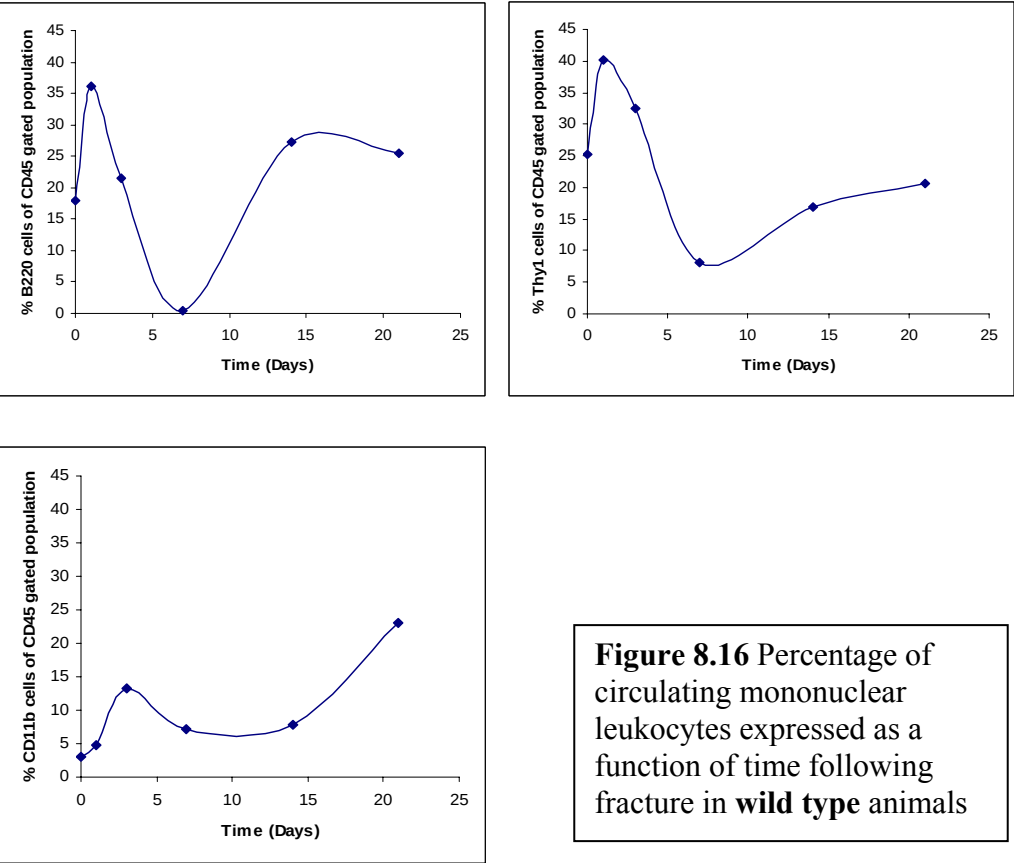


Figure 8.16 Percentage of circulating mononuclear leukocytes expressed as a function of time following fracture in **wild type** animals

8.5.2 SCID:

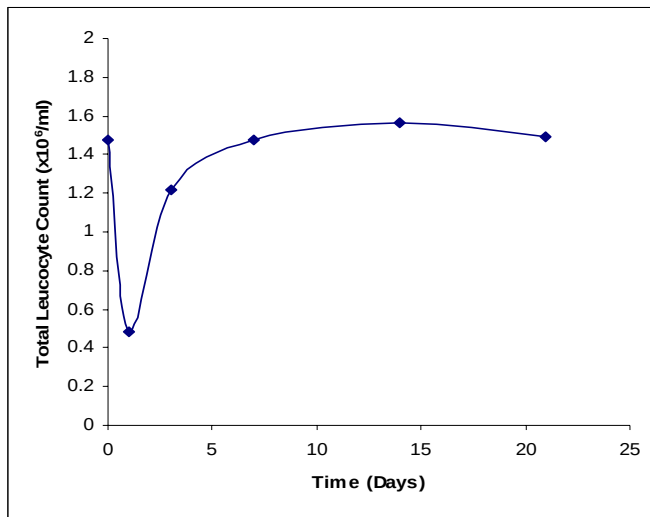


Figure 8.17 Total leukocyte count expressed as a function of time following fracture in SCID animals

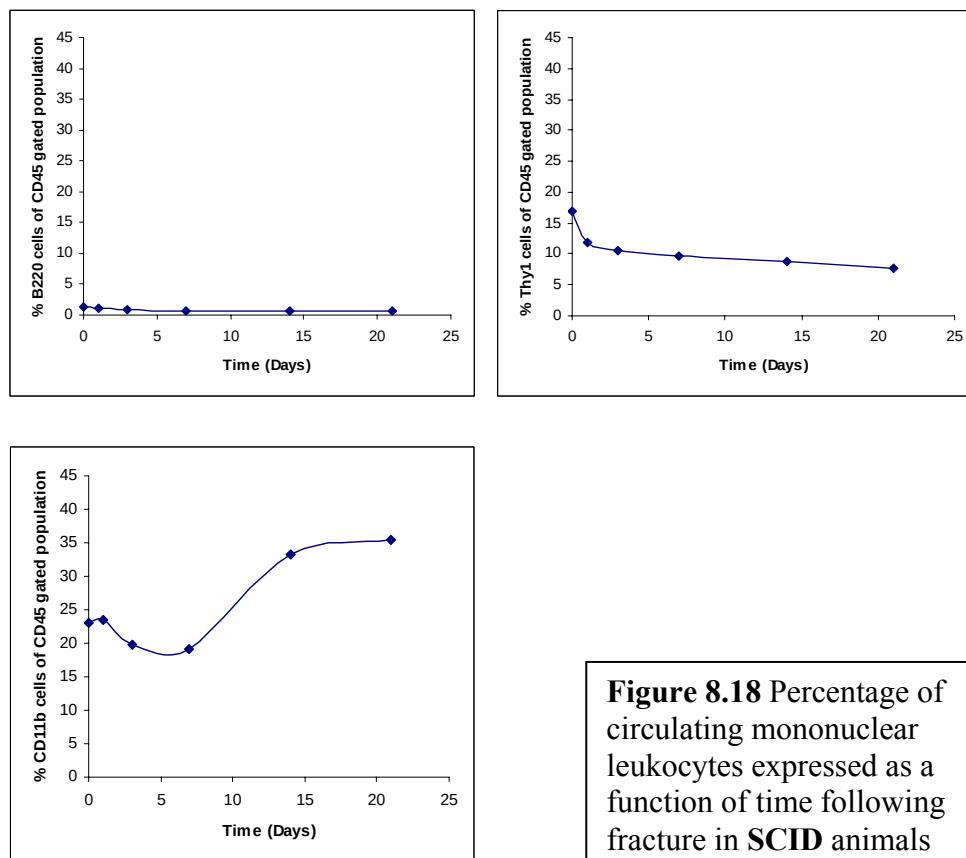


Figure 8.18 Percentage of circulating mononuclear leukocytes expressed as a function of time following fracture in SCID animals

8.6 Summary

The two groups of animal studied in this chapter both demonstrated an immediate depression of total leukocyte count following fracture. The total leukocyte count in the SCID animal was in general $0.4 \times 10^6/\text{ml}$ less than the wild type. However the post fracture trend with an initial drop to day 3 and the recovery over the next 10 days was the same in both groups of mice. The percentages of mononuclear leukocytes expressed following fracture showed that in wild type, despite an initial drop in total white cell number, the percentage of B and T cell rises to almost 75% of the lymphoid compartment. This is then followed by a dramatic drop in both these cells at day 7 post fracture. These compartments then gradually recover to pre-fracture levels over the next 2 weeks. The SCID animal showed insignificant levels of B cells at any stage. The T cells, known to be non-functional, showed some suppression in numbers which lasted for the duration of fracture repair.

With regard to monocytes-macrophages in wild type there was a rise in the percentage of these cells gradually to day 3 which then tailed off at day 7 before rising again at the end stages of fracture repair. The SCID animal shows a higher percentage of monocytes-macrophages pre-fracture. Following fracture there is a small initial increase, followed by a dip and then a rise in number. This pattern of the curve in the SCID is somewhat similar to the wild except that it occurs earlier *i.e.* the graph is perhaps shifted to the left, when compared to the wilds. It should also be noted that the *total* number of monocytes-macrophages is higher in SCID compared to wild type at days one and three following fracture, as high as threefold more at day one.

The implications of these findings are discussed in the next chapter.

Chapter 9

Discussion, Clinical Relevance and Future Research

9.1 Discussion

In the introduction to this thesis the importance that the immune system has on skeletal homeostasis was emphasised. For example, the importance of T cells on the activation of osteoclasts *via* TRANCE has been demonstrated (Kong, Feige *et al.* 1999). There has been limited research into the role of the cellular immune system in bone healing. The main aim of this thesis was to investigate the cellular role of the specific immune system in fracture repair. The following sections discuss the main findings from this thesis in the context of current published knowledge, followed by some limitations of the studies and the clinical relevance and future direction of this work

9.1.1 Summary of main findings from thesis and discussion

The work in this thesis was centred on the aims as detailed in Section 1.8. This work has generated a number of observations:

1. A closed tibia fracture model has been established in mice and has been shown to be repeatable, reliable and sufficient to produce samples for histological, histomorphometric, immunohistological and biomechanical testing to assess fracture repair. This model was relatively simple to create and could be used for similar studies dealing with fracture healing, eliminating the need for complex surgical procedures which interfere with the natural healing of fractures. The mouse is also an excellent tool for examining the effect of transgenesis on fracture repair as there are an infinite

number of transgenic strains now available. The immunodeficient mouse, as used in this study, is also an excellent model to accept xenographic material.

2. Chapter three has demonstrated the presence of immune cells locally during fracture repair in the wild type mouse. The T cells appear to be actively recruited into the periosteal region of fracture repair and are in high numbers during the early stages of healing. B cells were rapidly excluded from the fracture site and probably have no role in bone healing. The macrophage was retained in high numbers during the early stages of fracture repair. As the cartilage and bone forming stages of fracture repair progressed, both T cells and macrophages were excluded and numbers were diminished. The implications of this, in light of the current literature, are discussed further below, in point 6 of this section.

3. Comparison of SCID mice with wild type mice has revealed that the SCID mouse progressed through this process at a faster rate than the wild type control. This was evident in terms of earlier differentiation of tissue, earlier cartilage production and earlier endochondral ossification, on histological observations. The cellular process of repair did not appear to differ fundamentally except that the SCID was further along the repair pathway at any given time point. At the time of writing similar information has only been presented on one other occasion, to the author's knowledge. Yu *et al.* discussed healing in a bone defect model comparing wild type and *nude* mice – here *nude* mice were shown to have increased bone formation at days 7, 10 and 14 after creation of defect as measured histologically and histomorphometrically. They concluded that the 'immune response plays a role in the healing process' but no further

investigation than this was discussed (Yu, Yang *et al.* 2006). The nude mouse is a hairless mutant discovered in 1962 and is also immunodeficient as it congenitally lacks a thymus, which is essential for the production of T-cells. Therefore, the findings of Yu *et al.* are in keeping with the histological findings in chapter four of this thesis.

Further testing of fracture repair between the wild type and SCID as measured histomorphometrically, radiographically, biomechanically and by bone mineral density assessment also showed enhanced fracture repair in the SCID mice. The curve of the callus area and index graphs (Figures 4.19 – 4.21) in the radiographic assessment showed that the SCID callus size had actually started to decrease at the later time points. This was most likely as remodelling was well underway and this is indicative of the SCID strain have progressed further along the healing pathway. Both bone mineral density and mechanical testing indicated a more mature callus in the SCID mice, again indicating faster fracture repair. It was noted at this point that the intrinsic properties at three weeks of the unfractured bone were quite different to those of the fractured bone. This would be expected as the mechanical testing was carried out at post-fracture day 21, when the fractured bone has entered the remodelling phase but is still a long way from complete repair. The unfractured bone has higher stress at yield values and lower strain at yield values and is therefore acting as a stronger but more brittle material.

Implications of faster fracture repair in SCID mice:

It has been demonstrated that an animal model which lacks the specific immune system underwent accelerated fracture repair. While this is a novel finding in bone healing, previous work by Barbul *et al.* (1989) has demonstrated this finding in soft tissue,

incisional wounds in nude mice. They studied wound healing in congenitally athymic nude mice that lack a normally developed T cell system and share many of their immune deficient characteristics with SCID mice. Healing of incisional wounds, as assessed by wound breaking strength, was significantly stronger in nude mice compared with normal thymus-bearing animals. This was accompanied by a marked increase in the amount of reparative collagen synthesised at the wound site, assessed by the hydroxyproline content of subcutaneously implanted sponges (Barbul, Shawe *et al.* 1989). These authors proposed that the T lymphocyte system predominantly inhibited healing by altering the activity of macrophages, the main regulators of wound healing. Their finding of increased collagen production supports the finding of enhanced bone healing seen in SCID mice in this thesis. Similar thymectomy studies in rats have suggested an inhibitory role of T-suppressor lymphocytes on soft tissue wound healing (Barbul, Sisto *et al.* 1982). It has also been demonstrated that monoclonal antibody depletion of CD8+ T (suppressor) cells resulted in increased wound breaking strength and collagen levels as did combined depletion of CD4+ (T helper cells) and CD8+ cells, again in soft tissue incisional wounds. Depleting CD4+ T cells alone did not make any difference to the mechanical properties of the wound (Efron, Frankel *et al.* 1990). More recent work has also demonstrated mechanically stronger dermal wounds in nude mice at one and two weeks post injury (Chircop, Yu *et al.* 2002) and increased strength, resilience and elasticity constant in healing wounds of wild type mice depleted of T cells with antibodies which underwent subsequent laparotomy (Davis, Corless *et al.* 2001).

In light of the above literature, there were therefore several hypothesis for the enhanced fracture repair seen in the SCID mice:

- a) The T-lymphocyte system may exert an inhibitory role on fracture healing, accounting for the accelerated repair in SCID mice. This would be in keeping with findings of others in soft tissue healing, as detailed above.
- b) The observed changes may have been caused by an unrelated genetic aberration in the SCID mutant animal that directly affects and regulates fracture repair. This has not been defined or previously documented.
- c) The innate immune system in the SCID animal may have been intrinsically enhanced with up-regulation of macrophage or natural killer cell function. This may have been responsible for driving the enhancement of bone healing seen. It is important to note that upregulation of the innate immune system may actually be caused by lack of suppressor function from T-lymphocytes in these animals.

Barbul *et al.* (1989) also demonstrated in a further experiment with nude mice that T cell reconstitution of nude mice with syngenic splenic T lymphocytes resulted in a significant decrease in mechanical strength and collagen formation, to values similar to those observed in normal euthymic controls (Barbul, Shawe *et al.* 1989). This was to ensure that the effects of wound repair enhancement in nude mice were not due to an

inherent genetic difference which affected wound healing or to intrinsic differences in the innate immune system. Barbul *et al.* (1989) results supported the hypothesis that a subset of T cells normally plays a down-regulatory role in soft tissue wound healing (Schaffer and Barbul 1998). Further work looking at athymic nude mice demonstrated that these mice, *independent* of their genetic background, showed an enhanced ability for wound healing in an ear-punch wound model (Gawronska-Kozak 2004). One study in bone (not fracture repair) reported that nude mice did not lose bone mass after ovariectomy and therefore suggested that T cells are critical for this response (Cenci, Weitzmann *et al.* 2000).

Therefore, incisional skin wounds in animal models where the nude mouse was used or where T cell compartments were depleted with antibodies have shown enhancement of healing. They have shown both mechanically and immunohistochemically a suppressor role for T cells in wound healing. Barbul has reviewed lymphocyte function in soft tissue wound healing and concluded that “*T lymphocytes possess the capacity to regulate essential steps in the process of wound healing*”. Additionally, he noted that nude mice heal with stronger wounds at the same time point compared to wild-type mice, in his studies described above. These authors suggested that it was the CD8+ subset of T cells that has a role in the overall down regulation of wound healing, although the evidence for this has not been verified (Schaffer and Barbul 1998).

The healing process in soft tissue is undoubtedly affected by the cells of the specific immune system and it appears to be the T cell which is key in this role. It is therefore unsurprising that the initial work in this thesis reflects these findings in bone healing

where a T (and B) cell deficient SCID mouse shows more rapid fracture repair than the wild type control.

Reconstitution of SCID mice, similar to that described by Barbul *et al.* (1989), with specific immune cells was the next step in this study. The aim of this was to exclude causes (b) and (c) above as reasons for enhanced fracture repair.

4. Chapter five established bone marrow reconstitution of SCID mice, as determined by the production of antibody. Although it could not be concluded that T and B cell function was restored to normal, the mechanisms that produce and regulate these immunoglobulins come from both these groups of cells and require collaborative effort. The intricacies of reconstituting immune-deficient animals are discussed in Section 5.1 and the literature in this area guided the most appropriate way of achieving this in the SCID mice used (Zinkernagel, Ruedi *et al.* 1988). The results of chapter five demonstrated antibody production in experimental reconstituted mice, not possible in untreated SCID mice. Thus there seemed to be functional recovery of the B and T cell compartments in the reconstituted animals.

5. Chapter six demonstrated that reconstitution of SCID mice, as demonstrated by the ability to produce antibody, resulted in loss of the enhancement of fracture repair seen in SCID animals. This work further confirmed that it was actually the lack of the specific immune system that was causing enhancement and showed that it was not due to an inherent ability of SCID mice to repair bone faster or due to up-regulation of their macrophages (although this may have been the case *due* to a lack of the specific immune system). The results in chapter six also demonstrated verification of the results

in chapter four, confirming the enhancement of bony repair seen in SCID animals and repeating and confirming the viability of the methodology described in chapter two. The results in chapter six are similar to those seen in soft tissue incisional wounds by Barbul *et al.* (1989). As described above, they carried out reconstitution (with T cells) experiments in nude mice, which significantly lowered wound-breaking strength and collagen levels compared with untreated athymic nude mice, and produced similar results to the control, wild type mice (Barbul, Shawe *et al.* 1989). The outcomes shown here in bone healing reflects these results.

6. When one compares the findings of chapters three and seven with regard to the expression of mononuclear leukocytes locally at the fracture site there were very interesting observations made. In wild type mice there appeared to be selective recruitment of T cells, particularly into the periosteal region adjacent to the fracture site. This was even evident at day 1 where already T cells were seen within the periosteum – this would appear to be selective recruitment as there were fewer B cells in this region at this time (although abundant B cells are seen in the fracture haematoma).

Additionally, in the SCID animal, where the T cells were non-functional, there were none noted in the periosteum. With regard to macrophages at day 1 there were multiple numbers of these cells both in the haematoma and the periosteal region in both wild types and SCIDs. The numbers were approximately the same as T cells in wild types, indicating early recruitment of these cells to the periosteal region as well. Section 4.2 showed that fracture healing in the model used here started with increased cellular activity in the cambial layer of the periosteum with initial formation of a periosteal

cartilaginous callus. These findings indicate that both macrophages and T cells and the factors they produce are important in the early stages of fracture repair in the periosteal region. The production of TGF- β 1 by the macrophage has been shown to have a key role in osteogenesis (Champagne, Takebe *et al.* 2002). However, the finding of the T cell in the same area, in close association with callus formation is novel. Also, the selective migration of T cells compared to B cells, and compared to that seen in SCID mice, is a novel finding. By day 3 the numbers of T cells within the healing fracture of the wild type mice as a whole had slightly decreased. However, the numbers within the periosteum remained high again indicating an active, not passive role for these cells. No staining for T cells could be found in the SCID mice fracture area indicating that the non functional CD3 positive cells had been excluded from all areas at this time. In wild type animals there were now essentially no B cells within the periosteum and significantly less in the organising fracture gap and it is likely from the above findings that the B cells do not play an active role in fracture repair. Macrophage numbers remained high in the wild type in all areas at day 3, although were decreasing in number in the SCID in both the adjacent periosteum and fracture gap. By day 7 in wild type there was only sporadic staining for T cells in the organising fracture gap and next to the forming periosteal cartilaginous callus, but none seen within new, regenerate tissue. B cells were now absent from the fracture area. Macrophages were found adjacent to areas of cartilage and early ossification within the fracture regenerate and were most likely phagocytosing cell debris. At day 14 the numbers of T cells and macrophages had continued to decrease. They were in the same distribution as each other, essentially out with areas of new tissue formation, mostly within the periosteum. Both again showed a

slight decrease at day 21 in wild type but were found in the same regions. In the areas in which they were found, the macrophages tended to be in a perivascular location and this has been demonstrated previously (Henricson, Hulth *et al.* 1991). At days 7 to 21 in the SCID mice there was still no T cells found. The macrophages followed a similar pattern to that in the wild type, although the numbers in the periosteum and the overall numbers dropped at a quicker rate.

It can be concluded from the above findings that the T cell plays an active role in fracture repair. It appears to be recruited into the area of periosteal callus formation, the earliest active area of fracture repair in the model. It is likely that this role is inhibitory to fracture healing, given the previous points in this discussion. The macrophage, which has a known role in osteogenesis, is also selectively recruited to the initially active periosteal repair area but also shows more rapid exclusion from the areas of tissue regenerate in SCID mice compared to wild type, in keeping with a more rapid healing process in this strain.

7. Chapter eight revealed that both wild type and SCID mice demonstrated an immediate depression of total leukocyte number in the systemic circulation following fracture. This is a well documented phenomenon following any kind of injury, as seen in both in previous human and animal studies and is termed ‘injury-induced anergy’ of the immune system (Faist, Schinkel *et al.* 1996). This has been postulated to be the major factor in the increased susceptibility of the trauma patient to serious infection and multiple organ dysfunction syndrome (Schneider, Schwacha *et al.* 2006) and is thought to result from decreased entry of lymphocytes into the blood, rather than their increased

exit from the blood into the tissues or cell death (Bolton, Kirov *et al.* 1979). The leukocyte count in the SCID animal was, in general, $0.4 \times 10^6/\text{ml}$ less than in the wild type. This is possibly explained by the general decrease in T cells, which are non-functional, and the lack of B cells in SCID mice. The total leukocyte response over time did, however, show a similar pattern to the wild type, showing that the systemic response to trauma is maintained in SCID mice.

The percentages of mononuclear leukocytes expressed following fracture showed that in wild type, despite a drop in total white cell number, the percentage of B and T cells rose to almost 75% of the lymphoid compartment. A dramatic drop in both these cells then followed this at day 7 post fracture. These compartments then gradually recovered to pre-fracture levels over the next two weeks. This mobilisation of cells of the specific immune system into blood has been demonstrated early after thermal injury as has the rapid fall off in numbers at day 3 post burn (Organ, Antonacci *et al.* 1989). A similar finding has been demonstrated here post fracture and this has not previously been shown at the time of writing. The initial increase in B and T cells may occur in order to provide the fracture site, or other injured tissues, with these cells. Indeed the highest numbers of these cells recorded locally, at the fracture site, was seen at day 1 post fracture. However, the rapid fall off in these cells in blood is most likely related to the general systemic response to trauma and the expression patterns after day 1 did not match those seen at the fracture site. It may be that T cells (as the observations at the fracture site appeared to show significant involvement of these cells) are mobilised for recruitment to the fracture site at the early stages post fracture. This mobilisation then shuts down by day 3 as injury-induced anergy becomes relevant at this time, which

prevents their release to the circulation (Faist, Schinkel *et al.* 1996). The SCID animal showed insignificant levels of B cells at any stage. The T cells, known to be non-functional, showed some suppression in numbers which lasted for the duration of fracture repair.

With regard to the number of monocytes-macrophages in the blood of wild type mice post fracture, there was a rise in the percentage of these cells gradually to day 3, which then tailed off at day 7, before rising again at the end stages of fracture repair. This most likely represents the involvement of macrophages and their products in the initial response to injury which is well documented in the literature (Danon, Kowatch *et al.* 1989). This was then followed by further mobilisation of the monocyte lineage at the end stages of fracture repair, as these cells will differentiate into osteoclasts which are involved in the remodelling phase of fracture repair. The SCID animal showed a higher percentage and total number ($0.33 \times 10^6/\text{ml}$ in SCID compared to $0.045 \times 10^6/\text{ml}$ in wild) of monocytes-macrophages pre-fracture. Post-fracture there was a small initial increase, followed by a dip and then a rise in number which occurred earlier than in wild type mice. This pattern in the SCID was quite similar to the wild except that there was a greater number and the graph was perhaps shifted to the left *i.e.* earlier response - in keeping with the more rapid fracture repair seen in this strain. Comparing the initial quantity of macrophages at day 1 post fracture at the fracture site (Section 7.3) between the two strains interestingly did not show a higher count in the SCID, despite the higher circulating amount. This suggests that even at the earliest stages of fracture repair there is active control over the cells present rather than it being 'just a haematoma'.

The previous work in immune cells in fracture repair was discussed in Section 1.6. Here it was noted that it is likely that the macrophage has a key role in wound debridement, osteogenesis *via* elaboration of TGF- β 1 and BMP-2 and differentiation of uncommitted monocytes to osteoclasts for remodelling of the regenerate tissue. The findings here support this role by the observations:

- (1) the monocyte-macrophage are found locally at the active sites of bone formation during fracture repair
- (2) the systemic expansion of the lineage toward the end of fracture repair as these cells are required for differentiation to osteoclasts.

This work confirms findings from Andrew's work (Andrew, Andrew *et al.* 1994) in human fracture samples which concluded that macrophages were not simply acting as scavengers but are coordinating cells during fracture healing in a similar manner to that described in soft tissue healing. They were thought to be particularly important for steering the differentiation of mesenchymal stem cells according to the kind of tissue injury sustained. The macrophage will remain an important focus in fracture biology research and there are implications for the treatment of non-union of fractures by preventing the macrophage from going down a pro-inflammatory pathway as shown in Figure 1.15 – perhaps we should be attempting to direct the macrophage down the 'pro-wound healing' pathway.

With regard to lymphocytes one small, poorly described study looked qualitatively at T cells locally in the fracture callus in rats and described them as appearing in the fracture

haematoma at 3 hours, becoming more numerous at day 1 and decreasing by days 3-5, although no attempt was made to describe their location, relation to other leukocytes or function (Hulth, Johnell *et al.* 1985). The results from this thesis in chapter three confirm their findings, in particular the temporal distribution of T cells found locally at the fracture site. Andrew *et al.* (1994) investigated lymphocytes in human fracture samples post fracture. The samples were taken from healing fractures at variable sites at variable times. Despite this being an uncontrolled study in human samples, they also demonstrated selective recruitment of T lymphocytes to the initial fracture haematoma and subsequent exclusion from the areas of cartilage and bone formation. Santavirta *et al.* (1992) demonstrated a high number of CD⁴ T-lymphocytes in tissue samples of non-infected delayed union or non-union of diaphyseal bones (Santavirta, Konttinen *et al.* 1992) with only scattered macrophages, again from human samples. The combination of Andrew and Santarvita's studies in humans raises the question of whether T lymphocytes are important in the pathogenesis of failure of bone healing. The findings from this thesis also suggest that T cells inhibit fracture repair. It is possible that these cells are acting locally in a similar manner as to how they do systemically following a major trauma (see Section 1.5.7). Here they modify the hyperactivated innate immune response, dampening down the inflammatory response. In fracture repair it may be that T cells act to counter the effects of the macrophage to give a more ordered, dampened bony healing process in a microcosm of what happens systemically after trauma.

9.1.2 Conclusions

1. The cells of the specific immune system were present in the fracture callus of a closed murine model of fracture repair.
2. An immuno-deficient mouse, with no functioning T or B cells showed enhanced fractured repair with quicker healing times compared to the wild type and this enhancement was ablated with reconstitution of the specific immune system
3. There was selective recruitment of macrophages and T cells to active areas of fracture healing suggesting an active role for both these cells in this process.
4. There was early relative systemic mobilisation of the specific immune cells followed by rapid depression and then recovery. Macrophages/monocytes showed a biphasic systemic response with an early systemic rise which tails off and was then followed by an increase in systemic numbers at the end of fracture repair, presumably for osteoclast differentiation.

9.2 Limitations

9.2.1 Fracture model

In any study of fracture healing the best model to use is the human. However many impracticalities exist in attempting to use humans for studies such as this. Obtaining tissue from healing human fractures which have not required operative (therefore exposing the fracture to immunogenic stimuli) intervention raises ethical difficulties. Controlling for factors such as age, sex, co-morbidity and fracture configuration would have required recruitment of very large numbers. Mechanical property measurements,

one of the key outcomes used in this study, are not yet well defined in human fracture repair. However, this should not prevent human studies wherever possible and the analysis of human systemic markers. The development of new radiographic techniques should aid with this.

As discussed in Section 2.2, the mouse model used in the studies reported here overcomes most of the problems highlighted above. As with any animal model, limitations have to be accepted (Neyt, Buckwalter *et al.* 1998). The skeletal structure of murine bone is more primitive than human, has no Haversian system, and no Haversian remodelling during repair. Intramembranous bone formation occurs under the periosteum in the periosteal callus without endochondral ossification in humans while all bone formation in the mouse used here followed a cartilage intermediate (Bourque, Gross *et al.* 1992). The mouse is therefore a poor model to investigate the form or function of the Haversian system, but this was not important in this study. The mouse is however a good model for immunological studies and for mechanical testing of bone. A strength of this study was the closed nature of the fracture produced. This prevented contamination of the fracture site by any material which may have caused an immunological reaction. However, as the fractured tibia was stabilised in a cast, the alignment of fracture union was not 100% consistent in all limbs as anatomical reduction with internal fixation was not secured *i.e.* the fracture often healed with a small degree of displacement or angulation. However, this is in keeping with the typical scenario in a human, where the large majority of fractures are still managed by closed reduction and external stabilisation in cast, resulting in an imperfect healed fracture alignment in most cases. Of note, in the majority of animals the fibula remained intact,

acting as supplementary ‘internal fixation’ and this assisted in minimising the amount of displacement at the fracture site. The use of a multiple sections taken at variable parts of the fracture callus should have negated any effect of inconsistent alignment of fracture union in the studies in this thesis.

9.2.2 The SCID mouse

The disadvantages of using the SCID mouse strain are that it is a naturally occurring mutant and the full complement of effects this has on the biology of the animal are not known. The manifestations of the mutation of lymphopenia, agammaglobulinaemia and the very high susceptibility to infection with viruses, bacteria and other micro-organisms are well known. The susceptibility to infection may have been a confounding factor during fracture repair. However, all measures available to prevent this were used (*e.g.* individually ventilated cages and sterilised feed) and this was not an obvious problem throughout the study because of consistent results. Alternatively it could have produced a systematic error across all the results. The advantage of the SCID are that the non-lymphoid cells, including the macrophage, natural killer cell and granulocyte are all known to function normally. A known phenomenon in a small percentage of SCID is ‘leakiness’. This is the development of a few clones of functional B and T cells, capable of producing detectable levels of IgG and IgM. These animals still remain severely immunodeficient and incapable of responding to T and B cell mitogens (Carroll, Hardy *et al.* 1989; Hioki, Kuramochi *et al.* 2001). This affects less than 2% of SCID animals and from the ELISA estimation of IgG in chapter 5 (Section 5.3.3), this did not occur in the animals used in that experiment.

9.2.3 Outcome measures of fracture repair

The discussion around each of the techniques used to assess fracture repair and the references examining each of these techniques in greater detail are given throughout chapter 2. All techniques used are standard and accepted in the bone and mineral literature and the power analysis in Section 4.2 validates their use in this study.

9.2.4 Immunohistochemistry

The technique of immunohistochemistry in bony tissue presents many challenges and the intricacies of this have been discussed in Section 2.6. Several points were noted during the prolonged periods spent optimising each of the antibodies used for cell visualisation:

1. The addition of a commercially available protein block (Dako™ protein block) to reduce non-specific background labelling was key in producing useable results for each of the antibodies used in Chapters three and seven
2. The antigen retrieval step required refinement for each antibody used. Proteinase K (Dako™) proved the most useful agent for antigen retrieval but the duration of application, had to remain highly accurate. It should be noted that the lack of staining seen for any of the antibodies in the areas of cartilage and bone is most likely due to lack of these cells in these areas, but *could* have been due to insufficient antigen retrieval penetration in these areas. Adjusting the length of time of application of Proteinase K from that stated in Appendix 4 did not reveal positive staining in the regenerate areas, for any of the times used or in any of the sections.

3. When using the avidin-biotin complex detection system for cells in fracture samples, the counter-stain was often omitted or used very lightly to allow clearer visualisation of positive cells as the aim was to stain whole cells and carry out cell counts. The morphology of the fracture site could still be easily determined in all slides.

9.3 Clinical relevance

There are a high number of immune system related diseases and disorders which have an effect on the immune system (see Section 1.6). The findings from the animal experiments in this thesis raise concerns as to the effect these diseases may have on fracture healing. Any disease that changes the number of T cells or their function, will potentially have an adverse effect on bone healing. The emerging field of immunomodulation with agents such as infliximab (which induces apoptosis of T cells) in common diseases like rheumatoid and inflammatory bowel disease make the outcome of fracture repair in these patients less predictable, in light of these findings. Close observation of these patients will be required when such patients sustain a fracture. There may be implications for treatment of non-union of fracture with immunomodulating agents, although there is no current evidence for this.

The known alteration of the immune response with age (immunosenescence) as described in Section 1.6.2 has implications for the higher rate of non-union seen in the elderly and indeed there may be a direct link between T cell function and this phenomenon. Other clinical factors such as smoking (Section 1.6.7) may affect fracture healing through T cell function. Nicotine on its own has been shown to be immunosuppressive (Sopori and Kozak 1998) causing a lack of responsiveness in T

cells in culture but also to have a stimulatory effect on fracture healing (Presented ORS 2007, Betschold JE *et al.* Minneapolis). Smoking in human studies has been shown to increase numbers of T cells and change the ratios of subsets of T cells (Tollerud, Clark *et al.* 1989). In humans, smoking is well known to inhibit fracture repair (see Section 1.6.7). The clinical implications of the findings in this thesis are therefore multiple and will stimulate many different avenues of investigation aimed at preventing and treating non-union of fractures.

9.4 Future research

In endeavouring to find clinical solutions to the problem of fracture non-union, it is prudent to approach further work from this thesis in terms of developing a therapeutic modality. A soft tissue study in incisional rat wounds showed the CD⁴+:CD⁸+ ratio at 5 days approximates 2:1, which is lower than the 4:1 CD⁴+:CD⁸+ ratio found in peripheral blood (Fishel, Barbul *et al.* 1987). This is the result of both a decrease in the absolute CD⁴+ population and an increase in CD⁸+ levels. The pro-inflammatory effects of CD⁴+ T cell released cytokines and the suppressive effects of CD⁸+ T cells, combined with the temporal relation between the shifts in CD⁴+:CD⁸+ ratios, suggest an up-regulatory role for CD⁴+ lymphocytes and a down-regulatory role for CD⁸+ lymphocytes in wound healing (Boyce, Jones *et al.* 2000). Given the similarities of the findings in this thesis of immune cells in fracture repair with that in soft tissue, it is likely that these ratios are also altered in bone healing. Investigation of this following fracture locally and systemically would be prudent. In terms of therapy, a model of antibody depletion carried out at local and systemic levels, of CD⁴+ and CD⁸+ cells,

with monitoring of the effect on fracture repair would provide results that could potentially lead to a novel treatment. The labelling of these immune cells in the systemic circulation with tracking of their migration at the time of fracture, using a technique such as fluorescent in-situ hybridisation would also provide important information as to the source of the immune cells involved in fracture repair. These futures studies would prove an exciting forefront of fracture biology research.

Appendices

Appendix 1:

Mouse Monitoring Chart: see next page

Use 1 sheet for day 1 making regular post op recordings, and a recording in the evening at the time of analgesia administration and then a further sheet/s for the days following this

MOUSE IDENTITY NO.:

<u>SIGNS</u> ↓	TIME→ (Date & time)							
General Appearance/ Behaviour (0)Normal (1)Mildly abnormal (2)Grossly abnormal	Posture (<i>e.g.</i> Hunched)							
	Mobility							
	Fur (pilo-erection)							
	Eating and drinking							
	Total score (0-8)							
Body weight *	(Grams)							
Weight bearing of # limb	(Comment)							

Weight should be recorded on days 1 – 4 and weekly after that unless there is visual evidence of further weight loss or they have not regained their pre-operative weight by day 4

Analgesia given:	Drug	Amount	Date & Time

ALWAYS GET NACWO/NVS ADVICE IF ANY SUSPICIONS ABOUT THE WELFARE OF THE ANIMAL

Appendix 2: Haematoxylin and Eosin staining protocol for histology

There are several different types of haematoxylin available for the staining of nuclei and these include alum haematoxylins, iron haematoxylins, tungsten haematoxylins, molybdenum haematoxylins and lead haematoxylins. During this study Mayer's Haematoxylin was used - this is an alum haematoxylin chemically ripened with sodium iodate. It is suitable for both haematoxylin and eosin staining and also for the counter staining of nuclei after immunohistochemistry (Bancroft and Stevens 1996).

H&E Staining Method:

1. Place sections in a rack and dewax in xylene for 15 minutes
2. Rehydrate through graded alcohol: 100%, 100%, 90%, 70% for 2 minutes each
3. Rinse in running tap water and then in distilled water
4. Place in Mayer's Haematoxylin for 7 minutes
5. Rinse in running tap water and then in distilled water to remove excess stain
6. Blue section in 2% hydrogen carbonate for 2 minutes
7. Rinse in running tap water
8. Place in Eosin for 5 minutes
9. Rinse in running tap water
10. Dehydrate through graded alcohols: 70%, 90%, 100%, 100% for 2 minutes each
11. Place in xylene clearing and mount one at a time in DPX
12. Observe under light microscopy

I: Goldner's Modified Masson's Trichrome

Procedure:

13. Place sections in a rack and dewax in xylene for 15 minutes
14. Rehydrate through graded alcohol: 100%, 100%, 90%, 70% for 2 minutes each
15. Rinse in running tap water and then in distilled water
16. Stain with ferric chloride for 3 minutes
17. Rinse in running tap water and then in distilled water to remove excess stain
18. Stain with Mayer's haematoxylin (diluted in water 1:10) for 1 minute
19. Rinse in running tap water
20. Stain with Ponceau-acid fuchsin for 8 minutes
21. Rinse quickly with 0.5% acetic acid
22. Stain with 0.5% Phosphomolybdic acid for 3 minutes
23. Rinse quickly with 0.5% acetic acid
24. Stain with light green for 5 minutes
25. Rinse quickly with 0.5% acetic acid
26. Dehydrate through graded alcohols: 70%, 90%, 100%, 100% for 2 minutes each
27. Place in xylene clearing and mount one at a time in DPX
28. Observe under light microscopy

Stains mineralised bone green (nuclei will be visualised blue black from haematoxylin counter stain)

II: Safranin-O

Procedure:

1. Place sections in a rack and dewax in xylene for 15 minutes
2. Rehydrate through graded alcohol: 100%, 100%, 90%, 70% for 2 minutes each
3. Rinse in running tap water and then in distilled water
4. Stain with Mayer's haematoxylin (diluted in water 1:10) for 1 minute
5. Rinse in running tap water
6. Stain in 0.1% safranin O solution for 5 minutes
7. Rinse quickly with 1% acetic acid solution for no more than 10 –15 seconds.
8. Dehydrate through graded alcohols: 70%, 90%, 100%, 100% for 2 minutes each
9. Place in xylene clearing and mount one at a time in DPX
10. Observe under light microscopy

Stains cartilage orange to red, (nuclei will be visualised blue black from haematoxylin counter stain)

Appendix 4: Immunohistochemical protocol

Procedure (carried out at room temperature):

1. Place sections in a rack and dewax in xylene for 15 minutes
2. Rehydrate through graded alcohol: 100%, 100%, 90%, 70% for 2 minutes each
3. Rinse in running tap water and then in distilled water
4. Circle the section with a wax pen
5. Perform antigen retrieval with product and for time specific to antibody used
6. Wash in phosphate buffered saline (PBS) for 5 minutes
7. 3% hydrogen peroxide block (When ABC detection system used) for 15 minutes
8. Wash in phosphate buffered saline (PBS) for 5 minutes
9. Avidin block (When ABC detection system used) for 5 minutes
10. Wash in phosphate buffered saline (PBS) for 5 minutes
11. Biotin block (When ABC detection system used) for 5 minutes
12. Wash in phosphate buffered saline (PBS) for 5 minutes
13. Unspecific protein blocking with Dako™ protein block for 10 minutes
14. Pour off excess protein block
15. Immediately add primary antibody (at recommended dilution in PBS buffer with TWEEN) for 1 hour at room temperature.
16. Wash in phosphate buffered saline (PBS) for 5 minutes
17. Add appropriate secondary antibody (at recommended dilution in PBS buffer with TWEEN) for 30 minutes at room temperature.

18. Wash in phosphate buffered saline (PBS) for 5 minutes
19. Add Vector™ RTU avidin biotin complex (When ABC detection system used) for 30 minutes
20. Wash in phosphate buffered saline (PBS) for 5 minutes
21. Add diaminobenzidine (DAB) colour reagent until appropriate response seen on positive control (When ABC detection system used)
22. Wash in phosphate buffered saline (PBS) for 5 minutes
23. Counterstain (with Mayer's haematoxylin for 2 minutes for ABC sections or DAPI fluorescent nuclear stain for 5 minutes for fluorescent sections)
24. Rinse in running tap water and then in distilled water
25. Dehydrate through graded alcohols: 70%, 90%, 100%, 100% for 2 minutes each
26. Place in xylene clearing and mount one at a time in DPX
27. Observe under light/ fluorescent microscopy as indicated

NB. Fluorescent sections are best tapped on tissue and mounted in an aqueous mountant – do not allow sections to dry out at any point.

Antibodies used / modifications to protocol:

For detection of T cells:

Primary antibody: Monoclonal rabbit anti mouse T cell CD3 peptide (C7930, Sigma Ltd, USA). Diluted 1:100 and applied for 60 minutes at room temperature.

Secondary antibody: Alexa fluoro 488 goat anti-rabbit (Sigma-Aldrich, Steinheim, Germany). Diluted at 1:500 and applied for 30 minutes at room temperature.

Pre-treatment: Proteinase K (Dako™ S3020, Ely, UK) was applied for 5 minutes

Positive control tissue: Mouse spleen

Counter (nuclear) stain: DAPI (4',6-diamidino-2-phenylindole)

For detection of B cells:

Primary antibody: Monoclonal Biotin conjugated rat anti mouse CD45/B220 (553085, BD Biosciences, USA). Diluted 1:150 and applied for 60 minutes at room temperature.

Secondary antibody: Directly conjugated primary therefore no secondary required.

Pre-treatment: Proteinase K (Dako™ S3020, Ely, UK) was applied for 4 minutes

Positive control tissue: Mouse spleen Counter (nuclear) stain: Haematoxylin

For detection of monocytes-macrophages:

Primary antibody: Monoclonal rat anti mouse to F4/80 (ab16911, Abcam, Cambridge UK). Diluted 1:200 and applied for 60 minutes at room temperature.

Secondary antibody: Rabbit anti-rat immunoglobulins / Biotinylated (Dako™ E0468, Ely, UK). Diluted at 1:500 and applied for 30 minutes at room temperature.

Pre-treatment: Proteinase K (Dako™ S3020, Ely, UK) was applied for 7 minutes

Positive control tissue: Mouse spleen Counter (nuclear) stain: Haematoxylin

Appendix 5: Dulbecco's fetal calf complete media

100 Units/ml Penicillin

100 µg/ml Streptomycin

2 mM L-Glutamine

Dulbecco's Minimal Eagle's Media (Gibco)

10% Fetal Calf Serum

Appendix 6: ELISA protocol

1. **Coating:** Each well is coated with 100µl of rabbit anti-mouse IgG (DAKO Z0259,). Leave one pair of wells 'blank' (which gets diluting buffer only and then substrate and H₂SO₄ at end). Leave overnight at 4 °C. Coating antibody concentration is 1:1000 diluted in coating buffer – see reagents below for recipe for coating buffer.
2. **Blocking:** Pour off coating buffer and wash x 3 with washing buffer (see reagents). Removes excess buffer by hitting on to tissues on bench top after last wash. Block wells with 200 µl blocking buffer (5% BSA in PBS – bovine serum albumin;A-6793; Sigma). Incubate at room temperature for 1 hour. Pour off and wash x3 with washing buffer. Remove excess buffer.
3. **Samples:** Add 100µl of standard or sample to appropriate wells, incubate at room temp for 1 hour. For standard (which is purified mouse IgG (I5381; Sigma)) the stock concentration is 1mg/ml – Start at 1:5000 (200ng/ml), doubling dilutions to 1:163840000 (16 standards). Sample and standards dilution buffer: see reagents below. Apply serial dilutions of mouse serum samples, starting at 1:100. Wash plate as before with wash buffer, removing all traces of buffer after final wash.
4. **Detection antibody:** Add 100µl of second antibody (peroxidase conjugated rabbit antibodies to mouse IgG (DAKO P0260 1: 1000 in dilution buffer, see

- 5. Substrate:** Add 100ul of substrate to every well and incubate for 15 mins.

Substrate: OPD. Orthophenylenediamine – in tablet form, see reagents below,
prepare just before needed

- 6. Stop:** Stop reaction with 100ul to every well of 0.5M H₂SO₄, 0.5M, see
reagents below. Read at 490nm with MRX Microplate reader, DYN-Ex
Technologies

Reagents

A. Coating Buffer

0.01 M Phosphate Buffer, 0.15 M NaCl, pH 7.2

NaH₂PO₄, H₂O 0.35 g

Na₂HPO₄, H₂O₂ 1.34 g

NaCl 8.47 g

H₂O add 1 litre - check pH (7.2 ± 0.2)

Store at 4 °C. Stable for 2 months. (12ml of buffer A is needed for one microwell
plate).

B. Washing Buffer, Dilution Buffer

0.01 M Phosphate Buffer, 0.50 M NaCl, 0.1% Tween 20, pH 7.2

NaH₂PO₄, H₂O 0.35 g

Na₂HPO₄, H₂O₂ 1.34 g

NaCl 29.22 g

Tween 20 (Merck, No. 822184) 1 ml

H₂O add 1 litre - check pH (7.2 ± 0.2)

Store at 4 °C. Stable for 2 months. (1 to 3 litres of buffer B is needed for one microwell plate).

C. Chromogenic Substrate

Buffer-containing

OPD tablets of 2 mg 4 (DAKO OPD Tablets, Code No. S 2045)

H₂O 12 mL

Allow the tablets to dissolve, then add

30% H₂O₂ 5 µl

Prepare the reagent shortly before use. Remember that the chromogenic substrate should have reached room temperature before use. After the addition of H₂O₂ the reagent should be watched a few minutes to ascertain that it remains slightly yellow. If the reagent colours increasingly, it has been contaminated with peroxidase from unclean glassware or other source.

The solution is stable at 24 °C for at least 2 hours if protected from light. The given volume is suitable for one microwell plate.

D. 0.5 M Sulphuric Acid

28ml 95 - 97% H_2SO_4 is mixed into about

900ml distilled water.

Note: Do not mix the water into the concentrated acid (risk of boiling).

Adjust the volume to 1 litre with distilled water. (About 15 ml of reagent D is needed for one microwell plate).

Appendix 7: Typical ELISA plate plan, optical density results read out,

formation of standard curve and sample concentration determination to

include error determination from quality controls

The plate plan used for each assay was as follows:

	1	2	3	4	5
A	Std 1:5000 (200ng/ml)	Std 1:5000 (200ng/ml)	Std 1:1280000 (0.78ng/ml)	Std 1:1280000 (0.78ng/ml)	Blank
B	Std 1:10000 (100ng/ml)	Std 1:10000 (100ng/ml)	Std 1:2560000 (0.39ng/ml)	Std 1:2560000 (0.39ng/ml)	QC (10ng/ml)
C	Std 1:20000 (50ng/ml)	Std 1:20000 (50ng/ml)	Std 1:512000 (0.20ng/ml)	Std 1:512000 (0.20ng/ml)	Samp 1 (1:100)
D	Std 1:40000 (25ng/ml)	Std 1:40000 (25ng/ml)	Std 1:10240000 (0.1ng/ml)	Std 1:10240000 (0.1ng/ml)	Samp 1 (1:1000)
E	Std 1:80000 (12.5ng/ml)	Std 1:80000 (12.5ng/ml)	Std 1:20480000 (0.05ng/ml)	Std 1:20480000 (0.05ng/ml)	Samp 1 (1:10000)
F	Std 1:160000 (6.25 ng/ml)	Std 1:160000 (6.25 ng/ml)	Std 1:40960000 (0.025ng/ml)	Std 1:40960000 (0.025ng/ml)	Samp 1 (1:20000)
G	Std 1:320000 (3.13ng/ml)	Std 1:320000 (3.13ng/ml)	Std 1:81920000 (0.012ng/ml)	Std 1:81920000 (0.012ng/ml)	Samp 1 (1:40000)
H	Std 1: 640000 (1.56ng/ml)	Std 1: 640000 (78ng/ml)	Std 1:163840000 (0.006ng/ml)	Std 1: 163840000 (0.006ng/ml)	Samp 1 (1:80000)
	6	7	8	9	10
	Blank	Samp 2 (1:100)	Samp 2 (1:100)	Samp 3 (1:100)	Samp 3 (1:100)
	QC (10ng/ml)	Samp 2 (1:1000)	Samp 2 (1:1000)	Samp 3 (1:1000)	Samp 3 (1:1000)
	Samp 1 (1:100)	Samp 2 (1:10000)	Samp 2 (1:10000)	Samp 3 (1:10000)	Samp 3 (1:10000)
	Samp 1 (1:1000)	Samp 2 (1:20000)	Samp 2 (1:20000)	Samp 3 (1:20000)	Samp 3 (1:20000)
	Samp 1 (1:10000)	Samp 2 (1:40000)	Samp 2 (1:40000)	Samp 3 (1:40000)	Samp 3 (1:40000)
	Samp 1 (1:20000)	Samp 2 (1:80000)	Samp 2 (1:80000)	Samp 3 (1:80000)	Samp 3 (1:80000)
	Samp 1 (1:40000)	Empty	Empty	QC (10ng/ml)	QC (10ng/ml)
	Samp 1 (1:80000)	Empty	Empty	Empty	Empty

Key:

Std: Standard, purified mouse IgG

Samp: Sample. In each case sample 1 was from a wild type animal, sample 2 from a reconstituted animal and sample 3 from a SCID animal

QC: Quality control

An example of the optical density results from the reader were as follows:

	1	2	3	4	5	6	7	8	9	10
A	o	o	0.053	0.059	0.02	0.013	o	o	0.492	0.512
B	o	3.462	0.03	0.037	0.635	0.663	o	o	0.05	0.049
C	2.684	2.622	b	b	o	o	1.364	1.298	b	b
D	1.566	1.572	b	b	o	o	0.679	0.635	b	b
E	0.798	0.78	b	b	1.291	1.305	0.346	0.312	b	b
F	0.405	0.429			0.641	0.659	0.165	0.147	b	b
G	0.199	0.228			0.341	0.318			0.642	0.653
H	0.087	0.086			0.154	0.151				

Assay 4: Determination of mouse IgG concentration

Key:

o: over optical density readable

b: less than blank

From this the average blank optical density ($A5 + A6 \div 2$) was subtracted from

readable results (*i.e.* those not over optical density readable) to give the following:

	1	2	3	4	5	6	7	8	9	10
A			0.036	0.042					0.475	0.495
B		3.445	0.013	0.02	0.618	0.646			0.033	0.032
C	2.667	2.605					1.347	1.281		
D	1.549	1.555					0.662	0.618		
E	0.781	0.763			1.274	1.288	0.329	0.295		
F	0.388	0.412			0.624	0.642	0.148	0.13		
G	0.182	0.211			0.324	0.301			0.625	0.636
H	0.07	0.069			0.137	0.134				

Assay 4: Optical densities with mean blank subtracted

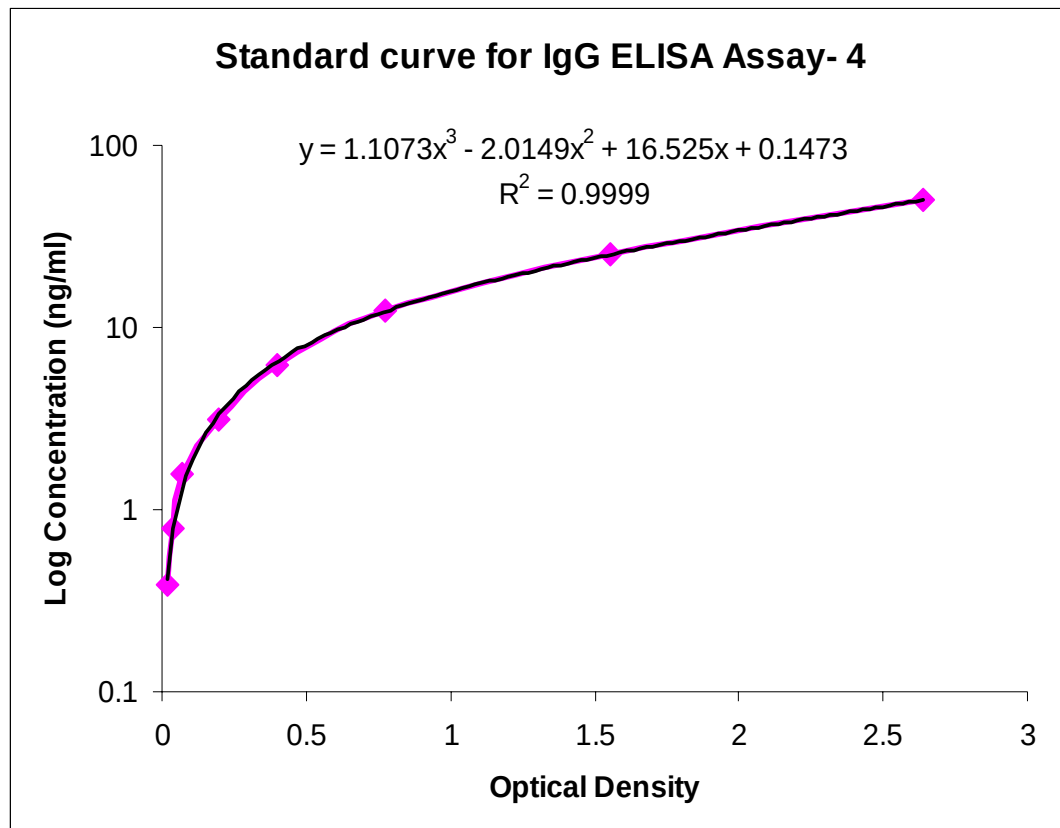
A standard curve was then constructed from the optical density results for the affinity purified IgG, taking the means of the optical densities and comparing this to the known concentrations of IgG.

In the above example there were 8 useable standards:

Conc (ng/ml)	Mean Optical Density Reading
50	2.636
25	1.552
12.5	0.772
6.25	0.4
3.13	0.1965
1.56	0.0695
0.78	0.039
0.39	0.0165

Assay 4: Mean results of standard IgG to allow standard curve to be formed

From this a standard curve was formed:



A typical standard curve used to ascertain total IgG concentration in reconstitution of SCID mice experiment

From this standard curve the concentration of the samples could be determined. The highest optical densities recorded for each sample used due to shape of curve (it is more accurate to use readings which will give a result from the straight (right) part of the curve). The quality controls (QC's) were used to determine error in sample concentration determination:

	Optical density	Conc (ng/ml)	Dilution factor	Real conc (mg/ml)
Samp 1: Average OD of E5 & E6 =	1.281	20.33707594	10000	0.203370759
Samp 2: Average OD of C7 & C8 =	1.314	20.89441543	10000	0.208944154
Samp 3: Average OD of A9 & A10 =	0.485	7.814295499	100	0.00078143
QCs to ensure accuracies of tests				
Average OD of B5 & B6	0.632	10.06582293	True conc= 10ng/ml	% Error: 0.7%
Average OD of G9 and G10	0.6305	10.0428656	True conc= 10ng/ml	% Error: 0.4%

Assay 4: Determination of sample concentrations and quality controls

Appendix 8: Statistical analysis of bone mineral density (BMD), water content and organic content results from Chapter 6

BMD

Statistical analysis revealed BMD data to be parametrically distributed. 1 way ANOVA revealed that there were significant differences in the data ($F=24.7$). The results (P values) of the *post hoc* Scheffé test are displayed in the table below, with significant results ($P<0.05$) highlighted in red:

	WT Exp	RECON Exp	SCID Exp	WT C	RECON C	SCID C
WT Exp		0.999938	0.000000	0.007667	0.095705	0.019714
RECON Exp	0.999938		0.000000	0.004245	0.058957	0.011218
SCID Exp	0.000000	0.000000		0.001162	0.000069	0.000434
WT C	0.007667	0.004245	0.001162		0.915366	0.999260
RECON C	0.095705	0.058957	0.000069	0.915366		0.986425
SCID C	0.019714	0.011218	0.000434	0.999260	0.986425	

Statistical analysis of BMD results using Scheffé test

Water content

Statistical analysis revealed the water content data to be parametrically distributed. 1 way ANOVA revealed that there were significant differences in the data ($F=4.5$). The results (P values) of the *post hoc* Scheffé test are displayed in the table below, with significant results ($P<0.05$) highlighted in red:

	WT Exp	RECON Exp	SCID Exp	WT C	RECON C	SCID C
WT Exp		0.998590	0.224136	0.703439	0.960644	0.999347
RECON Exp	0.998590		0.429896	0.461504	0.819663	1.000000
SCID Exp	0.224136	0.429896		0.009759	0.036380	0.395022
WT C	0.703439	0.461504	0.009759		0.986721	0.496624
RECON C	0.960644	0.819663	0.036380	0.986721		0.848022
SCID C	0.999347	1.000000	0.395022	0.496624	0.848022	

Statistical analysis of water content results using Scheffé test

Organic content

Statistical analysis revealed the organic content data to be parametrically distributed.

1 way ANOVA revealed that there were significant differences in the data ($F=24.6$).

The results (P values) of the *post hoc* Scheffé test are displayed in the table below,

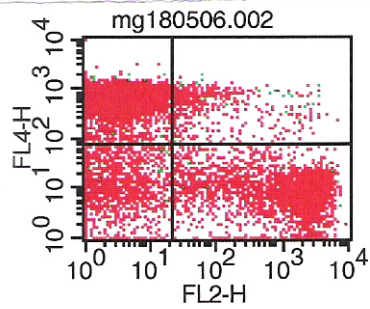
with significant results ($P<0.05$) highlighted in red:

	WT Exp	RECON Exp	SCID Exp	WT C	RECON C	SCID C
WT Exp		0.996067	0.015304	0.000027	0.000003	0.000002
RECON Exp	0.996067		0.053946	0.000111	0.000013	0.000010
SCID Exp	0.015304	0.053946		0.196431	0.055491	0.044111
WT C	0.000027	0.000111	0.196431		0.997567	0.994212
RECON C	0.000003	0.000013	0.055491	0.997567		0.999999
SCID C	0.000002	0.000010	0.044111	0.994212	0.999999	

Statistical analysis of organic content results using Scheffé test

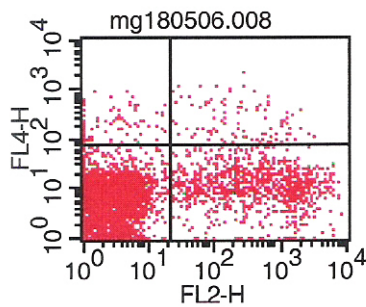
Appendix 9: Examples of scatter plots obtained from FACS analysis

The graphs below demonstrate the two dimensional histograms obtained for the antibodies as labelled on the plots following gating with CD45:



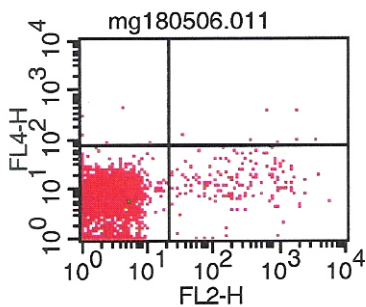
B220 APC
THy1 Pe

Wild type, day 1



B220 APC
THy1 Pe

Wild type day 14



B220 APC
THy1 Pe

SCID day 14

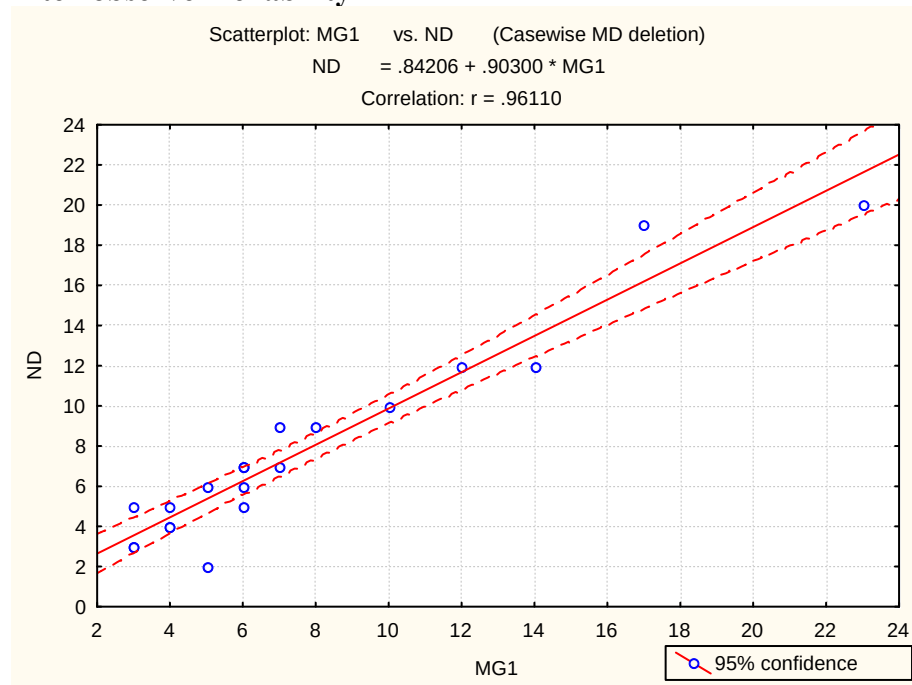
Appendix 10:

Reliability study for immunohistochemistry quantification

A random selection of the immunohistochemistry photographs captured for quantification were used to determine inter- and intra-observer reliability. Dr Nestor Demosthenous, a Research Fellow working in the lab kindly agreed to count the cells on these slides to determine the inter-observer reliability of the method – he was blinded to my original count result. I reassessed the same slides at a one month interval to determine the intra-observer reliability, blinded to both my original result and the result of ND.

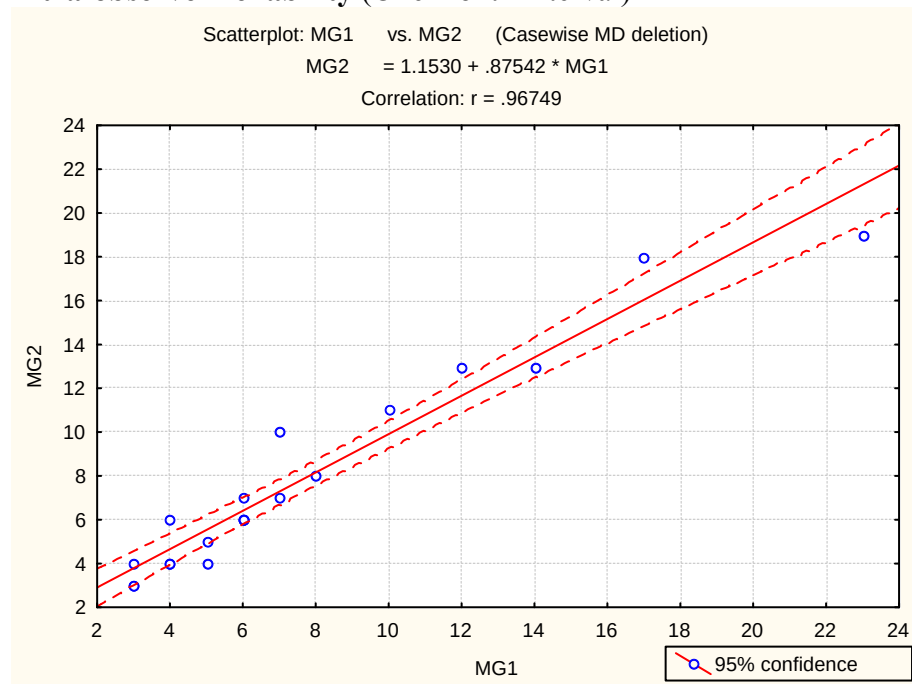
As the data were continuous – *i.e.* actual number of cells present, (and not categorised) a correlation coefficient was determined to ascertain inter- and intra-observer reliability. The Pearson Product Moment Correlation was determined using Statistica version 6.0 (Statsoft Inc, 2300 East 14th Street, Tulsa, O.K. 71404, U.S.A.). The results are described below:

Inter-observer reliability



Correlation coefficient $R = 0.96$
Statistically significant correlation $P < 0.05$

Intra-observer reliability (One month interval)



Correlation coefficient $R = 0.97$
Statistically significant correlation $P < 0.05$

Grants / Fellowships:

- Aug 2005 Pump Priming Support Grant for “The immune system has a pivotal role in the process of fracture repair”. Royal College of Surgeons of Edinburgh, (£6000 consumables).
- Dec 2005 Awarded Royal College of Surgeons of Edinburgh / **Cutner Orthopaedic Fellowship** for 1 year’s primary research in Orthopaedics – “The immune system has a pivotal role in the process of fracture repair”. (£45,000 salary).
- May 2006 RI Stirling award for travel to International Conference on Osteoimmunology, Crete, to present research from this thesis (£1200 travelling costs).
- Sept 2006 Small Research Support Grant, RCSEd for “T lymphocytes have an essential role in normal bone fracture repair” (£5800, consumables).
- Feb 2007 RI Stirling award for travel to 53rd Annual Meeting of the American Orthopaedic Research Society, San Deigo, to present research from this thesis (£1080 travelling costs)
- Oct 2007 **BORS/ORS Travelling Research Fellowship:** 4 week travelling fellowship to present research carried out in this thesis at orthopaedic centres in North America including UCSF, Mayo clinic, U Penn, Jefferson, HSS-NY and Mass General, Boston.

Prizes:

- BORS/BOTA prize at the BOA: 2nd place, best presentation (“The effect of knock-out of T and B cells in fracture repair”), Manchester. 26 September 2007.
- Roy Petrie Memorial Prize for best presentation (“Enhanced Fracture Repair *In Vivo* in the Absence of the Specific Immune System”) Queen Margaret Hospital, Fife. 12 June 2007.
- Awarded prize for best poster, (“Deficiency of the specific immune system enhances fracture repair *in vivo*”). Pan-Celtic Orthopaedic meeting. June 2006.

Appendix 12: Presentations at National and International meetings and Publications

Presentations to learned societies:

1. Oral presentation: “The effect of knock-out of T and B cells in fracture repair”. British Orthopaedic Association second podium prize at BOTA/BORS session, Manchester. 26 September 2007
2. E-Poster presentation: “Enhanced fracture repair *in vivo* in the absence of the specific immune system”. 8th EFORT Congress, Florence, Italy. 11-15 May 2007.
3. Oral presentation “Enhanced fracture repair *in vivo* in the absence of the specific immune system” American Orthopaedic Research Society, San Diego. 12th February 2007
4. Poster presentation “Deficiency of the Specific Immune System Enhances Fracture Repair *in vivo*” British Orthopaedic Association, Glasgow. Sept 27 – 29, 2006.
5. Oral presentation “Deficiency of the Specific Immune System Enhances Fracture Repair *in vivo*” British Orthopaedic Research Society, Southampton. 5th July 2006.
6. Poster presentation “Deficiency of the Specific Immune System Enhances Fracture Repair *in vivo*” Pan-Celtic Orthopaedic meeting, Ayrshire. 15th – 16th June 2006 – awarded best poster prize.
7. Oral presentation “Deficiency of the Specific Immune System Enhances Fracture Repair *in vivo*” 1st International Conference on Osteoimmunology: Interactions of the Immune and Skeletal Systems, Crete. May 28 - June 2, 2006.

Publications:

“Deficiency of the specific immune system enhances fracture repair *in vivo*” Gaston MS, Noble BS, Simpson AHRW. Journal of Bone and Joint Surgery - British Volume, Nov 2008 Vol 90-B: 365.

“Inhibition of Fracture Healing” Gaston MS, Simpson AH. Journal of Bone and Joint Surgery Br. 2007 Dec;89(12):1553-60. *Review written as summation of introduction to this thesis*

“Enhanced fracture repair *in vivo* in the absence of the specific immune system” Gaston MS, Noble BS, Simpson AHRW. Abstract: Transactions to Orthopaedic Research Society Vol.32, Number 357 San Diego 2007

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