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Inflammation in Cancellous and Cortical Bone Healing

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POPULÄRVETENSKAPLIG SAMMANFATTNING

För de allra flesta sker en association till ett brutet benskaft när man tänker på fraktur. Det har det även gjort för forskare. Men för många som haft en fraktur är erfarenheten en annan. Handledsfraktur, axelfraktur och överarmsfraktur är kliniskt vanliga exempel på så kallade metafysära frakturer. Detta är frakturer som uppstått i anslutning till en led. Det finns flera intressanta skillnader mellan en skaftfraktur och en metafysär fraktur. Över ett benskaft finns muskelbukar, som fäster in med senor nära leden. Muskeltäckning är viktigt för frakturläkning av ett skaft, men verkar inte vara behövligt vid metafysär fraktur där bara senor finns. Hos den vuxne finns det ingen blodbildande benmärg i mitten av ett rörbensskaft, men det finns det i metafysen. Det finns alltså uppenbara skillnader i de anatomiska villkoren för frakturläkning av skaftfraktur respektive metafysär fraktur. Vi vet experimentellt från djurmodeller att vanliga antiinflammatoriska läkemedel hämmar läkning av en skaftfraktur, men inte en metafysär fraktur. Varför det är så vet vi inte.

Denna avhandling försöker bidra till förståelsen kring metafysär fraktur och hur den skiljer sig från rörbensfrakturen.

I delarbete I kartlades den cellulära sammansättningen avseende immunceller vid metafysär benläkning med hjälp av flödescytometri. Cellsammansättningen i metafysära tibia studerades från skada till 10 dagar efteråt och jämfördes med dels oskadat ben, dels motsatta sidans ben hos samma mus. Cellsammansättningen var likartad i skadat ben och oskadat ben, men vissa skillnader kunde ses hos makrofager. En god uppfattning om naturalförloppet på cellnivå kunde etableras för metafysär skada och en panel för flödescytometri etableras.

I delarbete II kartlades skillnader i cellsammansättning hos kortikal och metafysär benläkning med hjälp av flödescytometri. Cellsammansättningen var likartad dag 3, men utvecklades i olika riktning till dag 5. Framförallt noterades att neutrofila granulocyter ökade i metafysärt ben medan monocyter och lymfocyter ökade i kortikalt ben.

I delarbete III utsättes metafysär och kortikal benläkning för det antiinflammatoriska läkemedlet indomethacin, vilket vi vet hämmar hållfasthet vid kortikal benläkning men inte vid metafysär benläkning. Vi kartlade cellsammansättningen med flödescytometri och proteinprofilen i cellmiljön med masspektrometri. Den huvudsakliga påverkan av indomethacin sågs i kortikalt ben dag 3, där proteinprofilen tydligt påverkades med ökat antal proteiner unika proteiner. Endast en skillnad noterades i cellsammansättningen, nämligen en tydlig ökning av inflammatoriska monocyter. Däremot sågs ingen enskild stor påverkan på kortikalt ben dag 5 eller på metafysärt ben dag 3 eller dag 5. Fyndet är förenligt med tidigare observation att hållfastheten i metafysärt ben inte påverkas av indomethacin, medan tidig indomethacinbehandling påverkar hållfastheten i kortikalt ben.

I delarbete IV studerades metafysär benläkning vid hämning av makrofager. Det kunde visas att utdragsmotståndet hos en skruv i benet blev lägre om man slog ut makrofagerna tidigt, men inte om makrofager slås ut vid senare tillfällen. Resultaten antyder att makrofager har en viktig roll i det tidiga skedet av metafysär benläkning. Med flödescytometri kunde det kartläggas att det framförallt var en viss typ av makrofager som slogs ut och sannolikt har delorsak till den sämre benläkningen.

Sammantaget redogör avhandlingen för benläkning på en detaljerad nivå avseende cellsammansättning och möjliga anledningar till vad som skiljer benläkningen i en skaftfraktur och en metafysär fraktur åt.

ABSTRACT

Fractures in humans most commonly occur near the joints, in the metaphyseal bone area mainly consisting of cancellous bone. Despite this, mainly cortical fractures, located in the diaphyseal bone area, have been studied in experimental models of bone healing. It is known from previous studies that the diaphyseal fracture is sensitive to anti-inflammatory treatment, while metaphyseal bone healing is more resistant. The aim of this thesis is to study the inflammatory response to bone trauma in cancellous and cortical bone. A flow cytometric method was established for the purpose of examining the cellular composition of the inflammatory process in models of bone healing

In paper I the cellular composition of metaphyseal bone healing was studied with flow cytometry. The proximal tibia was traumatized and then studied at day 1, 3, 5 and 10 afterwards and compared to healthy mice. The contralateral proximal tibia was also studied at the same time points to delineate the trauma site specific inflammation. A few changes could be noted that seemed specific to the trauma site in macrophage phenotype development. However, the cellular composition was similar at the trauma site and in the contralateral proximal tibia. This notion of a general skeletal response was confirmed with analysis of the humerus at day 5.

In paper II a model of cortical bone healing apt for flow cytometry was developed and compared to cancellous bone healing. A furrow was milled along the femoral cortex and the healing bone tissue analyzed. The earliest time point that enough cells were present for flow cytometry was day 3. The cortical and cancellous model of bone healing was compared at day 3 and 5 to study how they evolve in comparison to each other. It was noted that they were similar in cellular composition at day 3, but had diverged at day 5. The cancellous model increased in neutrophilic granulocytes, whereas the cortical model increased in lymphocytes.

In paper III the cancellous and cortical model were compared under experimental intervention of indomethacin. It is known that indomethacin leads to weakened biomechanical properties in cortical bone healing, but not in cancellous bone healing. The effect on cellular composition with indomethacin was studied with flow cytometry and the extracellular protein profile in the healing bone tissue with mass spectrometry. Unexpectedly, inflammatory monocytes were increased in the cortical model at day 3 with indomethacin, but otherwise the models were similar in cell composition at day 3 and 5. In mass spectrometry there was a large increase in detected proteins at day 3 in the indomethacin exposed cortical model, but otherwise the models were similar. This points to an early and model specific effect of indomethacin. The observed lack of indomethacin-induced effects in cancellous bone healing is in line with the previously noted lack of indomethacin-induced effects on bone weakening. The apparently increased inflammatory activity in the cortical model with indomethacin exposure at day 3 might indicate the healing process to be disturbed and not able to progress from the early proinflammatory state to a more anabolic, anti-inflammatory state.

In paper IV the effect of macrophage depletion on healing of metaphyseal bone was studied. Clodronate was given for depletion at different time points prior to surgery and the pull-out force of a screw or tissue phenotyping of macrophages was performed a varying number of days after surgery. It was noted that metaphyseal bone healing was to a large extent inhibited by macrophage depletion up to two days after surgery, but not if depletion was done more than two days after surgery. Thus, macrophages seem to be most important during the first two days after trauma in cancellous bone healing.

In summary this thesis provide insight to the natural development of bone healing. The findings emphasise that cancellous and cortical bone healing are different entities with differences in the inflammatory process leading to healing.

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First and foremost, I would like to thank my late supervisor Per Aspenberg. Per was very generous with his time. Per was very courageous to let me start with flow cytometry even though neither the group nor I had any prior experience. Per had a great sense of scientific research that is hard to gain by study, and I am thankful to have been let in on his thinking.

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Till mormor och morfar för livets visdomar.

Till moster med familj för umgänge och intressanta diskussioner.

Contents

List of Figures

List of Tables

List of Terms

CI

Confidence interval

Clodronate

A bisphophonate drug. Bisphosphonates inhibit osteoclasts and is widely used in treatment of osteoporosis. Clodronate inhibits macrophages and is used experimentally to deplete them and study the effect of their absence.

COX

Cyclooxygenase, formally known as prostaglandin-endoperoxide synthase (PTGS). Converts arachidonic acid to prostaglandins and thromboxanes. Exists in 2 isoforms, a constitutive and an inducible variant.

FMO

Fluorescense Minus One. A control in flow cytometry where all but one of the fluorchrome-conjugated antibodies are present. In this way the background fluorescence may be evaluated for a specific marker.

G-CSF

Granulocyte-colony stimulating factor. Also known as colony stimulating factor 3, CSF3. It promotes differentiation of neutrophilic granulocytes.

GM-CSF

Granulocyte-macrophage colony-stimulating factror. Also known as colony stimulating factor 2, CSF2. Its effect on myeloid cells is broader than M-CSF and G-CSF. It induces an M1 phenotype in macrophages.

HSC

Hematopoietic stem cell

IL-17

Interleukin 17. Family of proinflammatory cytokines secreted by Th_{17} cells.

Interferon-γ

A proinflammaty Th_1-associated cytokine

M2

Alternatively activated macrophages, also known as resident macrophages. Considered anti-inflammatory and important to the resolution of inflammation.

M-CSF

Macrophage-colony stimulating factor. Also known as colony stimulating factor 1, CSF1. It stimulates outgrowth of macrophages and induces an M2 phenotype. Its receptor is a known protooncogene, *c*-fms.

MSC

Mesenchymal stem cell

NSAID

Nonsteroidal anti-inflammatory drug. Old name to anti-inflammatory drugs that did not have a steroid ring structure, i.e. were not derived from glucocorticoids. With the expansion of anti-inflammatory drug products, it is today synonymous to COX-inhibitors.

PGE2

Prostaglandin E2

PMT

Photomultiplier tube. It is the detector of fluorescense in a flow cytometer.

$Rag^{-/-}$

Recombination activating gene. Rag deficient mice lack mature B and T lymphocytes and are used to model a state of no adaptive immunity.

RANKL

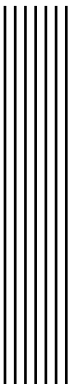
Receptor activator of nuclear factor ϰB ligand. Binds to osteoclasts and is a key regulator of osteoclast differentiation and activity.

STAT3

Signal activator and transducer of transcription 3

TNF

Tumor necrosis factor. Previously known as tumor necrosis factor α, lymphotoxin-α and cachexin. Hallmark proinflammatory cytokine to propagate inflammation. It can be produced by many cells.



List of Cell Types

γδ T cell

γδ T cell. Essentially an innate type of cell and not part of adaptive immunity. Its name derives from the T-cell receptor consisting of a γ- and δ-subunit instead of an α- and β-subunit found on conventional T cells of the adaptive immune system.

M1

Classically activated macrophages. Considered to be proinflammatory.

Mono

Short for monocytes and implies that they have been defined by CD45 expression and side scatter or morphology, but not specific lineage markers such as CD11b.

T_{Cyt}

T cytotoxic cells. These cells have the capability to kill cells directly with cytolytic enzymes. In contrast to T helper cells that are presented antigen, these cells inspect normal cells for foreign antigen. They are $CD3^+$ and $CD8^+$, but $CD4^-$.

T_{reg}

T-regulatory cells. Anti-inflammatory T cells.

T_{EMRA}

T-effector memory cells with RA-isoform of CD45. A subtype of $CD8^+$ T-cytotoxic cells found in humans but not in mice.

T_H

T-helper cells. These express CD4, but not CD8.

Th_{17}

T helper 17 cells. These cells produce interleukin 17.

List of Phenotypic Markers

CCR7

CCR7, also known as CD197, recognizes CCL19 and CCL21, which are produced in lymph nodes and aid homing. It can be used as an M1 marker for macrophages

CD115

Receptor to M-CSF. It is expressed mainly on monocytes and early osteoclasts.

CD11b

Myeloid cell marker. Integrin needed to attach to endothelium for diapedesis and migration. Prominent on monocytes, macrophages and granulocytes.

CD14

Lipopolysacharide (LPS) receptor. LPS is found on gram-negative bacteria. Marker for monocytes and macrophages.

CD16

FcγRIII. An F_C-receptor found on monocytes.

CD18

Integrin beta 2. A surface molecule that forms different heterodimers. When associated with CD11b it forms macrophage antigen-1 (Mac-1), which is a marker for macrophages. When associated with CD11a it forms lymphocyte functional antigen-1 (LFA-1), which is expressed by lymphocytes.

CD19

B cell marker. Expressed from early B cell development until plasma cell differentiation.

CD200

OX-2 membrane glycoprotein. Expressed mainly by B and T cells. Thought to provide an inhibitory signal to myeloid cells.

CD200R

Receptor for CD200. Inhibits inflammation by inhibiting expression of proinflammatory molecules such as TNF, interferons and inducible nitric oxide. Mainly present on myeloid cells such as macrophages, but also on certain T and B cells.

CD206

Mannose receptor. Mannose is present on some microorganisms and debris in inflammation. Commonly used as a marker for M2 macrophages

CD25

α-chain of IL-2 receptor. Activated lymphocytes have an increased expression of CD25.

CD3

T cell specific antigen. It is a coreceptor to the T-cell receptor and is critical to the activation of a T cell

CD4

Co-receptor to the T-cell receptor that is specific to T helper cells. It can be found on some myeloid cells as well. Myeloid cells, however, do not express CD3.

CD45

Found on all white blood cells, hence its usage as a general marker for leukocytes. Major leukocyte populations can be distinguished based on intensity of CD45 and side scatter (SSC).

CD68

Common marker for monocytes and macrophages. Also known as macrosialin. It is thought to be important to their migrating properties

CD8

Co-receptor to the T-cell receptor and found mainly on cytotoxic T cells.

CD80

Costimulatory signal to T cells found on presenting cells, i.e. dendritic cells, macrophages, and B cells. Also known as B7-1. CD86 has a similar function, and is also known as B7-2. These can both activate and inhibit T cells depending on which receptor it binds to (CD28 and CTLA-4, respectively).

F4/80

Mouse macrophage marker. Unknown function.

FOXP3

Forkhead box P3. Master regulatory transcription factor in T-regulatoy cells.

Gr-1

Antibody that binds to both Ly6C and Ly6G. It can therefore not distinguish between monocytes and granulocytes

IgD

Immunoglobulin D. Expressed during development and lost when the B cell leaves the bone marrow.

IgM

Immunoglobulin M. Monomeric IgM is expressed by B cells from late development in bone marrow until antigen stimulation and isotype switch.

Ly6C

lymphocyte antigen 6 complex, locus C1. Expressed by many hematopoietic cells, including neutrophils, monocytes, and subsets of lymphocytes. Commonly used to differentiate monocytes into $Ly6C^{lo}$ and $Ly6C^{hi}$ subsets together with other phenotypic markers

Ly6G

lymphocyte antigen 6 complex, locus G6D. Expressed by granulocytes and its expression increases with differentiation. It is useful to distinguish monocytes from granulocytes, which both are $CD45^{mid}$ and partially overlap in SSC. Both are $CD11b^{+}$.

NK

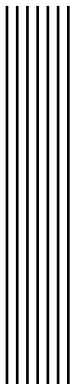
Natural killer cells. Lymphocytes that are innate, in comparison to T and B cells that are adaptive.

NK1

Natural killer cell receptor. Found on natural killer cell and some T cells, known as NKT cells. NK cells do not express CD3, which NKT cells do. NK1.1 is only present in the C57BL/6J strain of mice.

SSC

Side scatter. The side scatter is the reflection of cells in flow cytometer at an angle, i.e. the scattering of light from a cell. It is often used as a marker for cell complexity, in that complex cells reflects more light at an angle than normal cells. Neutrophils are typical complex cells that reflect light at an angle, while lymphocytes do so only to a small extent.



Thesis at a Glance

Table 1: Thesis at a Glance

	Question	Methods	Evaluation	Answer
I	What is the normal cellular response in metaphyseal injury? Is there a systemic effect?	Metaphyseal needle injury	Flow cytometry. Sacrifice day 1, 3, 5 and 10.	Macrophage polarization was rreversed with an initial M2 response, followed by M1. A systemic effect was seen.
II	What is the difference in cellular response in cortical and cancellous injury?	Metaphyseal needle injury. Femoral cortex milling	Flow cytometry. Sacrifice day 3 and 5.	Diverge from day 3 to 5. Lymphocytes and monocytes displayed a relative increase in cortical bone healing, vs. granulocytes in cancellous bone healing.
III	Is there a difference in cellular response with indomethacin in cancellous and cortical bone healing? Different protein environment with indomethacin?	Metaphyseal needle injury. Femoral cortex milling. Indomethacin injection. Sacrifice day 3 and 5.	Flow cytometry. Mass Spectrometry. Sacrifice day 3 and 5.	Increase in inflammation related cell population and proteins in cortical model was noted at day 3 with indomethacin.
IV	What is the effect of macrophage depletion in cancellous bone healing? Cellular composition in cancellous model with macrophage depletion?	Screw insertion proximal tibia. Metaphyseal needle injury.	Pull-out force. Flow cytometry	Depletion with clodronate resulted in decreased pull-out force and fewer resident phenotype macrophages.

List of Papers

I Isolated metaphyseal injury influences unrelated bones
— A flow cytometric study of tibia and humerus in mice

Tätting, L. Sandberg, O. Bernhardsson, M. Ernerudh, J. Aspenberg, P.
Acta Ortopaedica 2017

II Different composition of leukocytes in cortical and cancellous bone healing in a mouse model

Tätting, L. Sandberg, O. Bernhardsson, M. Ernerudh, J. Aspenberg, P.
Bone and Joint Research 2018

III Indomethacin Effects on Cellular Composition and Extracellular Protein Profile of Cancellous and Cortical Bone Healing

Tätting, L. Turkina, M. Bernhardsson, M. Eliasson, P. Ernerudh, J. Fahlgren, A.
in submission 2019

IV Temporal role of macrophages in cancellous bone healing

Sandberg, O. Tätting, L. Bernhardsson, M. Aspenberg, P.
Bone 2017

Relevant Work Not Included in This Thesis

Marrow Compartment Contribution to Cortical Defect Healing
Acta Orthopaedica 2017
Bernhardsson, M. Tätting, L. Sandberg, O. Schilcher, J. Aspenberg, P.

The cortical model with a cortical furrow in the femur was used (explained in section 3.1 on page 13). The marrow beneath the milled away cortex was removed. The adjoining intact bone marrow beneath intact cortex proximal and distal to the cortical defect was sealed off with silicone plugs. The healing cortical bone defect did not have any contact with bone marrow. The healing process was delayed compared to healing bone with sustained contact to bone marrow. Bone marrow does seem important to cortical bone healing.

Depletion of cytotoxic ($CD8^+$) T cells impairs implant fixation in rat cancellous bone
Journal of Orthopaedic Research 2019
Bernhardsson, M. Dietrich-Zagonel, F. Tätting, L. Eliasson, P. Aspenberg, P.

A subset of cytotoxic T cells has been shown to correlate with non-union of fractures. This paper tested the effect of $CD8^+$ cell depletion in a cancellous implant model. Contrary to our expectation, the pull-put force of treated mice was less than placebo treated controls. There was no difference in bone scan results. This points to a possible role of $CD8^+$ cells in implant related bone healing of cancellous bone.

Nomenclature

Anatomy vs. Physiology

The healing of bone can be described at the level of physiology or anatomy. “Cancellous bone healing” and “metaphyseal fracture healing” are used almost interchangeably, as is “cortical bone healing” and “shaft fracture healing”. “Metaphyseal” and “shaft” is used when emphasis need to be made on the anatomical or macrospopic aspect of bone healing, and “cancellous” and “cortical” when emphasis on the physiological aspect is needed. “Diaphyseal” is interchangeable with “shaft”. “Trabecula” is in between “cancellous” and “metaphyseal” and emphasizes physiology of the metaphyseal niche.

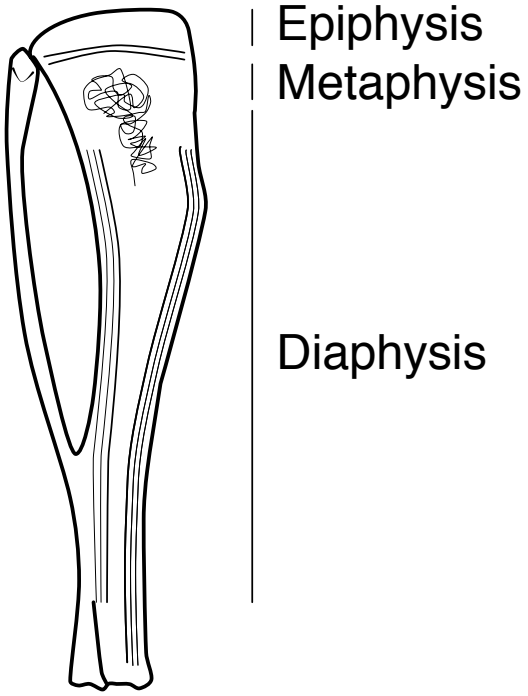


Figure 1: Nomenclature of Shaft Bones

A mouse tibia is shown as an example. The growth plate is still visible between the epiphysis and metaphysis. Cortical bone is lamellar and make up the circumference of shaft. Inside, trabecular (spongy) bone fills the inner volume of all three parts (only shown in upper metaphysis here). The growth plate separates the metaphysis from the epiphysis. Cancellous bone exists in the epiphysis as well. Note that the mouse fibula fuses with the tibia, which it does not do in humans.

1 Introduction

1.1 Previous Work

Much of what we know about bone healing is based on models of cortical bone healing. The principal model of cortical bone healing consists of an osteotomy of the femur of a rodent (further elaborated in section 3.1 on page 13). The mechanical strength of the healing fracture is then evaluated by simple bending until broken. This model is easy to interpret and use. However, this model is not representative of most fractures in the clinical setting. Most fractures in humans are close to the joint and characterized by trabecular bone damage (Donaldson et al. 2008; Singer et al. 1998). The shoulder, hip, distal radius and vertebrae mainly consist of trabecular bone. The thick cortical strength, abundance of surrounding muscle bellies and obligate instability of the femoral osteotomy does not model these fractures faithfully.

The Aspenberg group have previously studied the differences between cortical and cancellous bone healing (see Table 1 on page 2). These studies showed experimentally that metaphyseal fractures and shaft fractures are affected differently by anti-inflammatory agents. Mainly, the metaphyseal fracture seem not to rely on inflammatory stimuli as the shaft fracture is known to do.

1.2 Background and Rationale

Our group has shown that there are differences in healing of metaphyseal fractures and shaft fractures. However, it was not known how they were different. We assumed that the cellular response in the fracture was important in explaining differences between these two fracture types. Prior research had characterized fracture healing with mainly histological and immunohistochemical methods. These methods can describe the architectural progress of fracture repair. However, these methods lack the ability to faithfully describe the cellular compartment of a fracture since

they only see a small section of the fracture and only a minority of cell types may be stained for in the same section. Many cell types cannot be discriminated by morphology or dye staining, and need monoclonal antibodies to reveal expression of characteristic antigens. Immunohistochemistry allows this to some extent, but is much less capable than flow cytometry in phenotyping cells with multiple antibodies simultaneously. For subsets of immune cells, an array of antibodies are needed to delineate the phenotype properly.

Flow cytometry has been used in immunology for a long time and its capabilities made it appropriate to characterize the cellular composition of a fracture. We used flow cytometry to better understand the cell composition of healing bone.

Table 1: List of Prior Relevant Work

Relevant work from Aspenberg prior to and influential on the aims of this thesis.

Finding	Paper
Sclerostin antibody increases metaphyseal bone healing	Agholme et al. (2010)
TNF inhibitor etanercept does not impair metaphyseal bone healing	O. Sandberg, Eliasson, et al. (2012)
COX-2 inhibitor celecoxib has only a slight effect, if any, on metaphyseal bone healing	O. Sandberg and Aspenberg (2015*b*)
Dexamethasone does not impair, and maybe even aid, metaphyseal bone healing	O. Sandberg and Aspenberg (2015*a*)
A review on cancellous bone healing is written	O. Sandberg and Aspenberg (2016)
Alendronate is shown to affect metaphyseal and diaphyseal bone healing differently	O. Sandberg, Bernhardsson, et al. (2017)

1.3 Research Aims

The general aim of this thesis was to characterize cancellous and cortical bone healing.

PAPER I

i Establish a multi-color flow cytometric method that can assess the cellular inflammatory response during the process of bone healing

ii Elucidate which cell types are specific to the traumatized cancellous bone compared to uninjured contralateral cancellous bone in the same mouse

iii Elucidate if the cell composition differs between the traumatized cancellous bone compared to uninjured bone or if the effect of bone injury is systemic to other bones

PAPER II

i Compare and pinpoint potential differences in cell composition during the course of cortical and cancellous bone healing

PAPER III

i To assess if cancellous and cortical bone healing show different patterns of cell composition or extracellular protein profile during treatment with indomethacin

PAPER IV

i Assess the effects of indomethacin treatment on cell composition and extracellular protein profile in cancellous and cortical bone healing

ii Investigate if and when macrophage depletion may affect cancellous bone healing

2 Inflammation in Bone Healing

MANY cell subsets have been implicated in bone healing, including both myeloid and lymphoid cells. In the literature, data can be found at least for the following cell types — granulocytes (Chan et al. 2015; Kovtun et al. 2016), macrophages (Alexander et al. 2011; Chang et al. 2008; Levy et al. 2016; Vi et al. 2015; Wu et al. 2013), T cells and B cells (Könnecke et al. 2014; Nam et al. 2012; Toben et al. 2011), $CD4^+$ T-helper (T_H) cells (Nam et al. 2012; Sato et al. 2006), T-effector memory cells with RA-isoform of CD45 (T_{EMRA}) (Reinke et al. 2013) and T-regulatory (T_{Reg}) cells (Zaiss et al. 2007). All data on the influence of a particular cell subset on the outcome of fracture healing have been gained with models of shaft fractures. It is also from these models the phases of bone healing have been derived (Figure 1 on page 6). This thesis is mainly concerned with the initial inflammatory phase of bone healing.

2.1 Blood Counts Differ Between Humans and Mice

It should be noted that the relative proportion and to some extent morphology of major white blood cell populations differ between humans and mice. In humans, the dominating population in a normal blood sample is neutrophilic granulocytes followed by lymphocytes and then monocytes. In mice, the dominating feature is lymphocytes (70-80%) followed by granulocytes (20-30%). This is also true for the bone marrow where lymphocytes are more abundant than in other mammals. In addition, the cellularity of bone marrow is much higher in mice than in humans. While extramedullar hematopoiesis[1] is a pathological finding in humans, the spleen accounts for 30% of normal erythropoiesis in mice throughout life (O'Connell et al. 2015).

[1] Extramedullar hematopoiesis is the production of blood outside the bone marrow.

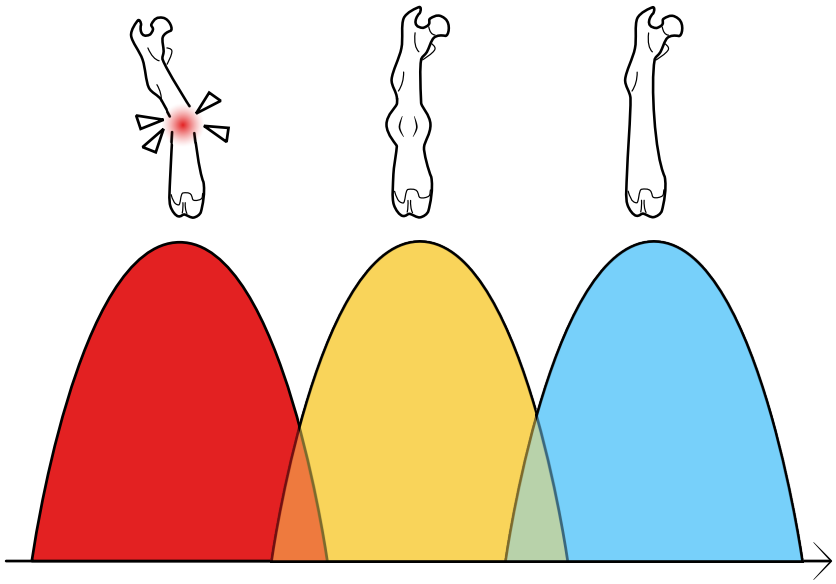


Figure 1: Phases of fracture healing

Simplified and modeled according to Baht et al. (2018), Edderkaoui (2017), Einhorn et al. (2015), and Ono and Takayanagi (2017). Initially, vivid inflammation characterizes the hematoma that develops in the fracture (red). In normal healing, this progress to anabolism of new bone and catabolism of old bone and debris. A callus usually develops in shaft bones and is made of cartilage giving rapid support to the healing bone. It is progressively ossified. Remodelling entails and shapes the bone microstructure to withstand the forces that acts on it. These phases are much more rapid in mice than in humans, with a large callus seen already at day 5. The phases are overlapping and not discrete.
Red) Inflammation.
Yellow) Callus.
Blue) Remodelling.

2.2 Myeloid Cells

Myeloid cells consist of granulocytes and monocytes. They share a common stem cell progenitor with megakaryocytes and erythrocytes. Granulocytes and monocytes depart at the stage of a granulocyte-monocyte specific progenitor (Akashi et al. 2000). Of particular note to bone healing, is the direct relationship between monocytes and osteoclasts (Göthlin et al. 1972). The differentiation of myeloid precursors, monocytes and granulocytes are stimulated by the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor (G-CSF), respectively. There is, however considerable overlap in their effect (Barreda et al. 2004). M-CSF is increased in fracture healing and correlates to bone resorption (Kon et al. 2001*a*; Sarahrudi, Mousavi, Thomas, et al. 2010). Osteoclast precursors from bone marrow (Jacquin et al. 2006; Xiao et al. 2013) and blood

(Galarza et al. 2013) have M-CSF receptor (CD115) as a common feature of their phenotype. This explains the phenotype of the *op/op* mouse — a mouse model with osteopetrosis — where a M-CSF mutation was found (Wiktor-Jedrzejczak et al. 1990). M-CSF and GM-CSF also have an effect on mature cells. M-CSF is present in biologically active concentrations in blood and tissues and seem to have a homeostatic effect on continued monocyte circulation (Stanley et al. 1997), and is stimulated by cytokines such as IL-4 and interferon gamma (IFN-γ) (Popova et al. 2011). *In vitro* stimulation of monocytes with GM-CSF and M-CSF shows a typical pro- and anti-inflammatory profile, respectively (Lacey et al. 2012). GM-CSF has a low basal circulating level and is mostly upregulated during inflammation to support accelerated myelopoiesis (Martinez-Moczygemba et al. 2003). Altogether, the genetic profiles and phenotype of stimulated monocytes correlate with the paradigm of macrophage polarization where GM-CSF induces an M1-phenotype and M-CSF an M2-phenotype (Hamilton et al. 2014). GM-CSF, M-CSF and G-CSF have all been indicated to aid fracture healing (Ishida et al. 2010; Moukoko et al. 2018; Sarahrudi, Mousavi, Grossschmidt, et al. 2009), but none is used in orthopedic clinical practice.

The common ancestry of monocytes and granulocytes leads to many common features. They are both phagocytes and able to clear pathogens, but in concept, the granulocyte is more aggressive and the monocyte more modulatory. This is thought to be the reason for their differing homeostasis. The granulocyte is short-lived in the circulation and the bone marrow carries great potential in increasing granulocyte production on demand. When demand increases, granulocytes are favored on behalf of lymphocytes (Ueda et al. 2005). There is debate and conflicting data as to how much monocytes contribute to renewal of tissue macrophages versus self-renewing peripheral macrophages from embryonic origin (Haldar et al. 2014; Zhao et al. 2018). The current conceptual paradigm on granulocytes and monocytes/macrophages is that monocytes survey tissues, initiate inflammation to recruit granulocytes but also suspend inflammation and clear debris after the fact. The simplistic view on granulocytes as simple inflammatory aggressors are challenged in that heterogeneity seems to exist in a similar manner to that of monocytes (Kumar et al. 2018).

Granulocytes Might Be Important Initiators of Fracture Healing

Neutrophils are early responders to inflammation in general. They extravasate quickly into inflamed tissue as seen on *s.c.* injection of tumor necrosis factor (TNF) (Tessier et al. 1997). Depletion of granulocytes with a monoclonal antibody towards lymphocyte antigen 6 complex, locus G6D (Ly6G) results in a decreased callus strength in the femoral osteotomy model (Kovtun et al. 2016). We could also see a rapid increase in granulocytes in bone healing tissue (paper I, II and III), indicating their importance to fracture healing. Ly6G is an antigen specifically present on mouse neutrophilic granulocytes (Fleming et al. 1993). Antibodies towards Ly6G specifically depletes neutrophils compared to Gr-1, which also depletes monocytes on account of affinity for lymphocyte antigen 6 complex, locus C1 (Ly6C) as well (Bruhn et al. 2016; Daley et al. 2008). Ly6G can be used to distinguish between monocytes and granulocytes with flow cytometry (Rose et al. 2012). The separation of monocytes and granulocytes can otherwise be hard as both may be high in side scatter (SSC) and positive for CD11b (Rose et al. 2012). An increase in granulocytes is a dominating feature of early inflammation in both cortical and cancellous bone healing (Tätting et al. 2018). It is not known, however, if depletion of Ly6G bearing cells would have a similar effect in a cancellous model. The exact contribution of granulocytes to the fracture healing cascade is unknown, but depletion results in an increased proportion of F4/80$^+$ macrophages in the fracture but decreased bending stiffness and bone volume (Kovtun et al. 2016). This is contradictory to clodronate depletion of monocytes showing a pronounced reduction in fracture healing in shaft as well as metaphyseal fracture models (O. H. Sandberg et al. 2017; Schlundt et al. 2018). The increase might thus be compensatory but does probably not have a causative effect on outcome. The increase might be explained by lack of feedback from entering granulocytes. In lack of negative feedback from granulocytes, monocytes might continuously enter the inflamed bone healing tissue (Kumar et al. 2018). This recruitment effect of granulocytes towards monocytes

might be important. Granulocytes can induce macrophages to an M2 phenotype(Butterfield et al. 2006; Headland et al. 2015; Kobayashi 2015; Soehnlein et al. 2009), which seem important to bone healing (further discussed in section 2.2 on page 8).

Monocytes Show a Continuum of Functionality

Monocyte have been most studied in humans where CD16 and CD14 were found in 1988 (Ziegler-Heitbrock et al. 1988) to distinguish blood monocytes into $CD14^{++}CD16^{-}$ and $CD14^{dim}CD16^{+}$ subsets. Their respective roles have since then been elaborated and are the mainstay of monocyte classification as classical (M1) and non-classical monocytes (M2) (Heitbrock 2007). Murine monocytes characterized by Ly6C are likely to develop from $Ly6C^{hi}$ to $Ly6C^{-}$ cells (Mildner et al. 2016). These cells correspond in transcriptional profile to human $CD14^{+}$ and $CD14^{dim}CD16^{+}$ monocytes (Ingersoll et al. 2010). In practice, it is important to define macrophages by both markers of exclusion and inclusion. Cells that need to be excluded are at least natural killer cells and neutrophilic granulocytes, which can overlap in the CD45-SSC window. A protocol has been published based on Ly6C and Ly6G (Rose et al. 2012). These two markers allow good discrimination between monocytes/macrophages and neutrophilic granulocytes, which are otherwise the hardest to distinguish as they overlap the most in CD45-SSC and share many myeloid antigens.

Monocytes develop to macrophages in tissues and the practical distinction in naming is based on whether the cell is blood or tissue derived. The continuum of macrophage polarization reaches from "classically activated" to "alternatively activated" macrophages, which are conceptually also known as "proinflammatory" and "anti-inflammatory" macrophages. The respective type is often simply termed M1 and M2. There are several classifications of macrophages, of which one suggests 7 distinct subtypes (Murray et al. 2014). These are defined by *in vitro* stimulation of different cytokines and the corresponding response seen with bone marrow derived macrophages. M1 is considered a proinflammatory phenotype and M2 an anti-inflammatory phenotype, but the polarization is more complex and not a dichotomy but a continuum. As the environment changes, so does the relative expression of genes correlated to each phenotype (Spiller et al. 2015). This also means that there is considerable overlap in phenotype marker expression of most macrophages as not all are at the extreme of the polarized continuum. To distinguish mouse M1 and M2 by phenotypic antigens, Jablonski et al. (2015) report only 70% success on dichotomized classification with an optimized panel of markers. At the level of gene expression, many papers have reported strong differences in key genes associated with M1 and M2 and gene expression profiling might represent a better way to classify macrophages (Gensel et al. 2017; Jablonski et al. 2015; Kigerl et al. 2009; Spiller et al. 2015).

M1 and M2 Macrophages in Bone Healing

Monocytes have been shown to enter the fracture from circulation in parabiotic[2] experiments (Göthlin et al. 1972). Thereby it was established that monocytes are recruited to fractures and that they develop into osteoclasts. Immunohistochemistry of the fracture callus has described macrophages to be present throughout in early inflammation, and later in proximity to bone formation (Andrew et al. 1994). In shaft fractures, macrophages seem to first be of M1 type ($CD68^{+}CD80^{+}$) and then as callus develops, M2 ($CD68^{+}CD206^{+}$) (Schlundt et al. 2018). Most studies on macrophages in fracture healing have focused on shaft models and mainly used markers that may be classified as "canonical" macrophage markers, such as the CD11b/CD18-complex (Mac-1), CD68, F4/80 and CD14, which largely lack discriminatory power on polarization. M2 have been reported to be increased in clavicle fractures of patients with traumatic brain injury, a clinically known state of increased fracture healing potential (R. Zhang et al. 2018). This indicates M2 macrophages to be of particular importance to bone healing.

[2]Parabiotic is the surgical connection of two organisms' circulation

Macrophages Appear Vital To Osteoblasts

Conditional knockout experiments in mice have shown that macrophage depletion does not alter osteoclast activity, but hinders osteoblast differentiation from mesenchymal stem cells (MSCs). At the molecular level, macrophages have been shown to induce signal activator and transducer of transcription 3 (STAT3) to promote osteoblast differentiation of MSCs (Vi et al. 2015). Macrophages seem to be important not only in fracture repair, but also during homeostatic conditions. In healthy bone, microscopy studies indicate a special niche for macrophages as lining cells between osteoblasts and bone marrow (Chang et al. 2008; Pettit et al. 2008). As macrophages also form the niche for hematopoietic stem cells (HSCs), it seems within the reach of their plasticity to form a niche for osteoblasts. Further, culture of calvaria osteoblasts are greatly hampered in mineralizing capacity if macrophages are depleted (Chang et al. 2008). This shows that macrophages support osteoblasts in an axis of metabolism seemingly disparate from that of bone fracture healing.

2.3 Lymphoid Cells

Lymphoid cells have generally gathered little attention in fracture healing compared to myeloid cell types. Some accounts on their potential importance do, however, exist.

Lymphocytes Are Indeed Present at the Fracture Site

Lymphocytes, especially T lymphocytes, have been known for a long time to enrich in fracture healing tissue (Andrew et al. 1994). T cells could be seen to enrich in the transition from the inflammatory to the bone anabolic phase of healing. With newer markers, both B cells and T cells have been shown in early fracture healing, then to disappear during the anabolic bone phase and then re-enter during remodelling (Könnecke et al. 2014). The exact contributions of adaptive immunity in fracture healing is unknown, however. Studies on $Rag^{-/-}$ mice have shown a better biomechanical outcome in stable femoral osteotomies (Toben et al. 2011), but less mineralization in calcein staining of tibial osteotomies (Nam et al. 2012). It is likely that T and B cells do have a role in fracture healing given these data, but how and when during the different phases of healing is unknown.

Subsets of Lymphocytes Might Have Specific Roles in Bone Healing

Addition of interleukin 17 (IL-17) to mesenchymal stromal culture from recombination activation gene negative ($Rag^{-/-}$) mice induce osteogenic differentiation (Croes et al. 2016). This cytokine is produced by γδ T cells and T helper 17 (Th_{17}) cells. Data do support γδ T cells to be important to fracture healing (Ono, Okamoto, et al. 2016). Addition of IL-17 led to an increase in osteoblasts in a cortical drill hole, but to a decrease of bone nodule formation and mineralization when added to culture of mouse (Ono, Okamoto, et al. 2016) and rat calvaria (Kim et al. 2014). This points to a potentially different effect of IL-17 depending on type of bone healing tissue.

T cytotoxic (T_{Cyt}) cells might have a role in fracture healing as well. A small subpopulation of T_{Cyt} cells only found in humans, T_{EMRA}, have been found to correlate with non-union of fractures (Reinke et al. 2013). This is a pathological condition of failed bone healing, and their effect in normal bone healing, however, is not known. Given that T_{Cyt} cells are important sources of inflammatory cytokines, especially TNF and IFN-γ (Best et al. 2013; Goldrath et al. 2004), they might contribute to normal bone healing by virtue of these cytokines. Depletion of T_{Cyt} cells with an anti-CD8 antibody reduced the pull-out force in a metaphyseal screw model, suggesting a role for T_{Cyt} cells at least in cancellous bone healing (Bernhardsson, Dietrich-Zagonel, et al. 2019). They might contribute with initial inflammation, as they seem to be attracted specifically

by granulocytes as seen from a reduction of T_{Cyt} cells on granulocyte depletion in a shaft fracture model (Kovtun et al. 2016).

The Role of B Cells in Fracture Healing Is Unknown

B cells do enter the callus of shaft fractures (Könnecke et al. 2014) and we have measured them to be present in both cancellous and cortical bone healing throughout paper I, II and III. However, their eventual mechanistic role in bone healing is unknown. A correlation has been shown between delayed fracture healing and regulatory B cells (Yang et al. 2015), but this needs to be experimentally confirmed.

2.4 Anti-inflammatory Agents

Several fracture studies have been done in the rat that show nonsteroidal anti-inflammatory drugs (NSAIDs) to cause weakened biomechanical outcome in fracture healing (Allen et al. 1980; Altman et al. 1995; Engesaeter et al. 1992; Sudmann et al. 1979).

Different NSAIDs have shown biologically meaningful effects on important cells in bone healing biology. Diclofenac have been shown to reduce the number of osteoblasts in histology after fracture (Krischak et al. 2007). In studies on mouse bone marrow, indomethacin lead to a dose-dependent decrease in osteoclast differentiation (Kellinsalmi et al. 2007). In knock-out mice lacking prostaglandin E2 (PGE2) production, osteoclast formation from bone marrow cultures is reduced and can be rescued by exogenous PGE2 (Okada et al. 2000). In mice osteoblast cultures, exogenous PGE2 had a sustained inhibitory effect on receptor activator of nuclear factor ϰB ligand (RANKL) secretion (X. Li et al. 2002), which would give a mechanism as to why indomethacin decreases osteoclast differentiation. The typical early proinflammatory cytokines IL-1 and IL-6 induce cyclooxygenase (COX)-2 expression in osteoblasts (Tai et al. 1997). As these cytokines are present in early fracture healing, it provides a mechanism as to how NSAIDs might have a negative effect on fracture healing. It might not be dependent on a decreased inflammatory infiltrate.

All studies on NSAIDs and fracture healing are experimental animal studies or retrospective human studies. One study, however, randomized patients with acetabular fracture needing prophylaxis for heterotopic[3] ossification to either local radiation or indomethacin treatment. The rate of non-union was significantly higher with indomethacin treatment. Even though non-union was not the primary variable, it is the only prospectively randomized study in humans to display an adverse effect of NSAIDs (A et al. 2003).

2.5 Nerves Supply Trophic Signals To Bone

The presence of nerves in bone is well known (Bjurholm 1991; Bjurholm et al. 1988; Calvo 1968; Imai and Matsusue 2002; Imai, Tokunaga, et al. 1997; Jones et al. 2004) and has been shown to influence fracture healing (Apel et al. 2009; Aro 1985; Hukkanen, Konttinen, Santavirta, Nordsletten, et al. 1995). The specific nerve supply of fractures include sensory nerves, expressing Substance P (SP) and Calcitonin-Gene Related Peptide (CGRP) (Hukkanen, Konttinen, Santavirta, Paavolainen, et al. 1993; J. Li, Ahmad, et al. 2001; J. Li, Kreicbergs, et al. 2007). These peptides have also been found in heterotopic bone formation (Bjurholm 1991). Bone marrow cells carry specific receptors to these peptides (Fernandez et al. 2001; Ho et al. 1997), and they have been shown to contribute to inflammation (Azzolina et al. 2003; Cuesta et al. 2002), affect osteoclasts (Sohn 2009), osteoblasts (Mrak et al. 2010) and bone marrow stroma cells (Mei et al. 2013). The immunostimulatory effects of sensory nerves are opposed to those of the autonomic

[3]Heterotopic bone formation is bone formation in the wrong place, such as in muscles

nervous system, which are mainly immunosuppressive (Nance et al. 2007; Rosas Ballina et al. 2009).

The phenomenon that an inflammatory stimulus is mirrored on the contralateral limb ("neurogenic reflex") is well known (Kelly et al. 2007; Levine et al. 1985; Q. Lin et al. 2000; Shenker et al. 2003). This phenomenon seems not to be a simple spinal arc reflex (Raghavendra et al. 2004). Spinal and supraspinal influence on inflammation has been shown experimentally (Boettger et al. 2010; Bong et al. 1996; Boyle et al. 2002; Sorkin et al. 2003). That some seemingly neural effect on bone healing exist has been known in orthopedics for a long time from the observation of fracture healing in brain trauma patients (Locher et al. 2015). The influence of the nervous system on bone healing could perhaps be explained by neuropeptide signaling (Song et al. 2012; D. Zhang et al. 2009).

All data on neural supply to bone fractures and their effect on bone healing have been gained from shaft fracture models.

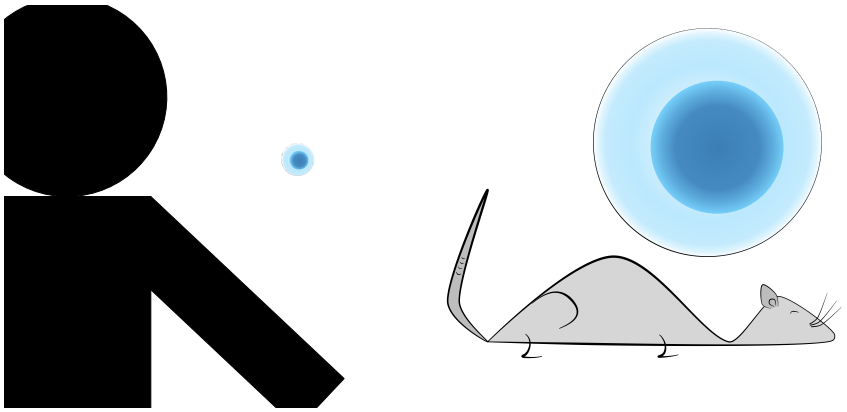
3 Comments on Material and Methods

3.1 Models of Fracture Healing

Do Mice Faithfully Model Human Bone Healing?

The purpose of a model is not necessarily to replicate the human situation exactly, but to understand a process in context of the model. A certain fracture in a mouse might therefore not be directly applicable to the corresponding fracture in humans, but the properties of the model and result of an experiment is likely to be applicable to a human fracture with similar properties. This is the main reason to be using animal models in the study of bone healing. An ankle fracture in a mouse could be a good model of fracture healing across a joint under loaded conditions, but a poor model of human ankle fractures as the mouse fibula does not extend to the ankle joint and the malleoli are not as prominent.

Mice are quadrupeds and humans bipedal, which puts the skeleton at a different strain. Mice are not proportionally smaller to humans in all aspects. Cell size is constant, which means that a long bone indeed is comparatively larger in relation to a bone cell in a human than in a mouse (Figure 1 on page 14). The length of a bone and size of a bone cell do not scale proportionally to each other. While the length of the femur is likely to scale linearly to the length of the organism, the volume of the femur is likely to scale with the cube of the weight. They serve different purposes in relation to the complete organism. As expected, certain differences therefore exist in comparative anatomy between humans and mice. Of note to the study of fracture healing are the different loading conditions that follow from quadrupedal gait, and different anatomy of the bones as muscle attach and exert forces differently. In fracture research, we believe the dominating qualities to be loading, stability and type of bone architecture. This needs to be evaluated on a model by model basis. General differences that affect all models do exist, however. Mouse microanatomy of bone is different. The cortex of mice develop through apposition. This leads to circumferential lamellae of cortical bone. This is in sharp contrast to humans where cortical bone remodels with formation of osteons. Osteons are absent in mice. However, porosities in



(a) A cell is small in comparison to a human

(b) A cell is small in comparison to a mouse, but relatively larger than it is to a human

Figure 1: Mouse as a human model. Properties of a human that one want to study might not have the same physiology, and might not scale proportionally to humans. The latter is probably the most important to orthopedic science. A bone does not scale proportionally to the body size in a human to a mouse, and some properties do not scale at all, such as a cell. Cell size differences are negligible in humans and mice.

the cortical bone develop in aging mice. The bone marrow of humans become fatty in the appendicular skeleton, whereas hematopoiesis persists in mice. However, both have woven and lamellar bone, and the trabeculae of mature mice consist of lamellar bone as in humans (Treuting et al. 2017).

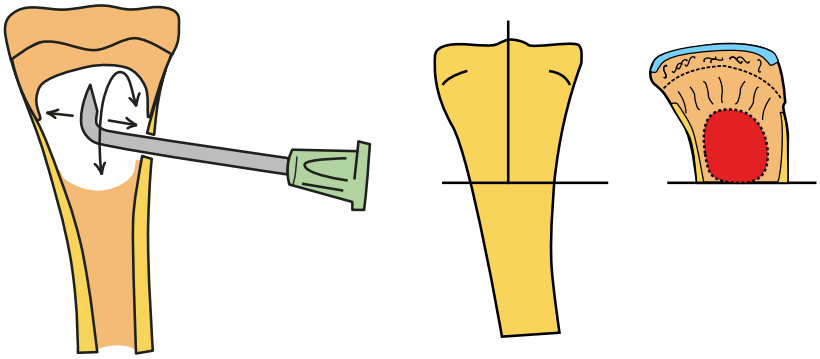
Fracture Models in Mice Are Synonymous with Shaft Fracture

The most commonly used models in experimental fracture healing research are likely to be shaft fractures on account of their long history. A standard method of a closed femoral fracture was described in 1984 by Bonnarens et al. A similar description was made of the tibia in 1993 by Hiltunen et al. Many animals have been used for different purposes and in general the closed femoral fracture model have been preferred, probably due to ease of use (Nunamaker 1998).

Cancellous Model

Few fracture healing models concern metaphyseal bone healing. A model was described in 2001 on a distal femur metaphyseal defect (Uusitalo et al. 2001). It was simple and consisted of a K-wire[1] inserted through the anterolateral cortex onto the opposing cortex through a small incision. It has a similar healing pattern to the proximal tibia defect used in our group for study of metaphyseal healing with bone appearing within the first week (Tätting et al. 2017). In our group the proximal tibia model has been preferred due to the ease of extracting healing tissue

[1]Kirschner wire (K-wire) is a sharp stainless steel pin



(a) The method for creating a fracture in the cancellous bone of the metaphyseal marrow. The proximal tibia is seen in anterior aspect. The growth plate is drawn as a wavy line. A bent needle was inserted below the growth plate and rotated to traumatize the metaphyseal bone.

(b) The proximal tibia in frontal and lateral aspect. The tibia was divided along the drawn lines (left). Tissue was harvested from the volume indicated by the dashed line (right).

Figure 2: The cancellous model used in all papers.

for flow cytometry. The model concerns mainly cancellous bone healing. It carries a small and manageable soft tissue trauma on reaching the proximal tibia. The proximal cancellous tissue of the bone is then traumatized with a bent needle (Figure 2a on page 15). The effect is a large cancellous bone injury, roughly $1/4\text{cm}^3$ in tissue volume, with minimal cortical and soft tissue trauma. This volume is easy to extract. It is enclosed within the tibia during extraction from the animal leaving a minimal trace of contamination. Separately, the tissue volume is retrieved by cleaving the bone (Figure 2b on page 15). The tissue is then easily scooped out with a bent needle as a spoon. It has been further characterized in rats with screw insertion for use of pull-out force in biomechanical testing (Bernhardsson, O. Sandberg, et al. 2015).

Cortical Model

In our experience, the femoral osteotomy model produces a large callus as a consequence of instability regardless the mode of fixation. This renders it intractable for methods of analysis that need cells in suspension, such as flow cytometry. Tissue lysis with enzymatic digestion does not allow faithful phenotyping with flow cytometry as surface antigens can both increase and decrease (Autengruber et al. 2012). We also found that the early hematoma is of considerable difficulty to extract in a consistent and well defined manner. Many times, no clear hematoma could be seen in the osteotomy as one would expect, and the surrounding muscle is quick to attach to the traumatized bone. To overcome these technical shortcomings in studying cortical bone healing, we devised a model where the femoral cortex was milled away longitudinally for a short distance (Figure 3 on page 16), first published in Tätting et al. (2018). The reaming injury to the cortex models trauma to bone with a major cortical component. It is consistent with the cancellous model in stability, and allow a consistent volume of tissue to be extracted.

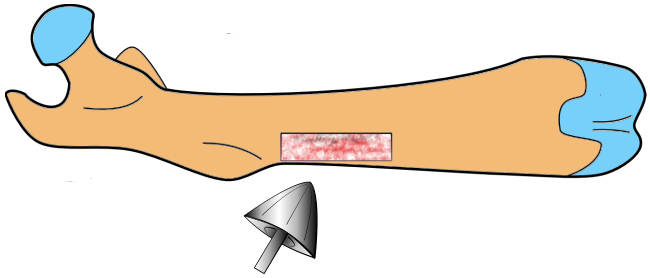


Figure 3: Cortical Model

The anterolateral aspect of the femur is accessed with incision and cleavage of the quadriceps from the hamstrings without traumatizing the muscle bellies. The cortex is milled away with a drill. A cortical defect with loss of bone marrow is left in an otherwise stable femur. The muscle is left intact to cover the defect. At tissue extraction, the cortical defect is accessed and soft tissue is extracted with a bent needle

Bone Healing Model *vs.* Fracture Model

The model of cancellous and cortical bone injury are better denoted as bone healing models than fracture models, as they do not accurately model a fracture. They do, however, reliably and consistently allow the study of cell- and protein composition of healing bone tissue of predominantly cancellous or cortical bone.

3.2 Flow Cytometry

Finding Cells in Bone

Flow cytometry is essentially a tool to phenotype cells. It relies on cells being in suspension and not of too large a size in diameter. We tried to use flow cytometry to phenotype the inflammatory cells found in bone healing tissue. The large collagenous callus of the femoral osteotomy in mice was inadequate to collect cells for phenotyping. For this reason, the cortical model was developed (section 3.1 on page 15). In both the cancellous and cortical model, cells could easily be retrieved in suspension and phenotyped with flow cytometry.

A problem with complete analysis of a volume without any reference in the instrument, is to know if comparisons across groups are adequate. In a microCT the volume of interest can be defined. This definition relies on the surrounding bone to be present as a reference in the microCT. One may then analyze a certain volume that is defined in relation to the bone as a whole, also part of the scan. Whole analysis of an extracted volume have no context during analysis. The operator extracting the tissue for flow cytometry is the independent variable to the variation in the volume of interest and is hard to measure (Figure 4 on page 17). It can only increase in consistency by having the same operator extract the tissue for all groups being compared. We have assumed that the volume of interest is homogenous in total leukocyte count, i.e. $CD45^{+}$ cells. If the volume extracted is homogenous in total leukocyte count, cells can be compared across groups as fractions of leukocyte count. This might not be a valid assumption for all subpopulations of cells. A certain subpopulation might have affinity toward the cortex or growth plate, and

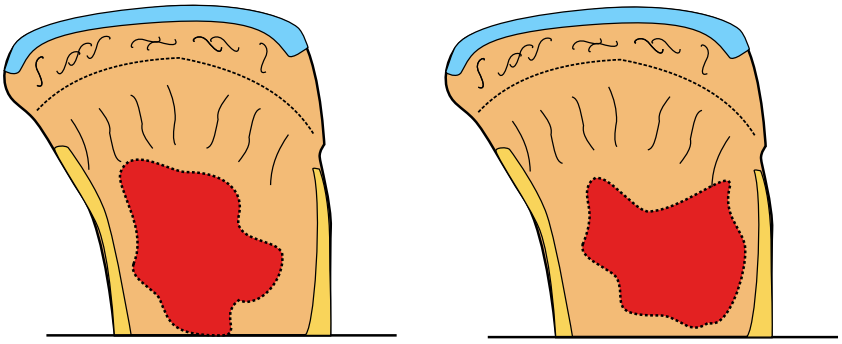


Figure 4: Variation of volumes extracted in the cancellous model

The proximal tibia after explantation and cleaving to reveal the traumatized tissue. Two samples are shown. During extraction of traumatized tissue volume from the cancellous proximal tibia the actually extracted volume is operator dependent. The volume extracted, indicated in red, is not identical to each another and contributes operator dependent variation to the analysis.

biology would then add to sampling variation in addition to operator dependent variation. They should, however, on average vary around a true mean. The choice of reporting subpopulations as a fraction to its parent or all leukocytes is contingent upon the investigators belief of what is relevant to the research question. If there is an increase in $CD3^+CD4^+$ T_H cells in relation to all leukocytes, but not in relation to all T cells, there indeed seem to be an absolute difference in T_H cells, but at the same time other T cells might have increased as well. In analysis with all subpopulations expressed as fractions of $CD45^+$ leukocytes, these subpopulations would show an increase, as would all T cells. In analysis by fraction of parent population, only T cells would display an increase. This rationale led us to report subpopulations in relation to total leukocyte count in paper II and III.

Finding Cells in the Flow Cytometer

The flow cytometer is an unstable and labor intensive instrument to use. It needs calibration on a daily basis to account for drift in the instrument, and each experiment need calibration that is specific to the current panel and origin of cell sample. A specifically arduous task is the setting up of new panels. The need for compensation is not only dependent on the fluorescense spectrum of each fluorochrome, but also on the biology in that the antigen expression levels vary wildly for different antigens.

The flow cytometer focuses cells in suspension to a stream that passes through lasers. Antibodies bound to the cell have a conjugated fluorochrome and fluoresce on passing through the laser. The emitted light is reflected on mirrors that pass on successively lower and lower frequencies of light to other mirrors, while some light pass through the mirror. Hence, slices of the emitted light spectrum from a given cell will reach different photomultiplier tubes (PMTs). This is the underlying principle to being able to interpret a phenotype. A detector corresponds to an interval of light, which corresponds to the maximum intensity of light emitted from a fluorochrome, which corresponds to its conjugated antibody being present on the cell, which corresponds to a certain antigen being present on that cell. This chain of logical deductions allow for some introduction of variation. However, the main error in practice is with the fluorochromes themselves as much as

with the technicalities of flow cytometry — they overlap in emission spectra. If two fluorochromes of overlapping emission spectra attach to the same cell, the relative contribution of light from each fluorochrome cannot be deduced from the readings. It can only be *compensated* for when knowing the exact contribution of *one* fluorochrome's light emission to *each* photomultiplier tube. This can cause great problems in interpretation if the need for compensation is high. The compensation is a simple linear arithmetic subtraction, but the biology and electron statistics of the amplification that takes place in the photomultiplier tube is exponential. As the intensity increases, so does the standard deviation in an exponential manner. The compensation lowers the median, but the standard deviation is kept constant. This makes it hard to discriminate populations of cells, as they widen. This is the main deterrent of compensation to faithful interpretation of data.

In clinical practice, the mainstay of panel design is a low count of different antibodies in several different panels with a common backbone marker. This puts compensation artifacts to a minimum. In paper I this methodology was used. However, as the compensation artefact only affects cells that *coexpress* antigens with overlapping fluorochromes, knowledge of the biology allow larger panels. In paper II and paper IV wider panels were used. Finally, in paper III a single panel of 12 antibodies could be used as intimate knowledge of the biology had been developed.

Where Does a Cloud Really End?

The resulting light scattering of events, assumed to be a cell each, show up as clouds when plotting the intensity in photomultiplier tubes against each other. Due to natural variation and the exaggeration of variation in the photomultiplier tube, some events will be more or less peripheral to this cloud. In standard analysis of flow cytometry, regions of lines, called gates, are put around events to define positivity of a certain marker. This method makes flow cytometry highly operator dependent in analysis. One may expect the measured percentage of positive cells to be variant across operators by several units of measurement. However, with a consistent strategy in gating and preferably a single operator, this can be alleviated in comparison across groups.

The biology of bone healing is mainly the biology of inflammation and normal bone marrow from the perspective of flow cytometry. Neither osteoblasts nor osteoclasts can be measured due to lack of antigens to target in flow cytometry and, probably, hardship in detaching them from bone and bring them to suspension. The normal bone marrow have cells with increasing intensity in common antigens to become fully intense on maturity. This can make it hard to distinguish cells recruited by inflammation and normally developing cells.

Some Phenotypes Are Bright and Some Dull

The phenotypic antigens are present in different densities on the target cells. Standard phenotypic markers of lymphocytes are easily distinguished in most setups, such as CD3, CD4, CD8 and CD19, especially in blood. They are somewhat harder in bone marrow due to the dimmer expression from developing cells, but generally easy to distinguish from negative cells. Some antigens are dim by nature and harder to distinguish from negative cells and does not form a cloud, but rather a smear. This can be especially troublesome in bone marrow and inflammation as the many stages of developing cells and dead cells that need to be accounted for in gating. If the population is small in percentage, discerning a population may be virtually impossible. A fluorescence minus one (FMO) directed gate may help in this instance. Among lymphocytes, the activation marker CD25 and T_{Reg} marker FOXP3 generally need to be free from compensation. Among myeloid cells, the standard markers for monocytes are generally dim to medium, such as CD11b, CD14, F4/80, CD206 and C-C motif chemokine receptor 7 (CCR7). Especially CCR7 can be hard to interpret, as it is known to differ by a great margin depending on the temperature during staining. In general, staining is otherwise robust to time and temperature if high affinity antibodies are used. Myeloid marker interpretation is further blurred by biology. In comparison

to major subsets of lymphocytes, which are virtually discrete clouds of different phenotypes, these markers are a continuum of expression from none to medium across subsets of macrophages. The dichotomization of these cell populations with these markers will inevitably either only include the most polarized cells of each end of the continuum, or cells of varying degrees of polarization. This further adds to the operator dependence of gating when reporting myeloid cells as populations with these markers. In mice, a set of markers have been validated for monocytes to be analogous to human monocytes of classical or alternate activation (see section 2.2 on page 8) with Ly6C. Together with Ly6G and CD11b, a good precision of granulocytes against monocytes is revealed. Further, $Ly6C^{hi}$ and $Ly6C^{lo}$ cells are easily separable due to the high density of this antigen. With CD11b to pregate myeloid cells, this strategy has shown itself easy to work with in the bone setting.

3.3 Mass Spectrometry

Mass spectrometry is a tool to measure charged masses. In the field of biology, these charged masses are chains of amino acids. A protein in itself is much too large and two distinct proteins could have equal mass/charge ratio yet be wildly different in amino acid sequence. For this reason, proteins are denatured, alkylated and digested prior to mass spectrometry. For technical reasons, cleaning of the peptide solution from salts and contaminants are also necessary with inevitable sample loss.

In mass spectrometry, highly complex mixtures of peptides are often analyzed. This makes the dynamic range of measurements extremely wide in both concentration of each type of peptide, and amino acid sequence of peptides. Common serum proteins, such as albumin, hemoglobin and antibodies are present in high concentrations throughout any tissue permeated with blood. This is true also for bone healing tissue. Further, the inflammation of an injured tissue adds complexity to the sample as many cells go through necrosis or apoptosis. Ribosomal proteins and common metabolic proteins may then also become of relatively high concentration, together making the yield for rare but biologically important signaling proteins low in the mass spectrometer.

During mass spectrometry, the entering of peptides for analysis is ordered by some chemical quality, usually polarity, in reverse phase chromatography that feeds the mass spectrometer. Still, great overlap is inevitable in complex mixtures such as serum due to the many different peptides present. The most common peptides that enter the mass spectrometer, will also be the ones to get sequenced. This introduces the bias that makes interpretation and comparison of mass spectrometry data hard. Any given peptide's probability of sequencing and thus detection, is not only dependent on its own concentration, but also on all the other peptides that have the same chemical quality in chromatography. It becomes virtually impossible to compare spectrum counts across samples of different background peptide composition as one cannot say if a peptide has increased, or if another, *suppressing* peptide, have decreased. In paper III, we have judged the background of indomethacin treatment or not to be comparable, but not the background of different bone healing models or the same model at different days.

3.4 The power of a p value

The papers in this thesis largely avoid the use of p values. Only paper III had a primary variable and a hypothesis to test. The other papers were exploratory, and p values have no interpretation in lack of a primary variable to statistically test.

In flow cytometry, we have looked at populations of cells. Since the number of populations needed to meaningfully represent inflammation is high, the number of tests that would need to be accounted for in statistical testing grows rapidly. This problem can be alleviated by correction of the significance value, that is, it is numerically corrected to convey the initially set level of

confidence. Such correction inevitably increases β, and the sample size or effect size needed grows quickly for this strategy to be feasible if the effect size is not very large. Another commonly employed method is to calculate the fraction of false rejections in a set of significant tests, i.e. instead of retaining p values which all honor α, a fraction of q many significant p values are falsely significant. This is the false discovery rate. It requires a null distribution to be known for the data. We have measured populations from different circumstances, and the FDR in our setup would be completely dependent on ascribed probability of a population to increase or decrease given no difference. It would also not be able to take into account closely related populations that are meaningfully changed in unison, and is thus not a good method in our papers.

In a clinical trial, our method of describing data would not be satisfactory. But no clinical trial would have been made without previous enquiry as to what would be a feasible trial to do in the first place. They are enquiries as to how *possibly* cancellous bone healing and cortical bone healing could be different in their inflammatory response. We know, with less than 5% left to chance (O. Sandberg and Aspenberg 2015*a*; O. Sandberg and Aspenberg 2015*b*), that anti-inflammatory treatment has an effect of clinically relevant effect size, and these papers aim at trying to establish reasonable clues as to the *why*. To conceptually reduce the multiplicity error, we as investigators have judged the data in reporting. Effects, for example, that are non-overlapping between groups and large in effect size, as well as concordant across several groups in a biologically meaningful way are more likely to represent a true difference. Technically this corresponds to effects that might would have passed a multiplicity test given the very small p value that arise from large effect sizes with non-overlapping and narrowly distributed groups. An argument could be made that some composite score on cell populations could be used, but this is to give an implicit interpretation to the data as the outcome would be dependent on the scoring system.

It is truly the trust in the data that underlies the confidence in the result of a significance test. In hypothesis testing, the confidence in the p value relies on the quality of the data in its attention to uncontrolled sources of variation. We have performed our analyses on data collected from experimental designs lacking only a prespecified primary variable in comparison to a trial. This reduces spurious sources of variation, and increases the confidence in our results. This allows knowledge to be derived from exploratory analysis with a certain confidence, but not in a formally defined manner as with hypothesis testing. We have presented confidence intervals of our data to still allow the reader to value the effect's confidence, given the reliability of the data as described in the methods section and the multiplicity given the number of measured populations. No statistic, however, will convey our results as good as looking at the plotted data. Technically, this corresponds to the dependence on the data as a whole that exploratory analysis entails, as the significance of one variable's effect is dependent on the whole data set (i.e. how many variables there are to test).

4 Results and Discussion

4.1 Metaphyseal Bone Trauma Causes Widespread Changes in Cell Composition of the Healing Cancellous Bone Tissue

Paper I

To better understand the natural healing of metaphyseal trauma, a descriptive study was performed (paper I). The proximal metaphyseal tibia was put through needle trauma as described in section 3.1 on page 14. The cell composition of the healing bone tissue was compared to the proximal tibia of healthy mice. Some differences are emphasized as judged by effect size and confidence intervals (Figure 1 on page 22). Among monocytes, inflammatory monocytes, mono/Gr-1^{hi}, was noted to be roughly halved in fraction of all monocytes throughout day 1 to 10 with a particularly large decrease at day 5. However, also at day 5, mono/CCR7^{+} cells could be noted to double in fraction of all monocytes, with no convincing difference at other time points. Mono/CD206^{+} cells could be noted to be consistently doubled with a narrow CI throughout the whole period of bone healing compared to healthy mice. These results indicate that the inflammatory infiltrate after trauma to the metaphyseal tibia is different from the canonical inflammatory response that shows an initial entering of inflammatory monocytes that progressively change to resident macrophages.

Among T cells a bimodal pattern could be noted. T cells overall (Lympho/CD3^{+}) showed a fluctuating and not confidently different fraction of all leukocytes at day 1 to 5, but a probable increase to double at day 5 and a convincing decrease to a fourth of healthy mice with a narrow CI at day 10. Neither B cells nor T_{Cyt} cells (CD3^{+}/CD8^{+}) showed a convincing difference compared to healthy mice throughout day 1 to 10. T_H cells (CD3^{+}/CD4^{+}) were convincingly decreased to a fourth at day 5 with a narrow CI.

A specific cell population, CD3^{+}CD200R^{+} cells, showed a marked increase throughout day 1 to 10. CD200R is known to be present on monocytes and is thought to relay anti-inflammatory

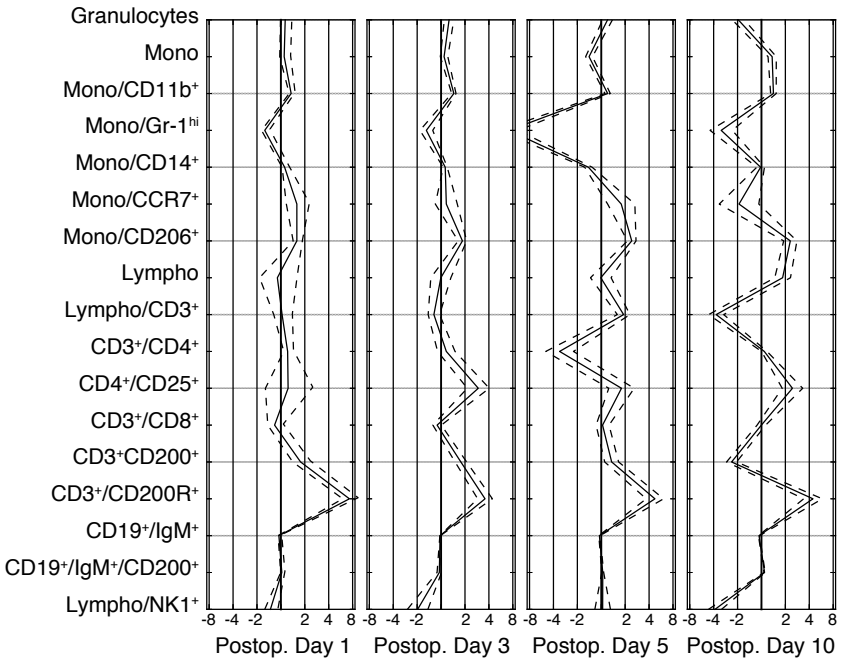


Figure 1: Comparison of Cell Composition in Cancellous Model and Healthy Bone

Cell populations in the injured tibia at different days after surgery compared with metaphyseal tibia in uninjured animals. A fold change of 2 indicates a doubling of the respective cell type compared to the proximal tibia of uninjured animals. The cell population names are indicated on the Y-axis. The straight line marks the mean fold change of observations from six traumatized mice compared to six healthy mice. The dashed lines mark the 95% t-distributed confidence interval without correction for multiplicity.
Postop.) Postoperative.

stimulus from $CD200^+$ lymphocytes (Jenmalm et al. 2006). However, CD200R is also present on a subset of T cells (Rijkers et al. 2008), but the significance of this is unknown. It has been shown that MSCs and T cells interact through CD200-CD200R signaling (Najar et al. 2012) and that metastatic melanoma cells grow much more rapidly in CD200R-deficient mice (Liu et al. 2016). This makes the CD200-CD200R axis of signaling potentially interesting to bone healing, and the finding that $CD3^+/CD200R^+$ T cells are consistently increased by a factor of 4 with a narrow CI in traumatized bone tissue compared to healthy mice is of potential significance to cancellous bone healing. Interestingly, $CD3^+CD200^+$ T cells showed a rough but consistent doubling day 1 to 5 with a medium sized CI, but a sharp decline at day 10 to being halved with a narrow CI. Even though the design of the study cannot reliable estimate the effect size at each day, the sharp decline from doubled to halved is unlikely to be by chanse and coincides with a shift from inflammation to bone anabolism. These cells might represent autoregulation of the initial inflammation followed by less need for regulation after bone anabolism has been started.

4.2 The Inflammatory Response in Cancellous Bone Healing Is Closely Mirrored in Other Bones

Paper I

The cell composition that was measured in the traumatized proximal tibia was unexpectedly similar to the contralateral proximal tibia. To see if this was a systemic and sustained effect, we additionally measured the cell composition of the humerus at postoperative day 5 (Figure 2 on page 24). Interestingly, the special population of $CD200R^+$ T cells is systemically upregulated 8-fold. T_H are convincingly downregulated in the traumatized tibia at almost 4-fold, but drift closer to the fraction of normal bone marrow in the contralateral tibia and then humerus, giving the appearance of a waning effect from the epicenter of trauma. The lower CI in the traumatized tissue is contiguous to the upper CI in the humerus. The same is true for $CCR7^+$ macrophages, which are roughly doubled in the traumatized tissue with a medium wide CI, but is normal in the contralateral tibia and almost halved in the humerus. The CI in traumatized tissue is not overlapping to the CI of the humerus with margin. Whether this seemingly distance related effect is by chance or does represent a biological effect is hard to decide but merits further study as it may indicate what differentiates inflammation in bone from early bone healing.

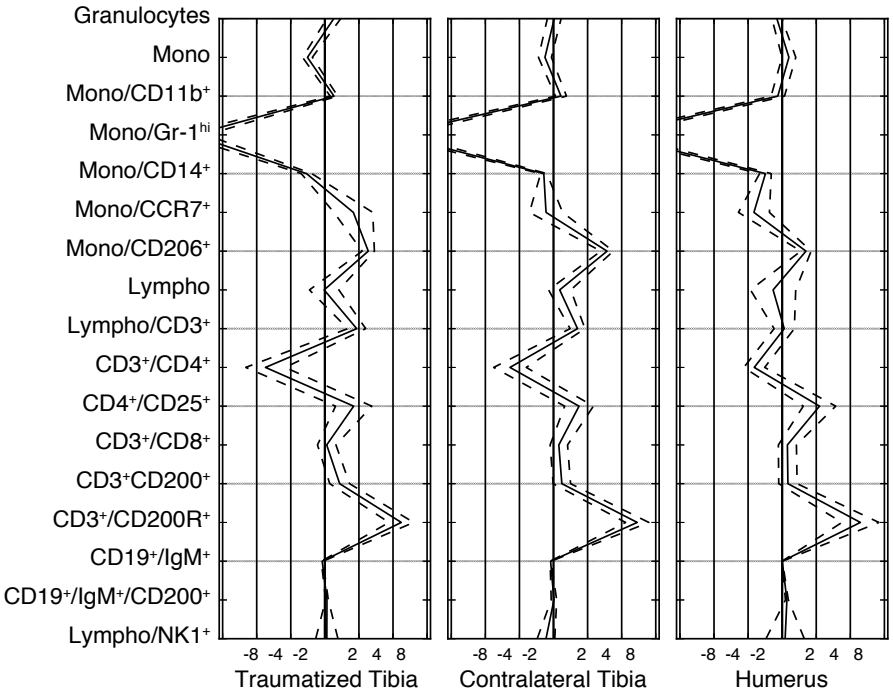


Figure 2: Cell populations in the injured tibia, contralateral tibia, and humerus in mice at postoperative day 5

The pattern of fold change among studied populations is similar between the tissues. The traumatized tibia and contralateral tibia are related to the tibia in uninjured mice, and the humerus to the humerus in uninjured mice. A fold change of 2 indicates a doubling of the respective cell type compared to the uninjured animals. Cell populations are indicated on the Y-axis. The straight line marks the mean fold change of six traumatized mice compared to six healthy mice. The dashed lines mark the 95% t-distributed confidence interval without correction for multiplicity.
Postop.) Postoperative

The Systemic Inflammatory Response on Metaphyseal Bone Trauma Seem Not to Be Neurally Mediated

Unpublished Data

To investigate if the widespread inflammatory bone response could be neurally mediated, we performed a study to evaluate interaction between ischiadic nerve transection and fracture on the contralateral cell response. Mice were subjected to metaphyseal trauma of the left tibia as described in section 3.1 on page 14. One group of mice received an additional transection of the left (ipsilateral) ischiadic nerve during surgery. A second group received transection of the right ischiadic nerve (contralateral) during surgery. A third group did not receive any nerve transection. There were 6 mice in each group (Table 1 on page 25).

After 5 days the mice were killed and the traumatized tissue and the contralateral metaphyseal tissue harvested for analysis with flow cytometry. There were no discernible difference in the groups with ipsi- or contralateral ischiadic nerve transection (Figure 3 on page 26). This suggests that the effect of the similar cellular response in other bones on bone metaphyseal trauma is not dependent on nerve signaling. Interestingly, in this study, the surgeon forgot to perform metaphyseal trauma in 2 mice, both in the afferent nerve transection group. These mice still received nerve transection. The cell composition in the left and right metaphyseal tibia of these mice were similar to those who had received bone trauma. Even though this number is low, it suggests that the response in unrelated bones on localized bone trauma is unspecific. Rather, the interaction between a general inflammation and traumatized bone may be the specific initiator of bone healing. By logic of exclusion, it would seem that the general inflammatory response seen in paper I is by humoral signaling.

Table 1: Study design of nerve transection in cancellous bone healing

It was a two-factor design with three levels for nerve transection including control, and one level for proximal tibia trauma. The table emphasizes that no trauma controls were included as the effect of bone trauma had already been studied.
n/n) number of animals that were analyzed in relation to the number of animals randomized.

	Ipsilateral Transection	Contralateral Transection	No Transection
Traumatized Tibia	4/6	6/6	6/6
Healthy Mice	0/0	0/0	0/0

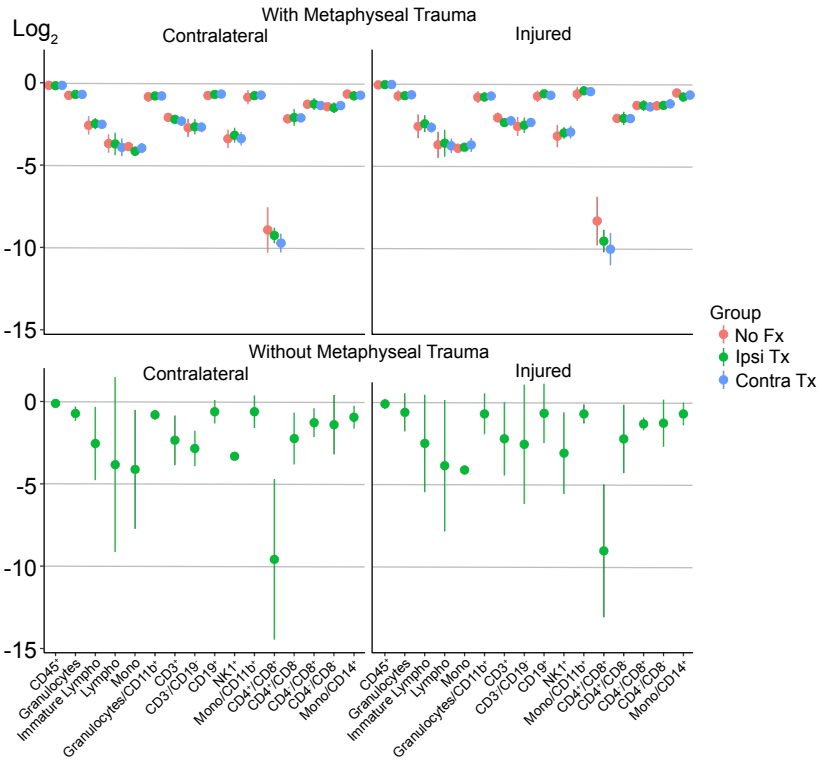


Figure 3: Cell composition with nerve transection and bone trauma

The mirrored inflammatory response in the contralateral metaphyseal tibia and humerus on metaphyseal trauma seem not to be neurally mediated. Mice were put through nerve transection of either the ipsilateral or contralateral ischiadic nerve in addition to metaphyseal trauma of the left tibia. A third group of mice only had metaphyseal trauma as nerve transection controls. No difference can be seen between the groups in the contralateral inflammatory response. All confidence intervals overlap and no indication of a relevant effect size can be seen. Two mice with nerve transection did not receive any fracture due to human error during surgery. Interestingly, these two mice show identical patterns of inflammatory cell composition as the mice with bone trauma, but with wide confidence intervals. Bars represent the t-distributed 95% confidence interval without correction for multiplicity. Log_2 axis.
No Fx) The mouse did not receive any metaphyseal trauma.
Ipsi Tx) The ischiadic nerve ipsilateral to the metaphyseal trauma was transected (the afferent nerve).
Contra Tx) The ischiadic nerve contralateral to the metaphyseal trauma was transected (the efferent nerve).

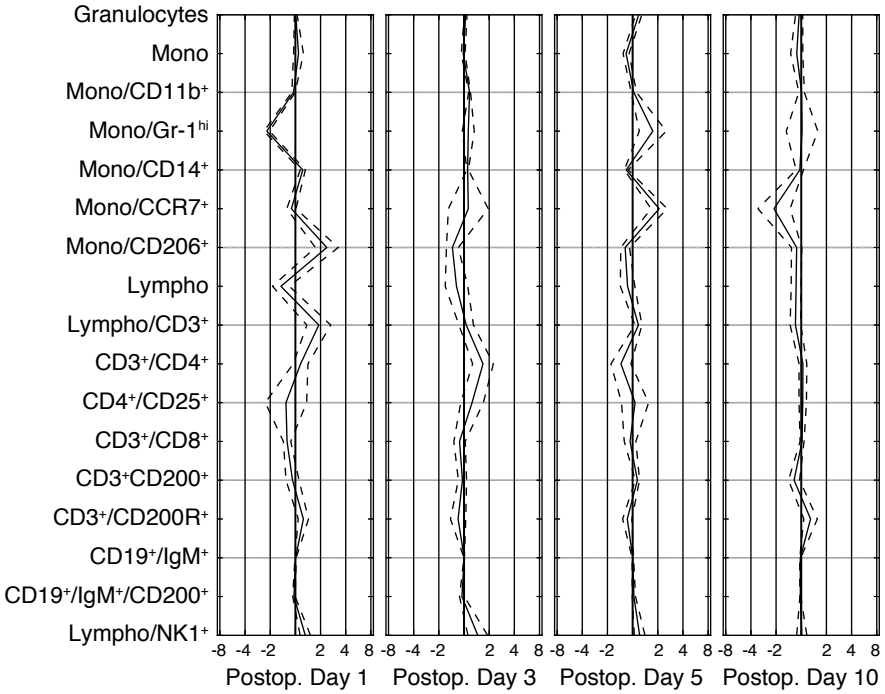


Figure 4: Cell populations in the traumatized tibia compared to the contralateral tibia in the same animals at days 1, 3, 5 and 10 after surgery

Note the generally smaller fold change values compared to Figure 1 on page 22. A fold change of 2 indicates a doubling of the respective cell type compared to the uninjured animals. Cell populations are indicated on the Y-axis. The straight line marks the mean fold change of six traumatized mice compared to six healthy mice. The dashed lines mark the 95% t-distributed confidence interval without correction for multiplicity.
Postop.) Postoperative

4.3 Some Cells Seem Specific to The Fracture Site in Cancellous Bone Healing

In Figure 4 on page 27 it was apparent that the inflammatory cell composition was very similar, but some differences could be discerned.

M2, and Then M1, Is Seen in Cancellous Bone Healing

Paper I

In a study by Schlundt et al. on cells in a mouse femoral osteotomy, an initial M1 phenotype ($CD68^+CD80^+$) was seen followed by M2 ($CD68^+CD206^+$) at 7 days. This is concordant with the general view of an initial inflammation followed by reconstitution. In our results on metaphyseal tibia healing, we could see a trauma specific (compared to contralateral proximal tibia) 2-fold

increase in M2 phenotype (mono/CD206^{+}) and a 2-fold decrease in mono/Gr-1^{hi} at day 1. At day 5, a 2-fold increase in M1 phenotype (mono/CCR7^{+} and mono/Gr-1^{hi}). The CIs for these observations did not overlap 0, but was wider for the observations on M1 cells at day 5 (Figure 1 on page 22). The Gr-1^{hi} population is likely to correspond to Ly6C^{hi} monocytes, although the lack of gating on Ly6G cannot rule out interference of weakly side scattering granulocytes. It is, however, strengthened in observation as the general population of granulocytes were not different in a similar manner, pointing to the need for a specific subeffect on SSC^{lo} granulocytes to bias the phenotype classification.

Lymphocytes Generally Decrease in Metaphyseal Fracture, but Some Subpopulations Increase

The normal effect on lymphocyte count in the bone marrow on trauma is a decrease. The lymphoiesis is lowered temporarily in favor of granulopoiesis to support acute inflammation (Moreau et al. 2015; Ueda et al. 2005). Lymphocytes (CD45^{hi}SSC^{lo} cells) had a small difference and an overlapping CI to 0 in the traumatized tibia to the contralateral tibia on all measured days. Among subtypes of lymphocytes, some small effects with non-overlapping CIs to 0 could be seen for NK1.1^{+} and T_H cells, but the effect size was too small to be interpreted as interesting. Our panel did not allow any further specification of which profile these T_H cells represented. However, lymphocytes exert influence by specialization and not by numbers. The effect size on number may be small and still biologically relevant, or the physiology of the cells change in an important way without a concomitant change in cell surface expression of markers.

4.4 Macrophages Seem Equally Essential to Metaphyseal Bone Healing as in Shaft Fractures

Paper IV

Depletion of macrophages has been shown to virtually halt fracture healing in shaft fractures, by means of systemic (H. N. Lin et al. 2017) and local clodronate administration (Alexander et al. 2011), as well as conditional knockout (Alexander et al. 2011). Interestingly, a long period (28 days) of clodronate injection prior to and during healing of shaft fracture does not have any negative effect (Madsen et al. 1998), indicating attenuation. In paper IV, one aim was to test if and when clodronate injection had an effect on pull-out force of a screw in the proximal tibia. Clodronate injection before screw insertion by -1 and -4 days both significantly decreased pull-out force compared to control (O. H. Sandberg et al. 2017). The other aim was to describe the macrophage population in the proximal tibia after clodronate injection and needle trauma. The cancellous model was used (detailed in section 3.1 on page 14) and the healing bone tissue analyzed at postoperative day 1 and 3. These mice received clodronate injection at day -3 and -2 preoperatively, respectively (Table 1 on page 25). The main effect at day 1 was depletion of resident phenotype macrophages, where $CD206^+$, $CD68^+$ and $F4/80^+$ macrophages had non-overlapping values and modest effect sizes. These changes were specific to day 1 except for $CD206^+$ macrophages that still had non-overlapping values at day 3 (Figure 5 on page 30).

Table 2: Study design clodronate injection in cancellous bone healing

Clodronate was injected i.p. at indicated days and subjected to screw insertion or metaphyseal trauma (cancellous model) The pull-out force of the screw and flow cytometry for macrophage phenotyping was done at indicated days. Days are in reference to the day of surgery.

	Pull-out					Flow Cytometry			
Injection (Day)	-4	-1	1	3	Control	-3	Control	-2	Control
Sacrifice (Day)	7					1	1	3	3
n	12					4	4	5	5

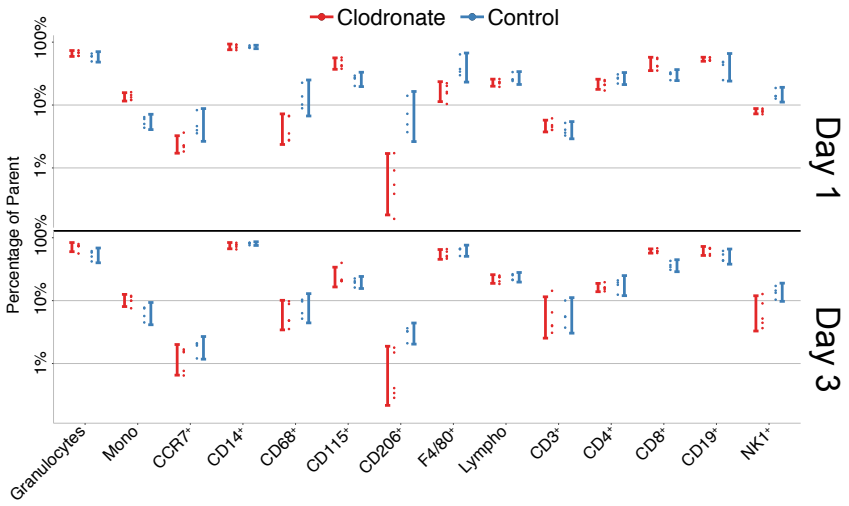


Figure 5: Macrophage composition in cancellous bone healing after clodronate injection

Macrophage composition in the cancellous model with clodronate injection. Logarithmic scale. Mice were subjected to the cancellous model of metaphyseal needle trauma and received clodronate before surgery i.p. Mice killed at day 1 received clodronate 3 days prior to surgery and mice killed day 3 received clodronate 2 days before surgery. A single clodronate liposome injection (0.2ml) was given intraperitoneally. The metaphyseal tibia was harvested and put through flow cytometry for analysis of macrophage phenotypes. Populations are indicated on the x-axis. The fraction of the parent gate (monocytes, $CD45^{mid}/SSC^{mid}$) is indicated on the y-axis. Bars indicate t-distributed 95% confidence interval. Please see Table 2 on page 29 for number of animals in each group.
Postop.) Postoperative.

4.5 Cancellous and Cortical Healing Recruit Different Lineages of Cells

Paper II

In paper II we compared the cellular composition in a cortical (detailed in section 3.1 on page 15) and cancellous (detailed in section 3.1 on page 14) bone healing model. Since these two models measure two different tissues we could not compare them directly against each other at day 3 and 5, but the comparison of relative difference in each model from day 3 to day 5 after trauma made each model its own control.

The cancellous and cortical model developed differently from day 3 to day 5 (Figure 6 on page 32). All cell populations changed with less than 10%, with most changes less than 5%. In the cancellous model, an increase could be seen in granulocytes, concomitant with a decrease in the cortical model. Both CIs were non-overlapping to zero. Monocytes were convincingly unaffected in the cortical model with a CI equilateral to zero, whereas monocytes decreased convincingly in the cancellous model with a CI well below zero. Lymphocytes showed an equally convincing increase in the cortical model, with a CI well above zero, whereas the same CI in the cancellous model overlapped with zero. Together, the shift in major populations show a clear line in the cortical model of decreased granulocytes and increased lymphocytes, and in the cancellous model of increased granulocytes, decreased monocytes, and maybe a slight increase in lymphocytes. The increase in lymphocytes in the cortical model seem to be general, but NK^+ cells (natural killer) increased the most. M1 phenotype macrophages somewhat increased in both models with a strictly positive CI, while M2 phenotype macrophages decreased in the cancellous model and were unaffected in the cortical model. This corroborates the findings from paper I.

It is unknown how the lymphocyte increase relative to cancellous bone healing is important in cortical bone healing. It is known that the cortical model is dependent on stem cells from the periosteum and surrounding muscle tissue for bone healing (Davis et al. 2015; Glass et al. 2011; Roberts et al. 2015), whereas the cancellous model is likely to recruit from locally abundant MSCs (Siclari et al. 2013). The lymphocytes might represent a modulation of the inflammation that is needed to orchestrate the complex process of recruiting stem cells. How come granulocytes increase as late as from day 3 to 5 in the cancellous model goes largely unanswered. Granulocytes are increasingly understood to have modulatory functions and not simply innate effector cells, and might have a specific function in cancellous bone healing relative to cortical bone healing (Kolaczkowska et al. 2013).

Table 3: Study design for comparison of cancellous and cortical bone healing at day 3 to day 5 after surgery. 3 animals were excluded from analysis in the cortical group at day 3 due to complete femoral fracture.
n/n) number of animals that were analyzed in relation to the number of animals randomized.

	Day 3	Day 5
Cancellous	6/6	6/6
Cortical	3/6	6/6

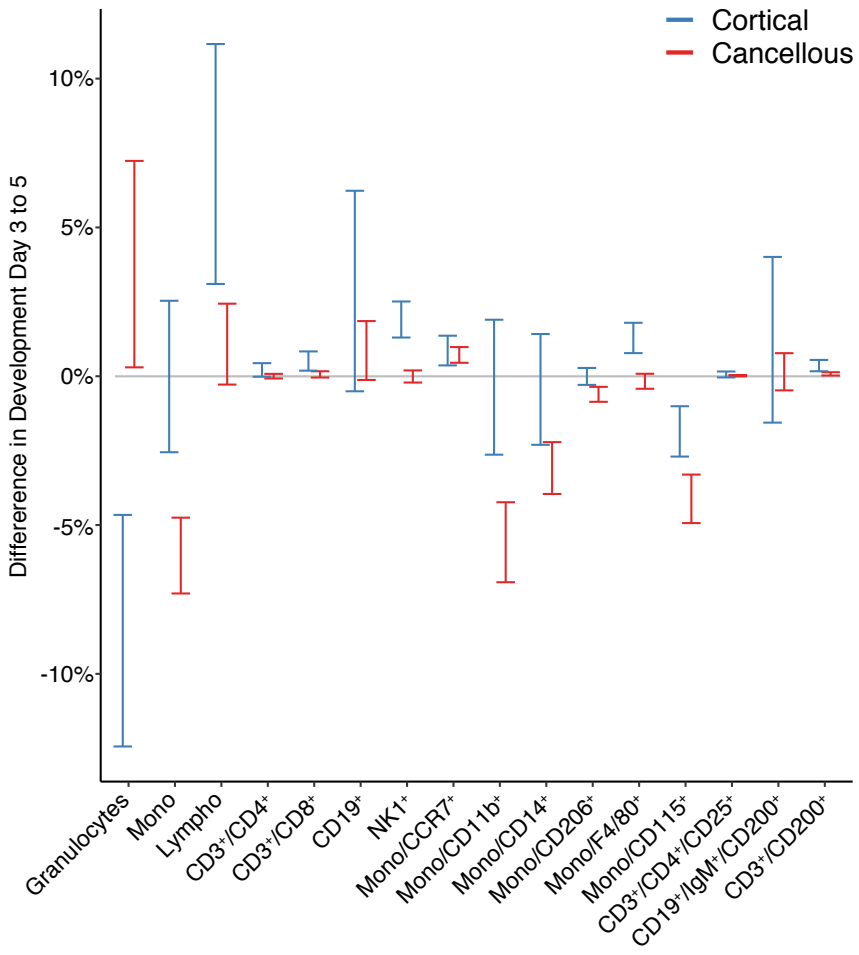


Figure 6: Cell Composition in Cancellous and Cortical Model Day 3 to 5. Cell populations are indicated on the x-axis. The fraction of each population to all leukocytes was calculated in both models at day 3 and day 5. The value at day 5 was compared with the value at day 3 to express the development of each cell population from day 3 to day 5. Higher values indicate that the cell population has increased in fraction at day 5 compared to day 3. Bars indicate the t-distributed 95% confidence interval without correction for multiplicity. 3 mice were excluded in the cortical group due to complete fracture of the femur.
$n_{cortical} = 3/6$
$n_{cancellous} = 6/6$

4.6 Different Response to Indomethacin in Cancellous and Cortical Bone Healing

Paper III

The inflammatory cytokine response of cortical bone healing has been well studied (Baht et al. 2018; Currie et al. 2014; Edderkaoui 2017; Gerstenfeld et al. 2003; Kobbe et al. 2008; Kon et al. 2001*b*; Ono and Takayanagi 2017; Schmid et al. 2009). In paper III we studied and compared cancellous and cortical bone healing in terms of cell composition and protein profile. The aim was to examine the cellular composition and protein profile with and without exposure to indomethacin in both models.

Mice were euthanised at day 3 and 5 to allow comparison of the models as in paper II (detailed in section 4.5 on page 31). All groups had a group size of 10 (Table 4 on page 33), but samples had to be excluded due to too few cells in flow cytometry or deviant protein concentration in certain groups.

Inflammation Is Seemingly Increased at Day 3 in the Cortical Model with Indomethacin

Contrary to our belief, the cellular composition was similar in indomethacin treated animals and controls in both the cancellous and the cortical model at both day 3 and 5. However, in the cortical model at day 3, one cell population was different enough to have a non-overlapping CI between treatment and control — inflammatory Ly6C^{hi} monocytes (Figure 7 on page 34). Mass spectrometry on healing bone tissue supernatants was used to describe the inflammatory milieu. Many proteins detected were of intracellular origin, and likely mark necrosis and apoptosis brought by inflammation. In analysis of indomethacin treated animals and controls, a large difference could be seen in the cancellous model at day 3, but only small differences at day 5 and in the cortical model overall. Many proteins were unique to indomethacin treated mice at day 3 with metaphyseal trauma. A literature review was performed on each protein to synthesize a higher understanding of the difference. Several proteins pertaining to eicosapentaenoic acid metabolism, inflammation and osteoclastogenesis could be found. Among pathways found in

Table 4: Study design in the study of the differential effect of indomethacin in cancellous and cortical bone healing as measured on day 3 and 5 after trauma. The collected tissue from each mouse was separated to a supernatant for mass spectrometry and the cells put through flow cytometry.
n/n) number of animals that were analyzed in relation to the number of animals randomized.

	Day 3		Day 5	
	Indomethacin	Control	Indomethacin	Control
	Flow Cytometry			
Cancellous	10/10	10/10	10/10	10/10
Cortical	6/10	6/10	10/10	7/10
	Mass Spectrometry			
Cancellous	8/10	1010	10/10	10/10
Cortical	9/10	9/10	9/10	7/10

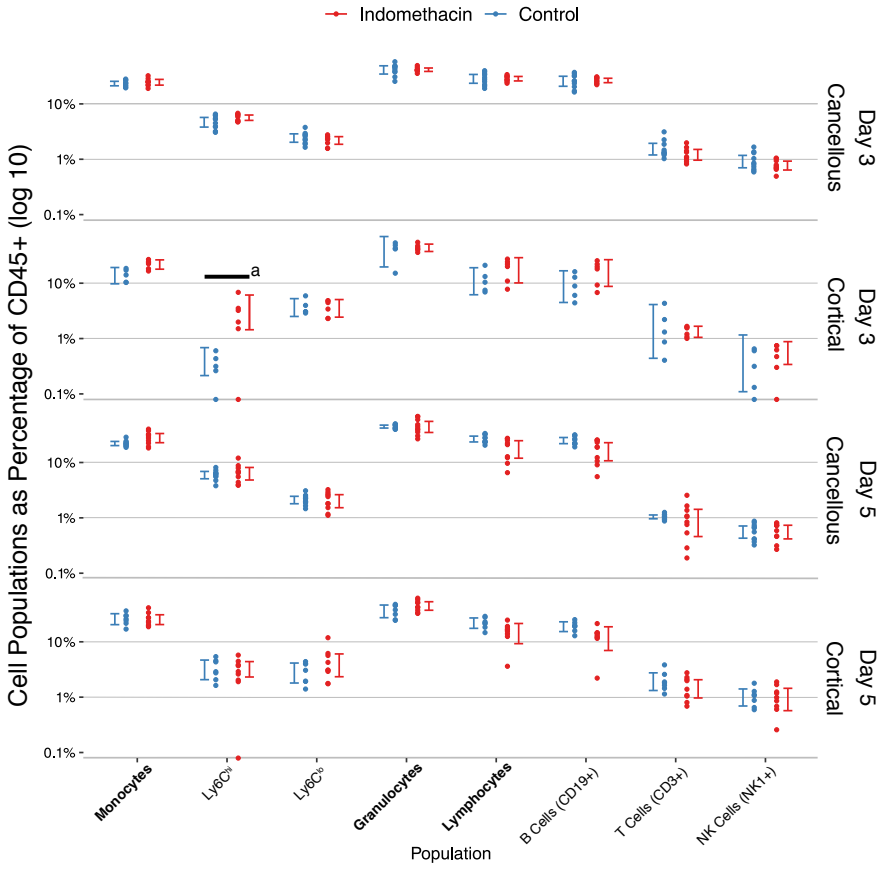


Figure 7: Cell populations in cancellous and cortical model at day 3 and 5 with or without indomethacin treatment. The bars denote the 95% t-distributed confidence interval without correction for multiplicity for each population as indicated on the x-axis. Only Ly6C^{hi} monocytes at day 3 in the cortical model had a non-overlapping confidence interval with indomethacin treatment compared to control.
a) non-overlapping confidence interval.

pathway enrichment analysis, immunologic pathways and inflammation were noteworthy. These results do show that the cortical model is affected at day 3 with indomethacin treatment in both cell composition and protein profile compared to cancellous bone healing. Yet, the direction of the results are hard to interpret given that indomethacin is an anti-inflammatory drug. However, it might represent an impeded cortical model that cannot progress from early inflammation, which have already started resolution and therefore less apparent in the cortical control. The feedback mechanisms of inflammation to start resolution might have been effectively inhibited in face of the strong inflammatory stimulus that a fracture undoubtedly constitutes. In immunologically restricted human patients (e.g. diabetes, alcoholism, autoimmune disease), Hoff et al. (2017) found an increased inflammatory response after fracture and surgery.

The Role of B Cells in Fracture Healing Is Unknown

No one has studied B cells' specific role in fracture healing. In paper I they were neither up- or downregulated with metaphyseal bone trauma, but in paper II they did increase from day 3 to day 5 in the cortical model. In paper III we could see that this is due to a likely increase of $CD19^{+}IgM^{+}IgD^{+}$ cells, which are immature. It is impossible to interpret whether these are part of normal regenerating bone marrow or the fracture callus, as histology of the cortical model in paper II indicated a segregation of these two volumes at day 5.

5 Concluding Remarks and Future

PAPER I

i A multicolor flow cytometry method was established to facilitate the mapping of leukocyte populations in models of bone healing.

ii Certain subpopulations showed differences with non-overlapping CIs to the no-change value (fold change; 1) and indicated an initial M2-inclined environment followed by M1 in the cancellous model.

iii Signaling along the CD200-CD200R^{+} axis might be important to cancellous bone healing.

iv The leukocyte composition was very similar in the traumatized tissue, contralateral proximal tibia and proximal humerus at day 5 indicating a systemic skeletal response.

PAPER II

i The cell composition in cancellous and cortical bone healing was similar at day 3, but diverged at day 5 with an increase in granulocytes in cancellous bone, while lymphocytes and monocytes increased in the cortical bone.

PAPER III

i Indomethacin treatment did not affect cell composition or extracellular protein profile in cancellous bone healing, whereas in cortical bone healing indomethacin at day 3 induced an increase in inflammatory Ly6C^{+} monocytes and a substantial increase in proteins involving pathways related to inflammation and osteoclasts. The inter-

pretation of these finding is unclear, but might explain the different susceptibility to indomethacin treatment seen in experimental bone healing.

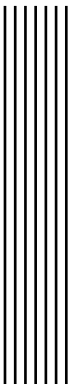
PAPER IV

i Cancellous bone healing is weakened if macrophages are depleted within the first two days after fracture, but not later

ii A decrease in resident phenotype (M2) macrophages in the healing tissue is noted with clodronate injection

The papers in this thesis show a distinct inflammatory response of cancellous bone healing that it is different compared with cortical bone healing, both its natural development and its response to anti-inflammatory treatment with indomethacin.

A good approach to further increase our understanding of bone healing, would be to profile the cells of early bone healing. What are the initiating factors and what behaviour do the cells develop on stimulus of either fracture type? It is known from other studies (Baht et al. 2018; Currie et al. 2014; Edderkaoui 2017; Gerstenfeld et al. 2003; Kobbe et al. 2008; Kon et al. 2001*b*; Ono and Takayanagi 2017; Schmid et al. 2009) that many of the foundational cytokines are readily expressed in fractured bone, and our results suggest that the dominating feature is not likely to be at the level of cell surface phenotype, but rather functional cell properties. The expression profiles of early responding cells, mainly macrophages, could further our understanding on how the healing is initiated differently in cancellous and cortical bone healing. However, these behaviours might not be solely captured by measurement of cytokines, as cell-cell signaling and response to tissue signals might play an important role. In addition to macrophages, neutrophilic granulocytes have been a conspicuous feature of the phenotype in both models at all time points. It is likely that they represent early cells that guide inflammation correctly. These basic science questions may have clinical implications. Pinpointing of the underlying mechanistic differences between cancellous and cortical bone healing would allow better approaches to confer the favorable healing characteristics of metaphyseal fracture to shaft fractures.



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