# The interaction between the immune and skeletal systems during bone repair: the role of mast cells

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*Para Isabella y los que vengan después,*
*quien serán como dijo Hernández,*
*el porvenir de mis huesos y de mi amor*

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# ABSTRACT

Mast cells are a sub-type of inflammatory cells of the constitutive immune system traditionally linked to inflammatory disorders, allergies and immunity against parasites, but they are increasingly recognized as important mediators of tissue repair. Their widespread functions include modulation of inflammation and promotion of neo-angiogenesis, which are critical components of tissue repair. Mast cells and their precursors are found in fracture callus, however, their role during bone healing remains obscure. Moreover, immune cell therapy has been proposed as an innovative approach for therapeutic intervention to modify bone repair and promote better healing outcomes in patients at high risk of fracture complications. The research presented in this thesis is based on the hypothesis that mast cells play a pivotal role in bone repair by coordinating the recruitment and activity of vascular, skeletal and immune cells at the site of healing. Its overall goal was to elucidate if mast cells played a role during bone healing in *Cpa3*$^{Cre/+}$ mice, in which the absence of mature mast cells is the only known defect, with the aim of identifying strategies to promote enhanced bone repair through their manipulation.

First, we quantified bone repair and vascular and inflammatory cell activity markers in bilateral window femoral defects of *Cpa3*$^{Cre/+}$ and *WT* mice. We identified defective capillary invasion, fibrous tissue accumulation, impaired mineralization and dysregulation of osteoclast activity and neo-vascularization patterning during bone healing in the absence of mast cells. Thus, a positive role of mast cells in bone healing was confirmed in a kit-independent mast cell deficient mouse model (Manuscript I). Next, we transplanted mature mast cells cultured *in vitro* to the mast cell deficient mice to perform phenotype rescuing analyses. We found that mast cell reconstitution

populates the normal cellular niches, however, no mature mast cells were found in healing bone. Afterwards, we tested whether transplanting mast cells immediately after the creation of the window defect would attract them to the healing tissue and promote repair. Unfortunately, we also found that mast cells migrate to their normal niches and are not attracted to healing bone. Through these experiments, we concluded that the mast cells observed during bone healing arise from precursors located *in situ* (Manuscript II).

Mast cell exhibits phenotypic plasticity; therefore, their activity can be modulated by changes in the environmental cues. Previous research demonstrated that granulated mast cell numbers were greatly diminished during bone repair under acute systemic inflammation induced by lipopolysaccharide (LPS). However, the effect of chronic systemic anti-inflammation on mast cell activity had not been tested. Moreover, because we also expected the change in the inflammatory environment to have some effect in bone repair, we sought to investigate if the chronic administration of diclofenac, a non-selective nonsteroidal anti-inflammatory drug, would rescue the phenotype of LPS-induced systemic inflammation. Our results show that chronic treatment with diclofenac promoted mast cell hyperplasia in the bone marrow due to the accumulation of non-degranulated cells and that it is cytotoxic to endothelial cells. Additionally, we found that the use of NSAIDs does not reverse the LPS-induced systemic inflammation phenotype, but rather adds to the dysregulation of the repair tissue (Manuscript III).

Finally, under the paradigm of mast cell immunogenic sensibilization and immune memory, we tested whether "priming" the immune system to repair broken bone may accelerate fracture healing in a subsequent fracture sustained shortly thereafter. Our results show enhanced bone healing characterized by more advanced bone remodelling in mice subjected to subsequent fractures, compared to controls. Based on our findings, we propose that mast cell immune memory may be used as an immunomodulatory treatment to enhance bone healing, but further studies to confirm this is required (Manuscript IV).

Taken together, the results presented in this thesis demonstrate that mast cells exert regulatory activity for immune cell, vascular cell and osteoclast activity regulation during bone healing; however, their effects are very much dependent on the inflammatory milieu. Nonetheless, we propose mast cells as a cellular target for immunotherapy directed towards promoting bone repair.

# RESUMÉ

Les mastocytes sont un sous-type de cellules inflammatoires du système immunitaire constitutif. Traditionnellement, ils sont liés aux maladies inflammatoires, aux allergies et donc l'immunité contre les parasites, mais ils sont de plus en plus reconnus comme des médiateurs importants de la réparation des tissus. Leurs fonctions répandues incluent la modulation de l'inflammation et la promotion de la néo-angiogenèse, qui sont des composants essentiels de la réparation tissulaire. Les mastocytes et leurs précurseurs se retrouvent dans les cales des fractures, mais leur rôle pendant la guérison des os reste obscur. De plus, la thérapie immunitaire cellulaire a été proposée comme une approche innovante d'intervention thérapeutique visant à modifier la réparation osseuse et à améliorer les résultats de la guérison chez les patients à risque élevé de complications fracturaires. La recherche présentée dans cette thèse est basée sur l'hypothèse selon laquelle les mastocytes jouent un rôle essentiel dans la réparation des os en coordonnant le recrutement et l'activité des cellules vasculaires, squelettiques et immunitaires sur le site de la guérison. Son objectif général était de déterminer si les mastocytes jouent un rôle lors de la guérison osseuse chez les souris *Cpa3*$^{Cre/+}$, dans lesquels l'absence de mastocytes matures est le seul défaut connu, dans le but d'identifier des stratégies pour promouvoir l'amélioration de la réparation osseuse par leur manipulation.

Pour la première étude, nous avons quantifié la réparation osseuse et les marqueurs d'activité cellulaire vasculaire et inflammatoire dans un modèle de fracture fémorale bilatérale chez les souris *Cpa3*$^{Cre/+}$ et *WT*. Nous avons identifié une invasion capillaire défectueuse, une accumulation de tissu fibreux, une minéralisation altérée et une dysrégulation de l'activité des ostéoclastes avec altération du patron de néovascularisation lors de la guérison osseuse en l'absence

des mastocytes. Ainsi, un rôle positif des mastocytes dans la guérison osseuse a été confirmé dans un modèle de souris déficientes en mastocytes indépendant de la mutation dans le récepteur kit (Manuscrit I). Ensuite, nous avons transplanté des mastocytes matures cultivés *in vitro* sur des souris déficientes en mastocytes pour effectuer des analyses de sauvetage de phénotype. Nous avons constaté que la reconstitution des mastocytes peuplait les niches cellulaires normales, mais aucun mastocyte mature n'a été trouvé dans l'os en cours de guérison. Nous avons ensuite vérifié si la transplantation de mastocytes immédiatement après la création du défaut osseause dans le fémur les attirerait vers le tissu en voie de guérison et favoriserait la réparation. Malheureusement, nous avons également constaté que les mastocytes migrent vers leurs niches normales et ne sont pas attirés par la guérison des os. A travers ces expériences, nous avons conclu que les mastocytes observés lors de la cicatrisation osseuse proviennent de précurseurs situés *in situ* (Manuscript II).

Les mastocytes présentent une plasticité phénotypique; par conséquent, leur activité peut être regulée par des changements dans les signaux environnementaux. Des recherches antérieures ont montré que le nombre de mastocytes granulés était considérablement réduit lors de la réparation osseuse dans les cas d'inflammation systémique aiguë induite par le lipopolysaccharide (LPS). Cependant, l'effet de l'anti-inflammation systémique chronique sur l'activité des mastocytes n'a pas été testé. De plus, comme nous nous attendions également à ce que la modification de l'environnement inflammatoire ait un effet sur la réparation osseuse, nous avons cherché à déterminer si l'administration chronique de diclofénac, un médicament anti-inflammatoire non stéroïdien (AINS) non sélectif, permettrait de sauver le phénotype de l'effet inflammatoire systémique induit par le LPS. Nos résultats montrent qu'un traitement chronique au diclofénac favorise l'hyperplasie des mastocytes dans la moelle osseuse en raison de l'accumulation de

cellules non dégranulées et qu'il est cytotoxique pour les cellules endothéliales. De plus, nous avons constaté que l'utilisation d'AINS n'inverse pas le phénotype d'inflammation systémique induite par les LPS, mais ajoute plutôt à la dysrégulation du tissu de réparation (Manuscript III).

Finalement, sous le paradigme de la sensibilisation immunogène des mastocytes et de la mémoire immunitaire, nous avons vérifié si le fait de "préparer" le système immunitaire à réparer un os fracturé pouvait accélérer la guérison osseuse lors d'une fracture subséquente subie peu de temps après. Nos résultats montrent une meilleure guérison osseuse caractérisée par un remodelage osseux plus avancé chez les souris soumises à des fractures ultérieures, par rapport aux témoins. Sur la base de nos résultats, nous proposons que la mémoire immunitaire des mastocytes puisse être utilisée comme un traitement immunomodulateur pour améliorer la réparation osseuse, mais des études supplémentaires sont nécessaires pour le confirmer (Manuscrit IV).

Pris ensemble, les résultats présentés dans cette thèse démontrent que les mastocytes exercent une activité régulatrice pour la régulation de l'activité des cellules immunes, des cellules vasculaires et des ostéoclastes lors de la réparation osseuse. Cependant, leurs effets dépendent beaucoup du milieu inflammatoire. Néanmoins, nous proposons les mastocytes comme une cible cellulaire pour l'immunothérapie visant à favoriser la réparation osseuse.

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During my Ph.D., I was fortunate enough to mentor several students that made internships in the lab. Teaching them also thought me many things and made me question our experiments with a set of fresh eyes. My thanks to Tim Wong, Daniel Chan, Levi Goldfarg, Jason Vu, Jacob Lee-Howes, Jaimie Clark and Tobi Olasubulumi. I hope I've been able to share a spark of joy for science.

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Finally, I would also like to acknowledge and thank all the people involved in the research projects that I undertook as a sideline to my thesis. They have been instrumental in helping develop a broad-based skill set and shaping my career. Special mention to Dr. Sheila Wang, Dr. Yunghan Au, Dr. Gregorio Zuniga, Dr. Mario Martinez, and Dr. Samuel Kolosovas; may these be the first of many more projects to come.

*Hic sunt dracones*

# CONTRIBUTION TO ORIGINAL KNOWLEDGE

The work in this thesis consists of several novel contributions to the field of osteoimmunology and bone repair. The contributions and their significance will be explained and discussed in the context of existing knowledge in the manuscripts and discussion chapters. Here, they are concisely summarized.

***OBJECTIVE I: Quantify bone repair at timed intervals in Cpa3$^{Cre/+}$ and WT mice with surgically induced defects that model human femoral fractures***

*Results*: We identified defective capillary invasion, fibrous tissue accumulation, impaired mineralization and dysregulation of osteoclast activity during bone healing in the absence of mast cells. However, no differences in alkaline phosphatase activity between strains were identified, thus suggesting that the dysregulation of anabolic cells probably is an event that affects mesenchymal stem cell recruitment and differentiation into pre-osteoblasts. A positive role of mast cells in bone healing was confirmed in a kit-independent mast cell deficient mouse model.

*Manuscript:* **Ramirez-GarciaLuna JL**, Chan D, Samberg R, Abou-Rjeili M, Wong TH, Li A, et al. Defective bone repair in mast cell-deficient Cpa3Cre/+ mice. *PLoS ONE.* 2017;12(3):e0174396.

***OBJECTIVE II: Identify markers of inflammatory and vascular cell function to investigate the temporal and functional role of mast cells in healing bone defects of Cpa3Cre/+ and WT mice***

*Results*: We found dysregulation of neo-vascularization and osteoclast activity, as well as fewer macrophages in mast cell deficient mice. In the absence of mast cells, the osteoclast dysregulation pattern was found to be bi-phasic, with downregulation of osteoclast and TRAP positive macrophage activity during the initial phases of bone healing and increase in osteoclast activity and intense bone resorption during the late phases. A macrophage – osteoclast – mast cell functional axis is suggested. Additionally, a micro-CT pipeline to reconstruct the blood vessel network inside of bone tissue was developed by me. Correlation with CD34 histological quantification was done and presented in the 2016 Bruker User Meeting and 3-D quantitative analysis of blood vessels using this technique have been published in several manuscripts (Drager et al., 2017; Ramirez-GarciaLuna et al., 2017; Ramirez-Garcia-Luna et al., 2019).

*Manuscript:* **Ramirez-GarciaLuna JL**, Chan D, Samberg R, Abou-Rjeili M, Wong TH, Li A, et al. Defective bone repair in mast cell-deficient Cpa3Cre/+ mice. *PLoS ONE.* 2017;12(3):e0174396.

***OBJECTIVE III: Develop a mast cell reconstitution model of Cpa3$^{Cre/+}$ mice to perform phenotype rescuing analyses and determine if exogenous mast cells enhance bone healing***

*Results:* Mast cell reconstitution populates the normal cellular niches, but they fail to appear in healing bone. Likewise, mast cells transplanted intrafemorally to mast cell deficient mice immediately after the creation of a bone defect populate the normal niches but fail to home into healing bone. Our results strongly suggest that mast cells that appear during bone healing are differentiated from *in situ* precursor cells. Furthermore, exogenously administered mature mast cells do not have the necessary receptors to target healing bone, thus disproving the theory that they can be used as a cellular therapy to enhance bone healing.

*Manuscript:* **Ramirez-GarciaLuna JL**, Abou-Rjeili M, D Chan, Goldfarb L, et al. Mast cells involved in fracture repair arise from precursors in bone marrow. *In preparation.*

***OBJECTIVE IV: Quantify bone repair and mast cell activity during the inflammatory phase of bone healing in WT mice treated with a non-steroidal anti-inflammatory drug (NSAID) with or without lipopolysaccharide (LPS) induced systemic inflammation***

*Results:* The results show impaired bone healing in the NSAID and NSAID + LPS treated mice with concomitant changes in the neo-vascularization pattern and changes in the mast cell distribution and granulation pattern. *In-vitro* analysis further confirmed cytotoxicity of diclofenac, the NSAID used in the study to vascular endothelial cells. We demonstrate that changes in the inflammatory environment are matched with changes in mast cell activity.

*Manuscript:* **Ramirez-Garcia-Luna JL**, Wong TH, Chan D, Al-Saran Y, Awlia A, Abou-Rjeili M, et al. Defective bone repair in diclofenac treated C57Bl6 mice with and without lipopolysaccharide induced systemic inflammation. *J Cell Physiol.* 2019 Mar;234(3):3078–87.

***OBJECTIVE V: Determine if "priming" the immune system to repair broken bone may accelerate fracture healing in a subsequent fracture sustained shortly thereafter.***

*Results:* We demonstrate enhanced bone healing characterized by more advanced bone remodelling in mice subjected to subsequent fractures, compared to controls. *In-vitro* analysis further showed increased mesenchymal stromal cell activity when co-cultured with mast cells in a 3D cell culture model. Based on our findings, we propose that mast cell immune memory may be used as an immunomodulatory treatment to enhance bone healing, but further studies to confirm this is required.

*Manuscript:* **Ramirez-Garcia-Luna JL,** Olasubulumi OO, Rangel-Berridi K, Abou-Rjeili M, et. al. Enhanced bone remodeling after fracture priming. *In preparation.*

# CONTRIBUTION OF AUTHORS

This thesis is written in standard format in accordance with the regulations put forth by the Faculty of Graduate and Postdoctoral studies at McGill University. It is written in the manuscript form and contains a list of original contributions to scientific literature, an introductory chapter, a literature review, four multi-authored manuscripts, a discussion chapter, and an appendix section.

The data for this thesis was collected during four years of laboratory work. I, JL Ramirez Garcia Luna, conducted the majority of experiments and data analysis and wrote the initial draft of each manuscript. However, none of this would have been possible without the guidance, support and edition of my supervisors, Dr. Janet E. Henderson and Dr. Paul A. Martineau.

Detailed contributions of each co-author of the manuscripts are provided at the end of each section. CA Lemarie, DH Rosenzweig, E Akoury, M Abou-Rjeili, and Y Al-Saran provided training for many of the experimental techniques I used, in addition to performing experiments. A Li provided training in histopathology and immunostaining. K Rangel Berridi, TA Wong, D Chan, L Goldfarb, AV Tran, and OO Olasubulumi, helped gather data in the form of micro-CT and histological images. TB Feyerabend and HR Rodewald contributed by providing the *Cpa3*$^{Cre/+}$ mouse strain, data interpretation, and by lending their considerable expertise as leaders in the field of mast cell biology.

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# LIST OF ABBREVIATIONS

aTB: acidified toluidine blue

ALP: alkaline phosphatase

BMD: bone mineral density

BV/TV: bone volume per tissue volume

Ch.N: vascular channel number

Ch.Sp: vascular channel separation

Ch.Th: vascular channel thickness

Ch.V/TV: vascular channel volume per tissue volume

Conn.Dn: connectivity density

DAMP: danger associated molecular pattern

DMEM: Dulbecco's Modified Eagle's Medium

EC: endothelial cell

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

FACS: fluorescence-activated cell sorting

FBS: fetal bovine serum

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

HE: hematoxylin / eosin

HIF: hypoxia-inducible factor

IL: interleukin

MC: mast cell

MEM: minimum essential media

micro-CT: micro computer tomography

MSC: mesenchymal stem cell

NSAID: non-steroidal anti-inflammatory drug

OPN: osteoprotegerin

PAMP: pathogen associated molecular pattern

PBS: phosphate buffered saline solution

PCR: polymerase chain reaction

PFA: paraformaldehyde

PMMA: polymethylmethacrylate

Po.Tot : total porosity

Po.V.op : volume of open pores

qPCR: quantitative polymerase chain reaction

RANK: receptor activator of nuclear factor kappa-B

RANKL: receptor activator of nuclear factor kappa-B ligand

ROI: region of interest

RT-PCR: reverse transcription polymerase chain reaction

SMI: structure model index

SCF: stem cell factor

TB: toluidine blue

Tb.N: trabecular number

Tb.Sp: trabecular separation

Tb.Th: trabecular thickness

TLR: toll-like receptor

TNF: tumor necrosis factor

TRAP: tartrate-resistant acid phosphatase

VEGF: vascular endothelial growth factor

VOI: volume of interest

# I. INTRODUCTION AND RESEARCH OBJECTIVES

Bone healing is composed by the sequential, yet interdependent phases of hemostasis, inflammation, repair, and remodeling. While there are some particularities of these processes for bone, they are not different from the wound healing phases observed in other tissues (Harper, Young, & McNaught, 2014). However, bone is remarkable because it is one of the few, if not the only, tissue that after an injury can regenerate and remodel to its native form, conserving its microarchitecture and minimal scarring (Claes, Recknagel, & Ignatius, 2012). This ability is probably an evolutive advantage, as fractures are one of the most common types of injury. For example, the estimated lifetime-risk for hip fractures in Canadian women is 12% (Hopkins et al., 2012), however, this figure increases to 50% in women with osteoporosis (Johnell & Kanis, 2005).

Even though fractures are so prevalent, the rate of non-union for the general population is between 5-10% (Zura et al., 2016), attesting to the extraordinary healing potential of bone. Nonetheless, major trauma, insufficient vascular supply due to injury or pre-morbid conditions, diabetes, or use of certain drugs that modify bone homeostasis increase the rate of fracture healing complications by approximately three to five-fold (Stegen, van Gastel, & Carmeliet, 2015). In order to achieve proper healing, bone needs three major components: **bone tissue itself, neo-vascularization of the tissue, and a competent immune system**. The adequate interplay of these components determines the success of the healing process and therefore, are amenable to targeted interventions to modify and promote repair in patients at high risk of fracture complications.

## 1.1 Bone tissue

Bone is a specialized type of connective tissue composed of three types of cells, anabolic osteoblasts, catabolic osteoclasts, and osteocytes that have mechanoreception properties. The balance between osteoblast and osteoclast activity determines the net bone mass change over time and is the basis of the bone remodelling process that is active throughout an individual's life. The inert component of bone is its matrix, whose biological component, termed osteoid, is composed of approximately 90% type I collagen and the remaining 10% of several different proteins, such as osteocalcin, osteopontin and osteonectin, that act as crystal nucleators and allow the deposition of a mineral lattice. This lattice, composed of hydroxyapatite crystals, constitutes the majority of the tissue itself and is perhaps its most salient characteristic (Limb, 2015).

Bone cells derive from two distinct stem cell populations. Osteoblasts arise from osteoprogenitor cells and mesenchymal stem cells (MSCs) located in the bone endosteum and periosteum (W. Huang, Yang, Shao, & Li, 2007). Osteoblasts deposit osteoid which mineralizes and becomes woven or primary bone. Some of the osteoblasts get encased in bone and mature into osteocytes, which compose approximately 90% of all the cells found in the skeleton. Osteocytes form an interconnected 3D network throughout the bone tissue and provide mechanotransduction signals for the full organ that allow its adaptation to mechanical stimuli (Graham, Ayati, Holstein, & Martin, 2013). Mesenchymal stem cells are a small fraction of multipotent stem cells that reside in the bone marrow. They are characterized by the positive expression of CD105, CD73 and CD90 and negative expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules, as well as exhibiting adherence to plastic culture flasks and the capacity to undergo differentiation to chondrocyte, osteoblast and adipocyte lineages *in vitro* (Dominici et al., 2006).

Under the influence of TGF-β and BMPs, particularly BMP-2, MSCs undergo differentiation into pre-osteoblasts, which in turn, mature into osteoblasts. As such, TGF-β and BMP-2 are some of the most potent chemotactic stimulators for MSCs and are required during fracture healing (Garg, Ryan, & Bowlin, 2011). Osteoclasts, on the other hand, derive from hematopoietic mononuclear precursors found in the bone marrow and are more closely related to macrophages than to other type of bone cells (Kurihara, Chenu, Miller, Civin, & Roodman, 1990). They are characterized by being large multinucleated cells that produce enzymes that erode the mineralized surface of the bone. Osteoclasts form by the union of several mononuclear precursors and are maintained by recruiting precursor cells into their syncytium (Miyamoto, 2011). The balance between osteoblast and osteoclast activity is maintained via the RANK – RANKL – OPN axis, which in turn, is dependant on several different mediators, including IL-1, IL-6 and TNF-a, all of which are active during bone repair (Nakashima, Hayashi, & Takayanagi, 2012). A special mention in this section is made to osteal macrophages or "osteomacs", which even though phenotypically resemble macrophages, reside within bone as a canopy structure that overlies mature osteoblasts and whose function is absolutely necessary to promote mineralization (Chang et al., 2008). Whether osteomacs constitute osteoclast precursor cells or are part of the bone immune surveillance cells is still debated. These cells are characterized by CD68+ expression, however, they lack the traditional murine F4/80 antigen that is common for macrophages, but expresses tartrate-resistant acid phosphatase (TRAP) activity which traditionally is a marker of osteoclasts. Moreover, they are able to phagocyte foreign material, detect bacterial products, and respond to antigen challenges (Miron & Bosshardt, 2016). What is clear is that these cells, although not traditionally belonging to the bone cell group are essential for the homeostasis of the tissue and highlight the blurring of the lines between the skeletal and immune system.

## 1.2 Neo-vascularization of the repair tissue

Because any insult that leads to tissue injury causes a disruption to the blood flow, the initial phase of wound healing is hemostasis (Harper et al., 2014). Nonetheless, once hemostasis has been achieved, perhaps the most critical step for achieving successful repair is the restoration of an adequate blood supply, which is done by the neo-angiogenesis process. Neo-angiogenesis is characterized by the budding of endothelial precursor cells from the existing blood vessels. These cells connect and form new vessels that bring oxygen, nutrients, precursor and immune cells to the wound tissue bed. In turn, once the repair has been achieved, part of the remodelling process consists in the pruning of the excess vascular tissue to maintain only the minimum required by the metabolic demand of the surrounding cells (Simons, 2005).

The most important factor that drives neo-angiogenesis is hypoxia. Hypoxia activates the Hypoxia Inducible Factor (HIF) pathway that stimulates the transcription of multiple response genes of which the vascular endothelial growth factor (VEGF) is a major target. In turn, VEGF promotes the survival of surrounding cells, the recruitment of angiogenic precursors, and the forming of tubules that will give rise to new blood vessels (Drager, Harvey, & Barralet, 2015). The bone microvasculature is characterized by the expression of CD34, CD31 and endomucin (Emcn) as markers of endothelial cells, however, the level of expression of the different markers can be used to differentiate cellular sub-populations. High expression levels of CD31 and Emcn are found in a very small population of endothelial cells that reside in the bone metaphysis and in the endostium. These cells are in close proximity to osteoprogenitor cells, forming a cellular niche that becomes active under the influence of the HIF pathway once the bone repair process has been started (Kusumbe, Ramasamy, & Adams, 2014). For this reason, the HIF pathway is also involved

in osteoblast survival and differentiation during bone repair. Pharmacological induction of the HIF pathway by iron chelators is associated with higher levels of neo-vascularization and enhanced bone production (Drager et al., 2017). However, the effect needs to be local, as chronically hypoxic fractures have very bad repair prognostic (Stegen et al., 2015).

Hypoxia and the HIF pathway are also major triggers for immunological responses (Halligan, Murphy, & Taylor, 2016). The HIF pathway is pro-inflammatory in itself through the activation of the nuclear factor-κB (NF-κB) pathway, which is a key player in the control of the proliferation of hematopoietic cells and the recruitment and maturation of mononuclear cells (D'Ignazio, Bandarra, & Rocha, 2016). Oxygen tension also plays a role in immune surveillance and the initial response to damage by neutrophils through the release of reactive oxygen species (ROS), which in turn create a positive feedback loop to the HIF pathway (Chandel et al., 2000). Taken together, these responses are called "inflammatory hypoxia" and are a hallmark of the inflammatory phase of wound healing (Taylor & Colgan, 2017).

Vascular remodelling and regression are needed once the repair process has been achieved to maintain the normal oxygen levels of the tissue. Vascular remodelling is triggered by low blood flow, as shear stress caused by normal flow inhibits endothelial cell apoptosis. Remodelling of this tissue is characterized by the occlusion of some of the blood vessel collaterals by hypertrophy of the endothelial cells and subsequent apoptosis, which leads to increase in the shear stress of the network and pruning of the excess tissue (Potente, Gerhardt, & Carmeliet, 2011). Failure to prune the excess blood vessels is associated with metabolic dysregulation and may exert a mechanical force that triggers remodelling of the surrounding tissue (Jain, 2003).

## 1.3 A competent immune system

Although the immune system plays a major role during bone healing, its contribution has been traditionally neglected in favor of research in areas of stem cells and angiogenesis. The field of osteoimmunology investigates the interactions between the skeletal and immune system. Its objective is to understand the crosstalk between the systems and how one affects the other, both under physiological and pathophysiological conditions (P. S. Schneider, Sandman, & Martineau, 2018). Beyond what was already discussed in the section of bone cells, the skeletal and immune system share several signaling pathways during normal bone functioning, such as the para-thyroid hormone (PTH) pathway and the RANK – RANKL – OPN axis (Pacifici, 2016). Moreover, under physiological states, the survival of osteoblasts is dependant on cues provided by T-helper lymphocytes, which also influence osteoclastogenesis (Ginaldi & De Martinis, 2016).

During bone repair, the immune system is the first-line responder to the injury. Neutrophils and monocytes infiltrate the wound bed and kick-start wound repair. Thus, an initial inflammatory response is absolutely needed to achieve proper healing. Late-comers are lymphocytes, macrophages and mast cells that shift the inflammatory balance and promote a shift in the inflammatory balance that is needed for progression in the repair process. Failure to control inflammation leads to non-healing chronic wounds that in bone are called non-unions (Beldon, 2010; Harper et al., 2014).

## 1.4 Rationale

Most of the literature on the role of the immune system during bone repair moves around lymphocytes and macrophages, however, there are several other cellular lines that may play regulatory roles during bone healing. One of those is mast cells, a component of the innate immune system. The primary role ascribed to mast cells is that of a protagonist in the pathology of inflammatory disorders, allergies and immunity against parasites, but they are increasingly recognized as important mediators of tissue repair (H.-R. Rodewald & Feyerabend, 2012).

Previous work from the Bone Engineering Labs of the RI-MUHC demonstrated for the first time impaired healing of cortical bone defects of mast cell deficient mice (Behrends et al., 2014). However, the results could have been confounded by the absence of c-kit, the receptor for stem cell factor, on other cells in the bone micro-environment. Because mast cells and their precursors are found in fracture callus, and immune cell therapy has been proposed as an innovative approach for therapeutic intervention to modify bone repair, the overall goal of my Ph.D. thesis was to confirm if mast cells played a role during bone healing in *Cpa3*$^{Cre/+}$ mice, in which the absence of mature mast cells is the only known defect, with the aim of identifying strategies to promote enhanced bone repair through their manipulation.

## 1.5 Hypothesis

Our research is based on the hypothesis that mast cells play a pivotal role in bone repair by coordinating the recruitment and activity of vascular, skeletal and immune cells at the site of healing. Moreover, we hypothesize that mast cell manipulation can be used as a strategy to enhance bone repair.

## 1.6 Thesis research objectives

***OBJECTIVE I: Quantify bone repair at timed intervals in Cpa3$^{Cre/+}$ and WT mice with surgically induced defects that model human femoral fractures***

*Approach:* Cortical window defects measuring 1mm x 2mm were drilled in the outer aspect at the mid-diaphysis of both femurs of skeletally mature mice. Cohorts were euthanized at 5 days postoperative to locate mature mast cells in and around the fracture. Additional cohorts were euthanized at 14-56 days postoperative to evaluate mineralized tissue using micro CT and von Kossa/toluidine blue staining of un-decalcified bone.

***OBJECTIVE II: Identify markers of inflammatory and vascular cell function to investigate the temporal and functional role of mast cells in healing bone defects of Cpa3Cre/+ and WT mice***

*Approach:* The quantity and spatial distribution of vascular endothelial cells, macrophages, osteoclast and osteoblast function were evaluated by histochemical and immune-histochemical staining in decalcified specimens.

***OBJECTIVE III: Develop a mast cell reconstitution model of Cpa3$^{Cre/+}$ mice to perform phenotype rescuing analyses and determine if exogenous mast cells enhance bone healing***

*Approach:* Mast cells were differentiated from bone marrow isolated from *WT* mice and transplanted into *Cpa3*$^{Cre/+}$ animals. Mast cell presence in the normal cellular niches was assessed at 8-weeks post-transplant, and after confirming engraftment of the cells, cortical window defects were generated as described in Objective I.

***OBJECTIVE IV: Quantify bone repair and mast cell activity during the inflammatory phase of bone healing in WT mice treated with a non-steroidal anti-inflammatory drug (NSAID) with or without lipopolysaccharide (LPS) induced systemic inflammation***

*Approach:* Cortical window defects were generated in skeletally mature *WT* mice as described in Objective I. Animal cohorts were treated with diclofenac, a non-selective NSAID, NSAID + LPS or placebo delivered via slow release pellets and intra-peritoneal injections, until the time of euthanasia at 14 days post-operative. Micro-CT and histochemical/immuno-histochemical analyses of molecular markers as in Objective II were used to assess vascular invasion of the callus in relationship to bone formation.

***OBJECTIVE V: Determine if "priming" the immune system to repair broken bone may accelerate fracture healing in a subsequent fracture sustained shortly thereafter.***

*Approach:* Cortical window defects were generated in skeletally mature *WT* mice as described in Objective I in the left and right legs two-weeks apart. Micro-CT and histochemical/immuno-histochemical analyses of molecular markers as in Objective II were used to assess bone formation, bone remodelling and vascular ingress to the repair tissue.

# II. LITERATURE REVIEW

This section has been adapted from the manuscript entitled *"The good, the bad and the ugly: a review of the role of mast cells in bone healing"*. To follow a logic order with the rest of the manuscripts that compose this thesis, the review has been divided into two chapters. The first chapter begins with the historic perspective of landmark research on mast cells since their discovery in the last quarter of the XIX century. Afterwards, mast cell biology, with emphasis on the different mediators that they synthesize and store, and their known role in tissue homeostasis and general wound healing is discussed. As such, the first part of the review provides the background and rationale for the presented investigations of this thesis.

The second part of the review, which will be presented in chapter seven of the thesis, delves on of the role of mast cells in bone healing, in other musculoskeletal tissues, and in biomaterial integration. Several of articles presented in this chapter correspond to original articles that compose the body of this thesis. The section ends with some perspectives and directions where we believe future research should be oriented and a general conclusion.

This review was an ongoing project started by Dr. Dominique Behrends and Mr. Desmond Hui, former students of the bone engineering lab in 2015, and continued by me under the supervision of Dr. Janet E. Henderson and Dr. Paul A. Martineau. Its aim is to review the body of knowledge related to mast cell functions beyond allergy and inflammation and to present our evolving understanding of their role in musculoskeletal tissue repair.

# THE GOOD, THE BAD AND THE UGLY: A REVIEW OF THE ROLE OF MAST CELLS IN BONE HEALING

## *Part I: Historic perspective, mast cell biology and the role of mast cells in wound healing*

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## 2.1 Abstract

Although mast cells have traditionally been linked to the development of allergy, asthma and immunity, there is a growing body of evidence showing that they also have a role in maintaining homeostasis during tissue repair. Mast cells are myeloid cells that reside in tissues in close proximity to the exterior of the body. They are characterized by a large number of granules that are replete with proteases, vasoactive compounds, growth factors and cytokines, and have the ability to synthetize several mediators *de novo* upon activation. Mast cells constitute a component of the innate immune system that is active during tissue repair. In the context of bone healing, mast cells have shown to be regulators of tissue mineralization and osteoclast activity, promotion of neo-angiogenesis, and modulation of the immune response. However, their specific role is obscured by some of their intrinsic properties, such as their plasticity that is strongly influenced by contextual cues in the surrounding tissues, and extrinsically by the use of different mouse models of mast cell deficiency or surgical techniques to assess healing. In the present work, we review the role of mast cells from their historical perspective, their biology and well-established role in wound healing in general, to their specific role in bone and musculoskeletal tissue healing and remodelling.

**Keywords:**

Mast cell, wound healing, fracture repair, tissue remodelling, osteoimmunology

## 2.2 Historic Perspective

The discovery of tissue mast cells (MC) in the late 1800s is attributed to physician scientist Paul Ehrlich who used the word "mastzellen" or well-fed to describe the large granular cells (Ehrlich, 1878). The metachromatic granules were visualized by histochemical staining with cationic dyes like toluidine blue, which is still used as a simple technique for accurate identification of mast cell granules in tissue sections and in isolated cells. Studies performed in parallel by other investigators were exploring the relationship between allergens and the intensity of a reaction in exposed tissue (Landsteiner, 1924).

### *Role in the immune system*

It was not until the 1950s that MC were identified as the major source of histamine in the body and that its release was responsible for allergic and anaphylactic responses in humans and animals (Riley & West, 1952). An enormous volume of work ensued using classic histological, biochemical and radiolabelling techniques to characterize MC as a repository for a wide variety of bioactive molecules including proteoglycans, proteases, cytokines and chemokines. Heparin glycosaminoglycans, which endow the granules with their metachromatic staining properties, were amongst the first to be identified (Jorpes, Odeblad, & Bostrom, 1953) along with trypsin (Glenner & Cohen, 1960) and chymotrypsin (Benditt & Arase, 1959) like enzymes that varied amongst species.

A landmark discovery in mast cell biology was made by Ishizaka and colleagues when they isolated a novel class of "reaginic" antibodies, designated IgE, as a minor but distinct component of Ig in serum harvested from humans suffering from ragweed pollen allergy (Ishizaka & Ishizaka,

1967). Classic genetic mutation and substitution analyses were used to identify the Fc region of the cognate receptor FcER1 as the binding site for IgE and to confirm that receptor aggregation triggered MC activation (Kulczycki, Isersky, & Metzger, 1974). IgE antibodies were later proposed to play a minor role in the protection of a host from parasitic disease (Mitchell, 1979) and to recognize an antigenic component in the cell wall of *S. Aureus* bacteria (Schopfer, Douglas, & Wilkinson, 1980).

### *Role in wound healing*

While the immunologists were working toward characterizing the role of MC in innate and acquired immunity, those working in other disciplines were exploring the relationship between MC and other cells involved in tissue homeostasis and repair. A positive role was attributed to MC in cutaneous wound repair when they were shown to discharge their granules during the inflammatory phase of repair in a rat model (Wichmann, 1955). Later it was shown that pre-treatment with compound 48/80 to promote mast degranulation resulted in a significant delay in skin wound healing, while chronic treatment with the MC products histamine and heparin expedited the repair process (Fenton & West, 1963).

### *Osteoimmunology*

A potential role in metabolic bone disease was suggested when experimentally induced changes in vitamin D metabolism increased the number of MC in bone marrow and along the endosteal surfaces of rat long bones *in vivo* (Urist & Mclean, 1957). Clinical evidence of heparin-induced osteoporosis in patients on long-term anti-coagulant therapy (Griffith, Nichols, Asher, & Flanagan, 1965), and osteosclerotic lesions in patients with systemic mastocytosis (Barer,

Peterson, Dahlin, Winkelmann, & Stewart, 1968), further supported the hypothesis that MC were implicated in the pathogenesis of osteopenic disorders (Frame & Nixon, 1968). These iconic studies were at the leading edge of the field now known as osteoimmunology, a term coined by immunologists Arron and Choi to initially describe T cell-mediated activation of osteoclasts (Arron & Choi, 2000). The field remains focused on RANKL- mediated bone destruction in osteoporosis, bone metastases and chronic inflammatory disorders like osteomyelitis, periodontitis and inflammatory arthritis. However, a growing body of evidence points to a critical relationship between the immune system, including MC, and the skeletal system in the context of bone homeostasis, remodelling and repair (Greene, Asadipooya, Corradi, & Akin, 2016; P. S. Schneider et al., 2018). Recent pre-clinical studies from our laboratory (Behrends et al., 2014, 2017; Ramirez-GarciaLuna et al., 2017; Ramirez-Garcia-Luna et al., 2019) clearly demonstrate a critical role for MC in bone healing that warrants further investigation of these cells as potential therapeutic targets in patients at risk of fracture mal-union. The remainder of this review will focus on an overview of MC biology relevant to skeletal tissues and the emerging role of MC in wound repair.

## 2.3 Mast Cell Biology

Prototypical MC are granular myeloid lineage cells that have been identified in all classes of vertebrates. In mammals, they express high affinity receptors for stem cell factor (SCF) and IgE and are stored in peripheral tissues in close proximity to mucosae, blood vessels and nerves. They exhibit distinctive characteristics that include proliferation in peripheral tissues, the capacity to re-granulate after de-granulation and cellular plasticity that results in phenotypic sub-populations within a single tissue. The increasing awareness of the complexity of mast cell biology has resulted in a paradigm shift in the role of MC in health and disease.

### *Migration and tissue heterogeneity*

Although it is generally agreed that MCs arise from committed myeloid progenitors in a series of steps that are tightly regulated at the transcriptional level, their precise origin in bone marrow is disputed to this day (Joakim S. Dahlin & Hallgren, 2015). Bi-potent mast cell/basophil precursors give rise to cells committed to the MC lineage known as MC progenitors (MCp). Unlike the majority of hematopoietic cells that circulate in their mature form MCp exit bone marrow and travel via the circulation to peripheral tissues like skin, gut, and lung that are in close contact with the external environment. MCp were originally quantified by limiting dilution assay and identified as cells capable of forming mature MC loaded with metachromatic staining granules (Takashi Sonoda, Ohno, & Kitamura, 1982). This laborious assay was later replaced by multi-channel fluorescence activated cells sorting (FACS) analysis to identify MCp. The authors found that a cell population in adult mouse bone marrow, characterized as Lin(-)c-Kit(+)Sca-1(-)-Ly6c(-)FcepsilonRIalpha(-)CD27(-)beta7(+)T1/ST2+, gives rise only to mast cells in culture and can reconstitute the mast cell compartment when transferred into mast cell-deficient mice. The experiments strongly suggested that these adult mast cell progenitors are derived directly from multipotential progenitors instead of common myeloid progenitors or granulocyte progenitors. (C.-C. Chen, Grimbaldeston, Tsai, Weissman, & Galli, 2005). The c-kit receptor for stem cell factor (CD117) is present at all stages of MC development, from pluripotent stem cell to fully differentiated MC residing in peripheral tissues. The interaction between kit ligand, also called SCF, released by fibroblasts and endothelial cells and its receptor CD117 is essential at all stages of the MC life cycle (Geissler, Ryan, & Housman, 1988). On the other hand, the FcεR1 for IgE is expressed at low levels in MCp and at higher levels in mature MC, identifying it as a useful maturation marker. In conjunction with a variety of integrin and cluster of differentiation (CD)

markers, CD117 and FcεR1 are used routinely to phenotype MCp grown in culture for molecular analyses and for adoptive transfer to MC deficient mice (Nakano et al., 1985).

The pattern of expression of cell surface receptors on MCp is proposed to change as they migrate across bone marrow sinusoids when they traverse the vascular endothelium in destination tissues and again when they reach their final destination where they proliferate and mature into MC (Collington, Williams, & Weller, 2011). Homing of MCp to destination tissues is mediated in large part by receptors for CC and CXC classes of chemokines like monocyte chemoattractant protein (MCP/CCL2) and stromal cell derived factor (SDF1/CXC12). MCp are also attracted to destination tissues by chemotactic agents like leukotriene (LT) B4, transforming growth factor (TGF)-β, and prostaglandin (PG) E2 (Gurish & Austen, 2012) and differentiate into mature granular MC under the influence of local factors (Kaieda et al., 2010). Homing of MCp was clearly demonstrated when bone marrow derived MC cultured *ex vivo* were transplanted into MC deficient W/Wv mice (Nakano et al., 1985). MC injected IP or IV into MC deficient mice migrate to tissues where they are commonly found in vivo, although the number and phenotype of adopted cells are seldom the same as native cells in congenic WT mice (Galli, Nakae, & Tsai, 2005).

Once they reach their destination and mature the phenotype and function of MC is dependent on factors present in the local microenvironment. Those found in connective tissues of skin, lung and gut are called connective tissue MC (CTMC) in rodents and $MC^{TC}$ in humans, the superscript referring to the protease content of tryptase and chymase in their granules (Irani, Bradford, Kepley, Schechter, & Schwartz, 1989; Kitamura, 1989). Those found in the tissue mucosa are called mucosal MC (MMC) in rodents and $MC^{T}$ in humans, as they contain only

tryptase. Targeted disruption of genes encoding proteins that regulate vesicular fusion and trafficking revealed additional heterogeneity in granule content. Bone marrow derived MC from vesicle associated membrane protein-8 (VAMP8) null mice revealed significant impairment in the release of β hexosaminidase but not histamine and tumor necrosis factor alpha (TNFα) (Puri & Roche, 2008). Confocal microscopic analysis of immunochemically stained cells confirmed the presence of distinct subsets of granules. It was later shown that VAMP8 is required for secondary granule-granule fusion events but not for primary membrane fusion events associated with degranulation (Behrendorff, Dolai, Hong, Gaisano, & Thorn, 2011).

### *Activation and mediator release*

Four different activation mechanisms have been identified in MC, which are associated with a wide variety of membrane-associated proteins implicated in granule trafficking and fusion events (Balseiro-Gomez, Flores, Acosta, Ramirez-Ponce, & Ales, 2016; Moon, Befus, & Kulka, 2014; Vukman, Försönits, Oszvald, Tóth, & Buzás, 2017). Intracellular trafficking and release of granules from MC are regulated at the level of calcium binding to ubiquitous proteins called synaptotagmins (Syt), which were discovered as calcium-dependent mediators of neuronal vesicular trafficking and exocytosis (Pang, Sun, Rizo, Maximov, & Südhof, 2006). This was effectively demonstrated in MC derived from $Syt2^{-/-}$ mice in which IgE-mediated degranulation was significantly reduced, but the release of cytokine and lipid mediators was the same as seen in WT cells (Melicoff et al., 2009).

The classic MC activation pathway leads to anaphylactic degranulation that occurs within seconds of IgE binding to FcεR1 (A. M. Dvorak et al., 1991). The Fc component of IgE antibody complexed with antigen binds to the extracellular domain of the alpha sub-unit of the tetrameric

FcεR1 on the cell surface, which triggers receptor aggregation (Siraganian, 2003). Aggregation initiates signal transduction by phosphorylation of tyrosine residues on the receptor beta and gamma sub-units, followed by a complex cascade of events that involves granule fusion and release, as well as processing and release of lipid mediators. In mice, IgE antibodies also bind to FcεR1 tetramers on MC and basophils independent of the presence of antigen. In humans, these stabilized receptors are also found on monocytes, macrophages, dendritic cells, platelets, eosinophils and Langerhans cells (Kraft & Kinet, 2007). Although IgE bound to FcεR1 on MC in the absence of antigen binding was originally thought to sensitize the cells to subsequent encounters with the same antigen, it was also shown to impact their survival. Treatment of primary bone marrow derived MC with a monoclonal antibody stimulated phosphorylation of signaling proteins downstream of FcεR1, promoted degranulation and arachidonic acid metabolism, induced cytokine expression and release and promoted survival of growth factor deprived cells (Kalesnikoff et al., 2001; J. Kitaura et al., 2003). These observations raised the possibility that the increased numbers of MC in people with allergies were due to the small increase in their serum IgE level.

A second pathway that involves intracellular vesicular transport and release of *de novo* synthesized cytokines like ILs and TNFα has been associated with the activation of toll-like receptors (TLRs). Unlike the MC restricted expression of FceRI, TLRs are widely expressed on immune cells including monocytes, macrophages and dendritic cells, as well as on fibroblasts and skeletal cells in connective tissue (Kawai & Akira, 2010). MC TLRs are located on the cell surface and on intracellular organelles like endosomes and lysosomes. They recognize primarily pathogen-associated molecular patterns (PAMPs) on bacteria and viruses but also bind to damage-associated

molecular patterns (DAMPS) on cellular and matrix components present in injured tissues (Sandig & Bulfone-Paus, 2012). Activation of TLRs on MC results in transcription, translation and secretion of cytokine, chemokine and phospholipid mediators. TLR4 was the first and remains the most extensively characterized of the ten TLR receptors identified in human and rodent MC that bind to a wide range of PAMPs including lipopolysaccharide (LPS), the major protein covering gram negative bacteria involved in septic shock (Poltorak et al., 1998). In chronic inflammatory conditions like osteoarthritis, TLR4 is implicated in sustaining the inflammatory milieu by binding to extracellular matrix degradation products like fibronectin, heparan sulfate, hyaluronic acid and tenascin (Midwood, Piccinini, & Sacre, 2009). Release of several ILs and TNFα 16 hours after LPS stimulation of MC derived from C57Bl6 mice was absent in cultures of cells derived from C57Bl6/ScNCr and C3H/HeJ mice which lack functional TLR4 receptors (Masuda, Yoshikai, Aiba, & Matsuguchi, 2002; McCurdy, Lin, & Marshall, 2001). Furthermore, activation of TLR2 and TLR4 on IgE primed MC is proposed to enhance IL-6, IL-13 and TNFα, but not PG production (Qiao, Andrade, Lisboa, Morgan, & Beaven, 2006).

The remaining two pathways are less well characterized and are reported to play roles in communication between MC and other cell types, rather than participate in classic immune responses (Vukman et al., 2017). The first involves piecemeal degranulation, which is associated with dysregulated MC activity and has been characterized solely on the basis of ultrastructural studies (Ann M. Dvorak, 2005). Prior to granule-granule fusion, small vesicles with sub-sets of mediators are proposed to progressively bud off of the main granule, migrate through the cell and fuse with the plasma membrane for release. Piecemeal degranulation is commonly seen in response to chronic inflammation, in the presence of infection and in autoimmune disease (Balseiro-Gomez

et al., 2016). The second pathway involves release of exosomes containing a variety of cell components including TGF-β (Hügle, Hogan, White, & Laar, 2011), TNF precursors and other vasoactive agents (Al-Nedawi Khalid, Szemraj Janusz, & Cierniewski Czeslaw S., 2005), in addition to RNA, via a proposed endocytosis-exocytosis pathway. Using time-lapse fluorescence microscopy in peritoneal mast cells it was shown that more than half of the labelled cell membrane was re-purposed for exocytosis within an hour of antigen-mediated degranulation (Balseiro-Gomez et al., 2016). This exosome pathway is proposed to account for the unique ability of MC to undergo repeated rounds of degranulation to sustain an allergic or inflammatory response.

### *Mediators and bioactivity*

Mature MC are characterized by their content of densely packed granules consisting of a serglycin core protein decorated with negatively charged chondroitin sulphate chains in MMC and heparin sulphate chains in CTMC (Kitamura, 1989). Ionic bonds loosely anchor the positively charged mediators of MC bioactivity to the negatively charged proteoglycan core, which as mentioned also forms a strong ionic bond with positively charged dyes like toluidine blue. The presence of these electrostatic complexes in the granules is supported by biochemical studies showing co-release of core protein with proteases and histamine from activated MC. Although heparin sulphate shares the same basic structure as the ubiquitous heparan sulphate, its sulphation pattern is restricted to MC. Targeted disruption of the gene responsible for heparin sulphation in mice revealed its critical role in the storage of histamine and mast cell specific proteases (Humphries et al., 1999).

Histamine and proteases are major constituents of MC granules. Targeted disruption of the gene encoding histidine decarboxylase in mice resulted in near complete absence of tissue histamine, a significant reduction in the number of MC and impaired storage of mediators in the remaining cells (Ohtsu et al., 2001). Subsequent studies revealed impaired IL-3 dependent differentiation of MC from bone marrow harvested from the same mice (Wiener et al., 2002). The biological activity of histamine is mediated via G-protein coupled receptors designated H1 to H4 that have been identified on target cells in most organ systems, notably integumentary, respiratory, digestive, nervous, cardiovascular and immune systems. The widespread tissue distribution of histamine receptors is reflected in symptoms like hives, skin rash, anaphylaxis, diarrhea and hypotension experienced by patients with mast cell activation disorders (MCAD) (Frieri, Patel, & Celestin, 2013). Systemic mastocytosis is a type of MCAD in which 90% of patients carry an activating mutation in c-kit. This results in unrestricted proliferation of MCp and accumulation of mature MC in bone marrow, skin, lymph nodes, gastrointestinal tract, liver and spleen (Valent, 2013). Related disorders in which normal numbers of MC are hyper-responsive to local stimuli include those associated with chronic inflammatory and autoimmune diseases like systemic sclerosis (Akin & Valent, 2014). A step-wise treatment approach to MCAD is initiated with H1 receptor antagonists or dual action agents like ketotifen, which blocks the H1 receptor while inhibiting MC de-granulation (Nurmatov, Rhatigan, Simons, & Sheikh, 2015).

MC also synthesize and store 16 different neutral proteases including tryptase (e.g. mMCP6), chymase (e.g. mMCP4) and carboxypeptidase A3 (Cpa3), which are considered to be mast cell specific (Wernersson & Pejler, 2014). They are stored in granules along with non-specific lysosomal enzymes required for pro-protein processing, and β hexosaminidase, which metabolizes

amino sugars and is used clinically as a marker for degranulation. Other proteases found in MC granules include metalloproteases MMP9 and ADAMTS5, which participate in collagen and proteoglycan degradation respectively.

In addition to histamine and proteases, MC store numerous cytokines and growth factors in their granules that impact inflammatory and angiogenic activity. Mature cells harvested from the peritoneum, as well as those derived from bone marrow in culture, exhibit a biphasic cytokine response (Wernersson & Pejler, 2014). The multi-functional inflammatory cytokine TNF-α was the first to be identified as having constitutive and inducible expression pathways in MC. IgE mediated activation of FcER1 resulted in the immediate release of TNF-α contained in granules as well as increased expression of TNF-α mRNA for several hours (Gordon & Galli, 1990). Other factors expressed constitutively and stored in granules for immediate IgE mediated release include vascular endothelial growth factor (VEGF) (Grützkau et al., 1998), basic fibroblast growth factor (bFGF) associated with heparin sulfate proteoglycan (Reed, Albino, & McNutt, 1995), interleukin 6 (IL-6) in the cytoplasm (Gagari, Tsai, Lantz, Fox, & Galli, 1997) and transforming growth factor beta (TGF-β) (Kendall, Li, Galli, & Gordon, 1997). Activated MC also release small heparin binding proteins called chemotactic cytokines, or chemokines, most of which have alternative descriptive names. For example, chemotactic cytokine ligand 2 (CCL2) is also known as monocyte chemoattractant protein 1 (MCP-1), monocyte chemotactic and activating factor (MCAF) and tumor-derived chemotactic factor (TDCF) (Deshmane, Kremlev, Amini, & Sawaya, 2009). Another example is CXCL8, also known as neutrophil chemotactic factor (NCF), neutrophil activating protein (NAP-1) and interleukin-8 (IL-8) (Turner, Nedjai, Hurst, & Pennington, 2014).

These small proteins attract other immune cells to sites of inflammation, for example in post-traumatic joint injury (Hildebrand, Zhang, Befus, Salo, & Hart, 2014).

Lipid mediators like PGD2 are released from MC in a biphasic manner to contribute to both the immediate-type hypersensitivity response and to a delayed response that requires gene transcription and occur hours after FcεR1 activation (Boyce, 2007). The immediate response follows rapid processing of membrane phospholipids by constitutive phospholipase A2 (PLA2) to generate arachidonic acid, which serves as substrate for the indomethacin-sensitive prostaglandin endoperoxide synthase type 1 isozyme (PGHS1) (Murakami, Matsumoto, Austen, & Arm, 1994; Nakatani, Murakami, Kudo, & Inoue, 1994). A second pathway involves the production of a heparin-sensitive PLA2 and *de novo* synthesis of the dexamethasone-sensitive PGHS2 to process arachidonic acid into PGD2 (Bingham et al., 1996). An elevation in the local heparin concentration following degranulation might, therefore, initiate signaling through the alternative inducible pathway.

## 2.4 Mast cells and wound healing

Wound healing involves a tightly regulated succession of overlapping phases. Hemostasis typically lasts minutes to hours, a limited period of inflammation followed by cellular proliferation to reconstitute the tissue and up to two years of remodelling to achieve the original structure. Each phase involves interactions between a wide variety of cells and components of the extracellular matrix (ECM) (Beldon, 2010). Only 0.3% of more than 80,000 publications since 1996 implicate MC in skin, muscle, bone, nerve or blood vessel repair. Much of this literature fails to discriminate

between sterile incisional wounds like those sustained during surgery and excisional wounds like penetrating traumatic injuries sustained in accidents in which microbes gain access to sub-cutaneous tissue. Furthermore, much of the literature fails to discriminate between regeneration, which involves the replacement of functional tissue, and the non-specific process of repair that can result in fibrous tissue formation or scarring in soft tissues.

Dysregulation of the orderly wound healing progression such as persistent inflammation, infection, impaired re-vascularization or excessive re-modelling leads to failed healing and development of chronic wounds like ulcers (Harper et al., 2014). In healthy young adults, tissue regeneration usually occurs in small and sterile wounds, whereas fibrous repair and scarring are more likely to occur in the case of large wounds and in older individuals. Fibrous encapsulation can also occur around non-degradable implants with smooth surfaces like micro-sensors, or orthopaedic hardware used to stabilize fractures. With these caveats in mind, we present a paradigm for wound healing that incorporates MC and their potential interactions with recognized effectors like platelets, neutrophils, macrophages and fibroblasts. For comprehensive reviews of the cells other than MC involved in wound repair the reader is referred elsewhere (B. Hinz, 2016; Italiani & Boraschi, 2014; Julier, Park, Briquez, & Martino, 2017; Reinke & Sorg, 2012; Semple, Italiano, & Freedman, 2011; Wang et al., 2018). For a recent review on the role of MC in skin wound healing, the reader is referred to the work by Komi et al. (Komi, Khomtchouk, & Santa Maria, 2019).

### *Hemostasis and thrombosis*

The process of wound healing is initiated when microorganisms and debris are flushed from damaged tissue by the efflux of blood and lymph from ruptured vessels, and platelets adhere to exposed collagen and ECM proteins at the site of injury. Adherent platelets undergo a rapid change from smooth to spiny morphology, which promotes aggregation and formation of a platelet plug, while the potent vasoconstrictor thromboxane A2 (TXA2) is processed from arachidonic acid at the cell membrane. MC are located near blood and lymphatic vessels where they are well positioned to regulate fluid flow and vascular permeability during hemostasis (Kunder, John, & Abraham, 2011). Alterations in the mechanical forces at a site of injury cause resident MCs to de-granulate and release their cargo, including histamine, tryptase and serotonin that increase vascular permeability. Together with platelet derived serotonin this facilitates entry of plasma-borne macromolecules like thrombin and fibrinogen involved in the coagulation cascade. Activated platelets release their cargo of stored molecules including fibrinogen, coagulation factors and calcium required for blood clot formation. Platelet derived ADP, cytokines and chemokines attract circulating platelets, neutrophils and monocytes whose passage into the site of injury is facilitated by the temporary increase in vascular permeability. Thrombin mediates the conversion of fibrinogen to fibrin, which then forms a mesh that traps activated platelets, red and white blood cells in a hemostatic plug to form a provisional platform for repair. Heparin and tryptase released from MC granules serve an important role in limiting platelet aggregation and thrombus formation by degrading fibrinogen and inactivating thrombin (Prieto-García, Castells, Hansbro, & Stevens, 2014).

### *Acute inflammatory response*

Once haemostasis is achieved a self-limiting inflammatory phase commences during which professional phagocytes like neutrophils and monocytes migrate into the wound bed where they work in partnership to remove pathogens, debris and dead cells (de Oliveira, Rosowski, & Huttenlocher, 2016). Large numbers of mature neutrophils are released from the bone marrow into the circulation on a daily basis where they constitute the largest population of white blood cells (Summers et al., 2010). When vascular permeability is increased during hemostasis their unique multi-lobular nucleus allows them to rapidly extravasate, followed shortly thereafter by circulating bone marrow derived monocytes. Neutrophils and monocytes then migrate through the tissue to join resident MC and platelets engaged in hemostasis and undergo further differentiation into pro-inflammatory N1 neutrophils (Selders, Fetz, Radic, & Bowlin, 2017) and M1 macrophages (Italiani & Boraschi, 2014), respectively. N1 neutrophils, M1 macrophages and to some extent MC, share a common mechanism of activation that triggers phagocytosis and release of antimicrobial peptides stored in their granules. Pattern recognition receptors (PRR) like TLR4 recognize DAMPs generated by cellular debris like nucleic acids and damaged matrix, as well as PAMPs like bacterial coat lipopolysaccharide (LPS) (X. Tang & Zhu, 2012). Engagement of PRRs triggers phagocytosis of pathogens and, in the case of macrophages, dead or dying cells at the wound site (Das et al., 2015). The phagosome membrane fuses with intracellular granules to release antimicrobial peptides like defensins into the phagosome where they permeabilize the outer membrane of target pathogens (Ganz, 2003).

In the early phase of wound healing, there is a progressive reduction in oxygen tension, due in part to disruption of the vascular feed and in part to the increased metabolic activity of

phagocytes (Hong et al., 2014). The hypoxic conditions, or oxidative stress, are proposed to result in increased production of reactive oxygen species (ROS), which serve a primary role in destroying pathogens phagocytosed by cells like N1 neutrophils and M1 macrophages (Babior, Kipnes, & Curnutte, 1973; Slauch, 2011). IgE/antigen-mediated activation of mouse bone marrow derived MC and circulating human MC have also been shown to increase their intracellular ROS and to promote degranulation (Swindle, Metcalfe, & Coleman, 2004). Adherent platelets at a site of tissue injury were also reported to increase ROS production, which was proposed to regulate the rate and extent of aggregation through autocrine mechanisms (Jang et al., 2013). Large quantities of anti-microbial ROS are generated via the assembly of the membrane-bound NADPH oxidase complex and transport of oxygen from the extracellular environment, accounting in part for the rapid drop in oxygen tension during acute inflammation. ROS also serve in signal transduction by promoting stabilization of hypoxia-inducible factor-1 (HIF-1) protein (Kumar & Choi, 2015). Under normal oxygen tension, the alpha sub-unit of the HIF-1 transcription complex undergoes constant ubiquitin-mediated degradation whereas the protein is stabilised under hypoxic conditions (L. E. Huang, Gu, Schau, & Bunn, 1998). HIF-1, in turn, promotes expression of genes involved in angiogenesis (VEGF), matrix metabolism (MMPs), glucose metabolism (GLUTs) and vascular tone (NOS) to restore the *status quo* (Holden & Nair, 2019).

Numerous growth factors and cytokines important for wound healing are amongst the biomolecules released by activated platelets (Golebiewska & Poole, 2015; Semple et al., 2011). In addition to their role in the recruitment of smooth muscle and endothelial progenitor cells for vessel repair, platelet derived chemokines facilitate the influx of neutrophils and monocytes to a site of injury. There is also evidence that platelet derived factors regulate the balance between pro- and

anti-apoptotic activities of cells in healing tissue, although the mechanisms remain poorly defined (Gawaz & Vogel, 2013). The presence of prototypical growth factors like PDGF, TGF beta, VEGF, EGF and IGH in platelet granules prompted interest in the use of platelet rich plasma (PRP) to expedite tissue repair in clinical practice (Martinez-Zapata et al., 2016; Roffi, Di Matteo, Krishnakumar, Kon, & Filardo, 2017). However, despite the accumulated biological data few clinical studies have demonstrated a significant benefit to the use of PRP to promote healing of musculoskeletal injuries.

Neutrophils receive survival and activation signals from mesenchymal and endothelial cells while producing CXC-based chemokines for self-preservation and CC-based chemokines to promote monocyte to M1 macrophage differentiation (Selders et al., 2017; Tecchio, Micheletti, & Cassatella, 2014). The chemokine CCL2/MCP-1 is primarily responsible for monocyte recruitment while CXL8/IL-8 and TNF alpha attract other neutrophils. Macrophage derived TNF-α is also proposed to promote the production of pro-inflammatory mediators like IL-1, ROS and the eicosanoid PGE2 in an autocrine/paracrine manner (Parameswaran & Patial, 2010). PGE2 is a smooth muscle relaxant that counteracts the TXA4 mediated vasoconstriction that occurred during hemostasis. It also plays a central role in the recruitment and activity of neutrophils, macrophages and MC in the context of immune disorders, rather than physiological wound healing (Kalinski, 2012). Neutrophil proteases modify the release and activity of these chemokines and regulate cell migration by cleavage of adhesion molecules (Pham, 2006). They also activate other proteases like the collagenase MMP-9 by releasing it from its endogenous tissue inhibitor, allowing for MMP-9 mediated release of stored VEGF from the extracellular matrix (Ardi, Kupriyanova, Deryugina, & Quigley, 2007). MC proteases serve similar functions in activating MMPs, as well as assisting in

matrix remodelling by degrading type III and type VI collagens in granulation tissue (Oskeritzian, 2012; Xue & Jackson, 2015).

### *Resolution of inflammation*

Given the potential toxicity of the inflammatory cytokines, proteases and ROS generated during acute inflammation to adjacent healthy tissue active resolution of inflammation likely represents the most critical phase of wound healing. As oxygen tension in the wound bed decreases pro-inflammatory NI neutrophils and MI macrophages undergo polarization to anti-inflammatory N2 and M2 phenotypes required for resolution of inflammation (Mantovani, Biswas, Galdiero, Sica, & Locati, 2013). Activated neutrophils undergo a form of programmed cell death called phagocytosis-induced cell death (PICD) (Kobayashi et al., 2003; McCracken & Allen, 2014). PICD involves alterations in gene expression that culminate in downregulation of the pro-inflammatory phenotype and induction of apoptosis. Activated neutrophils and MC also undergo a form of programmed cell death that differs from conventional apoptotic and PICD pathways, which involves the formation of extracellular traps (ET) for concentrated antimicrobial activity (Brinkmann et al., 2004; Köckritz-Blickwede et al., 2008). The process involves the production of reactive oxygen species (ROS), the intracellular release of enzymes from granules and the dissolution of the nuclear membranes. The nuclear and cytoplasmic contents then mix and the cell membrane ruptures to extrude the intracellular contents in the form of a DNA web decorated with antimicrobial proteins that trap microorganisms in the vicinity. NETs are eventually removed by DNase1 degradation and the debris removed by phagocytes during inflammation resolution. In areas of high neutrophil density, for example in the inflammatory phase of wound healing, NETs

have been shown to degrade cytokines and chemokines, thus limiting further recruitment and activity of inflammatory cells (Schauer et al., 2014).

The changes in shape and composition of the cell membrane identify dying neutrophils as targets for phagocytosis by M1 macrophages, which also limits inflammation by preventing neutrophil membrane rupture and release of their lethal contents. This mechanism of clearance is proposed by some to have the added advantage of promoting the transition of M1 macrophages to an anti-inflammatory M2 phenotype within the wound (Bratton & Henson, 2011). Although the contributions of catabolic M1 macrophages to wound healing through phagocytosis and production of pro-inflammatory cytokines is well established, their transition to an anabolic M2 phenotype during the resolution of inflammation remains less well defined. Accumulated data using isolated cells suggests the existence of four distinct sub-sets of M2 macrophages, only one of which is proposed to derive from M1 macrophages (Ferrante & Leibovich, 2012). There is however general agreement that the complement of mediators expressed by macrophages pre and post ingestion of dead and dying neutrophils differ significantly (Ortega-Gómez, Perretti, & Soehnlein, 2013). Generally speaking, it is believed that macrophage polarization from catabolic to anabolic phenotype is accompanied by a switch in production of TNF and iNOS to increased production of VEGF, TGF beta and the anti-inflammatory cytokine IL-10 (Galli, Borregaard, & Wynn, 2011).

### *Proliferation and remodelling*

In contrast to the conserved mechanisms involved in hemostasis and inflammation following tissue injury, repair and remodelling are characterised by tissue specific mechanisms that restore native form and function. The proliferative phase of wound healing is marked by a

steady increase in oxygen tension leading to robust angiogenesis and delivery of oxygen, nutrients and precursor cells destined to build a new tissue matrix. The growth factors, cytokines and ROS released by immune and other cells at the site of injury stimulate quiescent tissue fibroblasts and trigger their differentiation into contractile myofibroblasts that deposit ECM and promote angiogenesis (Wynn & Ramalingam, 2012). The resulting hyper-vascular collagen matrix called granulation tissue is common to all tissues undergoing repair. Granulation tissue forms a platform for subsequent tissue-specific phases of repair and remodelling, which can last for months and even years depending on the extent of the injury, tissue structure and composition (Harper et al., 2014).

The iconic cytokine TGF beta is recognized as the predominant factor involved in fibroblast proliferation and trans-differentiation to myofibroblasts during wound healing (Carthy, 2018). Numerous studies using pharmacologic and genetic manipulation of TGF beta availability and receptor activity are beginning to unravel the multi-faceted role of this cytokine in wound healing. It is stored in a latent form until dissociated from its binding proteins to activate heterdimeric receptors and activate signalling pathways that stimulate transcription of ECM proteins like collagen, glycosaminoglycans and proteoglycans (Finnson, McLean, Di Guglielmo, & Philip, 2013). Importantly the cytokine promotes the incorporation of alpha SMA into stress fibers and modification of focal adhesions that significantly enhance myofibroblast contractile function (Boris Hinz & Gabbiani, 2003). Myofibroblasts are also the major source of fibrillar collagens required for matrix remodelling and, in the case of soft tissues, scar formation (Xue & Jackson, 2015).

The cytokine IL-10 is highly expressed in fetal skin wounds, which are characterised by a shortened inflammatory response and the absence of scar tissue, suggesting it may have a dual mechanism of action in inflammatory and reparative phases of wound healing (King, Balaji, Keswani, & Crombleholme, 2014). Cells of the monocyte lineage are a major source of the anti-inflammatory cytokine IL-10, which serves to limit recruitment of neutrophils and macrophages to the site of healing by supressing production of pro-inflammatory mediators like MCP-1 and TNF alpha. Early pre-clinical studies supported this hypothesis by showing biphasic peaks in the expression of IL-10 RNA and protein as early as 3 hours, and again at 3 days post injury in mouse cutaneous wounds (Sato, Ohshima, & Kondo, 1999). Somewhat contradictory data has accumulated from rodent studies of mice lacking IL-10. In one study the absence of IL-10 led to enhanced contraction and skin wound healing accompanied by increased macrophage infiltration, myofibroblast differentiation and deposition of densely packed collagen (Eming et al., 2007). In another, the combined absence of IL-10 and IL-4 delayed skin wound healing and administration of recombinant IL-10 to rat skin wounds reduced inflammation and enhanced healing (Kieran et al., 2013). IL-10 is also expressed by MC in the wound healing micro-environment and proposed to contribute to delayed skin wound healing in a mouse model of lymphatic dysfunction in which MC numbers were increased and macrophage numbers decreased (Kimura, Sugaya, Blauvelt, Okochi, & Sato, 2013). Knowing that IL-10 is a pleiotropic cytokine expressed, perhaps at different times, by at least two of the many cells involved in wound healing indicate the use of conditional cell-specific knockout strategies will be required for a more mechanistic approach, as recently reported for mucosal repair (Quiros et al., 2017).

## 2.5 Conclusion

Mast cells may constitute late-comer cells to the wound tissue, but they participate throughout the phases of healing by secreting vasoactive substances, blood clotting factors, matrix remodelling proteases, growth factors, and pro-angiogenic molecules. These cells are equipped with a wide array of pre-formed compounds that can be immediately released to the surrounding tissues, as well as with the capacity to synthetize *de novo* compounds in response to paracrine signaling. In turn, their activity also influences the behaviour and recruitment of other cell types. Not surprisingly, it has been hypothesized that mast cells may play a role in wound healing in other types of tissue, especially in those where they are not normally found. For example, several reports have demonstrated that they help maintain tissue homeostasis after myocardial infarction (Cimini et al., 2007; Kwon et al., 2011; Shao et al., 2015) and may play a detrimental role in liver fibrosis (Bradding & Pejler, 2018) and implant rejection (L. Tang, Jennings, & Eaton, 1998).

In the context of bone healing, work from Domi Behrends, a previous student in the Bone Engineering Labs of the MUHC under the supervision of Dr. Janet E. Henderson and Dr. Paul A. Martineau, suggested that mast cells were also involved in bone repair (Behrends et al., 2014). However, the mast cell deficient mouse model that was used in that study, $Kit^{W\text{-}sh}$, exhibits defects in other cell types, including in the hematopoietic system and in osteoclasts. For this reason, no definitive conclusions were drawn. The next chapters in this thesis will present our research using the $Cpa3^{Cre/+}$ mouse strain, a c-Kit independent mast cell deficient mouse model. Our aim was to confirm the role of mast cells in bone healing, to determine the origin of the cells involved in the healing process, and to determine how can they be manipulated to enhance bone repair.

# III. DEFECTIVE BONE REPAIR IN MAST CELL-DEFICIENT *Cpa3*$^{Cre/+}$ MICE

The research presented in this section was published as *Ramirez-GarciaLuna JL, Chan D, Samberg R, Abou-Rjeili M, Wong TH, Li A, et al. Defective bone repair in mast cell-deficient Cpa3Cre/+ mice. PLoS ONE. 2017;12(3):e0174396*. This manuscript fulfills our two initial research objectives, quantify bone repair at timed intervals in *Cpa3*$^{Cre/+}$ and *WT* mice with surgically induced defects that model human femoral fractures, and identify markers of inflammatory and vascular cell function to investigate the temporal and functional role of mast cells in healing bone defects of *Cpa3*$^{Cre/+}$ and *WT* mice. The use of a kit-independent mast cell deficient model was needed to confirm the role of mast cells in bone healing without any potential confounding factors due to the mutation in the kit alleles. The *Cpa3*$^{Cre/+}$ mouse strain shows complete mast cell deficiency due to the genotoxic effects of the *Cre* recombinase that induce cellular death of mast cell precursors. Aside from a mild decrease in the number of circulating basophils, no other cellular or immune defect has been identified in this strain.

Our main findings are that in the absence of mast cells, dysregulation of osteoclast and vascular cell activity, lower number of macrophages in the repair tissue, and impaired mineralization of the osteoid that lead to defective bone healing can be observed. Thus, we confirm a positive role of mast cells in bone healing and propose a functional axis between macrophage – osteoclast – mast cell activity.

# DEFECTIVE BONE REPAIR IN MAST CELL-DEFICIENT Cpa3Cre/+ MICE

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**Conflict of interest**

The authors declare no conflict of interest.

**Ethical review committee statement**

All animal procedures were performed in strict accordance with a protocol approved by the McGill Facility Animal Care Committee, in keeping with the guidelines of the Canada Council on Animal Care.

## 3.1 Abstract

In the adult skeleton, cells of the immune system interact with those of the skeleton during all phases of bone repair to influence the outcome. Mast cells are immune cells best known for their pathologic role in allergy and may be involved in chronic inflammatory and fibrotic disorders. Potential roles for mast cells in tissue homeostasis, vascularization and repair remain enigmatic. Previous studies in combined mast cell- and Kit-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice ($Kit^{W\text{-}sh}$) implicated mast cells in bone repair but $Kit^{W\text{-}sh}$ mice suffer from additional Kit-dependent hematopoietic and non- hematopoietic deficiencies that could have confounded the outcome. The goal of the current study was to compare bone repair in normal wild type (*WT*) and $Cpa3^{Cre/+}$ mice, which lack mast cells in the absence of any other hematopoietic or non- hematopoietic deficiencies. Repair of a femoral window defect was characterized using micro CT imaging and histological analyses from the early inflammatory phase, through soft and hard callus formation, and finally the remodeling phase. The data indicate 1) mast cells appear in healing bone of *WT* mice but not $Cpa3^{Cre/+}$ mice, beginning 14 days after surgery; 2) re-vascularization of repair tissue and deposition of mineralized bone was delayed and dis-organised in $Cpa3^{Cre/+}$ mice compared with *WT* mice; 3) the defects in $Cpa3^{Cre/+}$ mice were associated with little change in anabolic activity and biphasic alterations in osteoclast and macrophage activity. The outcome at 56 days postoperative was a complete bridging of the defect in most *WT* mice and fibrous mal-union in most $Cpa3^{Cre/+}$ mice. The results indicate that mast cells promote bone healing, possibly by recruiting vascular endothelial cells during the inflammatory phase and coordinating anabolic and catabolic activity during tissue remodeling. Taken together the data indicate that mast cells have a positive impact on bone repair.

**Key Words:** Femoral window defect; fibrous mal-union; periosteal reaction; revascularization.

## 3.2 Introduction

It has been proposed that the discreet phases of bone repair in response to injury recapitulate those during development that gives rise to the adult skeleton (Vortkamp et al., 1998). It was recognized decades ago that cells of the immune system interact with those of the skeletal system during development and in the adult bone healing micro-environment. The term "osteoimmunology" was coined to define these complex interactions between lymphocytes, macrophages, mast cells, osteoclasts, osteoblasts and others (Lorenzo, Choi, Horowitz, Takayanagi, & Schett, 2016). In the long bones of the adult skeleton, the bone healing cascade is initiated with a blood clot and an inflammatory response during which cells migrate to the site of injury (T. A. Einhorn, 1999). The hematoma is replaced by granulation tissue to form a soft callus, metalloproteases cleave collagen and stored growth factors and cytokines are released. Angiogenic factors attract vascular endothelial cells, which form vessels throughout the repair tissue. Bone anabolic agents such as Wnt ligands, parathyroid hormone (PTH) and related protein (PTHrP) and bone morphogenetic proteins (BMPs) induce differentiation of mesenchymal stromal cells (MSC) into osteoblasts. Woven bone is deposited by these cells in and around the soft callus to form a hard callus, which is then remodeled by osteoclasts delivered through the new vessels.

Mast cells belong to the hematopoietic system and mast cell committed progenitors have been identified in fetal and adult mouse blood (J. S. Dahlin, Heyman, & Hallgren, 2013; H. R. Rodewald, Dessing, Dvorak, & Galli, 1996). They migrate to peripheral tissues such as lung, skin and intestine where mature mast cells are stored over the long term (Joakim S. Dahlin &

Hallgren, 2015). Mast cells are best known for their pathologic role in allergic diseases. Less well established are their suggested physiologic roles in tissue homeostasis and repair that include neo-vascularization (Blair et al., 1997). Mast cells contain the proteases tryptase and chymase along with a variety of cytokines and chemokines that contribute to allergic inflammation, but some of which may also act as mediators of tissue repair. These include TNFα, prostaglandin (PG) D2, leukotriene (LT) C4, monocyte chemoattractant protein (MCP), macrophage inflammatory protein (MIP) and a plethora of interleukins (ILs). The functional heterogeneity of mast cells is proposed to arise from differential activation of the FcεR1 by IgE or activation of a wide range of pattern recognition toll like receptors (TLR). Generally speaking, FcεR1 activation is proposed to trigger the release of proteases and cytokines stored in granules, whereas TLR activation results in de novo synthesis and release of a different sub-set of bioactive mediators (Moon et al., 2014).

An early study of fracture repair in young rats revealed mast cells adjacent to blood vessels in the soft callus at two weeks of healing and distributed throughout the hard callus near osteoclasts at six weeks (Banovac, Renfree, Makowski, Latta, & Altman, 1995), suggesting these cells played a role in the healing process. A study conducted under steady state on mast cell-deficient mice carrying a mutation in the receptor for stem cell factor Kit ($Kit^{W/Wv}$) revealed alterations in femoral bone mass and geometry leading to decreased mechanical strength (Cindik et al., 2000). However, the $Kit^{W/Wv}$ mice also exhibited anemia, neutropenia and other defects (H.-R. Rodewald & Feyerabend, 2012) that could have impacted the outcome. Our recent work on another strain of Kit mutant mice, $Kit^{W\text{-}sh}$, revealed that bone regeneration in a femoral defect was defective in these mice (Behrends 2014), raising the intriguing possibility that mast cells are

involved in bone repair. *Kit*$^{W\text{-}sh}$ mice were originally suggested to be mast cell deficient in the absence of other major hematopoietic cell deficiencies (Grimbaldeston et al., 2005). However, subsequent work showed that *Kit*$^{W\text{-}sh}$ mice suffer from a multitude of phenotypes beyond the mast cell deficiency (Feyerabend, Gutierrez, & Rodewald, 2016; Michel et al., 2013; Nigrovic et al., 2008).

Mouse mutants specifically lacking individual immune cell lineages are powerful resources to investigate the osteoimmunology of bone healing. Until now, mast cell deficient mice wildtype for Kit have not been used to this end. The goal of the current study was to characterize the impact of mast cell deficiency on bone repair using *Cpa3*$^{Cre/+}$ mice. Introduction of Cre recombinase into the gene encoding carboxypeptidase A3 (Cpa3), which is expressed at very high levels only in the mast cell lineage, results in the complete absence of mast cells in connective and mucosal tissues by genotoxicity (Feyerabend et al., 2011)[1]. This model of mast cell deficiency has been used by many investigators to probe the roles of mast cells under physiological and pathological conditions. Of note, in almost all instances, the suggested roles of mast cells could not be reproduced, thus contesting previous work in Kit mutant mice (Feyerabend et al., 2016; H.-R. Rodewald & Feyerabend, 2012). Because *Cpa3*$^{Cre/+}$ mice have no defects in the immune system other than the complete absence of mast cells and a partial reduction in basophils, we consider these mice an appropriate model to investigate the potential contribution of mast cells to bone repair in the absence of confounding factors arising from alteration in other cell lineages.

[1] For a full description of the Cre recombinase genotoxic effect, the reader is directed to Chapter VII Conclusion: the role of mast cells in bone healing.

## 3.3 Materials and Methods

### *Mouse model of mast cell deficiency*

Animal procedures were conducted in accordance with a protocol approved by the Facility Animal Care Committee of McGill University (AUP-7016), in keeping with the guidelines of the Canada Council on Animal Care. Animal surgery and post mortem analyses were performed essentially as described previously (Behrends et al., 2014; Gao et al., 2013; Henderson, Gao, & Harvey, 2011). Founder mice heterozygous for insertion of *Cre* recombinase in the gene encoding mouse mast cell Cpa3 (*Cpa3*$^{Cre/+}$ mice on the *C57BL/6* background) were obtained from the German Cancer Research Center, DKFZ, Heidelberg, Germany. A colony was established by mating with WT *C57BL/6* mice (Charles River Laboratories, Senneville, Qc H9X 3R3, Canada) and the offspring genotyped as described (Feyerabend et al., 2011) using PCR of DNA isolated from ear punch biopsies. *Cpa3*$^{Cre/+}$ mast cell-deficient male and female offspring were separated, and 3-4 mice/cage maintained with free access to food and water from 4.5 to 8 months prior to surgical intervention.

### *Surgical model*

Adult male and female mice were used for all experiments. Bilateral 1 mm x 2 mm defects were generated on the anterolateral aspect of the femora using the third trochanter as an anatomical landmark. Mice were anesthetized with isoflurane before shaving both hind limbs, disinfecting the skin with 70% ethanol and exposing the anterolateral aspect of the femora through a 3-mm skin incision extending from the third trochanter down the diaphysis. A 1 mm x 1 mm x 2 mm rectangular cortical window defect was generated with a 1 mm burr on Stryker

drill (50,000 RPM, Hamilton, ON, Canada). After gentle irrigation to remove bone shards the muscle and skin layers were re-apposed and sutured with PDS-II 4-0 thread. For pain control, an IP injection of 10 mg/kg carprofen with 0.1 mg/kg buprenorphine in 0.5 mL of sterile saline was administered immediately after wound closure, and 5 mg/kg carprofen injected for 3 days post-operative. Cohorts of mice were euthanized by $CO_2$ asphyxiation under anesthesia from 5-56 days post-operative. Femora were carefully dissected free of soft tissue before fixing for 24 hours in 4% paraformaldehyde. The bones were then rinsed x3 with sterile PBS and stored at 4° C until micro computed tomographic (micro CT) imaging.

### *Micro CT analysis*

Scans were performed on a Skyscan 1172 instrument (Bruker, Kontich, Belgium) with a 0.5 mm aluminium filter at a voltage of 50kV, a current of 200µA and a resolution of 5 µm/pixel. 2D images were reconstructed into 3D models using NRecon software v.1.6.10.4 (Bruker) and loaded into CTAn software v.1.16.4.1 (Bruker) for analysis. Quantitative data was recorded for bone and vessel regeneration in rectangular regions of interest (ROI) in the Cortex opposite the defect, measuring 1.5 mm long, 0.9 mm wide and 0.6 mm in depth and in the Defect/Medulla measuring 1.5 mm long, 1.0 mm wide and 1.3 mm in depth. Quantitative data for mineralized tissue includes bone volume/tissue volume (BV/TV %), trabecular thickness (Tb.Th. mm $x10^{-3}$), trabecular separation (Tb.Sp. mm $x10^{-3}$), open porosity (%) and open pore volume (Po.V Tot $mm^3$). Quantitative data for vascular channels (Bruker micro CT academy 2016 v5.3) includes vascular channel volume/tissue volume (ChV/TV %), vascular channel number (Ch.N), vascular channel thickness (Ch.Th mm $x10^{-3}$), and vascular channel connectivity density (Ch.Conn.Dn.).

### *Histological analysis*

Bones were either left un-decalcified and embedded in poly-methyl methacrylate (PMMA) plastic or decalcified and embedded in paraffin using established methodology. Skin and bone tissues were stained for mast cells using acidified toluidine blue (aTB) and anti-tryptase antibody (Abcam, Cambridge MA, USA ab 151757 1:300). CD34 (Abcam ab23830 1:300) immunohistochemistry was used to identify vascular endothelial cells in bone and soft tissue in regenerating bone. Sections of PMMA embedded bones were stained with von Kossa and counterstained with toluidine blue (VK/TB) to distinguish mineralized from soft tissue. VK/TB stained sections were compared with 2D micro CT images from the same region, or with sections of decalcified, paraffin embedded bone stained to identify alkaline phosphatase (ALP) activity in osteogenic cells. Tartrate resistant acid phosphatase (TRAP) histochemical staining was used to identify osteoclasts and F4/80 (Abcam ab6640 1:200) immunohistochemistry to identify macrophages in decalcified bone sections. Microscopic images were captured with a Zeiss Axioskop 40 microscope (Carl Zeiss, Toronto, ON, Canada) and stain intensity expressed as % of the ROI using ImageJ v.1.6.0 software (NIH, Bethesda, MD, USA).

### *Statistical analysis*

Quantitative data are expressed as mean ± SD and the open source statistical program R v.3.3.0 (R Core Team, 2015) used for Wilcoxon sum-rank tests. Analysis of variance (ANOVA) followed by Tukey post-hoc analysis was used for longitudinal and multiple comparisons between the different time points in the WT and *Cpa3*$^{Cre/+}$ mice. Differences were considered significant at $p < 0.05$.

## 3.4 Results

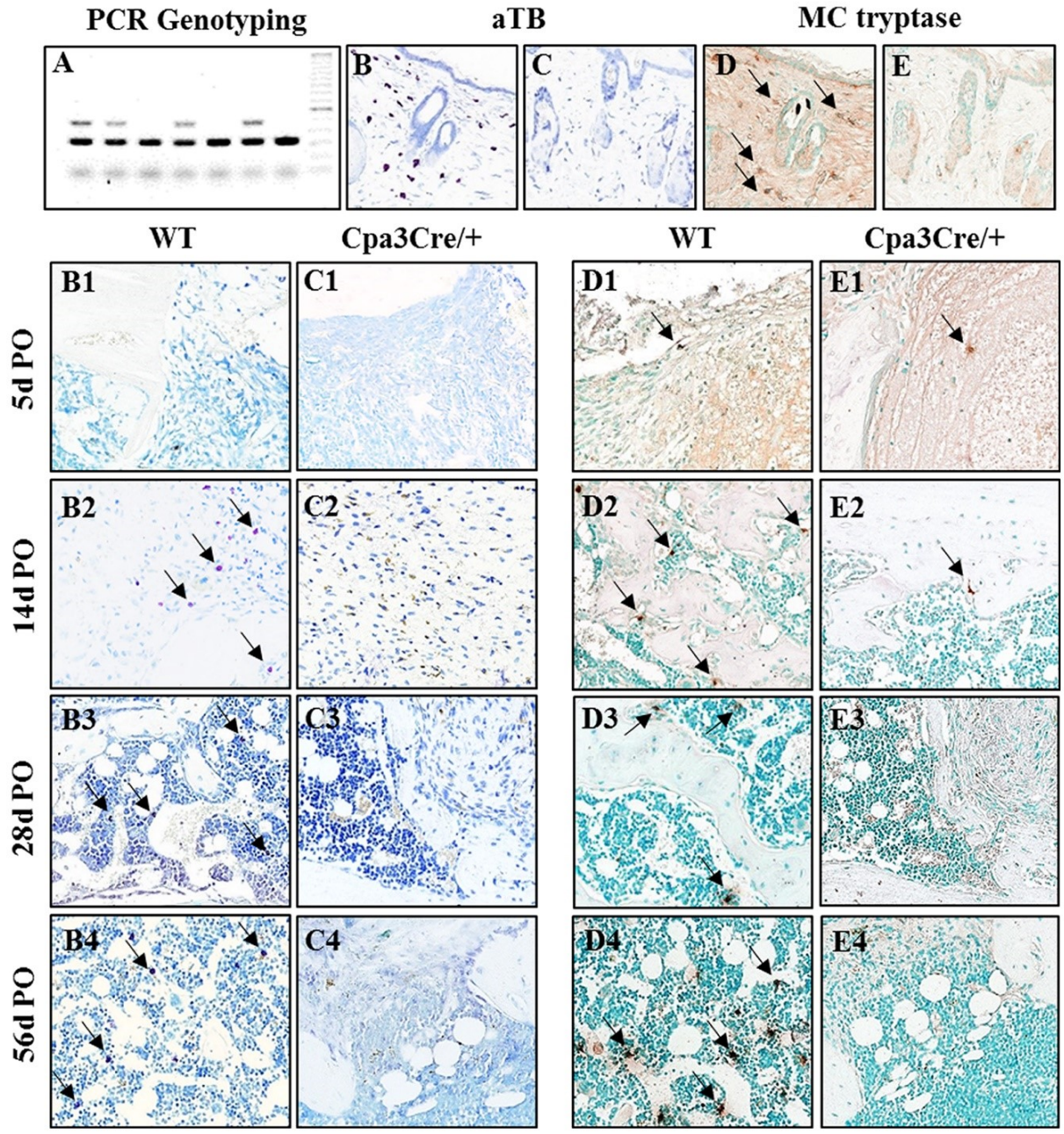
### *Distribution of mast cells during bone repair*

Mice were genotyped into *Cpa3*$^{+/+}$ (mast cell proficient; *WT*) and *Cpa3*$^{Cre/+}$ (mast cell deficient) littermates by PCR (Fig 1A). Mast cells are normally distributed throughout the dermis (20). Skin biopsies harvested from the backs of adult *WT* and *Cpa3*$^{Cre/+}$ mice were stained with aTB and for MC tryptase. Numerous aTB (Fig 1B) and MC tryptase positive (Fig 1D) cells were seen in the dermis, usually around hair follicles, in all *WT* (Fig 1A) but not in *Cpa3*$^{Cre/+}$ (Fig 1C, E) mice. The same strategy was used to identify mast cells in sections of regenerating bone harvested from mice euthanized from 5d-56d PO. At 5d PO, aTB positive cells were occasionally seen in *WT* mice on the periosteal surface of the proximal femur outside the region of the defect. At 14d PO they were seen in residual connective tissue in the defect/medulla of *WT* mice (Fig 1B2) and in the marrow, usually adjacent to vascular channels at 28d and 56d PO (Fig 1B3-B4). aTB positive cells were never seen in any of the bones harvested from *Cpa3*$^{Cre/+}$ mice (Fig 1C1-C4). Quantitative data for mast cell staining, expressed as the number of aTB positive cells/mm$^2$, is shown in Table 1. Non-granular, anti-tryptase antibody reactive cells of undefined lineage were seen in fibrous tissue at 5d and 14d PO in the defect/medulla of *WT* (Fig1D1-D2) mice and occasionally in *Cpa3*$^{Cre/+}$ (Fig1E1-E2) mice. At 28d and 56d PO, the distribution pattern of MC tryptase positive cells in *WT* mice resembled that of aTB positive cells, adjacent to bone and in the marrow next to vascular channels (Fig 1D3-D4). Tryptase positive cells were not seen in *Cpa3*$^{Cre/+}$ bone at these times (Fig 1E3-E4).

### *Table 3.1 Quantitative staining analysis of cellular activity*

| | 5 days postoperative | | | 14 days postoperative | | | 28 days postoperative | | | 56 days postoperative | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WT (n=6-10) | Cpa3Cre/+ (n=7-9) | p value | WT (n=6-13) | Cpa3Cre/+ (n=7-10) | p value | WT (n=8-9) | Cpa3Cre/+ (n=8-10) | p value | WT (n=5-6) | Cpa3Cre/+ (n=5-9) | p value |
| **Cortex** | | | | | | | | | | | | |
| aTB (#/mm$^2$) | 0 | 0 | 1.000 | 0.4 ± 1.1[b] | 0 | 0.268 | 1.6 ± 1.3[b] | 0 | **0.001** | 0.7 ± 0.7[b] | 0 | **0.016** |
| CD34 (%) | 0.35±0.20 | 0.24±0.21 | 0.309 | 0.69±0.35[a] | 0.70±0.28[b] | 0.942 | 0.80±0.19[b] | 1.31±0.26[b] | **0.002** | 0.63±0.18 | 1.02±0.28[b] | **0.046** |
| ALP (%) | 2.22±1.08 | 2.25±1.98 | 0.970 | 5.09±1.34[a] | 6.59±2.48[b] | 0.090 | 7.41±2.19[b] | 5.98±2.13[a] | 0.192 | 9.08±3.02[b] | 7.64±2.98[b] | 0.410 |
| TRAP (%) | 0.07±0.12 | 0.01±0.02 | 0.232 | 3.70±2.33[b] | 3.88±3.80[b] | 0.898 | 1.33±0.61 | 2.63±1.39[a] | **0.025** | 0.57±0.28 | 1.30±0.62[a] | **0.025** |
| F4/80 (#/mm$^2$) | 0.16±0.06 | 0.14±0.13 | 0.774 | 0.28±0.08 | 0.39±0.13[a] | 0.103 | 0.21±0.06 | 0.40±0.19[a] | **0.017** | 0.44±0.21[a] | 0.21±0.09 | 0.081 |
| **Defect/Medulla** | | | | | | | | | | | | |
| aTB (#/mm$^2$) | 0 | 0 | 1.000 | 10.0 ± 5.9[b] | 0 | **<0.001** | 8.0 ± 3.4[b] | 0 | **<0.001** | 4.9 ± 4.1[b] | 0 | **0.005** |
| CD34 (%) | 0.31±0.31 | 0.14±0.10 | 0.161 | 0.87±0.40[b] | 0.92±0.57[b] | 0.810 | 0.27±0.09 | 0.45±0.18[a] | **0.035** | 0.10±0.06 | 0.22±0.05 | **0.016** |
| ALP (%) | 0.01±0.01 | 0.02±0.03 | 0.384 | 5.16±1.92[b] | 4.37±2.11[b] | 0.378 | 3.54±1.25[b] | 2.14±0.84[a] | **0.014** | 2.09±0.78[a] | 2.11±1.54[a] | 0.983 |
| TRAP (%) | 0.35±0.44 | 0.01±0.02 | **0.047** | 5.56±2.99[b] | 3.68±2.13[b] | 0.122 | 1.10±0.63 | 1.63±0.85[a] | 0.161 | 0.29±0.15 | 0.82±0.30[a] | **0.002** |
| F4/80 (#/mm$^2$) | 0.72±0.51 | 0.36±0.29 | 0.171 | 3.59±1.24[b] | 2.27±0.67[b] | **0.047** | 4.03±0.74[b] | 2.96±0.64[b] | **0.009** | 3.05±0.49[b] | 3.38±0.76[b] | 0.486 |

*aTB mast cells; CD34 vascular endothelial cells; ALP osteoblasts; TRAP osteoclasts; F4/80 macrophages*


PCR Genotyping
aTB
MC tryptase
A
B
C
D
E
WT
Cpa3Cre/+
WT
Cpa3Cre/+
5d PO
14d PO
28d PO
56d PO
B1
C1
D1
E1
B2
C2
D2
E2
B3
C3
D3
E3
B4
C4
D4
E4

### *Figure 3.1 Identification of mature and immature mast cells*

*Mice heterozygous for knock-in of Cre recombinase in the first exon of the Cpa3 gene were generated by backcross of founder Cpa3$^{Cre/+}$ mice, shipped from the colony in Heidelberg, Germany, to C57Bl6 mice. PCR genotyping (A) of DNA isolated from ear punch specimens of 4-week-old mice yields 320bp and 450bp products for heterozygous Cpa3$^{Cre/+}$ mice. Back skin biopsies were harvested from N=3 wild type (WT, B, D) and N=3 Cpa3$^{Cre/+}$ (C, E) mice were embedded in paraffin and adjacent sections stained with acidified toluidine blue (aTB) or immunochemically with mast cell (MC) tryptase to identify mast cells. Representative images show numerous purple stained granular mast cells in WT (B) but not in Cpa3$^{Cre/+}$ (C) skin. MC tryptase immunopositive cells (D arrows) are also seen in WT but not in Cpa3$^{Cre/+}$ skin (E). Bilateral drill hole defects were generated in the femora of adult N= 37 WT and N = 35 Cpa3$^{Cre/+}$ mice and cohorts of animals euthanized from 5d to 56d post-operative (PO). Sections of bone stained with aTB show mature mast cells (arrows) in marrow adjacent to the defect in WT (B2-B4) but not in Cpa3$^{Cre/+}$ (C1-C4) mice. MC tryptase positive cells (arrows) are seen in WT tissue starting at 14d PO (D2-D4 but not in Cpa3$^{Cre/+}$ mice (E2-E4). Images are representative of N = 7 WT and N = 6 Cpa3$^{Cre/+}$ at 5d PO; N = 13 WT and N = 10 Cpa3$^{Cre/+}$ at 14d PO; N = 8 WT and N = 10 Cpa3$^{Cre/+}$ at 28d PO and N = 5 WT and N = 8 Cpa3$^{Cre/+}$ at 56d PO.*

### *Micro CT analysis of bone repair*

2D micro CT images were reconstructed into 3D models in which new bone (white) is distinguished from pre-existing bone (dark grey) (Fig 2A-H). A complex healing pattern was seen in the *Cpa3*$^{Cre/+}$ mice that differed from that seen in the *WT* mice. At 14d PO less of the periosteal surface opposite the defect was covered with new bone in *WT* (Fig2B-C) mice than in *Cpa3*$^{Cre/+}$ (Fig2F-G arrows) mice. By 56d PO, bridging of the defect was most often complete in *WT* (Fig2D) mice but remained incomplete with residual fibrous tissue in most *Cpa3*$^{Cre/+}$ mice (Fig2H arrow). Quantitative analysis of bone regeneration in the cortex and defect/medulla ROIs is shown in Table 2. By 14d PO the *WT* mice had less cortical bone with wider spaces between trabeculae (Tb.Sp.) and increased bone porosity (Po.Vop; Po.op) compared with *Cpa3*$^{Cre/+}$ mice, but with little difference in the defect/medulla. By 56d PO BV/TV was higher, with narrower spaces and less porosity, in WT than in *Cpa3*$^{Cre/+}$ mice in both cortex and defect/medulla.

### *Table 3.2 Quantitative micro CT analysis of bone architecture*

| | **5 days postoperative** | | | **14 days postoperative** | | | **28 days postoperative** | | | **56 days postoperative** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WT (n=7) | Cpa3Cre/+ (n=6) | p value | WT (n=16) | Cpa3Cre/+ (n=10) | p value | WT (n=8) | Cpa3Cre/+ (n=10) | p value | WT (n=6) | Cpa3Cre/+ (n=8) | p value |
| **Cortex** | | | | | | | | | | | | |
| BV/TV % | 33.6±4.7 | 30.8±3.3 | 0.112 | 34.4±4.1 | 41.7±4.6[b] | **<0.001** | 39.0±3.8[b] | 41.1±7.6[b] | 0.370 | 42.0±4.5[b] | 36.3±6.5 | **0.025** |
| Tb.Th. (mm x$10^{-3}$) | 20.7±2.0 | 19.2±1.2 | **0.031** | 17.7±2.4[b] | 16.8±2.0[a] | 0.242 | 17.1±2.7[b] | 16.00±2.6[b] | **0.001** | 18.0±1.9[a] | 16.4±2.5 | 0.133 |
| Tb. Sp (mm x$10^{-3}$) | 29.1±3.1 | 30.4±2.8 | 0.304 | 29.1±5.4 | 19.5±5.2[b] | **0.001** | 29.5±2.19 | 23.4±5.0[b] | **0.003** | 24.3±3.3[b] | 28.3±3.4 | **0.021** |
| Po.Vop ($mm^3$) | 0.54±0.04 | 0.56±0.03 | 0.117 | 0.54±0.05 | 0.47±0.06[b] | **0.001** | 0.49±0.04[a] | 0.48±0.06[b] | 0.601 | 0.47±0.04[b] | 0.56±0.06 | **0.002** |
| Po.op % | 66.4±4.7 | 69.2±3.3 | 0.113 | 65.6±4.1 | 58.3±4.6 | **0.001** | 60.9±3.8b | 58.9±7.4 | 0.373 | 58.0±4.5[b] | 63.7±6.5 | **0.025** |
| **Defect/Medulla** | | | | | | | | | | | | |
| BV/TV % | 0.95±0.73 | 0.53±0.69 | 0.163 | 9.23±6.16[b] | 8.44±5.39[b] | 0.660 | 22.9±8.4[b] | 16.3±6.5[b] | **0.018** | 22.6±4.5[b] | 16.8±7.09[b] | **0.032** |
| Tb.Th. (mm x$10^{-3}$) | 4.67±0.02 | 3.73±1.51 | 0.178 | 3.24±0.64[b] | 3.38±0.52 | 0.456 | 5.81±0.86[b] | 5.28±0.86[b] | 0.165 | 9.31±0.74[b] | 8.0±2.1[b] | 0.088 |
| Tb. Sp (mmx$10^{-3}$) | 59.7±3.1 | 61.7±1.8 | 0.075 | 28.5±10.3[b] | 29.9±14.4[b] | 0.707 | 20.5±5.6[b] | 21.7±4.5[b] | 0.584 | 25.1±4.0[b] | 30.7±4.6[b] | **0.016** |
| Po.Vop ($mm^3$) | 78.4±3.1 | 77.8±0.8 | 0.575 | 71.1±5.2[b] | 71.2±4.5 | 0.954 | 60.3±5.9[b] | 65.4±5.1 | **0.032** | 60.8±3.6[b] | 67.7±8.4[a] | **0.034** |
| Po.(op) % | 99.0±0.75 | 99.4±0.83 | 0.239 | 90.9±6.0[b] | 91.6±5.5[b] | 0.683 | 76.3±8.6[b] | 82.5±6.9[b] | **0.031** | 75.3±5.8[b] | 83.3±7.5[b] | **0.010** |

*BV/TV = bone volume/tissue volume; TbTh = trabecular thickness; TbSp.= trabecular separation; PoVop = open pore volume; Po(op) = open porosity. Significantly different from 5 days postoperative:* [a] *p <0.05 and* [b] *p <0.01*

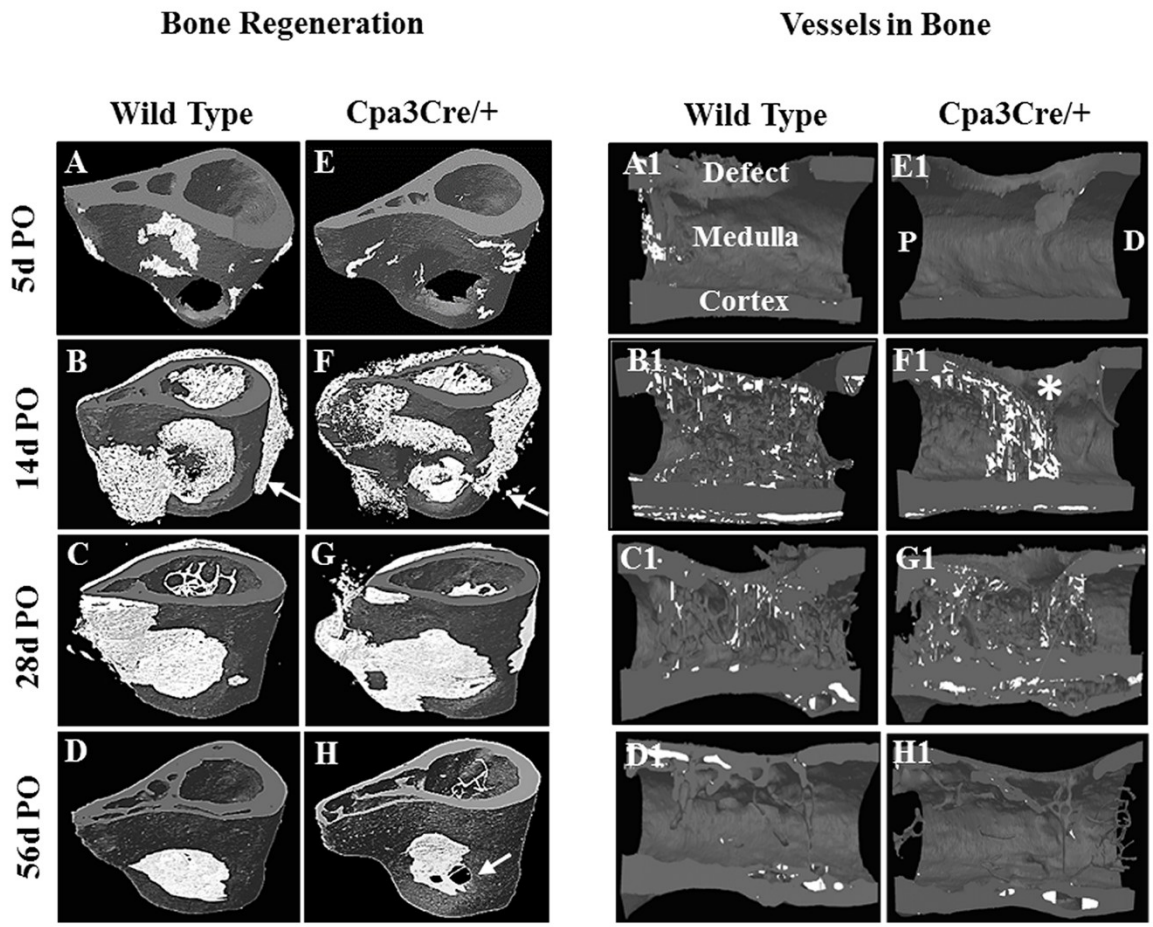



***Figure 3.2 Micro-CT analysis of bone and vessel regeneration***

*Femora harvested from WT and Cpa3$^{Cre/+}$ mice at the indicated time points were scanned at a resolution of 5μm on a Skyscan 1172 instrument. Representative 3D models (A-H), reconstructed from 2D images were tilted at a 45$^{o}$ angle to show healing of the defect over time. At 14d PO (B, F) significant new bone (white) is seen in the medullary canal and on the periosteal surface at the level of the defect (B, F arrows). By 56d PO, bone regeneration and remodelling have effectively closed the defect in the WT femur whereas mal-union is evident in the Cpa3$^{Cre/+}$ femurs, with holes penetrating the new cortical bone (H arrow). 3D models of hemi-femora (A1-H1) show the distribution of blood vessels (white) at the same time points. Revascularization of bone reaches a*

*peak at 14d postoperative, with a skewed distribution in Cpa3$^{Cre/+}$ mice (F1 asterix), and is restricted to the cortical bone by 56d PO (D1, H1).*

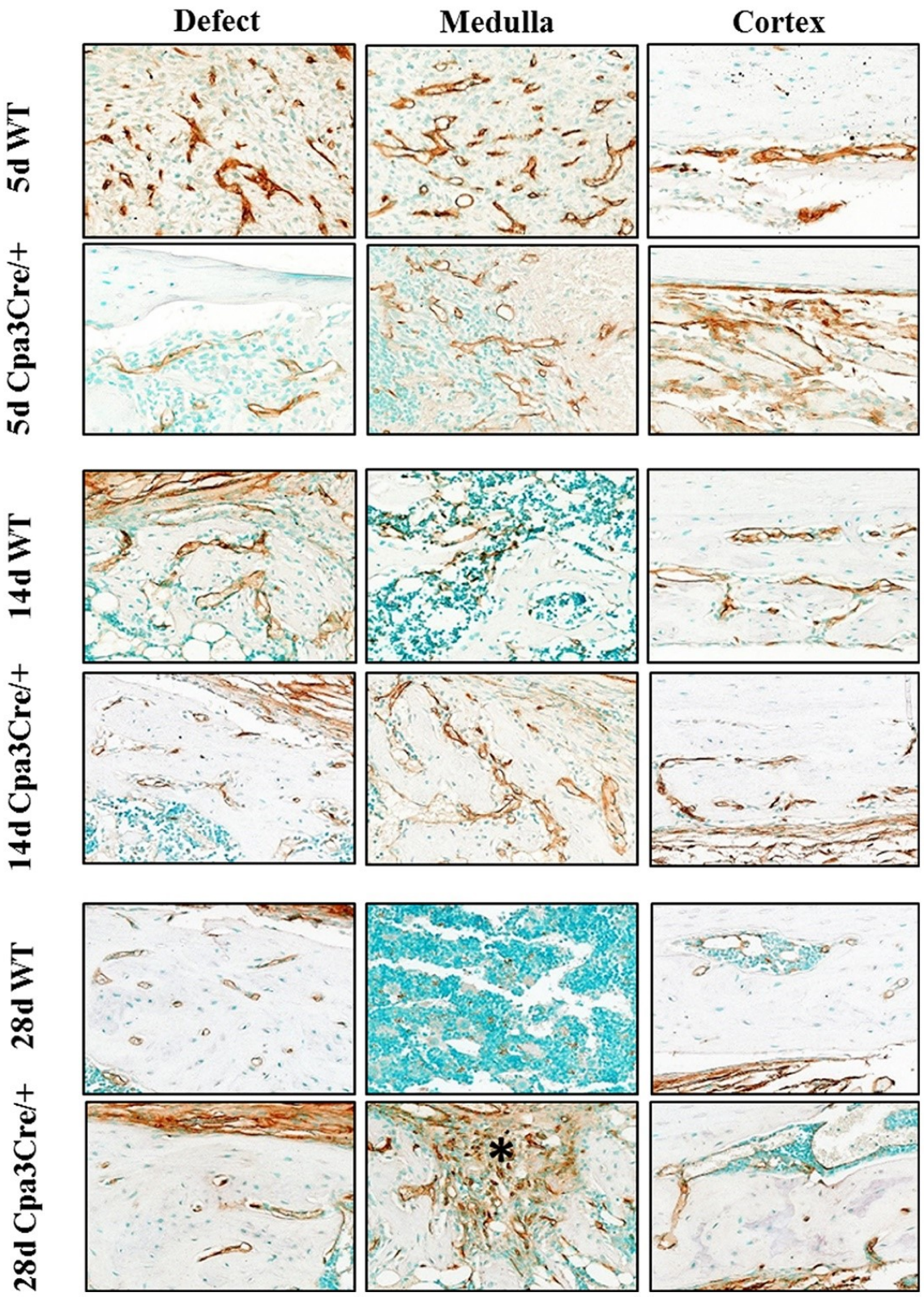
Modification of a new algorithm developed by Bruker was used to quantify vessels (Fig 2A1-H1) in bone (Bruker micro CT academy 2016 v5.3). At 5d PO vessels were starting to penetrate the repair tissue from the proximal femur in *WT* mice but were not yet visible in *Cpa3*$^{Cre/+}$ mice. By 14d PO there was a dramatic increase in vessels in both *WT* and *Cpa3*$^{Cre/+}$ bones. Whereas the distribution pattern was even in *WT* mice it was skewed to the proximal pole leaving a distal area with no penetration (asterix) and few cortical vessels in *Cpa3*$^{Cre/+}$ mice. By 56d PO the new vessels were effectively restricted to cortical bone. Quantitative analyses for new vessels is shown in Table 3. At 5d PO there were more vessels with a higher volume and better connectivity in *WT* than in *Cpa3*$^{Cre/+}$ bone, and the number, volume and thickness remained higher at 56d PO. Images are representative of N = 7 *WT* and N = 6 *Cpa3*$^{Cre/+}$ at 5d PO; N = 16 *WT* and N = 11 *Cpa3*$^{Cre/+}$ at 14d PO; N = 8 *WT* and N = 10 *Cpa3*$^{Cre/+}$ at 28d PO and N = 6 *WT* and N = 8 *Cpa3*$^{Cre/+}$ at 56d PO.

### *Table 3.3 Quantitative micro CT analysis of bone vasculature*

| | 5 days postoperative | | | 14 days postoperative | | | 28 days postoperative | | | 56 days postoperative | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WT (n=7) | Cpa3Cre/+ (n=6) | p value | WT (n=16) | Cpa3Cre/+ (n=10) | p value | WT (n=8) | Cpa3Cre/+ (n=10) | p value | WT (n=6) | Cpa3Cre/+ (n=8) | p value |
| **Cortex** | | | | | | | | | | | | |
| Ch.V/TV % | 0.43±0.4 | 0.26±0.2 | 0.264 | 9.8±5.8[b] | 11.2±4.8[b] | 0.482 | 2.04±1.7 | 4.5±2.4[b] | **0.003** | 1.73±0.8 | 1.83±1.1 | 0.814 |
| Ch.N. | 0.33±0.3 | 0.11±0.09 | 0.103 | 2.6±1.3[b] | 3.21.6[b] | 0.201 | 0.61±0.4 | 1.04±0.4[a] | **0.021** | 0.37±0.1 | 0.43±0.2 | 0.331 |
| Ch.Th. (mm x$10^{-3}$) | 16.8±7.4 | 19.7±12.0 | 0.537 | 32.6±9.4[b] | 36.1±9.0[b] | 0.224 | 30.8±8.0[b] | 41.0±12.8[b] | **0.009** | 46.1±14.7[b] | 40.8±15.4[b] | 0.471 |
| Ch.Conn.Dn. | 0.15±0.2 | 0.008±0.005 | **0.031** | 1.18±0.8[b] | 1.48±1.3[b] | 0.403 | 0.07±0.07 | 0.20±0.18 | **0.001** | 0.06±0.05 | 0.06±0.03 | 0.923 |
| **Defect/Medulla** | | | | | | | | | | | | |
| Ch.V/TV % | 9.04±8.7 | 3.5±3.1 | **0.039** | 38.61±7.2[b] | 28.71±13.4[b] | **0.006** | 10.74±4.7 | 12.12±3.7[a] | 0.401 | 3.58±2.25 | 1.44±0.8 | **0.022** |
| Ch.N. | 4.1±3.0 | 2.0±1.5 | **0.036** | 13.1±2.5[b] | 10.1±4.1[b] | **0.008** | 3.9±2.0 | 4.5±1.6[a] | 0.548 | 1.0±0.3[a] | 0.9±0.3 | 0.431 |
| Ch.Th. (mm x$10^{-3}$) | 19.3±6.9 | 18.0±3.2 | 0.667 | 31.1±2.8[b] | 25.9±7.4[b] | **0.007** | 28.6±8.8[a] | 27.9±5.9[b] | 0.801 | 35.6±16.2[b] | 23.1±5.8 | **0.039** |
| Ch.Conn.Dn. | 1.69±1.5 | 0.5±0.4 | **0.031** | 8.62±3.6[b] | 6.1±3.1[b] | **0.019** | 1.19±1.1 | 1.5±0.9 | 0.452 | 0.33±0.2 | 0.21±0.1 | 0.115 |

*Ch.V/TV % = channel volume/tissue volume; Ch.N. = channel number; Ch.Th. (mm x$10^{-3}$) = channel thickness; Ch.Conn.Dn. = channel connectivity density. . Significantly different from 5 days postoperative: [a] $p < 0.05$ and [b] $p < 0.01$*

The localization of vessels in regenerating bone was then compared with the results of CD34 immunohistochemistry, which was used as a sensitive marker for vascular endothelial cells in soft tissue and bone (Fig 3). At 5d and 14d PO, there was an extensive network of vessels in the defect, medulla and cortex of *WT* bones, compared with the less dense pattern of vessel distribution seen in $Cpa3^{Cre/+}$ bones. By 28d PO, there were few CD34 positive cells in the medulla of *WT* mice compared with numerous cells embedded in fibrous tissue remaining in the defect and medulla of $Cpa3^{Cre/+}$ bones (asterix). Quantification of CD34 staining, shown in Table 1, revealed peak activity at 14d PO in the defect/medulla and at 28d PO in the cortex of both *WT* and $Cpa3^{Cre/+}$ bones, but with fewer vessels at 28d and 56d PO in the *WT* mice.


Defect
Medulla
Cortex
5d WT
5d Cpa3Cre/+
14d WT
14d Cpa3Cre/+
28d WT
28d Cpa3Cre/+
*

### *Figure 3.3 CD34 immunohistochemistry in regenerating bone*

*Bones were decalcified, embedded in paraffin and 5μm sections stained immunochemically for CD34 expression. Representative images of the defect, medulla and contralateral cortex show robust staining of cells lining vessels at 5d PO in WT bone compared with weak, disorganised staining in $Cpa3^{Cre/+}$ bone. By 28 days PO CD34 immunoreactivity is restricted primarily to the periosteum in WT bone but persists in the remaining fibrous tissue in $Cpa3^{Cre/+}$ bone (asterix). Images are representative of N = 7 WT and N = 6 $Cpa3^{Cre/+}$ at 5d PO; N = 11 WT and N = 9 $Cpa3^{Cre/+}$ at 14d PO and N = 8 WT and N = 6 $Cpa3^{Cre/+}$ at 28d PO.*

### *Histological analysis of bone repair*

To further characterize the quality of regenerated bone we stained thin sections of un-decalcified bone harvested from the mid-saggital plane of the defect with von Kossa (Fig 4) to compare with 2D micro CT images. The morphological features seen in the 2D micro CT images (Fig 4A-H) were reflected in the VK/TB stained histological sections. Residual shards of old bone (Fig 4A and 4E arrows) were seen at 5d PO but no new bone was visible until 14d PO (Fig 4B and 4F). Bridging of the defect was evident at 28d PO in *WT* but not in $Cpa3^{Cre/+}$ mice (Fig 4C vs 4G) in which significant fibrous tissue remained (Fig 4G asterix). By 56D PO bone repair was effectively complete in most *WT* bones (Fig 4D) compared with $Cpa3^{Cre/+}$ bones, where the defect was filled with thin bone interspersed with fibrous tissue (Fig 4H asterix). The spaces between old and new bone seen in the cortex at 28D were retained only in $Cpa3^{Cre/+}$ bones (Fig 4H arrows).

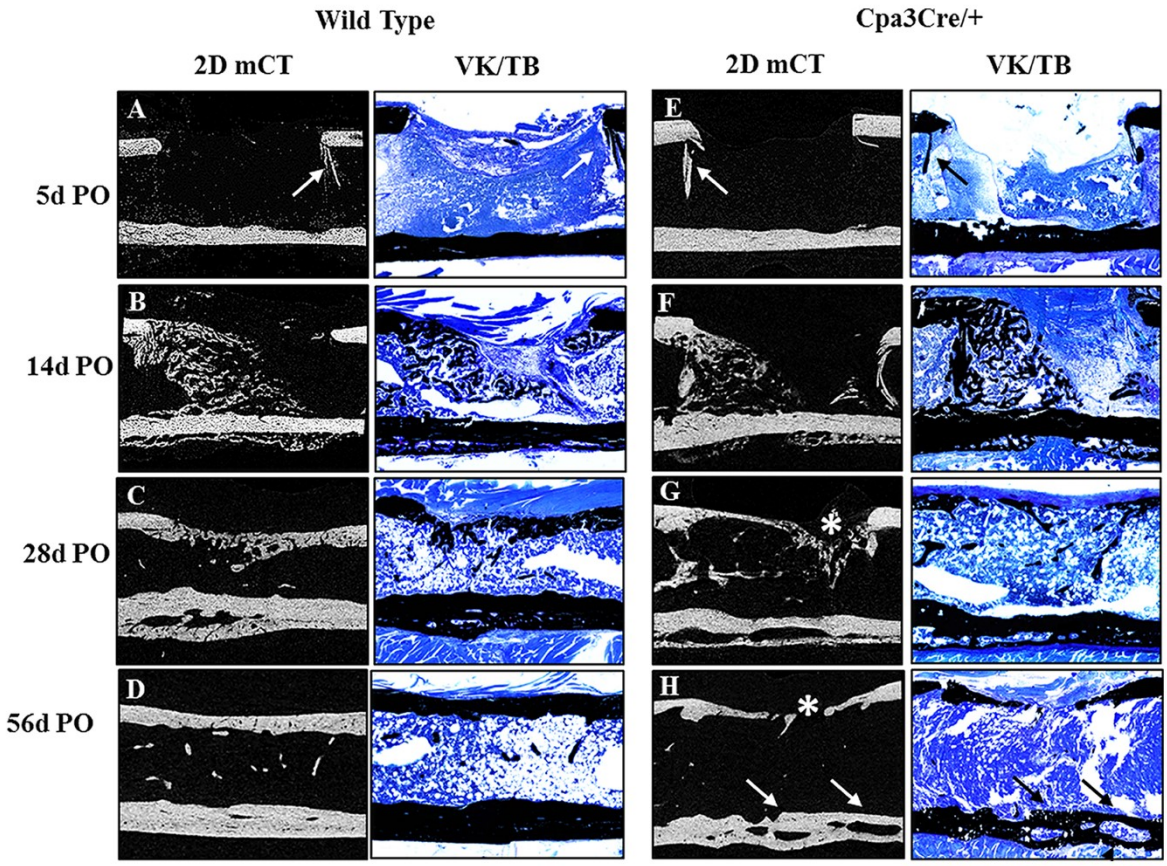



***Figure 3.4 Macroscopic evaluation of bone repair over time***

*2D micro CT images (A-H) were compared with 5 μm histological sections of un-decalcified bone stained with von Kossa and toluidine blue (VK/TB) to distinguish mineralised (black) from un-mineralized tissue (blue). Representative mid-sagittal images show shards of old bone (A, E arrows) remaining in the defect at 5d PO and significant new bone at 14d PO in the medullary canal and on the periosteal surface opposite the defect in both WT (B) and Cpa3$^{Cre/+}$ (F) femora. At 28 days PO, the defect is bridged with primary bone in many WT (C), but not Cpa3$^{Cre/+}$ (G asterix) mice. By 56d PO the majority of WT femora have assumed their pre-operative anatomy (D), whereas most of those from Cpa3$^{Cre/+}$ mice exhibit mal-union on the defect side (H asterix) and large channels separating old from new bone on the contralateral cortex (H arrows). Images*

*are representative of N = 7 WT and N = 6 Cpa3$^{Cre/+}$ at 5d PO; N = 16 WT and N = 11 Cpa3$^{Cre/+}$ at 14d PO; N = 8 WT and N = 10 Cpa3$^{Cre/+}$ at 28d PO and N = 6 WT and N = 8 Cpa3$^{Cre/+}$ at 56d PO.*

The cortical window defect is stable and therefore heals through intra-membranous bone formation, with no cartilage intermediate, so ALP is expressed only by anabolic cells of the osteoblast lineage. High magnification images of VK/TB and ALP stained sections from the corresponding region of the defect/medulla (Fig 5) revealed little bone forming activity at 5d PO in either *WT* (Fig 5A) or *Cpa3*$^{Cre/+}$ (Fig 5E) bones. At 14d PO there was an extensive network of new bone trabeculae at the proximal end of the defect and in the adjacent medulla, associated with intense ALP activity in both *WT* and *Cpa3*$^{Cre/+}$ bone (Fig 5B and 5F). At 28d and 56d PO, there was more bone and less osteoid (blue) spanning the defect in *WT* (Fig 5C) than in *Cpa3*$^{Cre/+}$ (Fig 5G) mice, while ALP activity appeared similar. Detailed examination of VK/TB staining in the cortex (Fig 6) revealed less pronounced fibrous periosteal tissue in *WT* (Fig 6A) than *Cpa3*$^{Cre/+}$ bone (Fig 6E asterix) at 5d PO, prior to active bone formation at 14d PO when *WT* (Fig 6B) and *Cpa3*$^{Cre/+}$ (Fig 6 B and 6F) bones looked similar. At 28d and 56d PO, the cortex of *WT* bones (Fig 6C and 6D) had fewer lacunae and less osteoid than of *Cpa3*$^{Cre/+}$ bones (Fig 6G and 6H).

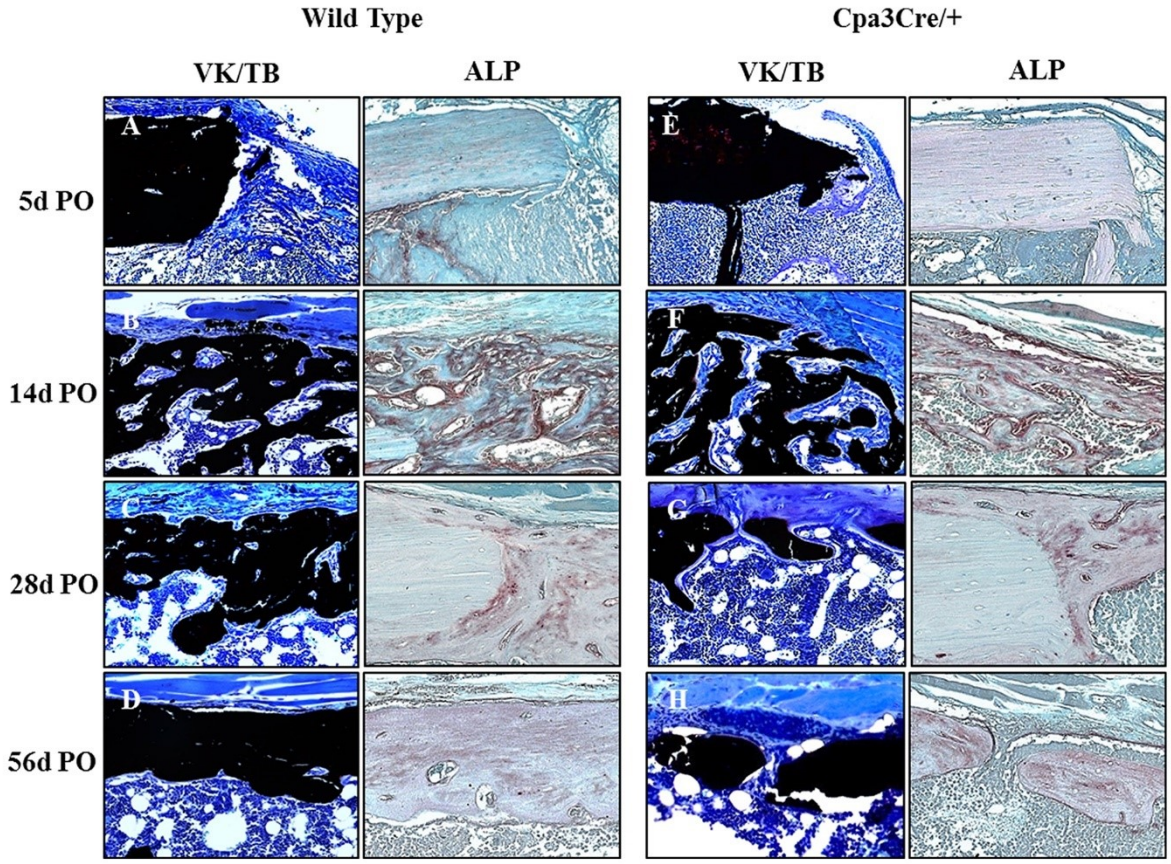



***Figure 3.5 Histochemical analysis of bone in defect/medulla***

*Representative images of 5 µm sections of un-decalcified bone stained with von Kossa and toluidine blue (A-H) were compared with the equivalent region of 5µm sections of decalcified bone stained with alkaline phosphatase (ALP). Prominent osteoblasts against osteoid seams can be seen at 14d (F) and 28d (G) PO in $Cpa3^{Cre/+}$ bones compared with WT bones (B-C). ALP activity peaks at 14 days PO in both WT (B) and $Cpa3^{Cre/+}$ (F) bones and declines thereafter. Images are representative of N = 7 WT and N = 6 $Cpa3^{Cre/+}$ at 5d PO; N = 16 WT and N = 11 $Cpa3^{Cre/+}$ at 14d PO; N = 8 WT and N = 10 $Cpa3^{Cre/+}$ at 28d PO and N = 6 WT and N = 8 $Cpa3^{Cre/+}$ at 56d PO.*

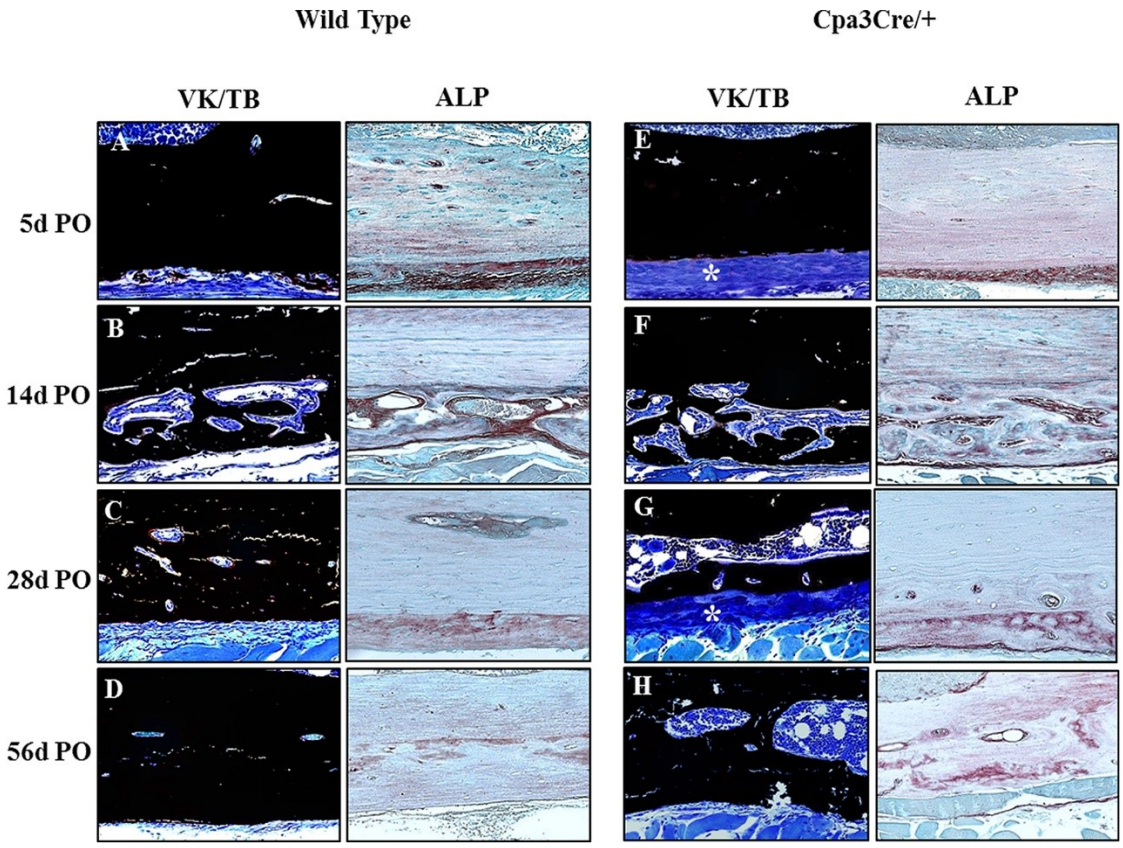



***Figure 3.6 Histochemical analysis of bone in contralateral cortex***

*Representative images of 5 µm sections of von Kossa stained un-decalcified bone (A-H) were compared with 5µm sections of decalcified bone stained with ALP. A thick, fibrous periosteum is apparent in Cpa3$^{Cre/+}$ bones (E, G asterix) in the absence of any significant difference in ALP activity. Bone formation with large osteoblasts adjacent to osteoid is apparent at 14d PO in WT (B) and Cpa3$^{Cre/+}$ (F) bones, accompanied by high ALP activity. Active bone formation is sustained at 28d (G) and 56d (H) PO in Cpa3$^{Cre/+}$ mice but is less apparent in WT mice (C, D). Images are representative of N = 7 WT and N = 6 Cpa3$^{Cre/+}$ at 5d PO; N = 16 WT and N = 11 Cpa3$^{Cre/+}$ at 14d PO; N = 8 WT and N = 10 Cpa3$^{Cre/+}$ at 28d PO and N = 6 WT and N = 8 Cpa3$^{Cre/+}$ at 56d PO.*

Adjacent sections of decalcified bone were stained with TRAP to identify osteoclasts or with F4/80 antiserum to identify macrophages (Fig 7). TRAP positive cells were seen at the proximal end of the defect and in the fibrous tissue filling the defect at 5d PO in *WT* bones (Fig 7A) but not in *Cpa3*$^{Cre/+}$ bones (Fig 7B). Peak TRAP activity occurred at 14d PO and was more intense in *WT* (Fig 7C) than in *Cpa3*$^{Cre/+}$ bones (Fig 7D), whereas it declined at 28d PO in *WT* (Fig7E) but not in *Cpa3*$^{Cre/+}$ bones (Fig 7F). F4/80 staining was prominent in connective tissue of *WT* (Fig 7A1) and *Cpa3*$^{Cre/+}$ (Fig 7B1) bones at 5d PO. By 14d PO prominent staining was seen adjacent to bone in *WT* (Fig 7C1) but not in *Cpa3*$^{Cre/+}$ (Fig 7D1) bones, where F4/80 positive cells remained scattered in connective tissue. By 28d PO, the F4/80 positive cells were seen in marrow adjacent to bone in *WT* mice (Fig 7E1) and persisted in fibrous tissue in *Cpa3*$^{Cre/+}$ bone (Fig 7F1). Quantitative analyses for ALP, TRAP and F4/80 staining (Table 1) confirmed similar patterns for ALP in *WT* and *Cpa3*$^{Cre/+}$ bones throughout the healing period, as well as higher TRAP activity in *WT* bone at 5d PO followed by lower levels than in *Cpa3*$^{Cre/+}$ bones at later time points. F4/80 immunoreactivity was generally similar except for transient reductions in *Cpa3*$^{Cre/+}$ bones at 14d and 28d PO.

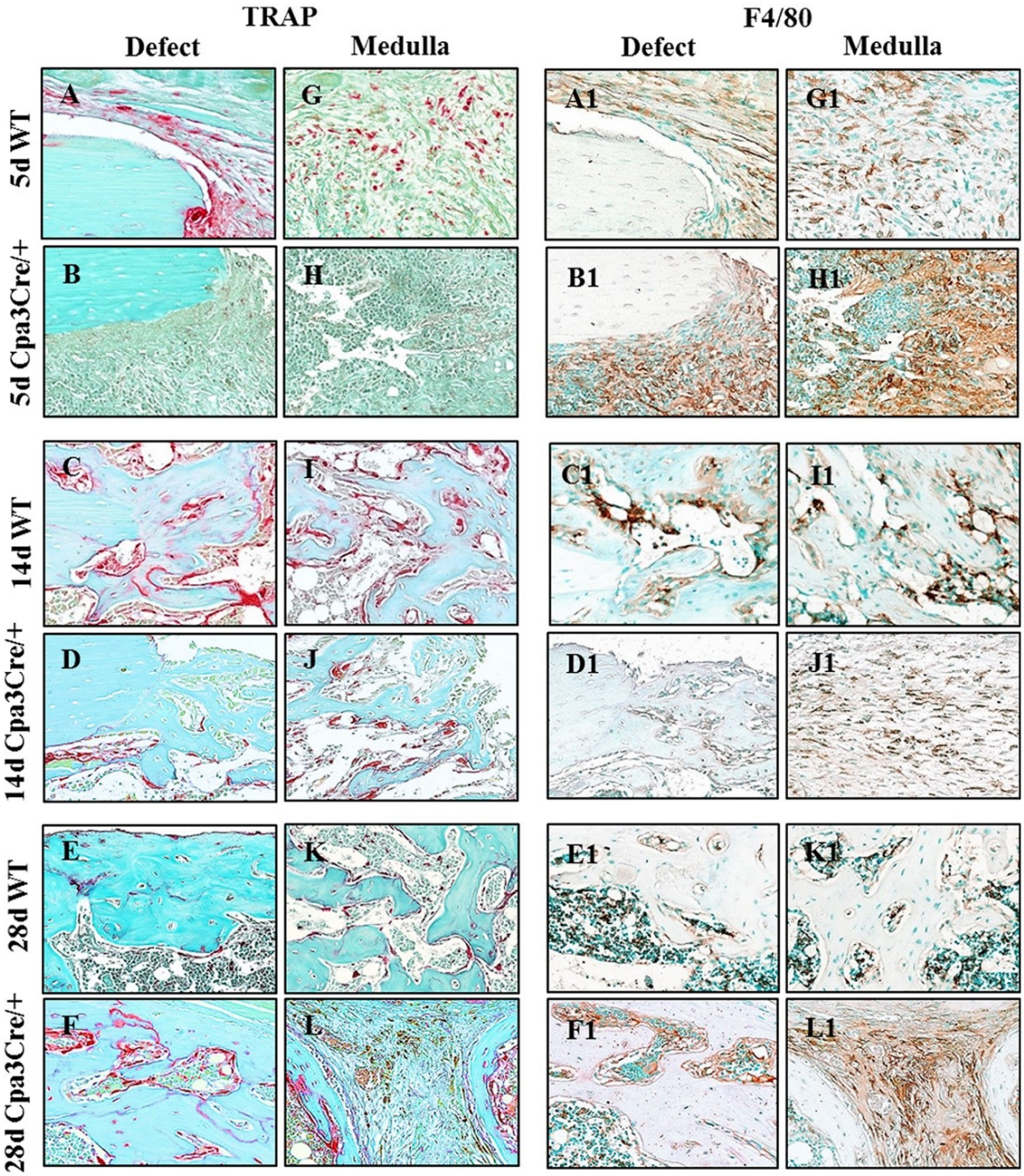

TRAP
F4/80
Defect
Medulla
Defect
Medulla
5d WT
5d Cpa3Cre/+
14d WT
14d Cpa3Cre/+
28d WT
28d Cpa3Cre/+
A
G
A1
G1
B
H
B1
H1
C
I
C1
I1
D
J
D1
J1
E
K
E1
K1
F
L
F1
L1

***Figure 3.7 Identification of osteoclasts and macrophages in regenerating bone***

*5 μm sections of decalcified bone were stained with tartrate resistant acid phosphatase (TRAP) or immunochemically with the macrophage marker F4/80. Representative images show more TRAP activity in WT than in $Cpa3^{Cre/+}$ bones at 5d (A vs B) and 14d (C vs D) PO, and less at 28d (E vs F) PO. F4/80 positive macrophages were seen in condensed mesenchyme filling the defect/medulla at 5d PO in both WT (A1) and $Cpa3^{Cre/+}$ (B1) bones. In WT bone, F4/80 positive cells can be seen lining vessels at 14d (C1) PO and scattered throughout bone marrow at 28d (E1) PO, whereas they were embedded in fibrous tissue in $Cpa3^{Cre/+}$ bone (F1). Images are representative of N = 7 WT and N = 6 $Cpa3^{Cre/+}$ at 5d PO; N = 16 WT and N = 11 $Cpa3^{Cre/+}$ at 14d PO; N = 8 WT and N = 10 $Cpa3^{Cre/+}$ at 28d PO and N = 6 WT and N = 8 $Cpa3^{Cre/+}$ at 56d PO.*

## 3.5 Discussion

The goal of the current study was to characterize the impact of mast cell deficiency on the repair of cortical bone defects using adult mast cell deficient *Cpa3*$^{Cre/+}$ mice. The *Cpa3*$^{Cre/+}$ strain is constitutively devoid of mast cells in connective and mucosal tissues and it has no known alterations in other cell lineages involved in bone repair. In *WT* but not in *Cpa3*$^{Cre/+}$ mice mast cells appeared in the repair tissue from 14d to 56d PO. Interestingly, bridging of the bone defect was complete in all (6/6) *WT* mice at 56d PO but only 3/8 *Cpa3*$^{Cre/+}$ mice. This incomplete bridging was associated with disruption of re-vascularization and impaired bone mineralization. Osteoclast activity was reduced in *Cpa3*$^{Cre/+}$ mice in the early phase of repair but increased at later stages, with no clear differences in macrophage activity. Taken together, the results indicate that mast cells have a positive impact on bone repair that is mediated in part by recruitment of vascular endothelial cells, as also suggested by the previous work of Boesiger *et al* (1998) (Boesiger et al., 1998), and in part by altered metabolism of newly formed bone. It was proposed more than two decades ago that mast cells are involved in tissue digestion and re-vascularization, which are early and essential steps in the bone healing cascade (Banovac et al., 1995). Mast cells reside over the long term in connective tissues where they are available locally and for potential trafficking via the vascular and lymphatic systems to sites of tissue injury and repair (Kunder et al., 2011). The surgical intervention used in this study would have temporarily disrupted existing hind limb vessels and caused local ischemia and hypoxia, which are the major stimuli for re-vascularization (Simons, 2005). Mast cells, identified by the use of metachromatic staining with aTB, were first seen in *WT* bone at 5d PO, which was the earliest time at which the soft callus could be preserved intact for histological analyses. At this time they were localized adjacent to vessels in muscle, in the bone marrow of the femur proximal to the site of injury, and at the

periosteal junction between soft tissue and bone. The appearance of aTB positive cells at the proximal, rather than distal, end of the femur suggests they migrated to the wound from soft tissue stores via the arterial or lymphatic vessels that supply the hind limb. This conjecture was supported by micro CT analyses of vessels in regenerating bone showing initiation of re-vascularization at 5d PO at the proximal end of the defect in *WT* bones. Quantitative analyses revealed peak numbers of aTB positive mast cells in the defect/medulla of *WT* bones at 14d PO, and in the cortex at 28d PO. This timeframe was supported by qualitative data showing MC tryptase positive cells in bone marrow starting at 14d in *WT* but not $Cpa3^{Cre/+}$ mice. Peak numbers of mast cells in regenerating bone coincided with peak numbers of vessels visualized by micro CT and with CD34 immunostaining of vascular endothelial cells.

Degranulation or secretion of factors by aTB positive mast cells would result in local release of angiogenic mediators like heparin, angiogenin, vascular endothelial growth factor and matrix metalloproteinases in *WT* mice (Theoharides, Valent, & Akin, 2015). Given their lack of mast cells, these mediators would be absent in $Cpa3^{Cre/+}$ mice, which could have accounted for the delay and disorganization of bone re-vascularization as evidenced by micro CT. In this and other studies of bone repair, we have used CD34 as a sensitive marker of endothelial cells, but it is also expressed on MSC, fibrocytes and other precursor cells capable of differentiating down the osteogenic lineage (Sidney, Branch, Dunphy, Dua, & Hopkinson, 2014). In the current work, CD34 cells were clearly organized into vascular channels in the condensed mesenchyme in defect/medulla and the cortex at 5d PO prior to bone formation. Consistent with delayed healing, CD34 cells persisted in the $Cpa3^{Cre/+}$ mice in the periosteum and in residual fibrous tissue in the defect/medulla at 56d PO, when re-vascularization was effectively complete in *WT* mice. The

absence of clearly defined channels and the location of CD34 positive cells at 56d PO suggest the cells were not endothelial cells but rather fibrocytes in the periosteum, or MSC that were unable to differentiate into bone-forming osteoblasts in the absence of mast cell mediators. The current literature identifies bone active agents like platelet derived growth factor, fibroblast growth factors, transforming growth factor and tumor necrosis factor, as well as matrix metalloproteinases as pre-formed components of stored granules that are released upon mast cell activation (Antebi et al., 2016; Kunder et al., 2011). After 14d PO mast cells were present in close proximity to vessel in the defect/medulla of *WT* mice but absent from *Cpa3*$^{Cre/+}$ mice. The absence of mast cell derived bone active agents could have impaired mineralization of newly formed bone at 14d and 28d PO despite similar levels of alkaline phosphatase activity in osteoblasts (Fig 6, 7). Furthermore, the absence of carboxypeptidase A, which is a major constituent of mast cell granules that targets the vasoconstrictor endothelin (Pejler, Knight, Henningsson, & Wernersson, 2009; L. A. Schneider, Schlenner, Feyerabend, Wunderlin, & Rodewald, 2007) could have resulted in excess endothelin-1 mediated vasoconstriction, increased oxidative stress and altered bone cell function (Pejler et al., 2009).

The importance of an adequate periosteal reaction to bone healing is emphasized by the current widespread use of vascularised fibular periosteal grafts to promote healing of large bone defects (Azi et al., 2016; Soldado et al., 2012) and in cell-based bone tissue engineering (van Gastel et al., 2014). Prior to skeletal maturity, the periosteum is thick and highly vascular, with active osteoblasts depositing intramembranous bone to increase the external diameter of growing long bones (Bisseret et al., 2015). The absence of mast cells in *Cpa3*$^{Cre/+}$ mice resulted in thickening of the periosteum, similar to that seen in adult *FGFR3-null* mice (Valverde-Franco et

al., 2004), and suggests an imbalance in FGF signaling might be involved in impaired bone regeneration in the *Cpa3*$^{Cre/+}$ mice. However, the similarity in ALP activity between *WT* and *Cpa3*$^{Cre/+}$ mice supports the hypothesis that the impairment was not mediated at an early stage of osteoblast differentiation.

The two principle cells responsible for catabolic activity in bone are osteoclasts and macrophages. Apart from being phagocytes, the lineages appear to share little in common. Osteoclast activity is restricted to the digestion of mineralized tissue in cartilage and bone whereas macrophages have been implicated in inflammatory disorders and fibrosis of most if not all major organs, including the brain. In our previous work that characterized bone repair in *Kit*$^{W\text{-}sh}$ mast cell deficient mice, osteoclast activity was shown to be elevated throughout the bone healing cascade (Behrends et al., 2014). This was not the case in the current study in which osteoclast activity was reduced in *Cpa3*$^{Cre/+}$ mice up to 14d PO and elevated thereafter compared with *WT* mice. A simple explanation for the discrepancy is that Kit is expressed on osteoclasts and their precursors, as well as mast cells, whereas Cpa3 expression is restricted to mast cells. Osteoclasts are considered to be tissue specific macrophages, distinct from bone marrow macrophages and "osteomacs" located on the periosteal and endosteal surface of resting bone (Chang et al., 2008). Using F4/80 immunostaining to identify all macrophages in healing bone it was surprising to find the lowest levels of expression during the inflammatory phase of repair at 5d PO, with an approximately 5- fold increase thereafter in both *WT* and *Cpa3*$^{Cre/+}$ mice. The relatively low abundance of macrophages under healing conditions in the absence of mast cells in *Cpa3*$^{Cre/+}$ mice in the current study, and previously in *Kit*$^{W\text{-}sh}$ mice, suggests an interdependence between the two lineages. This conjecture is further supported by the increase

in both mast cells and macrophages in bone defects in *WT* mice administered lipopolysaccharide (LPS) (Behrends et al., 2017). The timeframe and pattern of distribution of F4/80 positive cells in the current study closely resembled that of "osteomacs" in a similar model of cortical bone repair (Alexander et al., 2011). Given the proposed anabolic function of these cells in bone mineralization (Raggatt et al., 2014), their relative short supply in $Cpa3^{Cre/+}$ bone might explain the increase in osteoid compared with *WT* bone at 14d and 28d PO. Taken together the data suggest a functional relationship exists between mast cells, macrophages and MSC in the bone micro-environment that warrants further investigation. Only few suggested mast cell functions have been confirmed in studies using Kit-independent models of mast cell deficiency. Thus, the impairment of bone fracture healing in mice in the absence of mast cells appears to be a remarkable, largely unanticipated, non-immunological mast cell function.

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**Author roles**

Study design and execution: JRGL, JEH, PAM. Provision of mast cell-deficient mice: TBF, HRR Data collection and analysis: JRGL, DC, RS, MA, TW, AL. Data interpretation: JRGL, DC, RS, TBF, HRR, JEH, PAM. Manuscript draft: JRGL, HRR, JEH, PAM. All authors share responsibility for the integrity of the data analysis.

# IV. MAST CELLS INVOLVED IN FRACTURE REPAIR ARISE FROM PRECURSORS IN BONE MARROW

Having demonstrated that in the absence of mast cells bone repair is defective, we sought to develop a phenotype rescuing model to confirm our findings. Additionally, we had hypothesized that because mast cells exert a positive role in bone healing, their exogenous administration after a fracture would probably enhance bone healing and if so, that we could use them as a cellular therapy to promote better bone repair in patients at high risk of complications. Unfortunately, the mast cell reconstituted *Cpa3*$^{Cre/+}$ mice did not show granulated mast cells in the repair tissue, thus disproving our theory. However, we identified mast cell precursor cells in the bone marrow at levels comparable to both *WT* and non-reconstituted *Cpa3*$^{Cre/+}$ mice, thus concluding that the mast cells involved in bone healing did not come from peripheral tissues, but rather differentiate *in situ* from precursor cells. Furthermore, our inability to target the engraftment of mature mast cells to healing bone led us to abandon the idea that they could be exogenously administered and made us investigate other mast cell properties, such as immune memory. Fortunately, this approach resulted in positive findings of enhanced bone healing that will be presented in Chapter VI.

The research presented in this section aims to fulfill our research objective III, develop a mast cell reconstitution model of *Cpa3*$^{Cre/+}$ mice to perform phenotype rescuing analyses and determine if exogenous mast cells enhance bone healing. In contrast to the rest of our experiments, where we used both male and female mice, this experiment used only male mice as transplant recipients, following a protocol by Feyerabend et al. (Feyerabend et al., 2011).

# MAST CELLS INVOLVED IN FRACTURE REPAIR ARISE FROM PRECURSORS IN BONE MARROW

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## 4.1 Abstract

***Introduction:*** Mast cells play a regulatory role during wound healing. Previous research done in mast cell deficient mice have shown that they are involved in several processes during fracture repair. For these reasons, their exogenous administration could be used to enhance bone healing. This study had two-fold aims. First, to develop a model of mast cell reconstitution in mast cell deficient *Cpa3*$^{Cre/+}$ mice to perform phenotype rescuing bone healing studies and second, to determine if local exogenously administered mast cells promote better healing in mast cell deficient *Cpa3*$^{Cre/+}$ mice.

***Methods:*** Mast cells were differentiated and cultured from murine bone marrow. Mature *WT* mast cells were transplanted to *Cpa3*$^{Cre/+}$ mice and after 8-weeks, confirmation of the presence of cells was done histologically. Assessment of the presence of cells in bone repair was done in a uni-cortical femoral window defect model. Finally, transplantation of mast cells to *Cpa3*$^{Cre/+}$ mice immediately after generation of the bone defect and assessment of cellular presence at 3, 5, and 14 days of healing was done.

***Results:*** Transplanted mast cells populate their normal cellular niches. After induction of a fracture, no mature mast cells were identified in the repair tissue, however precursor cells were observed. Likewise, exogeneous administration of mast cells failed to demonstrate the homing of the cells to repair tissue.

***Conclusion:*** Mast cells observed during bone repair arise from *in situ* precursor cells. Bone repair tissue lacks the appropriate signals to trigger the retention of exogenously administrated mast cells.

## 4.2 Introduction

Mast cells are myeloid-derived cells, part of the innate immune system. Although their traditional roles are in allergy and inflammatory disorders, there is a growing body of evidence indicating that they also participate in tissue homeostasis and repair (Komi et al., 2019; H.-R. Rodewald & Feyerabend, 2012). Mature mast cells are characterized by the presence of multiple granules that contain a myriad of vasoactive substances, matrix remodelling proteases, growth factors and angiogenic factors (Wernersson & Pejler, 2014). In the context of bone healing, mast cells have demonstrated to have a regulatory role in tissue mineralization, macrophage and endothelial cell recruitment, and osteoclast activity modulation that is translated into defective bone repair in mast cell deficint mice (Behrends et al., 2014; Ramirez-GarciaLuna et al., 2017).

Fracture complications such as non-union or delayed unions are estimated to affect approximately 5-10% of all fractures, however, in wounds that compromise the vascular supply, or in patients chronically exposed to drugs that alter bone homeostasis, the complication rate can be as high as up to 45% (Stegen et al., 2015; Zura et al., 2016). Cellular therapy using exogenously developed mast cells cultured from the patient's bone marrow could be an innovative approach to treat patients at high risk of fracture complications if found to be effective. Under this paradigm, and with the hypothesis that mast cells differentiated from bone marrow precursors in culture can be attracted to healing bone to promote regeneration, the objective of this study was two-fold. First, to develop a model of mast cell reconstitution in mast cell deficient *Cpa3*$^{Cre/+}$ mice to determine if mast cell reconstitution promotes bone healing comparable to the observed in *WT*

littermates; and second, to determine if exogenously administered mast cells promote better healing in mast cell deficient *Cpa3*$^{Cre/+}$ mice.

## 4.3 Methods

### *Mouse model of mast cell deficiency*

Animal procedures were conducted following a protocol approved by the Facility Animal Care Committee of McGill University (AUP-7016) and in keeping with the guidelines of the Canada Council on Animal Care. Founder mice heterozygous for insertion of *Cre* recombinase in the gene encoding mouse mast cell Cpa3 (*Cpa3*$^{Cre/+}$ mice on the *C57BL/6* background) were obtained from the German Cancer Research Center, DKFZ, Heidelberg, Germany. A colony was established by mating with WT *C57BL/6* mice (Charles River Laboratories, Senneville, Qc H9X 3R3, Canada) and the offspring genotyped as described using PCR of DNA isolated from ear punch biopsies (Ramirez-GarciaLuna et al., 2017). Briefly, the ear punches were digested in 100 µl alkaline lysis buffer for 1 hour at 95°C and then neutralized with 100 µl neutralization buffer. Mice were genotyped by using a combination of three oligonucleotides (common 5': *GGA CTG TTC ATC CCC AGG AAC C*; 3'-WT: *CTG GCG TGC TTT TCA TTC TGG*; 3'-KI: *GTC CGG ACA CGC TGA ACT TG*), yielding 320 bp (*Cpa3*$^{+/+}$) and 450 bp (*Cpa3*$^{Cre/+}$) products. *Cpa3*$^{Cre/+}$ mast cell-deficient male and female offspring were separated, and 3-4 mice/cage maintained with free access to food and water.

### *Mast cell isolation and culture*

Bone marrow was isolated from 8-week old female *C57BL/6 WT* and *Cpa3*$^{Cre/+}$ mice as described before (Gao et al., 2013). Briefly, the soft tissue of the femora was removed under aseptic conditions before cutting the bones in half and placing them in Eppendorf tubes and centrifuging at low speed to dislodge the marrow. Bone marrow from individual mice (total 3) was -suspended in alpha-Minimal Essential Medium (α-MEM) containing 1% antibiotic/antimycotic (Sigma-Aldrich, St. Louis, MO, USA) and 10 % fetal bovine serum (FBS) (Wisent, St. Bruno, QC, Canada) and 1.5 x $10^6$ cells were plated in 75 $cm^2$ tissue culture dishes. After three days in culture at 37°C in a 5% $CO_2$ atmosphere, non-adherent cells were recovered, centrifuged, resuspended in Roswell Park Memorial Institute (RPMI) medium + 10% FBS, and plated in 75 $cm^2$ plates at a density of 3 x $10^6$. Suspension cells were then differentiated into MC as described by Feyerabend et al. (Feyerabend et al., 2005) by supplementing with 10ng/mL rIL-3 (R&D Systems, Minneapolis, MN, USA) and 25ng/ml stem cell factor (R&D Systems) for 4 weeks. Assessment of differentiation into MC lineage was performed by acidified toluidine blue staining (aTB) and immunostaining with CD117 (Bioss bs-0672R, RRID:AB_2725777, 1:200) and tryptase (Abcam ab134931, RRID:AB_2783526, 1:300) anti-sera.

### *RNA extraction and quantitative real time PCR (qPCR)*

Total RNA was isolated from cells in culture at the end of week 1, 2 and 3 using Trizol (Thermo Scientific). DNA was eliminated using DNA-free TM kit (DNAfree TM DNA removal kit, Life Technologies), RNA concentration was measured using a Nanodrop machine (Thermo), and cDNA was synthetized from 1µg of RNA using a reverse transcription kit (QuantaBio). Amplification of specific cDNA was afterwards performed using a thermal cycler (Applied

Biosystems) programmed for 35 cycles of denaturation at 94°C for 30s, annealing at 54°C for 30s and extension at 72°C for 1 min. PCR products were measured in a Nanodrop machine and aliquoted at a concentration of 1mg/50µL. Finally, five µL of PCR products were fractionated by electrophoresis on a 1.5% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen) before being photographed under UV light. All the primers used for qPCR are listed in Table 1.

*Table 4.1 qPCR primers*

| Gene | Forward primer | Reverse primer | Size (bp) | Reference |
|---|---|---|---|---|
| **Cpa3** | GGA CTG TTC ATC CCC AGG AAC C | WT: CTG GCG TGC TTT TCA TTC TGG<br>KI: GTC CGG ACA CGC TGA ACT TG | WT: 320<br>KI: 450 | PloS One. 2017;12(3):e0174396. |
| **mMCP-1** | CTT CAA CTT TAT CCC ACC | CAC AAA ACC TGC ACT ATT C | 500 | Blood. 2005;105(11):4282-9. |
| **Fcer-1** | TGC CCA GCT GTG CCT AGC AC | CCA CCT GCC TAA GAT AGC CC | 527 | Blood. 2005;105(11):4282-9. |
| **C-kit** | CAT CCA TCC AGC ACA ATC AG | AAC ACT CCA GAA TCG TCA ACT G | 759 | Blood. 2005;105(11):4282-9. |
| **GAPDH** | GGT GAA GGT CGG TGT CAA CG | CAA AGT TGT CAT GGA TGA CC | 496 | Hum Mol Genet. 2004;13(3):271–84. |

### *Cell characterization by flow cytometry*

After 4 weeks in culture, cells were harvested, counted and $1.5x10^6$ transferred to FACS tubes. The cells were washed with FACS buffer and centrifuged at 689g for 5 minutes. The cells were resuspended in 25 µL of FACS buffer containing anti-CD117 (BD Biosciences) and anti-Fcer1 APC (Thermo Fisher) and incubated for 15 minutes at room temperature in the dark. Samples were washed once with FACS buffer (689g for 5 minutes), followed by a wash with PBS (689g for 5 minutes). Cells were incubated with a Fixable Viability Dye (Thermo Fisher) for 30 minutes at 4oC. Samples were washed once with PBS followed by a 10-minute incubation with 2% paraformaldehyde in PBS at room temperature in the dark. Finally, cells were washed twice with FACS buffer (689g for 5 minutes) and analyzed on a BD Biosciences LSR-II flow cytometer.

### *Adoptive mast cell transfer*

Mast cell reconstitution of *Cpa3*$^{Cre/+}$ mice was performed using a modified procedure by Feyerabend et al. (Feyerabend et al., 2011). Briefly, after confirming mast cell differentiation by FACS, cells from individual animals were pooled and aliquots of $5x10^6$ cells in 200 µL of serum and cytokine free RPMI were prepared and stored on ice until use. 8-week old male *Cpa3*$^{Cre/+}$ mice (n = 9) were used as transplant recipients of the mast cells via intra-peritoneal injection. No more than 15 minutes were elated from the preparation of the cell aliquots to their injection.

Eight weeks after the transplant, three animals were euthanized and skin, peritoneum, small intestine, lungs, and femora were harvested, fixed in 4% PFA and embedded in paraffin for histological sectioning. The remainder 6 mice underwent a skin biopsy under anesthesia and the

skin was prepared for histological analysis as described before. 5-micron thick sections were prepared from all tissue samples and stained with aTB to identify granulated mast cells.

### *Assessment of mast cells in healing bone*

Bilateral femoral defects measuring 1mm x 2mm were drilled on the anterolateral aspect of the femora of three *WT* mice, the six remaining mast cell reconstituted *Cpa3*$^{Cre/+}$, and three *Cpa3*$^{Cre/+}$ mice under anesthesia, as described previously (Behrends et al., 2017; Ramirez-Garcia-Luna et al., 2019). For pain control, 10 mg/kg carprofen with 0.1 mg/kg buprenorphine in 0.5 mL of sterile saline was administered IP immediately after wound closure, and 5 mg/kg carprofen was injected daily for three days post-operative thereafter. The cohorts were euthanized by $CO_2$ asphyxiation under anesthesia at 14 days post-operative and the femora fixed overnight in 4% paraformaldehyde. Bones were decalcified, embedded in paraffin, sectioned and stained using established protocols (Henderson et al., 2011). Serial 5 µm mid-sagittal sections were cut and stained with aTB to identify granulated mast cells and with CD117 and tryptase antisera to identify mast cells.

### *Mast cell intrafemoral transplant*

Aliquots of $3x10^6$ mast cells were prepared as described before, resuspended in a volume of 50 µL and stored on ice. Bilateral femoral defects measuring 1mm x 2mm were drilled on the anterolateral aspect of the femora of nine *Cpa3*$^{Cre/+}$ male mice and immediately after surgery, the cells were transplanted via intrafemoral injections, as described before (Mazurier, Doedens, Gan, & Dick, 2003). Briefly, the knee was flexed at the full range of motion and the cells were injected with a 25-G needle through the intercondylar region of the joint into the femur. For pain control,

10 mg/kg carprofen with 0.1 mg/kg buprenorphine in 0.5 mL of sterile saline was administered IP immediately after wound closure, and 5 mg/kg carprofen was injected daily for 3 days post-operative thereafter. The three animals per cohort were euthanized by $CO_2$ asphyxiation under anesthesia at 3, 5, and 14 days post-operative. The femora, skin and peritoneum samples were harvested and embedded in paraffin for histological analysis as described before.

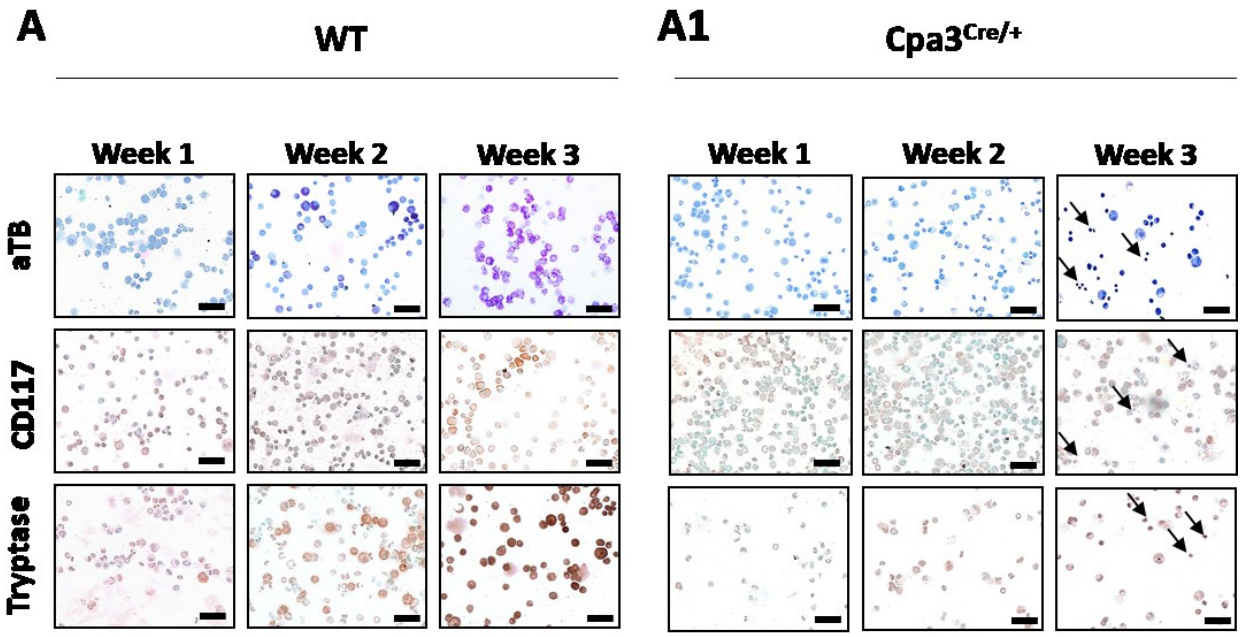
### *Statistical analysis*

This is a qualitative report. No quantitative data are presented in it.

## 4.4 Results

### *WT and Cpa3$^{Cre/+}$ mast cell precursors are isolated in culture but only WT cells reach maturity*

Bone marrow non-adherent cells from *WT* and *Cpa3$^{Cre/+}$* mice were cultured in RPMI supplemented with 10% FBS, IL-3 and SCF. After one week in culture, aTB staining did not show granulated mast cells in any strain, whereas CD117 and tryptase immunostaining showed the presence of cells positive for both markers in both strains. After two weeks in culture, more CD117 and tryptase positive cells, along with some granulated mast cells, were observed in *WT* cultures; while *Cpa3$^{Cre/+}$* cultures showed considerably fewer positive cells and no granulated mast cells. Finally, after three weeks in culture, most *WT* cells stained positive for aTB, CD117 and tryptase. *Cpa3$^{Cre/+}$* cells still showed reduced expression of CD117 and tryptase and absence of granulated aTB positive cells, however, multiple structures consistent with apoptotic cells were also identified (Fig. 1A). Gene analysis of other markers associated with mast cells showed a similar increase from week one to week three in *WT* cells, while *Cpa3$^{Cre/+}$* cell gene expression was considerably diminished, and no expression pattern was observed (Fig 1B). Taken together, the results strongly suggest that starting on week 1, mast cell precursors can be differentiated in culture for both strains, but that the development of mast cell granules, and thus, the culmination of the differentiation process, only happens in *WT* cells. In contrast, *Cpa3$^{Cre/+}$* mast cell precursors exhibit apoptosis and do not reach maturity, as described before (Feyerabend et al., 2011). However, precursor cells stain positive for CD117 and tryptase and show the gene expression of several mast cell proteins.


A
WT
A1
Cpa3Cre/+
Week 1
Week 2
Week 3
Week 1
Week 2
Week 3
aTB
CD117
Tryptase

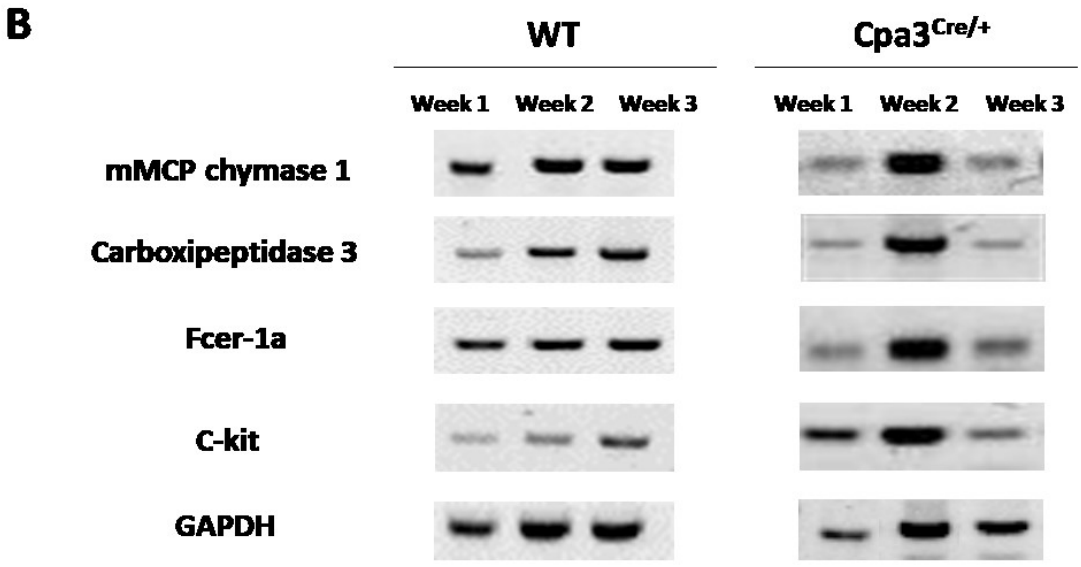

B
WT
Cpa3Cre/+
Week 1
Week 2
Week 3
Week 1
Week 2
Week 3
mMCP chymase 1
Carboxipeptidase 3
Fcer-1a
C-kit
GAPDH

***Figure 4.1 In vitro mast cell culture and differentiation***

*Bone marrow non-adherent cells from female WT or Cpa3Cre/+ mice (n = 3 per strain) were isolated and cultured in RPMI medium supplemented with IL-3 and SFC for 3 weeks to differentiate into mast cells. Staining and immunostaining of WT cells at 1, 2 and 3 weeks of culture (A) showed that granulated mast cells (aTB) started appearing after week 2, while CD117 and tryptase expression was observed starting in week 1 and peaking at week 3. In contrast, in Cpa3Cre/+ mice (A1) granulated mast cells never developed. However, CD117 and tryptase expression can be observed during the three time points, with diminishing expression, probably due to cell death, as time progress. At week 3, multiple apoptotic cells can be observed (arrows). Gene analysis for several mast cell markers (B) shows gradual increase in WT cells and erratic expression in Cpa3Cre/+ cells probably due to the fluctuating number of viable cells. Scale bar represents 50 μm.*

### *Cpa3$^{Cre/+}$ mast cell reconstitution populates the normal mast cell niches*

After four weeks in culture, all *WT* cells showed viability of 98% and almost all were found to be mast cells, as assessed by FACS (Fig. 2A, B). Aliquots of 5 x $10^6$ cells were injected intraperitoneally to nine *Cpa3$^{Cre/+}$* male mice and after eight weeks, three mice were euthanized and skin, peritoneum, small intestine, lungs, and femora were harvested, and tissue sections were prepared and stained with aTB to identify granulated mast cells. Mast cell distribution in the repopulated animals showed a similar distribution to the reported in *WT* animals. Granulated cells were observed in all organs, except for bone marrow (Fig 2. C to H). In the remaining six animals, skin biopsies from the dorsum confirmed the presence of granulated mast cells (images not shown), thus confirming the successful engraftment of the transplanted cells. Taken together, the results indicate that mast cell reconstitution in *Cpa3$^{Cre/+}$* mice populates the normal mast cell niches.

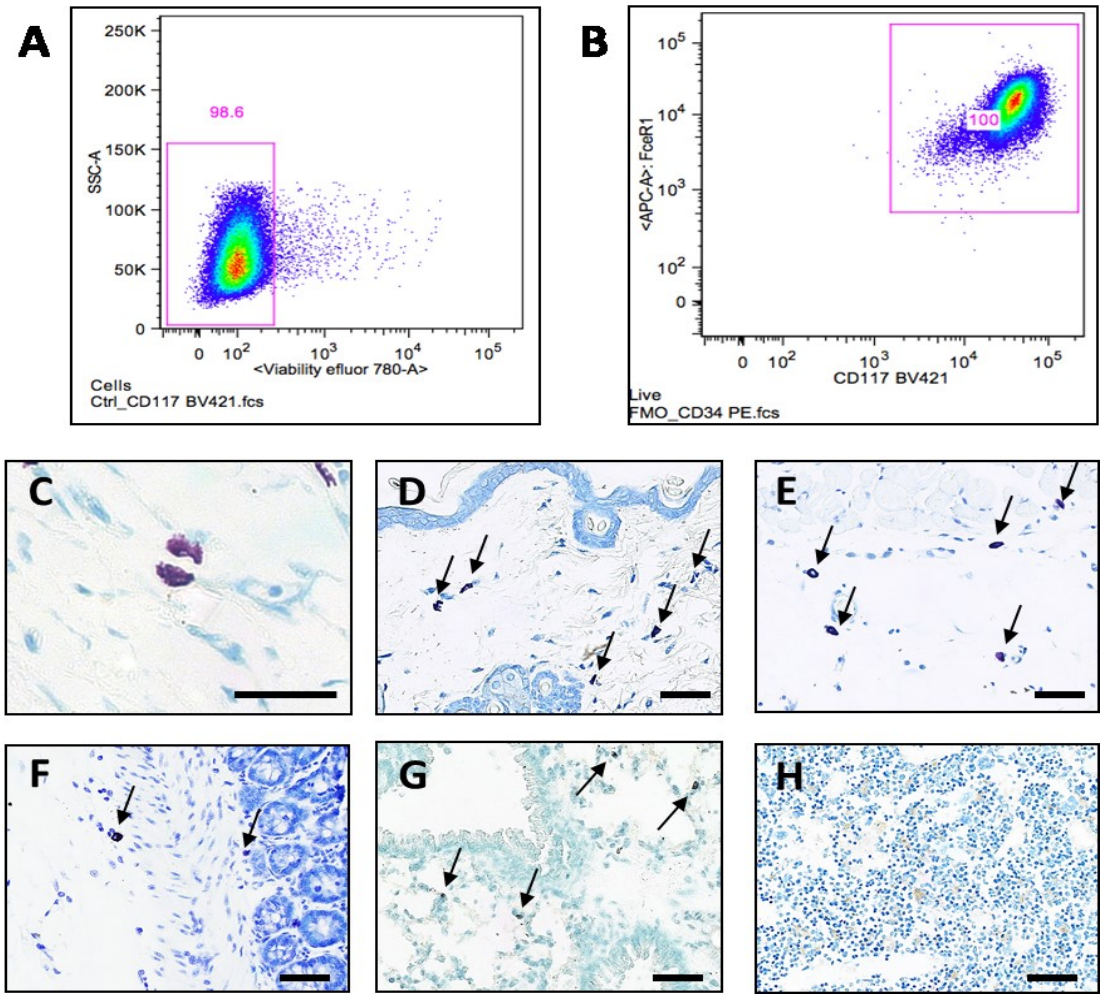



***Figure 4.2 Cpa3Cre/+ mast cell adoptive transfer***

*Mast cells were cultured and differentiated from female WT bone marrow cells (n = 3, pooled). After four weeks in culture, the cells had a viability of 98% (A) and all of them exhibited high levels of expression of CD117 and Fcer-1, as assessed by FACS analysis (B). Five million cells were injected intra-peritoneally to Cpa3Cre/+ male mice (n = 4), and the animals were euthanized 8 weeks later. Numerous granulated mast cells (C, arrows) were seen after aTB staining in the skin (D), peritoneum and abdominal musculature (E), small intestine (F), and lung (G), confirming the appropriate engraftment of the cells. No mast cells were observed in the bone marrow (H), as expected. Scale bar in C represents 20 µm. All other scale bars represent 50 µm.*

### *Granulated mast cells do not migrate into healing bone*

To determine if mast cells residing in peripheral organs would contribute to the mast cell population observed in healing bone, bilateral femoral window defects were drilled in the remaining six mast cell reconstituted *Cpa3*$^{Cre/+}$ mice 12 weeks after the transplant. As a positive control for this experiment, three *WT* littermates were used, and as a negative control, three *Cpa3*$^{Cre/+}$ mice were used. After 14 days of healing, granulated mast cells were found in the repair tissue (Fig. 3A to A2) and the medullary canal (Fig 3B to B2) of *WT* mice, however, no cells were identified in reconstituted *Cpa3*$^{Cre/+}$ mice nor in control *Cpa3*$^{Cre/+}$ mice. Immunostaining with CD117 (Fig. 3C to C2) and tryptase (Fig. 3D to D2) antisera showed numerous positive cells for both markers in all three groups. Taken together, the results suggest that the mast cells observed in healing bone do not migrate from the peripheral organs, but rather develop from precursor cells residing in the bone marrow. Granulated mast cells were observed in the skin of reconstituted *Cpa3*$^{Cre/+}$ mice, as well as CD117 and tryptase positive cells in post-mortem analysis; however, none of these were found in control *Cpa3*$^{Cre/+}$ mice (data not shown).

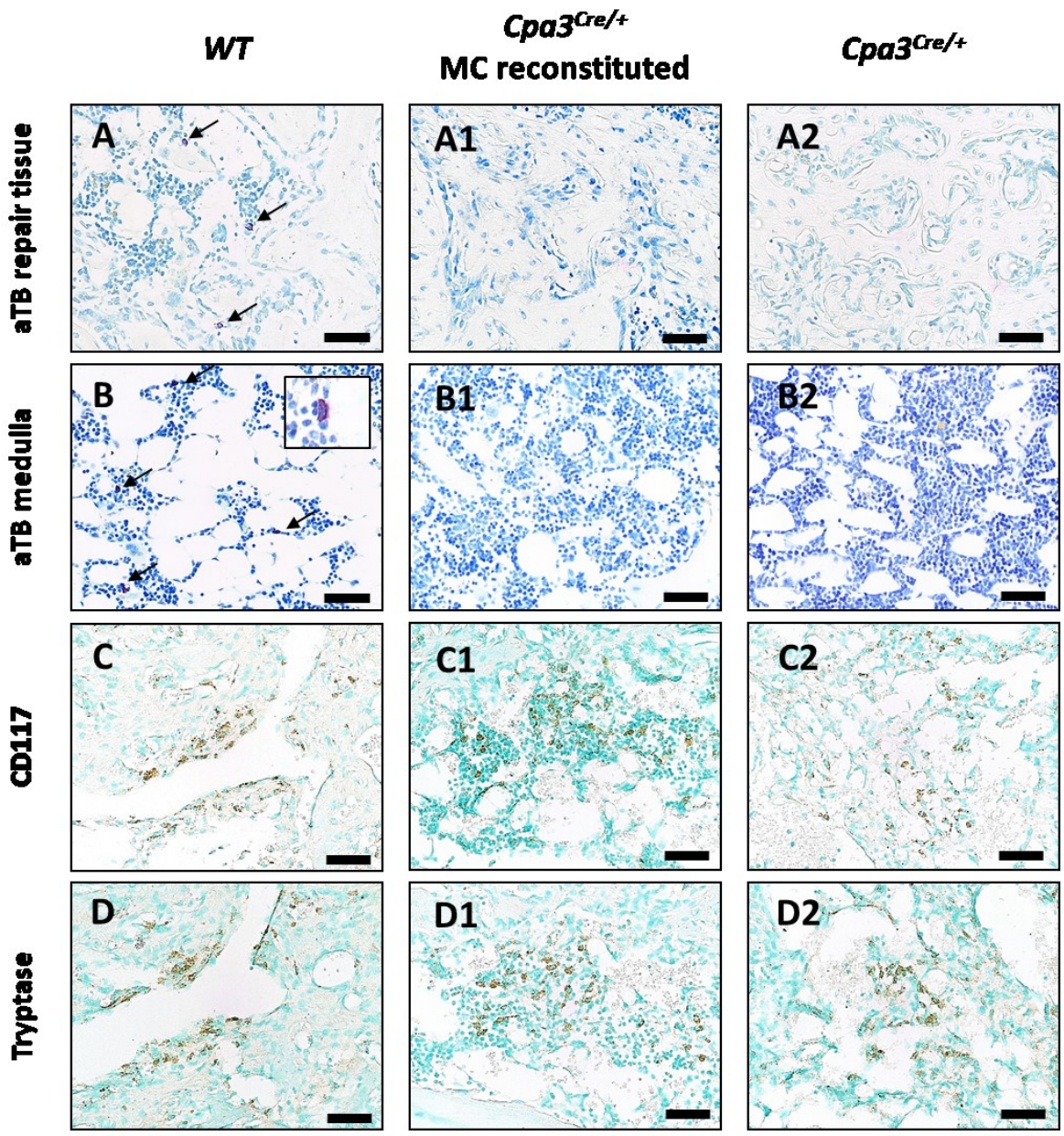



*Figure 4.3 Mast cells in healing bone*

*Bilateral femoral window defects were drilled in WT (n = 3), Cpa3Cre/+ mast cell reconstituted (n = 6) and Cpa3Cre/+ mice (n = 3). After 14 days of healing, the presence of mast cells was assessed using aTB staining. Granulated cells were identified in the repair tissue (A) and the adjacent medullary canal (B and insert) of WT mice. However, no granulated cells were observed*

*in Cpa3Cre/+ mast cell reconstituted (A1, B1) or Cpa3Cre/+ mice (A2, B2). Immunostaining using CD117 (C, C1, C2) and tryptase antisera (D, D1, D2) in the medullary canal adjacent to the defect showed positive cells in the three groups. Scale bars represent 50 µm.*

### *Mast cells transplanted after a fracture do not migrate to bone*

Finally, to determine if mast cells transplanted immediately after a fracture would be triggered to migrate into the repair tissue, bilateral femoral window defects were drilled in male *Cpa3*$^{Cre/+}$ mice and 3 x $10^6$ mast cells were transplanted intrafemorally immediately after surgery. Cohorts of three animals per group were euthanized at 3, 5, and 14 days post-operative. Granulated mast cells were not observed in the repair tissue or proximal medullary canal in any group. In contrast, mast cells were identified in the skin at all time points, thereby confirming the engraftment of the cells (Fig. 4). Thus, the results indicate that exogenously administrated mast cells do not migrate into healing bone.

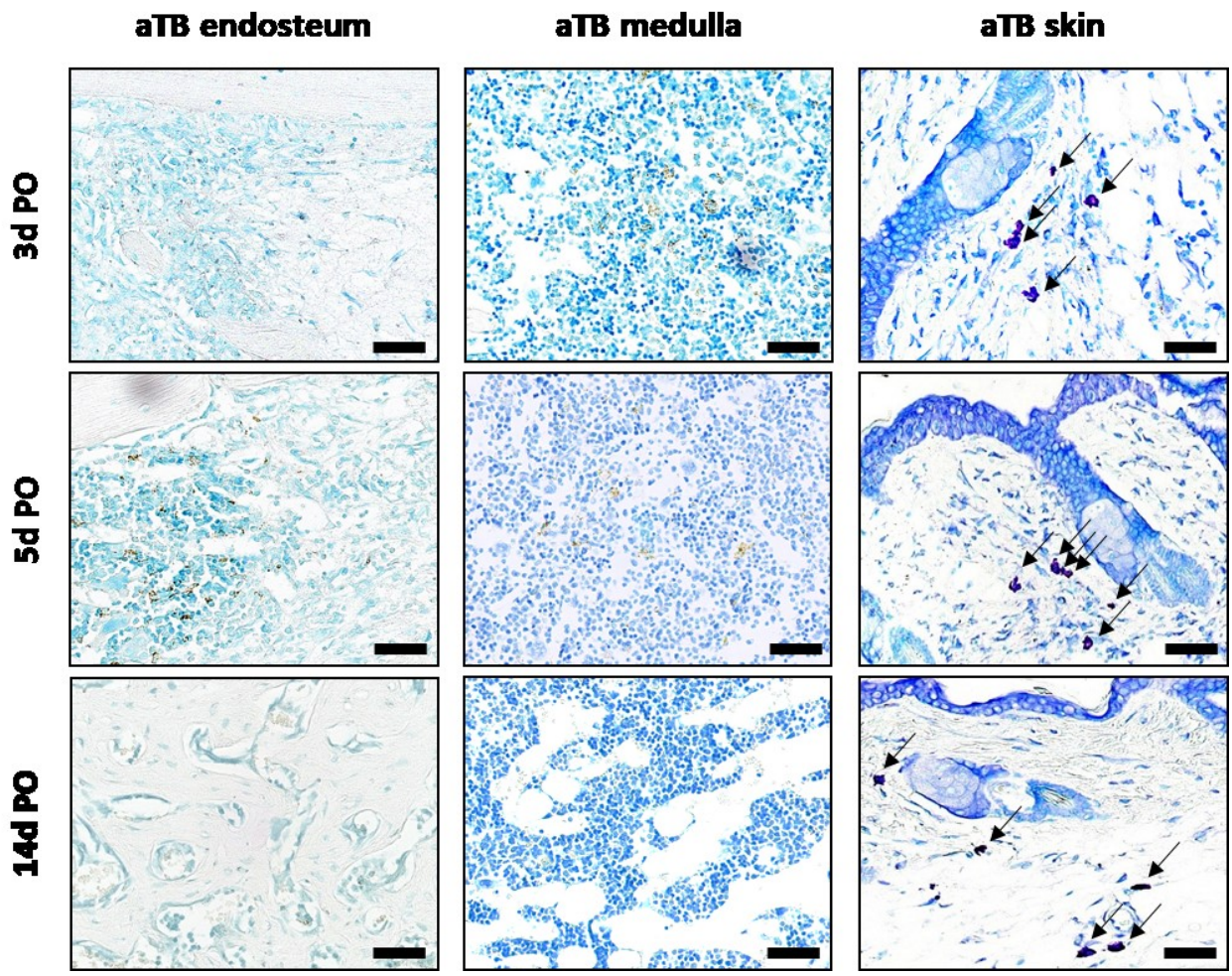



***Figure 4.4 Mast cell intrafemoral transplant***

*Bilateral femoral window defects were drilled in Cpa3Cre/+ mice (n = 3 per time point) and 3x106 mast cells were injected intrafemorally on each side immediately after surgery. After 3, 5, and 14 days of healing, the presence of mast cells was assessed using aTB staining. Granulated mast cells were not found in the repair tissue or in the medullary canal of the femora. However, they were observed in skin, confirming the transplant of the cells. Scale bars represent 50 µm.*

## 4.5 Discussion

Our previous research done in c-kit and c-kit independent models of mast cell deficient mouse strains demonstrated that bone repair is deficient in the absence of these cells (Behrends et al., 2014; Ramirez-GarciaLuna et al., 2017). However, the hallmark of any knock-out study is recuing of the observed phenotype by *in vivo* complementation (Eisener-Dorman, Lawrence, & Bolivar, 2009), and to our best knowledge, no studies of bone repair in mast cell reconstituted mice have been published. In this paper, we aimed to develop a model of mast cell reconstitution in mast cell deficient $Cpa3^{Cre/+}$ mice with the double objective of performing phenotype rescuing bone repair analyses and determining if exogenously administered mast cells could be used to promote better bone repair. While we demonstrate that mast cell repopulation of $Cpa3^{Cre/+}$ mice is achieved through the injection of exogeneous mast cells through intra-peritoneal or intra-femoral injections, we failed to observe any mature mast cells in the bone repair tissue, and thus, could not accomplish our initial objectives.

Mast cells are derived from myeloid precursors independent of monocyte or granulocyte precursor cells. Mast cell precursors exit the bone marrow and travel to peripheral tissues where they finish their maturation process and become granulated mast cells (Joakim S. Dahlin & Hallgren, 2015). Although mast cells in the peripheral tissues are considered to be terminally differentiated cells, they have demonstrated to have very long life spans and to be able to undergo repeated cycles of de-granulation and re-granulation events (Padawer, 1974; Xiang, Block, Löfman, & Nilsson, 2001). Increases in mast cell number in peripheral tissues observed both in human and animal experimental models of disease have been demonstrated to be partly due to *in*

*situ* proliferation (Kambe, Kambe, Kochan, & Schwartz, 2001; T. Sonoda et al., 1984), however it is considered that the majority of the cells arise from mast cell precursors that are recruited to the tissue. For example, increases in lung mast cell content during allergic inflammation has been found to be mediated by up-regulation of the expression of VCAM-1 by endothelial cells that bind to α4-integrin expressed in the membrane of mast cell precursors, thereby enabling them to enter the tissue. Once there, the precursors undergo mitosis and differentiation under the regulation of other inflammatory cells, such as T lymphocytes and macrophages (Abonia et al., 2006).

In line with these findings, our results strongly support the notion that the mast cells observed during bone repair arise from precursor cells *in situ* and do not come from cells migrating from peripheral organs. The *Cpa3*$^{Cre/+}$ mast cell deficient mouse model relies upon the co-expression of the *Cre* recombinase during the expression of carbopeptidase-3 during mast cell maturation, which then induces genomic damage due to the cleavage of pseudo-Lox flanked DNA of the cells, thereby promoting apoptosis (Feyerabend et al., 2011). Our *in vitro* results show that culturing bone marrow cells of *Cpa3*$^{Cre/+}$ mice promotes their differentiation into mast cell lineage, as shown by the positive expression of CD117 and tryptase, and the RNA expression of several other mast cell specific genes such as Fcer-1. However, precursor cells do not reach maturity, as demonstrated by the lack of granulated mast cells and the appearance of structures compatible with apoptotic cells (Fig. 1). Based in our *in vitro* results, this is an early event during mast cell maturation, probably within the first 7 days. In agreement with these observations, once mature mast cells are transplanted into *Cpa3*$^{Cre/+}$ mice, they populate the organs where they are traditionally found (Fig. 2). Nonetheless, once a fracture is induced, no granulated mast cells are

observed in the healing tissue, but CD117 and tryptase positive cells can be observed (Fig. 3), thus confirming the presence of precursor cells.

Mature mast cells activated by inflammatory stimuli have been found to be attracted and activated in injured tissue through the recognition of damage-associated molecular patterns (DAMPS) by toll-like receptors (TLRs) in their cell surface. Activation of TLRs results in the synthesis and secretion of several cytokines and inflammatory mediators that trigger extracellular matrix remodeling processes (Sandig & Bulfone-Paus, 2012). Our previous findings have demonstrated increases in mast cell content and degranulation in healing bone after the systemic administration of lipopolysaccharide (LPS) (Behrends et al., 2017; Ramirez-Garcia-Luna et al., 2019), highlighting the fact that the inflammatory environment provides cues that modulate mast cell behavior. In this context, we hypothesized that exogenous local mast cell administration after a fracture would promote mast cell recruitment through the recognition of DAMPS and that these cells may assist in the healing process. However, our results disprove this hypothesis and show that mast cells transplanted intrafemorally migrate to their normal niches and are not retained by the healing tissue. Whether the local delivery of mast cells via a biomaterial implant, the transplant of previously-activated mature mast cells or of mast cell precursors can trigger the recruitment of these cells into bone repair tissue is yet to be elucidated. Nonetheless, the results disprove our theory that exogenous mature mast cell administration could be used to enhance fracture repair in patients at high risk of fracture complications. Probably the use of other mechanisms unique to mast cells, such as immunogenic memory and antigen priming could be used to deliver their factors to broken bone.

In conclusion, our results show that the mast cells involved in bone healing locally arise from bone marrow precursor cells and that mature mast cells exogenously administered after a fracture do not home to the repair tissue. Phenotype rescuing experiments to confirm the role of mast cells in bone healing is still needed. Reconstitution of the mast cell precursor pool by full bone marrow transplant in irradiated mice would probably be an appropriate alternative to conduct this experiment.

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**Author roles**

Study design and execution: JRGL, MA, JEH, PAM. Provision of mast cell-deficient mice: TBF, HRR Data collection and analysis: JRGL, MA, DC, LG, AVT. Data interpretation: JRGL, TBF, HRR, JEH, PAM. Manuscript draft: JRGL, JEH, PAM. All authors share responsibility for the integrity of the data analysis.

# V. DEFECTIVE BONE REPAIR IN DICLOFENAC TREATED *C57BL/6* MICE WITH AND WITHOUT LYPOPOLYSACCHARIDE INDUCED SYSTEMIC INFLAMMATION

This section aims to fulfill our fourth research objective, quantify bone repair and mast cell activity during the inflammatory phase of bone healing in *WT* mice treated with a non-steroidal anti-inflammatory drug (NSAID) with or without lipopolysaccharide (LPS) induced systemic inflammation. Previous work done in the lab demonstrated that granulated mast cell numbers were greatly diminished during bone repair under acute systemic inflammation induced by LPS (Behrends et al., 2017). However, the effect of chronic systemic anti-inflammation on mast cell activity had not been tested. Moreover, because we also expected the change in the inflammatory environment to have some effect in bone repair, we sought to investigate if the chronic administration of NSAID would rescue the phenotype of LPS-induced systemic inflammation. These research questions were based on the observation that mast cell plasticity allows them to express different mediators and interact in different ways with the surrounding tissue based on environmental cues. Interestingly, our results showed that chronic treatment with diclofenac, a non-selective NSAID, promoted mast cell hyperplasia in the bone marrow, probably due to the accumulation of non-degranulated cells. Additionally, we found that the use of NSAIDs does not reverse the LPS-induced systemic inflammation phenotype, but rather adds to the dysregulation of the repair tissue. This section has been published as Ramirez-Garcia-Luna JL, Wong TH, Chan D, Al-Saran Y, Awlia A, Abou-Rjeili M, et al. Defective bone repair in diclofenac treated C57Bl6 mice with and without lipopolysaccharide induced systemic inflammation. *J Cell Physiol*. 2019 Mar;234(3):3078–87.

# DEFECTIVE BONE REPAIR IN DICLOFENAC TREATED *C57BL/6* MICE WITH AND WITHOUT LYPOPOLYSACCHARIDE INDUCED SYSTEMIC INFLAMMATION

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## 5.1 Abstract

Bone repair following trauma or surgical intervention involves a tightly regulated cascade of events that starts with hemostasis and an inflammatory response, which are critical for successful healing. Non-steroidal anti-inflammatory drugs (NSAID) are routinely prescribed for pain relief despite their potential inhibitory effect on bone repair. The goal of this study was to determine the impact of administration of the non-selective NSAID diclofenac in the inflammatory phase of bone repair in mice with or without lipopolysaccharide-induced systemic inflammation.

Repair of femoral window defects was characterized using micro CT imaging and histological analyses at 2 weeks post-operative. The data indicate 1) impaired bone regeneration associated with reduced osteoblast, osteoclast and macrophage activity; 2) changes in the number, activity, and distribution of mast cells in regenerating bone and 3) impaired angiogenesis due to a direct toxic effect of diclofenac on vascular endothelial cells. The results of this study provide strong evidence to support the conjecture that administration of NSAIDs in the first 2 weeks after orthopaedic surgery disrupts the healing cascade and exacerbates the negative effects of systemic inflammation on the repair process.

## 5.2 Introduction

Successful wound healing following trauma or surgical intervention involves a tightly regulated cascade of events that starts with hemostasis and an inflammatory response, then proceeds through phases of repair and remodelling (Steen, Gerstenfeld, & Einhorn, 2016). Circulating neutrophils are first to arrive and release stored mediators including anti-bacterial agents to fight pathogens and enzymes like peroxidase, matrix metalloproteinase and collagenase to remove damaged matrix. Circulating monocytes extravasate and migrate into the wound where they undergo differentiation into M1 pro-inflammatory macrophages. Resolution of this acute inflammatory reaction to tissue injury, mediated primarily by neutrophils and M1 macrophages, is arguably the most critical stage of wound healing (Claes et al., 2012). Early reports suggesting mast cells play a key role in the inflammatory phase of bone repair (Banovac et al., 1995; R. Lindholm et al., 1969) have recently been substantiated (Behrends et al., 2014, 2017; Ramirez-GarciaLuna et al., 2017; L. Zhang et al., 2017). Taken together these studies suggest that impaired recruitment of mast cells during bone repair leads to disruption of macrophage, vascular endothelial and bone cell function, culminating in failure to heal.

Non-steroidal anti-inflammatory drugs (NSAID) such as diclofenac are routinely prescribed for pain relief after surgical reconstruction of orthopaedic injuries, despite their reported inhibitory effect on bone repair (Dodwell et al., 2010). Pre-clinical studies suggest that NSAID inhibition of cyclooxygenase-2 (COX2) and PG production during the inflammatory phase of bone healing results in 1) impaired communication between osteoclasts and osteoblasts via the RANK/RANKL mechanism (Haversath, Catelas, Li, Tassemeier, & Jäger, 2012); 2) suppression of canonical Wnt signalling in osteoblasts (Nagano, Arioka, Takahashi-Yanaga, Matsuzaki, &

Sasaguri, 2017) and 3) impaired endothelial progenitor cell proliferation, which is necessary for angiogenesis (Colleselli, Bijuklic, Mosheimer, & Kähler, 2006). An early study on fracture healing also suggested a negative impact of NSAID might be mediated by delayed recruitment of mast cells to repair tissue (Banovac et al., 1995). The goal of this study was to determine the impact of NSAID administration during the inflammatory phase of bone healing in a stable cortical window defect in the absence and presence of lipopolyssacharide (LPS)-induced systemic inflammation.

## 5.3 Methods

### *Mice and treatment groups*

All live animal procedures were conducted in accordance with a protocol approved by McGill MGH Facility Animal Care Committee in keeping with the guidelines of the Canada Council on Animal Care. Male and female *C57BL/6* mice (Charles River Laboratories, Senneville, Qc H9X 3R3, Canada) were housed 3-4 mice/cage with free access to food and water up to 5 months of age before using in experiments. Bilateral femoral defects measuring 1mm x 2mm were drilled on the anterolateral aspect of the femora of skeletally mature (5-8 months) anesthetized mice essentially as described previously (Gao et al., 2013). Mice were then randomly assigned to one of the following treatment groups: **1) Placebo** treated mice received a placebo pellet (Innovative Research of America, Sarasota, FL) implanted under the dorsal neck skin and daily IP injections of sterile PBS; **2) Diclofenac** treated mice received 5 mg diclofenac/kg/day via sustained release pellet to maintain a circulating level of 0.238 mg/ml (Innovative Research); **3) Diclofenac + LPS** treated mice received a diclofenac pellet with IP injection of 25µg LPS (Sigma-Aldrich,

Oakville ON) diluted in 0.1ml of sterile PBS for 7 days, which represents 1/10 of the lethal dose documented for mice. For pain control, 10 mg/kg carprofen with 0.1 mg/kg buprenorphine in 0.5 mL of sterile saline was administered IP immediately after wound closure, and 0.1 mg/kg buprenorphine injected twice a day for 3 days post-operative thereafter. The cohorts were euthanized by $CO_2$ asphyxiation under anesthesia at 14 days post-operative and the femora fixed overnight in 4% paraformaldehyde prior to micro CT imaging.

### *Micro CT analysis*

Micro CT analyses of bone and vascular channels were performed on a Skyscan 1172 instrument (Bruker, Kontich, Belgium) at a resolution of 5 microns using NRecon v.1.6.10.4 to generate 3D models and CTAn v.1.16.4.1 software for quantitative analysis (Bruker) as described previously (Ramirez-GarciaLuna et al., 2017). Quantitative data for bone and vessel regeneration was recorded in a region of interest 1.5 mm long, 1.0 mm wide and 1.3 mm in depth spanning the defect and medullary canal. Bone parameters include bone volume/tissue volume (BV/TV %), trabecular number (Tb.N /$mm^{-1}$), trabecular thickness (Tb.Th μm), trabecular separation (Tb.Sp μm), total porosity (Po.Tot %) connectivity density (Conn.Dn $mm^{-3}$). Vascular parameters include: channel volume/tissue volume (ChV/TV %), channel number (Ch.N $mm^{-1}$), channel thickness (Ch.Th μm), and connectivity density (Conn.Dn. 1/$mm^{-3}$).

### *Histological analyses*

Bones were decalcified, embedded in paraffin, sectioned and stained using previously described protocols (Henderson et al., 2011). Serial 5 μm sections were cut and stained for alkaline phosphatase (ALP) activity in anabolic cells, tartrate resistant acid phosphatase (TRAP)

activity in catabolic cells and acidified toluidine blue (aTB) to identify granules in mast cells. Immunohistochemistry was performed using the following antisera: CD117 (Bioss bs-0672R, RRID:AB_2725777, 1:200) in mast cells; CD34 (Abcam ab23830 1:300) in vascular endothelial cells; F4/80 (Abcam ab6640 1:200) in all macrophages and Arginase-1 (Santa Cruz sc-271430, RRID:AB_626697, 1:300) in M2 anabolic macrophages. A Zeiss Axioskop 40 microscope (Carl Zeiss, Toronto, ON, Canada) was used to capture high resolution images and staining intensity analyzed using ImageJ v.1.6.0 software.

### *Isolation, culture and metabolic activity of cells*

Endothelial cells were isolated from the lungs of 8 week old WT C57BL/6 mice using two rounds of ICAM-2 immuno-selection as described (Robins et al., 2013). Isolated cells were cultured in 50% endothelial cell basal medium (EBM-2, Lonza, Walkersville, MD, USA) supplemented with endothelial cell bullet kit (EGM-2, Lonza) and 50% DMEM/F12 (Wisent, QC, Canada) supplemented with 10% fetal bovine serum (FBS, Wisent, QC, Canada). Mast cell precursors were isolated from the non-adherent fraction of bone marrow flushed from the femora and tibia of 8-week old WT C57BL/6 mice as described (Valverde-Franco et al., 2004). Suspension cells were differentiated into mast cells as described by Feyerabend et al. (Feyerabend et al., 2005). Cells were seeded at a density of 2.5 x $10^6$ in RPMI supplemented with 10% FBS (Wisent), 10ng/mL rIL-3 (R&D Systems, Minneapolis, MN, USA) and 25ng/ml stem cell factor (R&D Systems). After 4 weeks cells were subjected to flow cytometric analyses and smears prepared for staining to determine the number of mast cells. For experiments, endothelial cells were used between passages 3 and 5 and mast cells between week 4 and 5 of differentiation. Metabolic activity was assessed using the Alamar Blue assay (Thermo-Fisher, Nepean, ON,

Canada) on cells seeded at a density of 1 x $10^4$ cells/well in 96-well and cultured overnight in complete medium. Cells were fasted for 24 hours in the appropriate medium before adding diclofenac (Sigma-Aldrich) to reach concentrations of 1 x $10^{-2}$ M, 1 x $10^{-3}$ M or 1x$10^{-4}$ M, using basal medium as control, and using Alamar Blue according to the manufacturer's directions.

### *Statistical analysis*

Quantitative data are expressed as Mean ± SD and R v.3.4.1 (R Core Team, 2017) statistical program used for analysis of variance (ANOVA), with Tukey post-hoc analysis for multiple comparisons. Differences were considered significant at $p < 0.05$.

## 5.4 Results

### *Micro CT analysis of bone and vessel repair*

Tissue regeneration in the femoral defects of skeletally mature C57Bl/6 mice was assessed at 14 days post-operative when the soft callus is being replaced by primary bone. Mid-saggital micro CT images (Fig 1A) and quantitative data (Fig 1B) showed less bone with a proportional reduction in vessels in mice treated with diclofenac, with or without LPS, compared to placebo control. Quantitative analysis of bone and vessel volume relative to the volume of the ROI (white rectangle) confirmed a significant reduction in bone (grey) and vessel (red) volume per tissue volume in diclofenac treated mice, which was marginally increased in the presence of LPS-induced inflammation. Quantification of parameters of bone and vessel architecture (Table 1) revealed diclofenac mediated thickening of trabeculae and vascular channels, accompanied by increased porosity. In mice with LPS-induced systemic inflammation bone and vessel architecture was further compromised by treatment with diclofenac, with notable reductions in bone and vessel connectivity (Conn.D).

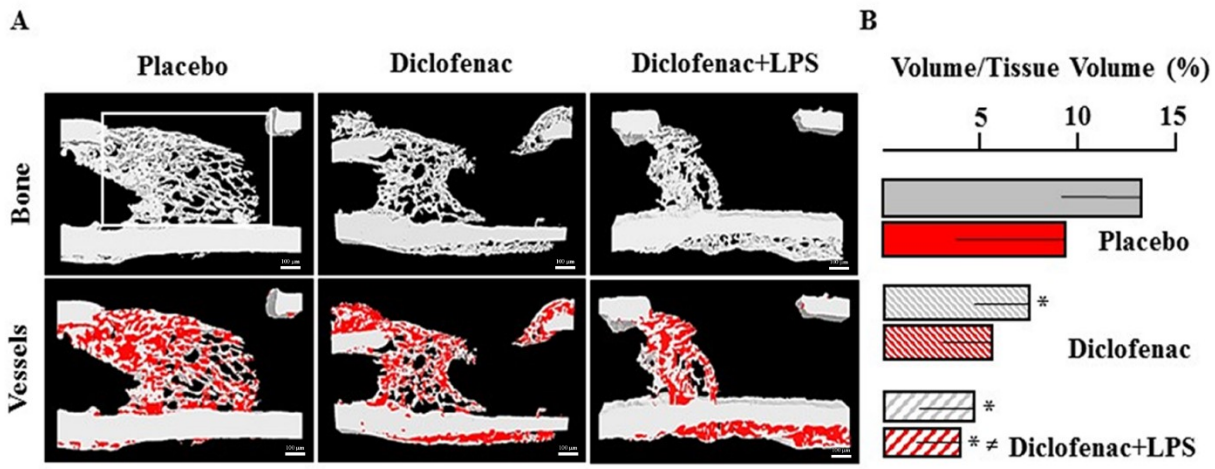


***Figure 5.1 2D micro CT images and 3D volumetric analysis of healing bone***

*Femora harvested at 2 weeks post-operative were scanned at a resolution of 5 microns on a Skyscan 1172 instrument. Representative 2D mid-saggital images (A) show reductions in bone and vessels in mice treated with diclofenac compared with placebo control, and a further reduction in those treated with diclofenac + LPS. Quantitative data (B) captured in the region of interest (white rectangle) confirmed the reductions in bone (grey) and vessel (red) volume in both treatment groups compared with control. ANOVA with Tukey post-hoc test was applied to data from N=9 placebo, N=10 diclofenac and N=10 diclofenac + LPS treated mice. Significantly different from placebo control * $p<0.01$; significantly different from diclofenac treated mice ≠ <0.05. Scale bars in panel A represent 100 µm.*

*Table 5.1 Quantitative micro CT analysis at 2 weeks post-operative*

| Parameter | Placebo | Diclofenac | Diclofenac + LPS |
|---|---|---|---|
| | N=9 | N=10 | N=10 |
| **Medulla Bone** | | | |
| Tb.N | 3.6 ± 1.2 | 2.4 ± 1.2* | 1.8 ± 1.3** |
| Tb.Th | 45.0 ± 12.6 | 30.7 ± 4.4** | 32.7 ± 8.8** |
| Tb.Sp | 336 ± 166 | 397 ± 136 | 665 ± 180**†† |
| Po.Tot | 86.0 ± 6.6 | 92.7 ± 3.7** | 94.6 ± 3.9** |
| Conn.D | 1.7 ± 0.9 | 1.1 ± 0.5 | 0.8 ± 0.6** |
| **Medulla Vessels** | | | |
| Ch.N | 2.7 ± 1.7 | 1.7 ± 0.9* | 1.4 ± 1.0* |
| Ch.Th | 29.6 ± 5.5 | 38.5 ± 3.7** | 27.2 ± 5.8†† |
| Ch.Sp | 454 ± 199 | 557 ± 239 | 678 ± 171* |
| Conn.D | 1.8 ± 1.2 | 1.2 ± 0.7 | 0.7 ± 0.6** |

*Analysis of regenerated bone and blood vessels was performed in the region of interest indicated in Figure 1A. ANOVA with Tukey post-hoc test was applied to data from N=9 placebo, N=10 diclofenac and N=10 diclofenac + LPS treated mice. Significantly different from placebo control * p <0.05, ** p<0.01; significantly different from diclofenac treated mice: †† p<0.01. Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Po.Tot = total porosity. Conn.D = connectivity density; Ch.N channel number; Ch.Th = channel thickness; Ch.Sp = channel separation; Conn.D = connective density.*

### *Recruitment of bone cells and macrophages to regenerating tissue*

A low power micrograph (Fig 2A) indicates regions of interest in the proximal (ROI 1) and distal (ROI 2) medulla adjacent to the gap. Adjacent sections were stained for ALP activity in osteoblasts, TRAP activity in osteoclasts, Arginase-1 in M2 alternatively activated macrophages and F4/80 in all macrophages (Fig 2B). ALP and TRAP activity was focused in ROI 1, Arg-1 staining at the bone/marrow interface between ROI 1 and ROI 2, and F4/80 staining in ROI 2. Compared with placebo controls, staining was reduced in all mice treated with diclofenac, with or without LPS treatment. Quantitative analyses (Fig 2C) confirmed a significant reduction in staining for both anabolic (ALP, Arg-1) and catabolic (TRAP, F4/80) markers in diclofenac treated mice and further reduced in macrophages in mice treated with LPS.

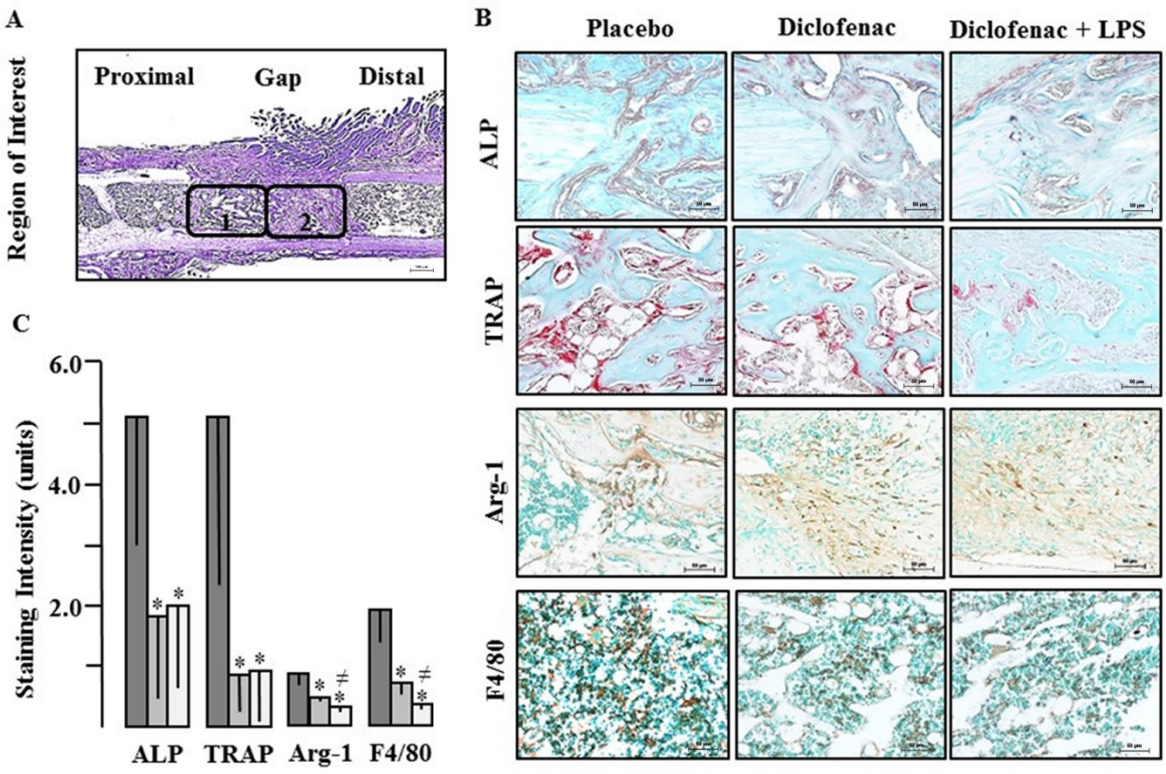


***Figure 5.2 Cellular activity in regenerating bone***

*After micro CT analyses femora were decalcified, embedded in paraffin and 5-micron sections stained with toluidine blue (A) to demonstrate the proximal (1) and distal (2) Regions of Interest (ROI) where micrographs of specific stains (B) are localised. Alkaline phosphatase (ALP) stains osteoblasts and tartrate resistant acid phosphatase (TRAP) stains osteoclasts adjacent to bone spicules in ROI 1. Images for Arginase-1 (Arg-1) in primarily M2 macrophages and F4/80 in all macrophages were captured in ROI 2. Quantitation of staining intensity (C) shows the highest level in Placebo control (dark grey) and lower levels in mice treated with diclofenac (medium grey) and diclofenac + LPS (light grey). ANOVA with Tukey's post-hoc test was applied to measurements from sections from N=4 to N=12 mice. Significantly different from placebo control * $p<0.01$; ≠ significantly different from diclofenac treated mice: $p<0.01$. Scale bar in panel A represents 100µm and in panel B 50µm.*

### *Recruitment of mast cells to regenerating tissue*

The number and location of MC in regenerating bone and marrow was quantified on adjacent sections of femora stained with aTB, to identify mast cell granules, or with CD117 antiserum to the c-kit membrane receptor (Fig 3). More than 90% of mast cells were distributed approximately evenly throughout healing tissue (Fig 3A squares) in the medullary canal, with the remaining cells located adjacent to the defect (red triangles) between the periosteum and muscle. Quantitative data (Fig 3B) showed a significant increase in mast cells, stained by either method, in mice treated with diclofenac alone compared with those receiving placebo or diclofenac + LPS. Of note was the discrepancy between aTB stained cells and CD117 stained cells in mice treated with diclofenac + LPS, which had fewer aTB positive cells and similar numbers of CD117 positive cells as placebo control. Fig 3C shows representative, high magnification images of mast cells stained with aTB (upper) and CD117 (lower).

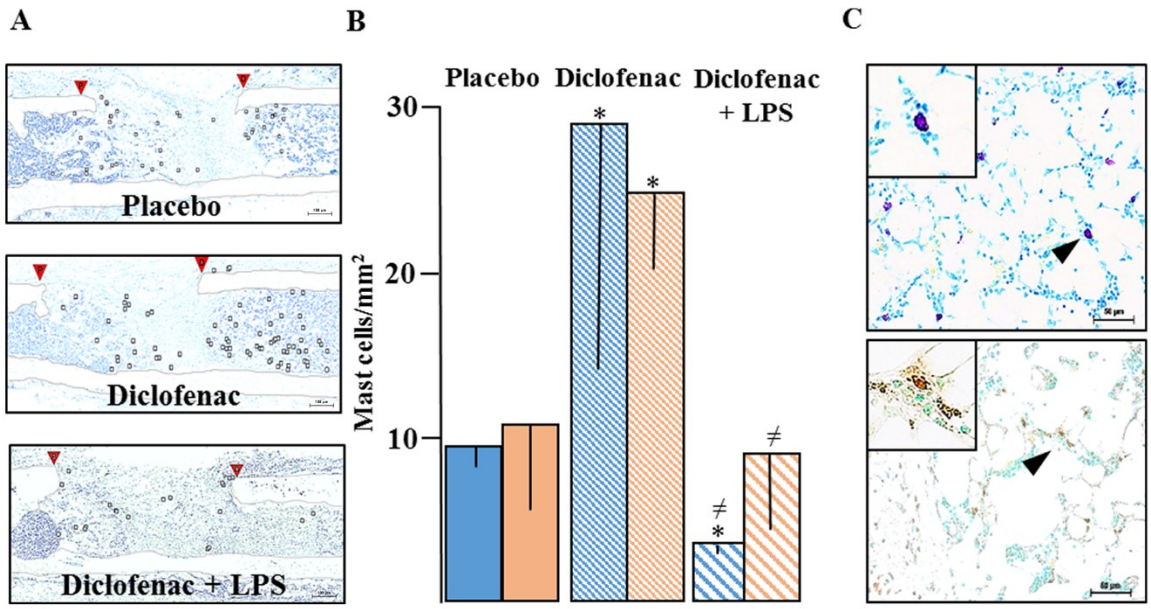


***Figure 5.3 Localisation of mast cells in healing bone***

*Serial 5-micron sections of decalcified bone were stained with acidified toluidine blue (aTB) (A) to quantify mast cells in the medullary canal in relationship to the defect (red arrowheads). Quantification of mast cells (B) stained with aTB (blue) or immunostained with CD117 antiserum to the c-kit membrane receptor (orange) revealed more mast cells in mice treated with diclofenac alone compared with placebo or those treated with diclofenac and LPS. High magnification of aTB stained (C upper) and CD117 stained (C lower) mast cells are representative of those from N=8 placebo, N=8 diclofenac and N=5 diclofenac + LPS treated mice. ANOVA with Tukey's post-hoc test: significantly different from placebo control * $p<0.01$; significantly different from diclofenac treated mice ≠ $p<0.01$. Scale bars in panel A represent 100µ and in panel C 50µm.*

### *Re-vascularization of regenerating tissue*

CD34 immunohistochemistry was used to identify vascular endothelial cells lining channels at the bone/marrow interface in the medullary canal and in periosteal bone adjacent to the femoral defect (Fig 4). CD34 positive immunoreactivity was seen in well-defined channels penetrating newly formed bone in the proximal region of the medulla and aligning with the longitudinal axis of the femur in periosteal bone (Fig 4A). In mice treated with diclofenac alone, there are fewer CD34 positive cells associated with wider channels, while mice treated with diclofenac and LPS appeared to have few defined channels and many isolated CD34 positive cells scattered throughout the marrow. Quantification (Fig 4B) confirmed significant reductions in staining intensity between placebo control and diclofenac treated mice, but no significant difference between mice treated with diclofenac alone and those receiving diclofenac + LPS.

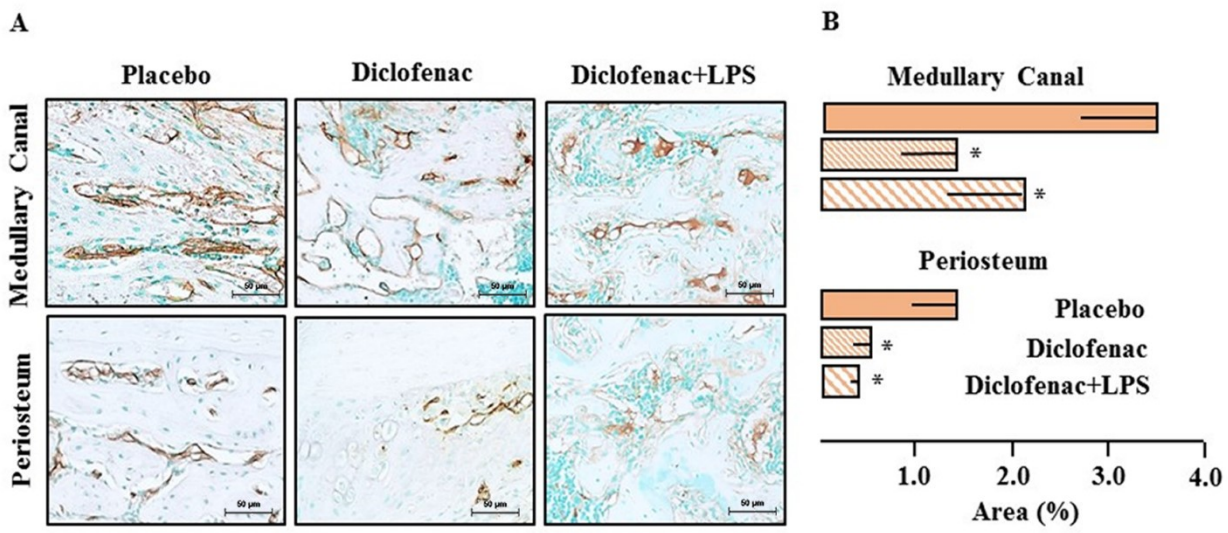


***Figure 5.4 Localization of vascular channels in regenerating bone***

*Vascular channels were identified by CD34 immunohistochemistry to identify vascular endothelial cells in regenerated bone in the medullary canal and periosteum. Representative images (A) show robust CD34 immunoreactivity in well-organized channels at both locations in placebo controls, compared with reduced and disorganized staining in mice treated with diclofenac and diclofenac + LPS. Results were confirmed by quantification of staining intensity (B) on N=5 to N=6 mice. ANOVA with Tukey's post-hoc test significantly different from placebo control * $p<0.01$. Scale bars in panel A represent 50 µm.*

### *Susceptibility of vascular endothelial and mast cells to diclofenac in vitro*

The preceding experiments suggested that chronic administration of diclofenac to mice during the first 2 weeks of bone healing resulted in an increase in mast cells and an apparent decrease in vascular endothelial cells in repair tissue. We, therefore, examined the susceptibility of primary bone marrow derived mast cells and lung-derived vascular endothelial cells to diclofenac toxicity in vitro using the AlamarBlue metabolic assay (Fig 5). The metabolic activity of primary mouse mast cells was approximately 1/10 that of primary vascular endothelial cells. Both cell types maintained ~ 75% activity in the presence of $10^{-3}$ M diclofenac but was reduced to 25% at 24 hours in endothelial cells exposed to a ten-fold higher concentration.

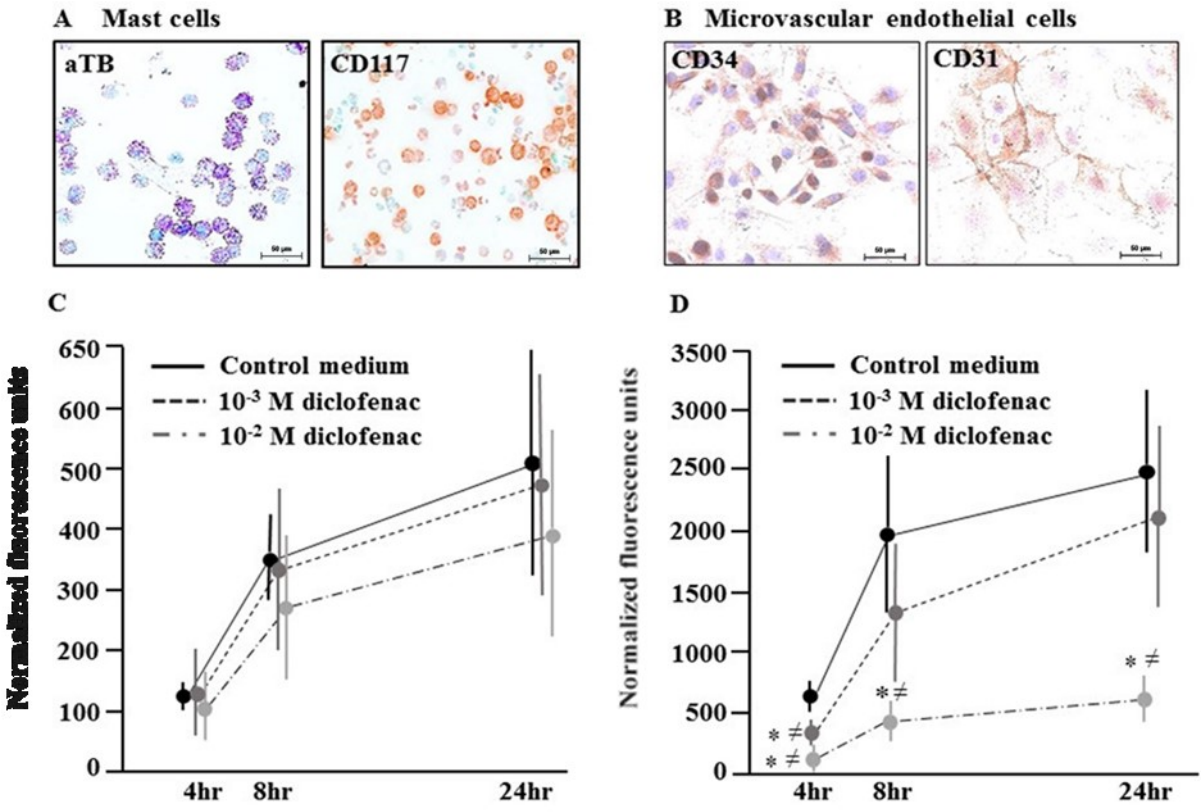


***Figure 5.5 Identification and metabolic activity of isolated cells exposed to diclofenac***

*Mouse bone marrow derived mast cells (A) grown for 4 weeks in the presence of SCF and IL-3 were stained with acidified toluidine blue (aTB) or immunostained with CD117 antiserum. Microvascular endothelial cells (B) were isolated from mouse lung tissue by immuno-selection with ICAM-2 antibody and immunostained with CD34, used clinically as an angiogenesis marker, or with CD31, which marks intercellular junctions. Metabolic activity of mast cells (C) and vascular endothelial cells (D) exposed to increasing concentrations of diclofenac was assessed using the AlamarBlue assay. Both cell types maintained a relatively high level of metabolism in the presence of $10^{-3}$ M diclofenac compared with control medium. Although $10^{-2}$ M diclofenac had little effect on mast cells it severely compromised the metabolic activity of vascular endothelial cells. ANOVA with Tukey's post-hoc test significantly different from control medium * $p<0.01$; significantly different from diclofenac treated mice ≠ $p<0.01$. All scale bars represent 50 µm.*

## 5.5 Discussion

Despite mounting evidence of potential toxic side effects, NSAIDs remain as one of the most common classes of analgesic drugs used to relieve pain associated with injury and surgical intervention, and to treat chronic inflammatory conditions in most soft tissues (Lands & Stanojevic, 2016; McGeer, Rogers, & McGeer, 2016; Moore & Scheiman, 2018; Schmeltzer et al., 2016; Tacconelli, Bruno, Grande, Ballerini, & Patrignani, 2017; Yingjun Zhou, Boudreau, & Freedman, 2014). Despite this extensive and widespread documentation, there are surprisingly few pre-clinical studies that have investigated how therapeutic doses of NSAIDs mediate their negative impact cells and tissues. The adverse effects of systemic administration of NSAIDs on bone repair are well established and date back decades in both the clinical and pre-clinical literature (Hernandez, Do, Critchlow, Dent, & Jick, 2012). The goal of the current study was to determine if systemic administration of a therapeutic dose of the NSAID diclofenac would 1) alter bone repair when administered alone and 2) improve bone repair in the presence of LPS-induced systemic inflammation. Analysis of bone repair was performed in 16 male (5 placebo, 6 dicolfenac, 5 diclofenac + LPS) and 13 female (4 placebo, 4 diclofenac, 5 diclofenac + LPS) skeletally mature mice aged 5-8 months. Analysis of micro CT and histochemical data using two-way ANOVA showed no significant differences between male and female mice in any of the three groups. At 2 weeks post-operative diclofenac treated mice show a significant reduction in vascularized bone compared with placebo control, which was associated with significant reductions in activity of bone, inflammatory and vascular cells. Diclofenac treatment of mice with LPS-induced systemic inflammation appeared to exacerbate rather than reverse the negative impact of LPS in the bone healing micro-environment.

### *PGE2, OC and OB activity*

Nonselective NSAID like aspirin, ibuprofen and indomethacin inhibit the cyclooxygenase (COX) enzymes that regulate the conversion of arachidonic acid into prostaglandins (PG) like PGE2. PGs mediate their biological activity by binding to receptors EPR1-4 and cAMP mediated signaling in a wide variety of tissues (Pilbeam, Choudhary, Blackwell, & Raisz, 2008). The COX-1 isoenzyme is expressed constitutively in a wide variety of cells while COX-2 expression is generally induced in response to inflammatory signals (Harder & An, 2003). Reports of adverse effects arising from COX-1 inhibition in kidney, liver and gut prompted the development and large scale clinical trials of the "coxib" family of drugs, which specifically inhibit the COX-2 enzyme, in patients with chronic inflammatory disorders like arthritis (Silverstein et al., 2000). Unfortunately, many of these COX-2 inhibitors were withdrawn from the market due to concerns associated with increased risk of cardiovascular complications (Mukherjee, Nissen, & Topol, 2001).

The COX-2 enzyme is upregulated in response to bone injury resulting in synthesis and local release of PGE2, which binds to EPR2 and EPR4 on osteoclasts and osteoblasts in the early phase of healing (Simon & O'Connor, 2007). Although the prevailing biologic effect of EPR2 and EPR4 activation in bone cells during fracture healing is proposed to be anabolic, the precise signaling pathways remain to be defined. PGE2 receptor activation is proposed to be mediated through upregulation of RANKL and OPG inhibition in osteoblasts, leading to enhanced osteoclast formation from monocytes (Blackwell, Raisz, & Pilbeam, 2010). COX-2 would have been inhibited by sustained release of diclofenac, sufficient to maintain a circulating concentration equivalent to that recommended for pain relief in human subjects. Subsequent PGE2 reduction in

the bone healing environment of diclofenac treated mice would result in impaired osteoclast activation via RANK/RANK/OPG signaling compared with placebo treated controls. This was in fact, the case in mice treated with diclofenac, where TRAP activity in osteoclasts in regenerating bone was reduced six-fold, in the absence and presence of LPS-induced systemic inflammation. Macrophages derive from the same myeloid progenitor as osteoclasts in bone marrow and are differentially activated into primarily catabolic M1 or anabolic M2 phenotypes in wound repair (Ferrante & Leibovich, 2012). Macrophage polarization is a consequence of environmental cues that lead to the acquisition of functional characteristics by the cells. Classical activation of resting macrophages by toll like receptor (TLR) ligands and increased production of the cytokine IFN-γ in the early inflammatory phase of bone repair leads to M1 ploarization. Subsequent exposure to cytokines like IL-4 and IL-13 leads to M2 polarization (Mosser & Edwards, 2008). The cell surface glycoprotein F4/80 is used as a marker for M1 and M2 macrophages whereas arginase-1 expression is restricted to M2 macrophages. Similar to TRAP positive osteoclasts, F4/80 positive macrophages were reduced six-fold and arginase-1 positive macrophages twofold in diclofenac treated mice, with further reductions in mice with LPS-induced systemic inflammation. In our previous work, we showed the administration of LPS alone resulted in elevated circulating TNFα in the first week of healing, a transient increase in TRAP positive osteoclasts and increased F4/80 activity in macrophages (primarily M1) up to 2 weeks post-operative in regenerating bone (Behrends et al., 2017). Taken together these data suggest that sustained treatment with a therapeutic dose of NSAID will inhibit basal and LPS-induced catabolism by osteoclasts and macrophages during bone repair.

Diclofenac-mediated disruption of PGE2 signaling could also account for the 65% reduction in ALP activity in osteogenic cells in the current study. In addition to its prominent role in osteoclast formation, osteoblast derived PGE2 is proposed to act in an autocrine loop by upregulation of Runx2/Osx mediated transcription of differentiation associated genes like ALP during bone turnover and fracture repair (Haversath et al., 2012). Early studies investigating PGE2 as a potential anabolic agent showed direct delivery of a highly selective EPR2 receptor agonist promoted bone regeneration and cortical bridging of critical sized defects in canine tibia and ulna (Paralkar et al., 2003). Systemic delivery of PGE2 in aged rodents was also shown to increase trabecular bone formation in vivo and to enhance mesenchymal stromal cell differentiation and bone nodule formation *ex vivo* (Keila, Kelner, & Weinreb, 2001). Conversely, targeted ablation of the gene encoding COX-2 led to fibrous mal-union of stabilized fractures of the tibia in young adult mice, in association with reduced osteoblast formation (X. Zhang et al., 2002). A subsequent study on the same COX-2 null mouse strain showed the administration of an EP4 receptor agonist, led to significant improvement in bone and vascular repair, quantified by micro CT, at 2 weeks post-operative (Xie et al., 2009). Taken together our results suggest diclofenac mediated inhibition of COX-2 in the early phase of bone healing had a negative impact on osteoblast, osteoclast and macrophage activity.

### *Vascular endothelial cells and angiogenesis*

Restoration of a vascular network to transport cells, biologics and waste materials to and from the site of healing is a prerequisite for effective bone repair (Grosso et al., 2017). In previous work, we showed fibrous mal-union associated with impaired angiogenesis, which was attributed to the absence or depletion of mast cells and the pro-angiogenic factors that they release (Behrends

et al., 2014, 2017; Ramirez-GarciaLuna et al., 2017). In the current study defective angiogenesis and fibrous mal-union was shown by both micro CT and CD34 immunoreactivity in mice treated with diclofenac. The fact that these mice had more or equal numbers of mast cells compared with PBS treated controls indicates alternative inhibitory pathway/s must have been involved. This could have occurred directly by diclofenac mediated inhibition of COX-2 leading to cell cycle arrest and apoptosis of bone marrow derived endothelial progenitor cells as reported previously (Colleselli et al., 2006). In cultures of primary micro-vascular endothelial cells, we showed decreased metabolic activity in response to diclofenac, but not in cultures of mature mast cells, which have a slower metabolic rate. Alternatively, impaired re-vascularization of the repair tissue in diclofenac treated mice could have been a consequence of altered signaling mediated by hypoxia inducible factor-1 (HIF-1). Under normal circumstances, the hypoxic environment of early bone repair would result in activation of the HIF-1 transcription complex, consisting of alpha and beta sub-units, and upregulation of genes involved in inflammation, matrix metabolism and angiogenesis, including VEGF (Wan et al., 2008). HIF-1 regulation of angiogenesis has been identified as a key regulator of bone development (Rankin, Giaccia, & Schipani, 2011) but its role in fracture repair is less well defined. Femoral fractures in mice heterozygous for *Hif1α* expression, which results in decreased HIF-1 protein, were reported to heal better than those in wild type mice (Komatsu, Bosch-Marce, Semenza, & Hadjiargyrou, 2007). Using the same mouse femoral fracture model, vascularity and callus size were also reported to be increased in wild type mice treated with inhibitors of the key enzyme that mediates HIF-1 degradation, which would result in more protein (Shen et al., 2009). In cell based studies, NSAIDs like diclofenac are reported to inhibit HIF-1 expression and activity in the gastro-intestinal tract (Yokoe, Nakagawa, Kojima, Higuchi, & Asahi, 2015). In the current study administration of a therapeutic dose of diclofenac

could, therefore, have inhibited HIF-1 and subsequent VEGF mediated angiogenesis in the repair tissue. This supposition is supported by an in vivo study conducted to determine the functional outcome of local delivery of angiogenesis inhibitors from an intra-medullary nail on bone repair in a 1mm rat tibia osteotomy (Fassbender et al., 2011). At two weeks post-operative there were ~50% fewer vessels stained with smooth muscle actin in the repair tissue of legs treated with fumagillin, a potent angiogenesis inhibitor, compared with control. Despite the differences in animal species, surgical model and molecular marker of vascular channels, these results are remarkably similar to our own in the current study. They also suggest that direct inhibition of vascular endothelial cells, rather than depletion of mast cell derived growth factors, lead to a similar outcome of fibrous mal-union.

### *Mast cell accumulation, degranulation and cell death*

In contrast to our previous studies in which mast cells were absent or decreased in the early phase of bone healing, there was a significant increase in mast cells, identified by aTB or CD117 immunohistochemistry, in diclofenac treated mice. More than 90% of these cells were located in regenerating tissue within the medullary canal with very few in periosteal bone. In placebo treated controls 15% of the mast cells were located in the proximal medulla occupied by primary bone, whereas only 4% were in the equivalent location in diclofenac treated mice. The remainder were distributed approximately evenly between the gap region and distal medulla in all mice. The accumulation of mast cells in the medullary canal distal to regenerating bone suggests that they arise from bone marrow precursors, rather than being delivered via the systemic circulation, which runs proximal to distal. The significant increase in mast cells in diclofenac treated mice could be due to impaired vascular regeneration (discussed above) at the bone/marrow interface, thus

preventing dissemination of mast cells from distal to proximal regions. An alternative explanation would be that NSAID inhibition of the Cox-1 enzyme increased leukotriene D4 production and smooth muscle contraction leading to altered blood flow in adjacent existing vessels (Greenberger, 2006). Regardless of the mechanism, if blood flow in an around the defect was restricted in diclofenac treated mice, we would expect to see a similar accumulation of mast cells in the mice treated with diclofenac and LPS. In fact, mast cells were significantly reduced in those mice compared with mice treated with diclofenac alone, with the greatest reduction seen in specimens stained with aTB. A similar loss of aTB positive cells in repair tissue was seen in our previous work in which treatment with the same dose of LPS was sufficient to increase circulating TNFα but insufficient to cause toxicity (Behrends et al., 2017).

There is an emerging and somewhat controversial literature that documents diverse pathways activated by LPS in target cells (Tan & Kagan, 2014). LPS on the outer membrane of bacteria interact with a variety of receptors that include membrane bound TLR4. Initiation of signaling downstream of TLR4 in mast cells is reported to result in degranulation as well as de novo synthesis and release of cytokines, chemokines and prostaglandins in an inflammatory response (Sandig & Bulfone-Paus, 2012). LPS-induced mast cell de-granulation could explain the apparent discrepancy in staining between aTB, which stains only the granules, and CD117 which stains the cell itself. It is also possible that LPS-induced inflammatory cell death, or pyroptosis, accounting for the reduction in mast cells in mice treated with diclofenac and LPS compared with those treated with diclofenac alone. Intracellular LPS triggers the formation of multi-protein complexes called inflammasomes and activation of caspase mediated cell death (Hagar, Powell, Aachoui, Ernst, & Miao, 2013; Kayagaki et al., 2013). Inflammasomes in mast cells are also

recognized as key effectors of the innate immune response to invading pathogens via caspase-mediated activation of inflammatory cytokines like IL-1β, which then acts in an autocrine manner to perpetuate the response (Bonnekoh, Scheffel, Kambe, & Krause, 2018).

In conclusion, the results of this study provide strong evidence to support the conjecture that administration of NSAIDs in the first 2 weeks after orthopaedic surgery disrupts the healing cascade and also appears to exacerbate the negative effects of systemic inflammation on the repair process. In our previous studies, similar defects at 2 weeks post-operative were predictive of failure of cortical bridging and fibrous mal-union at 12 weeks post-operative. Whereas mast cell depletion appeared to be the primary defect in the previous work, the negative impact of diclofenac appeared to be mediated primarily on vascular endothelial cells and osteoblasts.

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**Author roles**

Study design and execution: JRGL, JEH, PAM. Data collection and analysis: JRGL, THW, DC, YAS, AA, MA, SO. Data interpretation: JRGL, EA, CAL, JEH, PAM. Manuscript draft: JRGL, CAL, JEH, PAM. All authors share responsibility for the integrity of the data analysis.

# VI. ENHANCED BONE REMODELING AFTER FRACTURE PRIMING

Mast cells are memory cells. Once they have been primed to "remember" an epitope, they will react with a more powerful response to repeated exposures to it. This mechanism is the basis for allergies and anaphylactic reactions (LoVerde, Iweala, Eginli, & Krishnaswamy, 2018). Based on this paradigm and in the research presented in the previous chapters, where we demonstrate that mast cells react to the inflammatory environment of healing bone and interact with other cell lines to promote repair, we hypothesized that if mast cells and other immune cells were sensitized to recognize broken bone, they would be able to mount a more powerful response to a subsequent exposure that may be translated into enhanced healing. To test this hypothesis, we created a window bone defect on the left leg of anesthetized mice and two weeks later, we created a second one on the right leg. Bone repair in the right leg was compared to control mice that underwent the creation of bilateral window bone defects at the same time. We selected a 14-day time point as it is the end of the inflammatory phase of bone healing and coupled remodelling of the newly formed bone is well under way. A 56-day time point was also analyzed because by then, the bone has already bridged, and the tissue just needs to complete its remodelling process. Our results show how this "double fracture" approach does not modify the amount of bone formed but promotes faster remodelling that is translated into better bone quality after 56 days of healing. We believe this experiment shows how immunomodulation can be used to promote healing and paves the way for the identification of molecular targets that may be used some day to create a "fracture vaccine".

# ENHANCED BONE REMODELING AFTER FRACTURE PRIMING

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## 6.1 Abstract

***Introduction:*** The immune system is an active component of all the phases of bone repair. Mast cells, a component of the innate immune system, influence the recruitment of macrophages, osteoclasts and blood vessels into the repair tissue. Based on the observations that modifying the inflammatory environment through immunotherapy leads to tissue remodeling, our aim is to test the hypothesis that "priming" the immune system to repair broken bone may accelerate fracture healing in a subsequent fracture sustained shortly thereafter.

***Methods:*** Mice were randomly allocated to one of two groups, control where bilateral defects were drilled simultaneously, or double fracture, where the first femoral defect was made on the left leg two weeks before the creation of the defect on the right leg. Bone repair was assessed only in the right legs of the animals through micro-CT and histology at two and eight weeks of healing.

***Results:*** We did not find significant differences in the amount of bone produced at 14- or 56-days post-operative, however significant differences in the bone tissue quality were observed. Increases in the content of blood vessels, osteoclasts, the total number of macrophages and M2 macrophages were observed at 14 days of healing in the mice belonging to the double fracture group. At 56 days of healing, these animals showed signs of more advanced remodelling and better-quality repair tissue compared to controls.

***Conclusion:*** Our results provide strong evidence that a transient increase in the inflammatory state of a healing fracture promotes faster bone remodeling and increased neo-angiogenesis.

## 6.2 Introduction

The process of fracture healing is composed of the sequential, yet interdependent phases of hemostasis, inflammation, repair, and remodeling. Fracture healing is initiated through the hemostatic and inflammatory response to injury, which directs the ensuing processes of repair and remodeling by the recruitment of precursor cells and the release of cytokines and growth factors (P. S. Schneider et al., 2018). An appropriate balance of localized inflammation and a systemic inflammatory reaction is critical to achieving successful bone healing, as the appropriate resolution of the inflammatory phase is necessary for the progression of the healing process into the repair and remodeling phases (Harper et al., 2014). The immune system plays a critical role in bone healing, as it is the first-line component for the hemostasis and inflammation phases, as well as being an active player throughout the repair and remodeling stages, often acting as paracrine agent that influences neo-vascularization, stem cell recruitment, and precursor cell differentiation (Bodnar, 2015; Claes et al., 2012).

Recent reports have highlighted the positive impact of emerging immunotherapies in cancer survival (Banerjee et al., 2018; Blum, Martins, & Lübbert, 2017; Sampson, Maus, & June, 2017). This treatment modality relies on manipulating immune cells to recognize and destroy cancer cells, either through boosting the activity of immune cells or by enabling the recognition of neo-antigens, which are peptides that are exclusively expressed in diseased tissue. In the context of bone healing, a recent paper by Wu et al. demonstrated that the systemic administration of *Staphylococcal* enterotoxin C2 (SEC2) induced better bone repair in a rat fracture model (Wu et al., 2018). Their results showed increased bone callus, mineral tissue content, and better

remodelling characterized by higher mechanical stability of the fractures of the animals treated with SEC2 compared to controls. This paper highlights the feasibility and impact of a non-targeted approach to modify bone repair through inducing a mild systemic inflammatory response.

Mast cells are a subgroup of innate immune system cells that traditionally have been linked to the development of asthma and allergies (Andersson, Tufvesson, Diamant, & Bjermer, 2016), but emerging literature has demonstrated a role in the regulation of the inflammatory, repair and remodeling phase of bone healing. Mast cells have proved to influence the recruitment and polarization of macrophages into M2 anabolic cells, osteoclast differentiation and activity, and neo-vascularization of wound tissue (Behrends et al., 2014; Kroner et al., 2017; Ramirez-GarciaLuna et al., 2017). Based on the observations that modifying the inflammatory environment through immunotherapy leads to tissue remodeling, in our previous observations that modifications in the inflammatory environment modify the outcome of bone repair (Behrends et al., 2017; Ramirez-Garcia-Luna et al., 2019), and in mast cell biology, we have hypothesized that "priming" the immune system to repair broken bone may accelerate fracture healing in a subsequent fracture sustained shortly thereafter. Henceforth, the objective of the present study was to determine this in a mouse model of a stable bone window defect.

## 6.3 Methods

### *Mice and treatment groups*

All live animal procedures were conducted in accordance with a protocol approved by McGill MGH Facility Animal Care Committee (7016) and in keeping with the guidelines of the Canada Council on Animal Care. Skeletally mature male and female C57BL/6 mice (Charles River Laboratories, Senneville, QC, Canada) were used for this experiment. The animals were housed 3-4 per cage with free access to food and water. Bilateral femoral defects measuring 1mm x 2mm were drilled on the anterolateral aspect of the femora of 5-6-month-old anesthetized mice essentially as described previously (Gao et al., 2013). Mice were then randomly assigned to one of the following treatment groups: **1) Control** mice underwent the creation of both defects at the same time; **2) Double fracture** treated mice underwent the creation of the first femoral defect on the left leg two weeks before the creation of the defect on the right leg. For pain control 10 mg/kg carprofen with 0.1 mg/kg buprenorphine in 0.5 mL of sterile saline was administered IP immediately after wound closure, and 5 mg/kg carprofen injected once a day for three days post-operative thereafter. The cohorts (n = 6 per group or time point) were euthanized by $CO_2$ asphyxiation under anesthesia at 14- or 56-days post-operative and the femora fixed overnight in 4% paraformaldehyde prior to micro CT imaging. All analyses were performed using only the right legs.

### *Micro CT analysis*

Micro CT analyses of bone were performed on a Skyscan 1172 instrument (Bruker, Kontich, Belgium) at a resolution of 5-microns using NRecon v.1.6.10.4 to generate 3D models and CTAn v.1.16.4.1 software for quantitative analysis (Bruker) as described previously (Ramirez-GarciaLuna et al., 2017). Quantitative data for bone and vessel regeneration was recorded in a region of interest 1.0 mm long, 0.5 mm wide and 1.5 mm in depth spanning the defect (gap region). Analyzed parameters include bone volume/tissue volume (BV/TV %), trabecular number (Tb.N /$mm^{-1}$), trabecular thickness (Tb.Th µm), trabecular separation (Tb.Sp µm), connectivity density (Conn.Dn mm-3), total porosity (Po.Tot %), number of closed pores (Po.N.cl /$mm^{-1}$), and tissue mineral density (TMD gr/$cm^{-3}$), which was normalized to the TMD of the adjacent healthy cortex and thus is expressed as %.

### *Histological analyses*

Bones were decalcified, embedded in paraffin, sectioned and stained using previously described protocols (Henderson et al., 2011). Serial 5-µm sections were cut and stained for alkaline phosphatase (ALP) activity in anabolic cells, tartrate resistant acid phosphatase (TRAP) activity in catabolic cells and acidified toluidine blue (aTB) to identify granules in mast cells. Immunohistochemistry was performed using the following antisera: CD34 (Abcam ab23830 1:300) in vascular endothelial cells; CD117 (Bioss bs-0672R, RRID:AB_2725777, 1:200) in mast cells; F4/80 (Abcam ab6640 1:200) in all macrophages; and Arginase-1 (Santa Cruz sc-271430, RRID:AB_626697, 1:300) in M2 anabolic macrophages. A Zeiss Axioskop 40 microscope (Carl Zeiss, Toronto, ON, Canada) was used to capture high resolution images, and staining intensity

analyzed using ImageJ v.1.6.0 software. Quantitative analysis units represent the percentage of stained area relative to the total region of interest area and are expressed as % of the total area.

### *Isolation and culture of cells*

Bone marrow was isolated from the femora of 8-week old C57BL/6 mice, as described previously (Gao et al., 2013). Briefly, the soft tissue of the femora was removed under aseptic conditions before cutting the bones in half and placing them in Eppendorf tubes and centrifuging at low speed to dislodge the marrow. Bone marrow from individual mice (total 3) was pooled, re-suspended in alpha-Minimal Essential Medium (α-MEM) containing 1% antibiotic/antimycotic (Sigma-Aldrich, St. Louis, MO, USA) and 10 % fetal bovine serum (FBS) (Wisent, St. Bruno, QC, Canada) and 1.5 x $10^6$ cells were plated in 75 $cm^2$ tissue culture dishes. After three days in culture at 37°C in a 5% $CO_2$ atmosphere, non-adherent cells were recovered, centrifuged, resuspended in Roswell Park Memorial Institute (RPMI) medium, and plated in 75 $cm^2$ plates at a density of 3 x $10^6$. Suspension cells were then differentiated into mast cells as described by Feyerabend et al. (Feyerabend et al., 2005) by supplementing with 10% FBS (Wisent), 10ng/mL rIL-3 (R&D Systems, Minneapolis, MN, USA) and 25ng/ml stem cell factor (R&D Systems) for 4 weeks. Adherent cells continued to be grown under the described conditions and used at the second passage to prepare mesenchymal stromal cells (MSC) seeded collagen microgels.

### *Assessment of the interaction of MSC and MC in vitro*

To assess if the culture of MSCs was affected by their co-culture with MC, we used a microgel droplet 3D culture system using a modification of a previously described method (Moraes, Simon, Putnam, & Takayama, 2013). Briefly, a 3 mg/mL type-I bovine collagen (Sigma-Aldrich) solution was adjusted to 2 mg/mL in a sterile solution of 10% v/v 10x DMEM (Sigma-Aldrich), 1% v/v 3M NaOH, and 15% v/v dextran T500 (DEX) (Pharmacosmos, Holbaek, Denmark) solution (to a final concentration of 3% dextran) and 7.3% v/v additional buffer. Depending on the experiment performed, the buffer solution was either α-MEM or a suspension of MSCs (1 x $10^6$ / mL) in fully supplemented α-MEM. Neutralized collagen-DEX solutions were stored on ice for a maximum of 30 min before use. To create the microgel droplets, a 20% w/w polyethylene glycol 35k (PEG) (Sigma-Aldrich) in fully supplemented α-MEM was pippeted into a 96-well plate and kept at 37°C until use. To create the droplets, the collagen-DEX solutions were maintained on ice, and 1 µL was rapidly pipetted directly into the PEG-enriched media in the center of the well. Following dispensation of the collagen-DEX solution, the plates were placed at 37 °C for 30 min to allow the collagen to crosslink. The PEG-enriched medium was then carefully aspirated, the droplets were washed once with 1x PBS, and fully supplemented α-MEM was pipetted rapidly to dislodge the collagen droplet from the underlying surface, before culturing the microgels for 72 hours with or without 1 x $10^4$ mast cells.

After three days in culture, the media was removed, the droplets were washed in 1x PBS, the microgel contraction rate was assessed by comparing the size of the droplet to its initial size, and then was fixed for 20 minutes in 4% PFA. Afterwards, the droplets were stained for ALP, high-resolution images were acquired, and staining intensity was analyzed using ImageJ v.1.6.0

software. Quantitative analysis units represent the percentage of stained area relative to the total droplet area and are expressed as % of the total area. All experiments were done by triplicates.

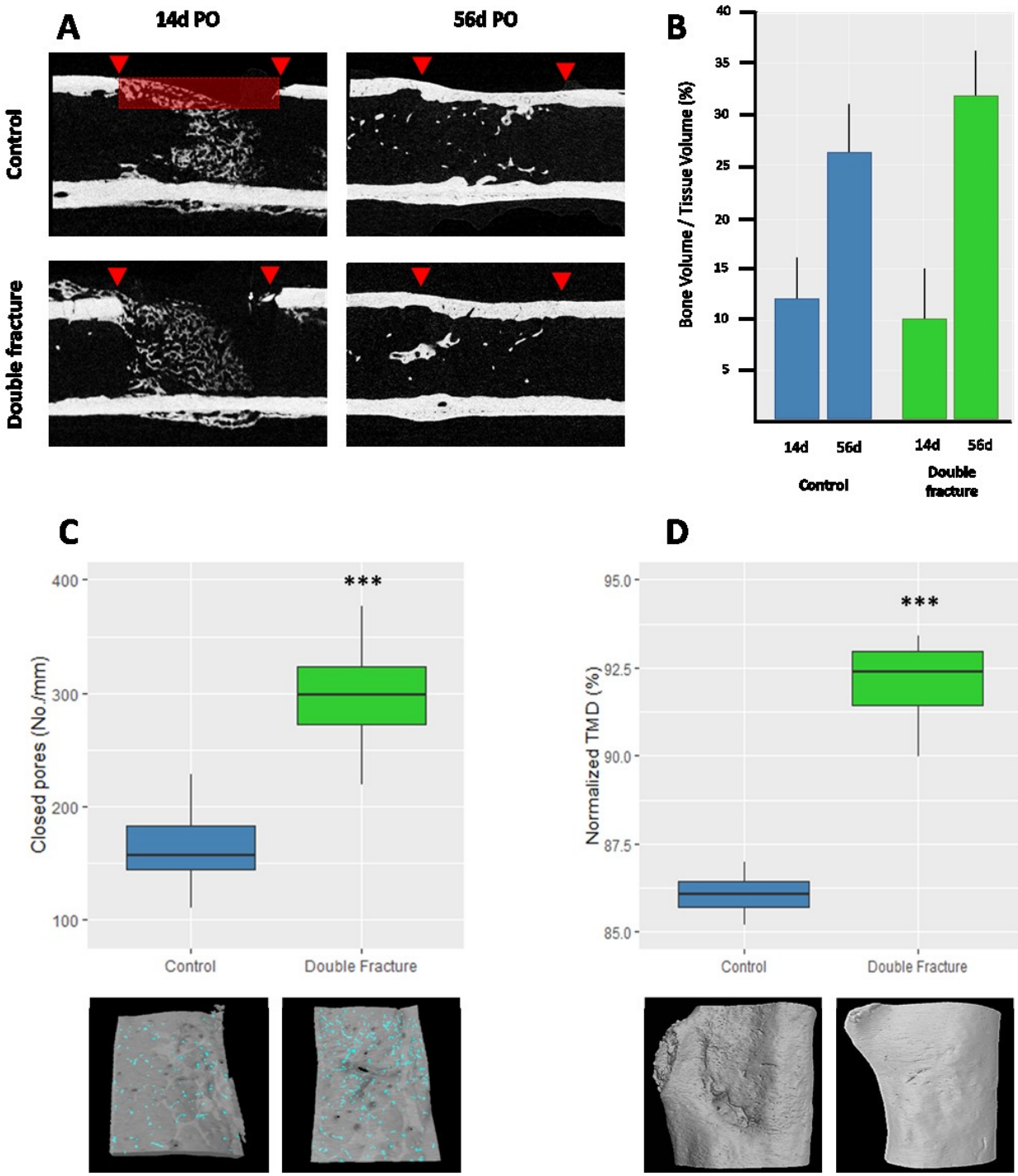
### *Statistical analysis*

Quantitative data are expressed as Mean ± SD and R v.3.4.1 (R Core Team, 2017) statistical program used for unpaired t-tests. Tukey post-hoc analysis for multiple comparisons was used when appropriate. Differences were considered significant at $p < 0.05$.

## 6.4 Results

### *Micro CT analysis of bone repair*

Tissue regeneration in the femoral defects of skeletally mature C57/Bl6 mice was assessed at 14- and 56-days post-operative. Mid-sagittal micro CT images (Fig 1A) showed similar bone healing patterns and quantitative data (Fig 1B) confirmed no significant differences in the amount of bone formed in the gap region of interest of mice in the double fracture group, compared to controls. Quantification of parameters of bone micro-architecture (Table 1) revealed thickening of trabeculae, accompanied by increased connectivity density and a higher number of closed pores, which represent osteocyte lacunae, in the double fracture mice at 14 days of healing. At 56 days of healing, connectivity density was lower in the double fracture group, while the number of closed pores remained significantly higher (Fig 1C). 3D reconstruction of the bones revealed significantly less scarring and better remodeling of the repair tissue at 56 days of healing in the double fracture group (Fig 1D). This finding was matched with significantly higher tissue mineral content (TMD).


A
14d PO
56d PO
Control
Double fracture
B
Bone Volume / Tissue Volume (%)
40
35
30
25
20
15
10
5
14d
56d
14d
56d
Control
Double fracture
C
Closed pores (No./mm)
400
300
200
100
***
Control
Double Fracture
D
Normalized TMD (%)
95.0
92.5
90.0
87.5
85.0
***
Control
Double Fracture

### *Figure 6.1 Bone micro-CT analysis*

*Femora harvested at 2- and 8-weeks post-operative were scanned at a resolution of 5 microns on a Skyscan 1172 instrument. Representative 2D mid-sagittal images (A) showed similar healing patterns between mice in the control and double fracture group. 3D morphometric analyses were conducted in the gap region (red box, arrowheads mark the edge of the defect). Quantitative analysis showed no significant differences in bone content at 14 or 56 days of healing (B). However, after 8-weeks of healing more closed pores, representing osteocyte lacunae (C), higher tissue mineral density (TMD) and les scarring (D) were observed in the double fracture group, compared to controls. TMD in the gap region was normalized to TMD in the adjacent healthy cortex. Unpaired t-test was applied to data from N=6 animals per group. Significantly different from control *** $p<0.001$.*

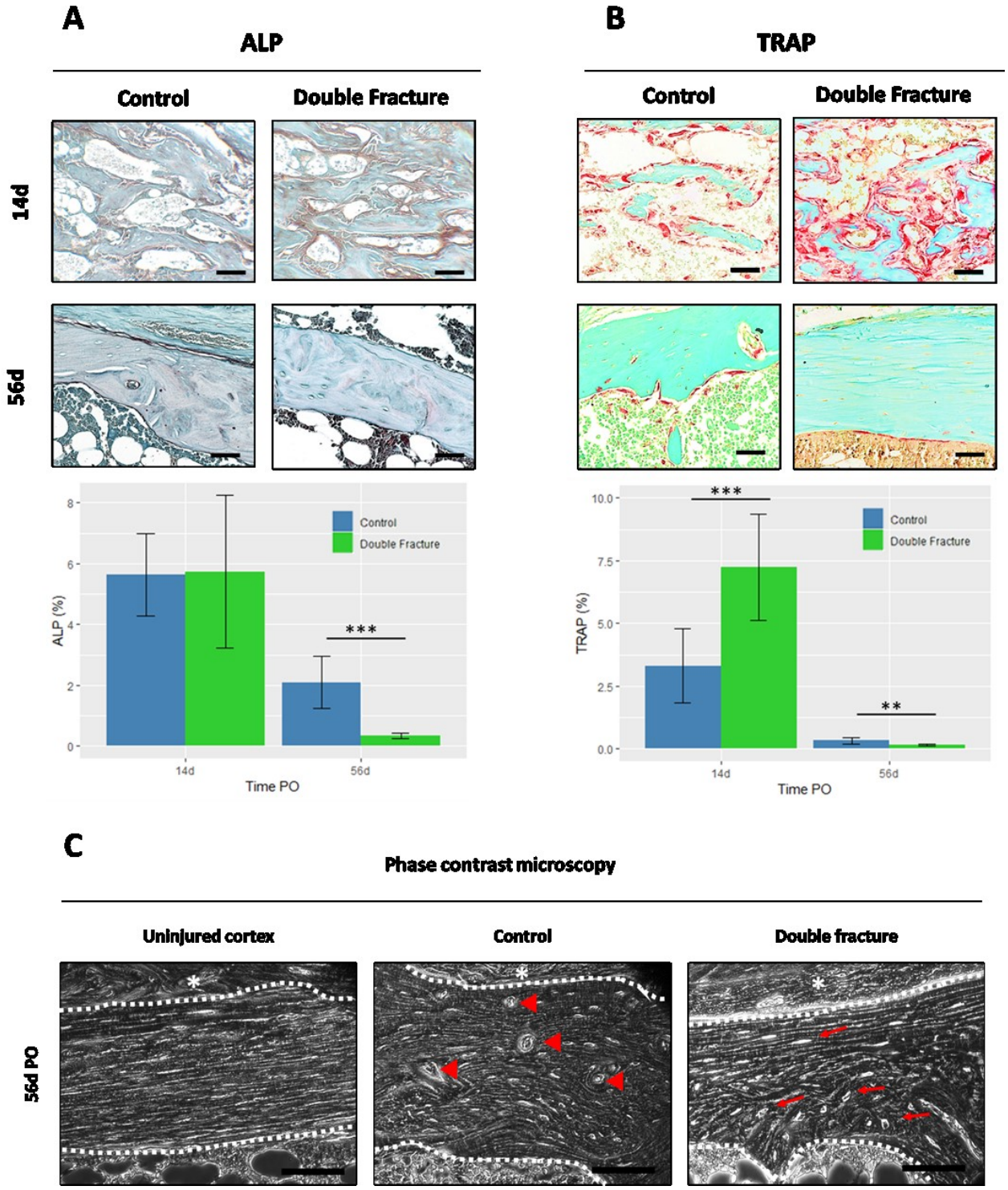
*Table 6.1 Quantitative micro CT analysis at 2- and 8-weeks post-operative*

| Variable | 14d PO | | | 56d PO | | |
|---|---|---|---|---|---|---|
| | Control | Double fracture | p value | Control | Double fracture | p value |
| **BV/TV (%)** | 12.5 ± 4.6 | 9.9 ± 4.3 | 0.23 | 26.1 ± 5.9 | 32.6 ± 6.2 | 0.15 |
| **Tb.N (No./mm$^{-1}$)** | 3.6 ± 1.2 | 3.4 ± 1.6 | 0.75 | 2.5 ± 0.4 | 2.6 ± 0.5 | 0.90 |
| **Tb.Th (µm)** | 45.0 ± 12.6 | 29.2 ± 8.4 | <0.001 | 100.5 ± 14.2 | 101.3 ± 11.9 | 0.92 |
| **Tb.Sp (µm)** | 336 ± 166 | 274 ± 128 | 0.40 | 236 ± 42 | 236 ± 39 | 0.99 |
| **Conn.Dn (1/µm)** | 1.4 ± 0.7 | 2.3 ± 0.9 | 0.02 | 0.04 ± 0.02 | 0.001 ± 0.01 | 0.01 |
| **Po.Tot (%)** | 85.9 ± 6.5 | 90.1 ± 4.3 | 0.16 | 73.9 ± 5.9 | 72.8 ± 8.5 | 0.83 |
| **Po.N.cl (No./mm$^{-1}$)** | 31 ± 20 | 54 ± 24 | 0.04 | 165 ± 44 | 296 ± 69 | <0.001 |

*Analysis of regenerated bone was performed in the region of interest indicated in Figure 1A. Unpaired t-test was applied to data from N=6 animals per group. BV/TV = bone volume / tissue volume; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Conn.Dn = connectivity density; Po.Tot = total porosity; Po.N.cl = Number of closed pores.*

### *Bone cellular activity in regenerating tissue*

Adjacent 5-micron histology sections were stained for ALP activity in osteoblasts, TRAP activity in osteoclasts or left unstained for phase contrast microscopy. In line with our previous micro-CT results, ALP content at 14 days of healing was not found to be different between groups (Fig 2A). However, TRAP activity was almost three-fold increased in the double fracture mice (Figure 2B). At 56 days of healing, both cell markers were significantly lower in the double fracture group, compared to controls. Phase contrast microscopy was used to reveal the alignment of the tissue in the gap region of interest of the healing bone after 56 days post-operative (Fig 2C). Double fracture animals showed a better organized repair tissue, more similar to uninjured cortical bone. Additionally, more numerous and larger osteocyte lacunae were observed in the tissue (Fig 3C, arrows). Control mice tissue showed more structures resembling blood vessels inside of the repairing bone (Fig 3C, arrowheads).


A
ALP
Control
Double Fracture
14d
56d
ALP (%)
Control
Double Fracture
***
14d
56d
Time PO
B
TRAP
Control
Double Fracture
TRAP (%)
***
**
14d
56d
Time PO
C
Phase contrast microscopy
Uninjured cortex
Control
Double fracture
56d PO

### *Figure 6.2 Cellular activity in regenerating bone*

*After micro CT analyses femora were decalcified, embedded in paraffin and 5-micron sections stained with alkaline phosphatase (ALP) (A) to identify osteoblast activity in the regenerating bone. Quantitative data at 14 days of healing did not show significant differences in ALP activity, but significantly less activity was observed at 56 days post-operative in the double fracture group. Osteoclast activity was identified using tartrate-resistant acid phosphatase (TRAP) staining (B). Significantly more TRAP positive cells and TRAP activity was observed in the double fracture healing bone at 14 days, which then reversed at 56 days post-operative. Phase contrast microscopy of the gap region in unstained samples at 56 days of healing (C) showed significantly more blood vessels (arrowheads) in the repairing bone. The alignment of the tissue of the animals in the double fracture group appears to be more similar to that of uninjured cortical bone and larger osteocyte lacunae (arrows) can be observed. Dotted lines represent the cortex boundary and the asterisk marks the periosteal side. Unpaired t-test was applied to data from N=6 animals per group. Significantly different from placebo control ** $p<0.01$, *** $p<0.001$. Scale bars represent 50 µm.*

### *Re-vascularization of regenerating tissue*

CD34 immunohistochemistry was used to identify vascular endothelial cells lining channels at the gap region of interest and inside of the medullary canal adjacent to the femoral defect (Fig 4A). At 14 days post-operative, double fracture mice showed more numerous vascular channels aligning with the longitudinal axis of the femora in the gap region, while more intense CD34 positive immunoreactivity was observed in the medullary canal, compared to controls. Significantly more CD34 positive cells were also observed adjacent to the vascular channels in the double fracture group. At 56 days of healing, CD34 immunohistochemistry confirmed a higher presence of vascular channels in the gap region repair bone of control mice, whereas stronger positive CD34 immunoreactivity remained in the double fracture mice. Quantitative analyses (Fig 4B) confirmed higher CD34 expression at both time points in the double fracture mice, compared to controls.

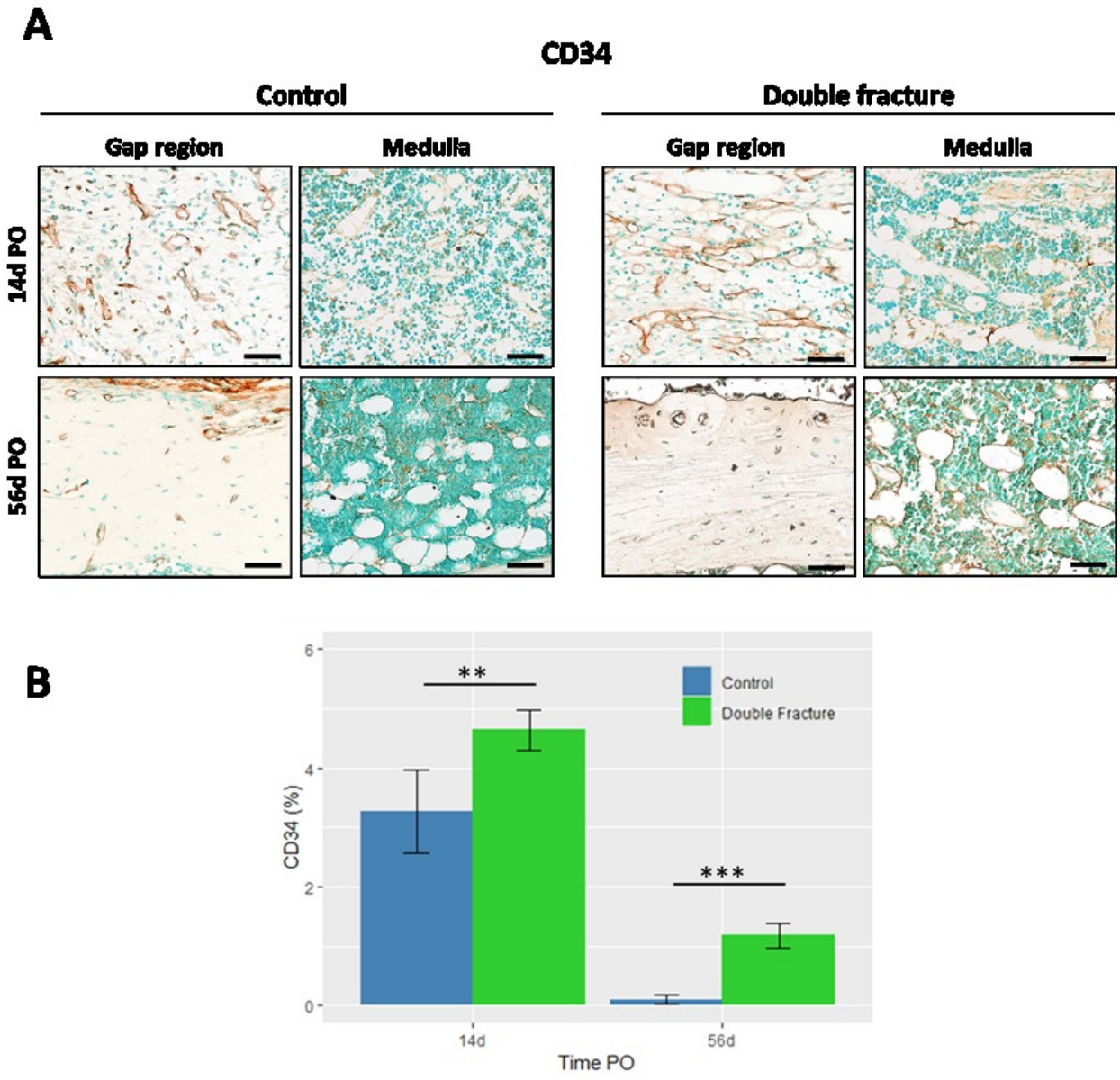


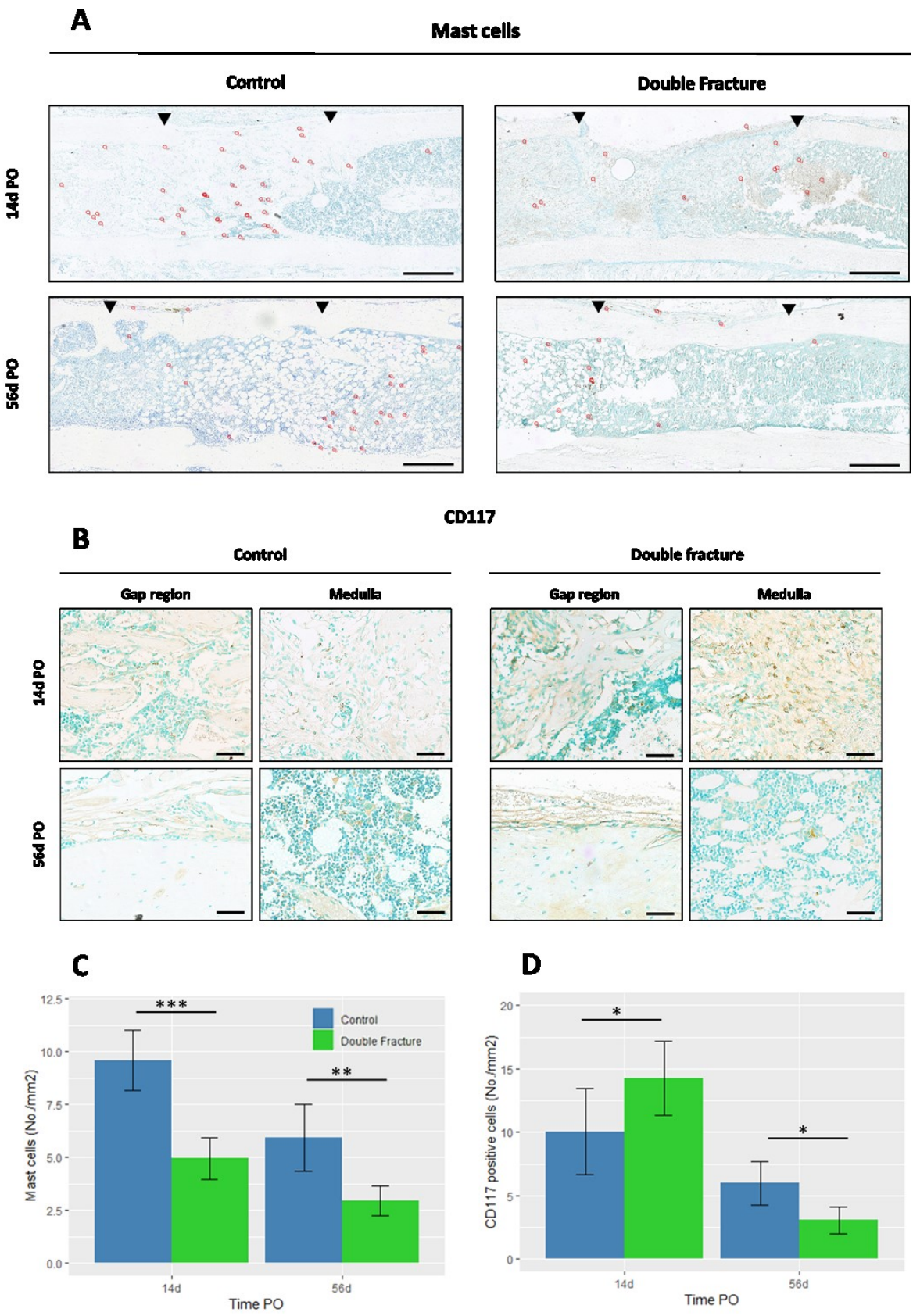

*Figure 6.3 Vascular activity in regenerating bone*

*Vascular channels were identified by CD34 immunohistochemistry to recognize vascular endothelial and precursor cells in regenerated bone in the gap region and medullary canal. Representative images (A) and quantitative data (B) show robust CD34 immunoreactivity and better organized vascular channels in both locations in the double fracture group, compared to controls. Likewise, in the medullary canal of the former group, more positive CD34 cells can be*

*observed adjacent to the vascular channels at 14 days post-operative. After 56 days of healing, more vascular channels embedded in the gap region bone can be observed in the control group, as well as less vascular channels in the medullary canal. Unpaired t-test was applied to data from N=6 animals per group. Significantly different from placebo control *** $p<0.001$. Scale bars represent 50 µm.*

### *Recruitment of mast cells to regenerating tissue*

The number and location of MC in the regenerating tissue of the medullary bone channel were quantified on adjacent sections of femora stained with aTB to identify mast cell granules, or with CD117 antiserum to the c-kit membrane receptor. Significantly less granulated mast cells were found in the tissue of double fracture mice, compared to controls (Fig 4A and C). However, at 14 days post-operative, significantly more CD117 positive cells were observed in the double fracture group, predominantly in the medullary region of the bones (Fig 4B and D). At 56 days of healing, significantly less CD117 positive cells were found in the double fracture group, consistent with the previous finding of less granulated mast cells. Noteworthy was the finding that control animals exhibited significantly more CD117 positive cells adjacent to the periosteum.


A
Mast cells
Control
Double Fracture
14d PO
56d PO
CD117
B
Control
Double fracture
Gap region
Medulla
14d PO
56d PO
C
D
Control
Double Fracture
Mast cells (No./mm2)
CD117 positive cells (No./mm2)
Time PO
14d
56d
***
**
*

### *Figure 6.4 Mast cell activity in regenerating bone*

*Serial 5-micron sections of decalcified bone were stained with acidified toluidine blue (aTB) (A) to localize and quantify granulated mast cells in the medullary canal in relationship to the defect (arrowheads). Quantitative data (C) shows significantly fewer granulated mast cells at 14 and 56 days of healing. Immunostaining (B) and quantification (D) of mast cells with CD117 antiserum to the c-kit membrane receptor showed significantly more CD117 positive cells at 14 days of healing in the double fracture group, predominantly due to staining of cells in the medullary canal. However, at 56 days of healing, control animals showed more CD117 positive cells both in the periosteal side of the gap region and in the medullary canal. Unpaired t-test was applied to data from N=6 animals per group. Significantly different from placebo control ** p <0.01, *** p<0.001. Scale bars in A: 500 µm, in B: 50 µm.*

### *Recruitment of macrophages to regenerating tissue*

Adjacent sections of femora were immune-stained with F/40 or arg-1 antisera to identify all macrophages or M2 alternatively activated macrophages, respectively (Fig 5). Double fracture animals showed significantly more F4/80 and arg-1 immunoreactivity in the regenerating tissue and medullary canal at 14 days post-operative. Quantitative analysis confirmed an increase in both anabolic (F4/80) and catabolic (arg-1) markers at 14 days post-operative in double fracture mice, which reversed at 56 days of healing. Table 2 shows a summary of all cellular markers in the experiment.

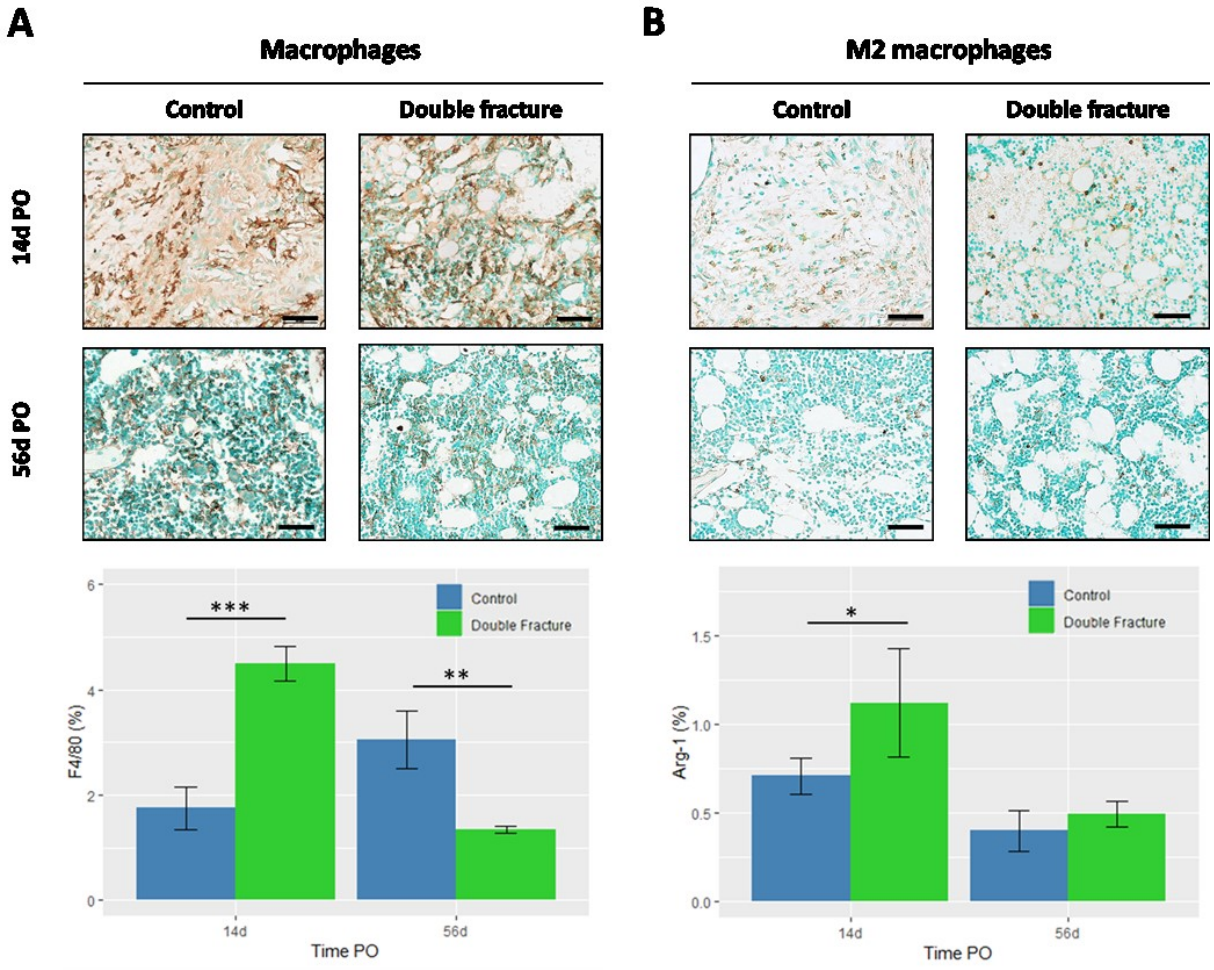



***Figure 6.5 Macrophage activity in regenerating bone***

*Immunostaining of serial 5-micron sections of decalcified bone with F4/80 antiserum to identify all macrophages (A) or arginase-1 antiserum to identify M2 macrophages (B) in the medullary canal showed significantly more positive cells at 14 days of healing in the double fracture group compared to controls. At 56 days of healing, significantly more F4/80 positive macrophages were found in the control group, while no significant differences in the M2 macrophage content between groups were observed. Unpaired t-test was applied to data from N=6 animals per group. Significantly different from placebo control * p <0.05, ** p<0.01, *** p<0.001. Scale bars represent 50 µm.*

*Table 6.2 Quantitative staining analysis of cellular activity*

| | 14d PO | | | 56d PO | | |
|---|---|---|---|---|---|---|
| **Variable** | **Control** | **Double fracture** | **p value** | **Control** | **Double fracture** | **p value** |
| **ALP (%)** | 5.6 ± 1.3 | 5.7 ± 2.5 | 0.919 | 2.1 ± 0.8 | 0.3 ± 0.08 | <0.001 |
| **TRAP (%)** | 3.3 ± 1.4 | 7.2 ± 2.1 | <0.001 | 0.3 ± 0.1 | 0.1 ± 0.03 | 0.027 |
| **CD34 (%)** | 3.2 ± 0.6 | 4.6 ± 0.3 | 0.018 | 0.1 ± 0.07 | 1.1 ± 0.2 | <0.001 |
| **aTB$^+$ cells (No./mm$^2$)** | 9.5 ± 1.6 | 4.9 ± 0.9 | <0.001 | 5.9 ± 1.5 | 2.9 ± 0.7 | 0.007 |
| **CD117$^+$ cells (No./mm$^2$)** | 10.0 ± 3.3 | 14.3 ± 2.9 * | 0.037 | 6.0 ± 1.7 | 3.1 ± 1.0 | 0.016 |
| **F4/80 (%)** | 1.7 ± 0.4 | 4.5 ± 0.3 | <0.001 | 3.0 ± 0.5 | 1.3 ± 0.07 | 0.004 |
| **Arg-1 (%)** | 0.7 ± 0.1 | 1.1 ± 0.3 | 0.024 | 0.4 ± 0.1 | 0.5 ± 0.07 | 0.198 |

*Unpaired t-test was applied to data from N=6 animals per group. All units represent % of stained area relative to total area. Significantly different from acidified toluidine blue * p <0.05.*

*ALP = osteoblasts; TRAP = osteoclasts; CD34 = vascular endothelial cells; aTB = granulated mast cells; CD117 = mast cells; F4/80 = macrophages; Arg-1 = M2 macrophages.*

### *Assessment of the interaction of mesenchymal stromal cells with mast cells in vitro*

The preceding experiments suggested that mast cell activity was not related to increased bone production, but rather was associated with the remodelling process and probably to the recruitment of precursor cells. We, therefore, examined the interaction of primary bone marrow derived mesenchymal stromal cells (MSCs) and mast cells (MC) *in vitro* using a 3D microgel culture system (Fig 6). After three days of culture in the microgel, the droplets containing MSCs showed similar rates of contraction, regardless of if they had been co-cultured with MC or not. However, ALP expression in the droplets co-cultured with MC was significantly higher.

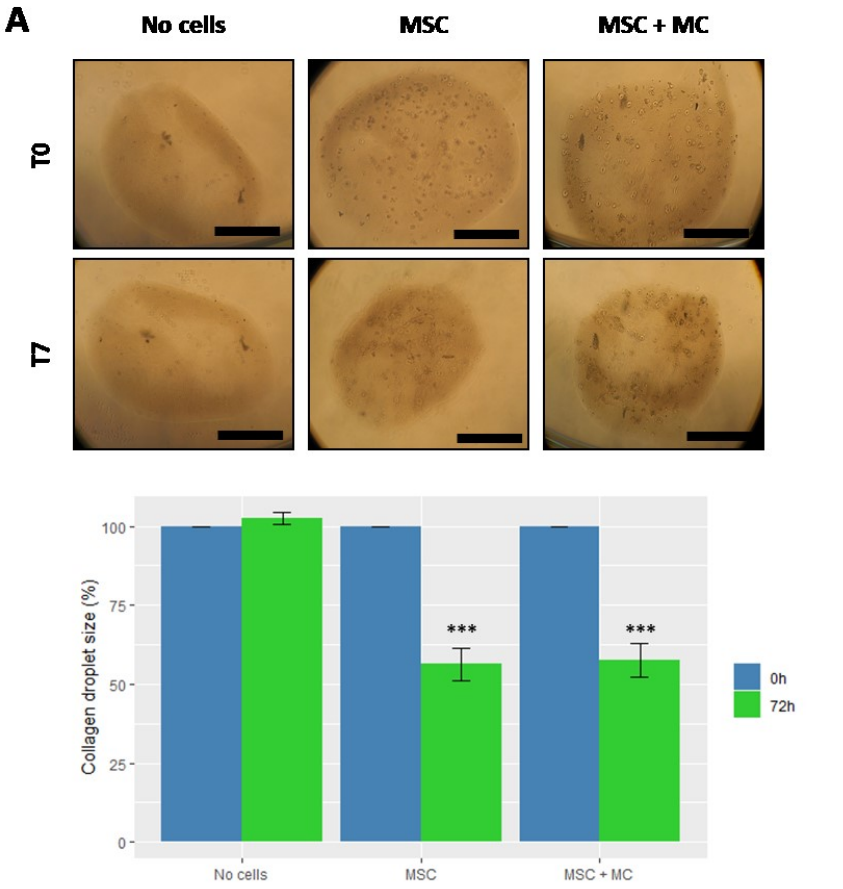

A
No cells
MSC
MSC + MC
T0
T7
Collagen droplet size (%)
100
75
50
25
0
***
***
No cells
MSC
MSC + MC
0h
72h
B
MSC
MSC + MC
ALP stain area (%)
15
10
5
0
###
***
***
No cells
MSC
MSC + MC

***Figure 6.6 Interaction of mesenchymal stromal cells with mast cells in vitro***

*Collagen microgels were prepared either without cells, murine mesenchymal stromal cells (MSC) or co cultured with mast cells (MSC + MC). After 72 hours of culture, the microgels containing cells experienced significant contraction without differences between the MSC or MSC + MC gels (A). Alkaline phosphatase (ALP) staining (B) showed significantly higher expression of this marker in the microgels of MSC co-cultured with MC. Unpaired t-test was applied to data from N=3 animals per triplicate. *** $p<0.001$ compared to acellular microgels, ### $p<0.001$ compared to MSC. Scale bars represent 50 µm.*

## 6.5 Discussion

Osteoimmunology is an emerging field dealing with the interactions between the immune and skeletal system under healthy or pathological states. Not surprisingly, the bone is highly influenced by the immune system, as it acts as a primary lymphoid organ where stem cells are maintained. As such, the immune interactions are bidirectional, and bone metabolism can also influence immune regulation (Ponzetti & Rucci, 2019; Tsukasaki & Takayanagi, 2019). In the context of bone healing, the effect of the inflammatory environment is well known. Inflammation plays a crucial role in maintaining the normal healing process through the activity of soluble factors, cell to cell interactions and paracrine signaling (Gerstenfeld et al., 2003; Ono et al., 2016; Toben et al., 2011). Despite this extensive documentation, there are almost no pre-clinical studies that have investigated if and how immunotherapies can affect bone healing. The goal of the current study was to determine if the repair of an initial fracture would improve bone repair in a second fracture sustained shortly after that under the paradigm that the initial fracture would "prime" the immune system to recognize broken bone neo-epitopes or induce a better inflammatory response.

Analysis of bone repair was performed in both male and female skeletally mature mice aged 5-6 months. Analysis of micro-CT data showed surprisingly no significant differences between the mice groups in the quantity of bone produced at 14 or 56 days of healing. However, the bone quality presented significant differences. At 14 days postoperative, mice in the double fracture group showed more branched and thinner trabeculae that had more osteocyte lacunae; and at 56 days, the connectivity density of the repairing bone was significantly lower than the observed in controls, however the number of osteocyte lacunae remained significantly elevated and higher

mineral content was identified in the tissue. 3D models of the bone and gap region confirmed the findings and showed a better remodeling profile, with less scarring and an appearance that resembled uninjured bone. Taken together these findings strongly suggest that the animals in the double fracture group do not show an increase in the mineralized tissue production nor an increase in the healing speed, as both groups already show almost complete bridging of the defects, but rather an increase in the rate of bone remodeling.

Interleukin-6 (IL-6), Tumor Necrosis Factor (TNF)-α, Receptor-Activator of Nuclear factor Kappa B (RANK), and its Ligand (RANKL) are some of the molecules that not only are associated with an inflammatory response, but also are critical components in the bone homeostasis, particularly at the osteoclast level (Nakashima et al., 2011). In contrast to Wu et al. report, where they found an increase in the bony callous volume after administration of SEC2 in their rat model (Wu et al., 2018), our results do not indicate an increase in the activity of osteoblasts. In line with our previous findings, ALP activity, a marker of bone anabolism, was not significantly different in the double fracture mice at 14 days of healing compared to controls. Nonetheless, TRAP activity was significantly increased at this time point and furthermore, more and larger TRAP positive cells could be identified in the former group (Fig 2B). Osteoclastogenesis is a process regulated by the RANK-RANKL-OPN axis. Previous studies have consistently failed to provide *in vivo* evidence showing that RANKL can be substituted by other molecules (Tsukasaki et al., 2017), but in turn, the RANK expression can be induced by several molecules and cell types (H. Kitaura et al., 2004). One of the main drivers of RANK expression is TNF-α, a potent pro-inflammatory molecule (Nakashima et al., 2012), thus the finding of a higher inflammatory state in the double fracture mice, revealed by the presence of significantly more total

number of macrophages and of alternatively activated M2 macrophages, can partly explain the increase in TRAP activity. By 56 days of healing the inflammatory response had subsided, as evidenced by significantly less macrophages in the double fracture mice, and the tissue showed signs of greater remodelling, such as less coupled ALP and TRAP activity and an orientation of the repair tissue that resembled the uninjured cortical bone more than the observed in the control group. Therefore, our findings further support the notion of an increase in the bone turnover rate probably fueled by a transient increase in the inflammatory state.

Increased inflammatory activity at 14 days of healing in the double facture group is also suggested by the differences observed in the number of granulated mast cells and CD117 positive cells. Mast cell granules contain a wide array of extracellular matrix remodelling enzymes, chemokines and growth factors, which when released modify the activity of surrounding cells, promote neo-angiogenesis and have immunomodulatory effects (Baram et al., 2001; Wernersson & Pejler, 2014). Our results show that the double fracture group had significantly less granulated mast cells than the controls. However, the absolute number of CD117 positive cells was increased. In contrast, control animals had a similar number of granulated cells and CD117 positive cells. CD117 is also expressed in progenitor and hematopoietic cells to a lesser extent (Joakim S. Dahlin & Hallgren, 2015; Yan Zhou et al., 2010, p. 117), thus we believe that taken together, the results suggest that the mast cells present in the repair tissue of the double fracture group are more active. In accordance with this line of thought, our previous research has shown a mismatch in the granulated to the total number of mast cell ratio in mice exposed to lipopolysaccharide (LPS) during bone repair (Ramirez-Garcia-Luna et al., 2019), highlighting the impact that shifting the systemic inflammatory environment has on mast cell activity.

Neo-angiogenesis and restoration of adequate blood supply are essential for successful fracture repair (Stegen et al., 2015). The CD34 immunohistochemistry demonstrated a significant increase in this marker in the double fracture group animals both at 14 and 56 days of healing. The bones in this group not only showed significantly more vascular channels in the gap and medullary canal regions at 14 days of healing, but they also presented an increase in the number of CD34 positive cells in the bone marrow, a finding that was consistent at 56 days post-operative as well. Because the CD34 surface marker is also expressed by hematopoietic and a subgroup of mesenchymal stromal cells (Sidney et al., 2014; Viswanathan, Kulkarni, Bopardikar, & Ramdasi, 2017, p. 34), we believe that the increase in total CD34 expression observed in the double fracture group of the present study may be in part due to a larger network of newly formed blood vessels, but also because increased number of progenitor cells. In previous work, we showed defective bone repair associated with impaired angiogenesis, which was attributed to the depletion of mast cells and the pro-angiogenic factors that they release (Behrends et al., 2014; Ramirez-GarciaLuna et al., 2017). Our finding of increased mast cell activity at 14 days of healing in the double fracture mice may explain in part the increase in the recruitment of blood vessel and stromal cells. This hypothesis is further substantiated by our *in vivo* finding of increase ALP expression in mesenchymal stromal cells co-cultured with mast cells.

In conclusion, the results of this study provide strong evidence to support the conjecture that a transient increase in the inflammatory state of a healing fracture induced by another fracture previously sustained within a short period promotes faster bone remodeling and increased neo-angiogenesis. This strategy needs to be validated in larger animal models and using a full fracture model, as our animals heal by intramembranous ossification. However, if found to be effective, this strategy could be used to promote better repair tissue in patients at high risk of fracture complications.

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**Author roles**

Study design and execution: JRGL, JEH, PAM. Data collection and analysis: JRGL, OOO, KRB, MA. Data interpretation: JRGL, DHR, JEH, PAM. Manuscript draft: JRGL, DHR, JEH, PAM. All authors share responsibility for the integrity of the data analysis.

# VII. CONCLUSION: THE ROLE OF MAST CELLS IN BONE HEALING

This section has been adapted from the manuscript entitled *"The good, the bad and the ugly: a review of the role of mast cells in bone healing"*. In this second chapter of the manuscript, the role of mast cells in bone healing, in other musculoskeletal tissues, and in biomaterial integration is reviewed. The section ends with some perspectives and directions where we believe future research should be oriented and a general conclusion of the topic. It also serves to summarize the work presented in the previous sections and to discuss it in light of the current literature about mast cells and bone healing.

This review was an ongoing project started in 2015 by Dr. Dominique Behrends and Mr. Desmond Hui, former students of the bone engineering lab, and continued by me under the supervision of Dr. Janet E. Henderson and Dr. Paul A. Martineau. Its aim is to review our current understanding of the role of mast cells in musculoskeletal tissue repair and how they may affect biomaterials used for osteointegration, and to present a general conclusion of the body of work that we have created in the past years. As such, this constitutes the final chapter of this thesis and will serve to propose questions that could be addressed to continue where the work presented here ended.

# THE GOOD, THE BAD AND THE UGLY: A REVIEW OF THE ROLE OF MAST CELLS IN BONE HEALING

## *Part II: Mast cells, musculoskeletal repair and response to biomaterials*

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## 7.1 Mast cells and fracture repair

Despite significant advances in the management of orthopaedic trauma reconstruction of open fractures of the appendicular skeleton, particularly those that involve extensive soft tissue damage, remains a significant challenge to orthopaedic surgeons. The term non-union is assigned to fractures considered to have no potential to heal without further intervention. A recent analysis of health research data in the United States (Zura et al., 2016), the United Kingdom (Court-Brown, Bugler, Clement, Duckworth, & McQueen, 2012) and Denmark (Larsen et al., 2015) revealed that while the incidence of fracture non-unions is around 5%, the highest incidence is in open fractures of the tibia in patients under the age of 50. In addition to anatomical location, risk factors for impaired healing included the amount of bone loss, the extent of soft tissue injury and the degree of wound contamination. Other factors known to compromise fracture healing include chronic inflammatory pathologies, age related disorders like osteopenia and chronic use of medications like nonsteroidal anti-inflammatories (NSAID) in patients with arthritis and corticosteroids used in the treatment of cancer (Zura et al., 2016). Autologous bone grafts remain the "gold standard" for adjunct therapy to assist healing of long bone defects but are limited by insufficient supply, donor site morbidity and extended operating time. Allograft bone is more plentiful but carries the risk of infection and is relatively inert, so does not integrate with native bone, while recombinant growth factors are used at prohibitive cost and with serious risks to patients.

### *Physiologic bone healing*

In Chapter II, we outlined the diversity of cells and their inter-dependence in modulating the overlapping phases of hemostasis, inflammation, repair and remodelling of soft tissue wounds. The early phases of fracture healing occur through a similar, highly ordered sequence of events that is heavily dependent on interactions between mesenchymal, vascular and immune cells in a time dependent and spatially restricted manner. Fracture healing is, however, distinguished from all other connective tissue healing by its requirement for mineral deposition to achieve its original form and function. After the formation of a soft connective tissue callus, osteogenic cells mature from bone marrow precursors and deposit woven bone to form a hard callus, which is remodeled by osteoclasts that mature from blood borne mononuclear cells. The woven bone is then replaced slowly over time by *bona fide* bone composed of osteocytes organized into interconnected osteons. Two distinct processes of the forming of the soft callus are recognized: intramembranous healing, in which mineralization of a collagen scaffold called osteoid and the extracellular matrix occurs, or endochondral bone repair, in which a cartilaginous template is formed first and undergoes secondary ossification. From both processes, intramembranous healing achieves better results as it directly restores the anatomical structure and physiological function of bone. However, this type of bone repair rarely takes place under physiological conditions because it requires a stable bone defect and an adequate blood supply (Marsell & Einhorn, 2011). The normal healing process lasts 8 to 12 weeks in humans, but to restore the previous architecture of the bone and its load-bearing capacities, the remodeling phase can last up to two years (Thomas A. Einhorn & Gerstenfeld, 2015).

The presence of MC in fracture healing was first documented in adult rats with crude, unfixed transverse fractures of the tibia and fibula (R. Lindholm, Lindholm, & Liukko, 1967). MC stained with toluidine blue were shown to increase in number in undifferentiated mesenchymal repair tissue for up to 2 weeks post-operative, and then decline concomitantly with osteogenic differentiation. A similar study in rabbits showed a peak number of MC in the periosteal callus at 1-week post-operative, with some evidence of MC in the endosteal region (R. V. Lindholm & Lindholm, 1970). The rat model of blunt force trauma was later refined to generate more reproducible closed transverse fractures of the femur stabilized with an intramedullary pin (Sarmiento, Schaeffer, Beckerman, Latta, & Enis, 1977). This model was used to demonstrate localization of chymase-positive MC in the vicinity of blood vessels at 2 weeks post-fracture and in connective tissue occupying the intramedullary space at 8 weeks (Banovac et al., 1995). The results from these histochemical analyses were in keeping with previous biochemical analyses of cell and matrix vesicles, harvested from fracture callus from one to four weeks post fracture (Thomas A. Einhorn et al., 1989). The peak in neutral peptidase and alkaline phosphatase activity at 2 weeks of healing coincided with the early peak in MC numbers and suggested to facilitate angiogenesis in the early phase of healing.

Microarray analyses of the fracture callus over time suggested peak accumulation of MC at four weeks rather two weeks post fracture in rat femur fractures. In young adult rats, signals for MC chymase and Cpa3 RNA were significantly increased at four and six weeks in the callus of unstable femoral fractures fixed with an internal pin, as in the original model, and to a lesser extent in those immobilized with plate and screw fixation (Heiner et al., 2006). Similar results were seen for peak MC tryptase and Cpa3 at four weeks of healing in RNA harvested from fracture callus of

juvenile, adult and aged rats (Meyer et al., 2006). Our own results in mice femora show no granulated mast cells in the medullary canal of uninjured bone during the first five days after sustaining a fracture, a gradual increase in mast cell number at 14 days after the injury, the peaking of the cells at 4 weeks of healing, and a gradual decrease in cell numbers at 8 weeks after the injury (Ramirez-GarciaLuna et al., 2017). The apparent discrepancy in timing is most likely attributable to differences in the methodology, aimed at assessment of MC granules or protein in one case and RNA, which may or may not be transcribed in the other.

### *Bone healing in the presence of mast cell excess*

Adult onset systemic mastocytosis is classified by the World Health Organization as a heterogenous neoplastic disease characterised by monoclonal proliferation of MC in extra-cutaneous tissue, most commonly bone marrow (Horny, Sotlar, & Valent, 2014). It differs from the pediatric form of mastocytosis, which is restricted to skin and commonly resolves as the child matures. In contrast to the large, round granular cells found under normal circumstances, transformed MC tend to be spindle shaped and express common T cell markers CD25 (Il-2 receptor) and/or CD2 (cell adhesion), along with CD117 and tryptase. Retrospective analysis of bone biopsies from more than 150 patients with untreated indolent systemic mastocytosis revealed neoplastic cells situated around blood vessels, dispersed in a diffuse pattern throughout the bone marrow or concentrated in granulomas (Seitz et al., 2013). Quantitative analyses indicated a reduction in trabecular bone volume and an increase in osteoid volume were associated with an increase in osteoclasts and osteoblasts. These observations are supported by clinical reports of an increased incidence of vertebral fragility fractures, as well as focal lytic and sclerotic lesions in patients with indolent systemic mastocytosis (Rossini et al., 2016). Chronic, unstimulated release

of mediators like histamine and TNF alpha from transformed MC in the bone marrow are proposed to cause the bone pathology. MC histamine has been proposed to promote osteoclast recruitment and activity during the physiologic turnover of the rat maxillary bone after tooth extraction (Dobigny & Saffar, 1997) and in the skull of young rats after parathyroid hormone (PTH) injection (Nakamura, Kuroda, Narita, & Endo, 1996). Moreover, the use of teriparatide, a recombinant PTH, has demonstrated an increase in angiogenesis with a coupled reduction in the number of large blood vessels (arteriogenesis) in a mouse calvarial bone defect model (L. Zhang et al., 2017). When the mast cell inhibitor sodium cromolyn is injected into the animals, inhibitory effects on arteriogenesis are also observed. For both cases, Zhang et al. showed that the inhibition of arteriogenesis is characterized by a reduction of mast cells and prevention of fibrotic scarring in the defects, which was translated into better bone healing.

Mast cell involvement in fibrosis of soft organs is a controversial topic, although most of the evidence points to a pro-fibrotic role (Bradding & Pejler, 2018). Mast cells secrete both pro and anti-fibrotic molecules and have the capacity of tissue heterogeneity, thus, are subjected to cues from the surrounding tissue that directs and guide their activity. Fibrodysplasia ossificans progressiva (FOP) is a genetic disorder characterized by progressive heterotopic ossification associated with dysregulated production of bone morphogenetic protein 4 (BMP4). The hallmark of this disease is uncontrolled inflammation and tissue repair after minor trauma that leads to fibrotic scarring and calcification of the repair tissue (Connor & Evans, 1982). Mast cells have been found to be present at every stage of the development of FOP lesions, being the most active at the fibroproliferative stage that is characterized by an excess of neo-angiogenesis. Mast cell density at the periphery of FOP lesion tissue was found to be up to 150-fold greater compared to

uninvolved skeletal muscle from FOP patients. Thus, mobilization and activation of inflammatory mast cells appear to be part of the physiopathology of FOP lesions (Gannon et al., 2001). This hypothesis was recently tested through the use of a mouse model of FOP (Brennan et al., 2018). Using this model, Brennan et al. showed that mast cell inhibition by cromolyn sodium, a mast cell stabilizer that prevents de-granulation, reduced the amount of heterotopic bone formed in a dose-dependant manner, and reduced the number of mast cells in the lesioned tissue. Furthermore, it has also been shown that FOP tissue highly expresses the proinflammatory cytokines TNF-α, IL-1β, and IL-6, but that depletion of mast cells and macrophage populations lowers the cytokine content and prevents the appearance and progression of FOP lesions (Convente et al., 2017). A recent paper showed the interdependency of a niche of stem cells with vascular endothelial cells, macrophages and mast cells that upon excessive activation of the BMP signaling pathway form a unique pro-inflammatory environment that promotes the deposition of excessive repair tissue and the development of FOP (Kan et al., 2019). Finally, in human biopsy samples of calcific tendinitis, a disease characterized by the calcification of tendons that frequently affects shoulders and is not associated to FOP, researchers found a twofold to eightfold increase of nerve markers, neovascularization markers, macrophages, and mast cells compared to control biopsies or biopsies of people with rotator cuff tear injuries (Hackett, Millar, Lam, & Murrell, 2016). Taken together, these findings highlight the impact of the excess of mast cell numbers and activity in inflammation, fibrosis, fibrotic scarring and the development of heterotopic ossification.

Mast cell accumulation during bone healing has also been found in animal models with delayed healing of fractures induced by non-steroidal anti-inflammatory drugs (NSAIDs) (Banovac et al., 1995; Ramirez-Garcia-Luna et al., 2019). In this context, hyperplasia of mast cells

and cell degranulation was often seen in close proximity to osteoclasts and areas of bone resorption, as well as in the bone marrow. The authors of these reports concluded that mast cell hyperplasia was probably reactive to the anti-inflammatory environment and Banovac et al. were able to characterize the mast cells in the fracture callus as being chymase-positive, thus belonging to the connective tissue mast cell type. In contrast, significant reductions in skeletal cells, endothelial cells, macrophages and M2 alternatively-activated macrophages were observed in the NSAID treated animals, compared to controls (Ramirez-Garcia-Luna et al., 2019). However, it is not clear what is the clinical meaning of the mast cell hyperplasia, as NSAID treatment affects multiple cell lines, including osteoblasts and endothelial cells (García-Martínez, De Luna-Bertos, Ramos-Torrecillas, Manzano-Moreno, & Ruiz, 2015; Lucena et al., 2016; Ramirez-Garcia-Luna et al., 2019), which make the interpretation of the results obscure.

### *Bone healing in the absence of mast cells*

A wide variety of MC deficient transgenic mice exist, but they are traditionally grouped as Kit or Kit-independent models (H.-R. Rodewald & Feyerabend, 2012). Mice with spontaneous and targeted mutations in genes that alter MC numbers have made significant contributions to our knowledge of their role in bone healing. The white spotting (*W*) mutation results from a spontaneous deletion on mouse chromosome 5 that encodes amino acids 513-590, which includes the transmembrane domain of the c-kit receptor (Geissler et al., 1988). $W^v$ is a spontaneous point mutation in the sequence encoding amino acid 660 in the membrane proximal kinase domain that results in about 90% reduction in the receptor's kinase activity (Nocka et al., 1990). The receptor tyrosine kinase Kit maps to the mouse W locus; thus, Kit mutations are causal for the phenotypes of W mice, including their mast cell deficiency (H.-R. Rodewald & Feyerabend, 2012). Adult mice

heterozygous for the $W/W^v$ mutation known as $Kit^{W/Wv}$ have no mature peritoneal or connective tissue MC (Cindik et al., 2000). However, $Kit^{W/Wv}$ mice are severely affected by their Kit deficiency in several tissues, including severe hematopoietic abnormalities that compromise the fitness of stem cells, the reproductive system and the immune system (Russell, 1979; Waskow, Terszowski, Costa, Gassmann, & Rodewald, 2004; J. S. Zhou, Xing, Friend, Austen, & Katz, 2007). As such, the femora of $Kit^{W/W\text{-}v}$ mice were found to be lighter and thinner than in their wild type littermates. The bone mass and geometry were significantly altered, but bone density and microstructure were normal. This was associated with a lower load bearing capacity of the femora, which was most likely caused by the low total bone mass (Cindik et al., 2000). In contrast, $Kit^{W\text{-}sh}$ mice have fewer cellular defects, especially at the hematopoietic level (Grimbaldeston et al., 2005). However, they still suffer from neutrophilia, megakaryocytosis and thrombocytosis that is associated with cardiac, lymphoid and splenic defects (Nigrovic et al., 2008). Nevertheless, in the femora of mast cell deficient $Kit^{W\text{-}sh}$ mice, no morphological differences in length, width or cross-sectional area at the mid-diaphysis were found compared to wild type mice (Behrends et al., 2014), thus it seems to be the best Kit MC deficient model to assess bone repair.

In recent work, we demonstrated impaired bone healing and fibrous mal-union in mast cell null $Kit^{W\text{-}sh}$ mice (Behrends et al., 2014). Healing of a cortical bone defect in these mice was significantly impaired as indicated by a high rate of mal-unions and decreased quality of the new bone. The defective healing was associated with premature and excessive expression of osteoclasts and tartrate-resistant acid phosphatase (TRAP) positive cells embedded in fibrous tissue with little change in osteoblast activity. Additionally, we found reduced vascular endothelial cell and macrophage activity at two weeks post-operative. Impaired bone healing was, therefore attributed

to altered catabolic activity, impaired re-vascularisation and compromised replacement of osteoid with bone (Behrends et al., 2014). However, since Kit mutant mice also exhibit defects in stem cells and skeletal cells, particularly osteoclasts (Lotinun & Krishnamra, 2016), the involvement of mast cells in these processes was inconclusive.

Kit-independent MC deficient mouse models have been developed through the expression of Cre recombinase under the control of mast cell protease genes. The Cre recombinase is a tyrosine bacteriophage recombinase enzyme that carries out site specific recombination events between two DNA recognition sites, called LoxP sites. The enzyme's recombination system can be used to manipulate genes in such a way that allows the knock out or knock in of specific genes (Nagy, 2000). The Kit-independent MC deficient mouse strains developed to date differ in the selected gene loci, the methods to drive gene expression in mast cells, and in the MC depletion mechanisms (H.-R. Rodewald & Feyerabend, 2012). Some of them use the diphtheria toxin (DT) system for the depletion of the mast cell lineage (Dudeck et al., 2011; Otsuka et al., 2011), while others ablate mast cells constitutively by exploiting the genotoxicity of Cre-recombinase (Feyerabend et al., 2011), or through depletion of mast cells by Cre-mediated deletion of the *Mcl-1* apoptosis suppressor gene (Lilla et al., 2011). Interestingly, since all these mouse models vary in the Cre gene target, MC deficiency can be either selectively triggered in specific MC populations, for example in the *Mcpt5-Cre* x iDTR mouse where connective tissue MC are ablated but not mucosal MC, or the full MC lineage can be ablated, such as in the $Cpa3^{Cre/+}$ mouse strain. Additionally, except for the $Cpa3^{Cre/+}$ mouse strain, all other Kit-independent MC deficient models show a rebound of the MC populations after 7 to 20 days after the administration of DT, or after inflammatory events (H.-R. Rodewald & Feyerabend, 2012).

To more precisely define the cellular targets impacted by mast cells in healing bone, we characterized the repair process in mast cell deficient *Cpa3*$^{Cre/+}$ mice (Ramirez-GarciaLuna et al., 2017). *Cpa3*$^{Cre/+}$ mice exhibit mast cell deficiency and up to a 40% circulating basophil deficiency in the absence of any other documented cellular defect (Feyerabend et al., 2011). The *Cpa3* locus is very strongly expressed in mast cells, and Cre expression from this locus leads to p53-dependent apoptosis of mast cells due to chromosomal lesions brought about on endogenous pseudo-loxP sites in the mouse genome (Feyerabend et al., 2011; H.-R. Rodewald & Feyerabend, 2012). Morover, under physiological conditions, the skeletal phenotype of *Cpa3*$^{Cre/+}$ mice is indistinguishable from their *WT* littermates. Using this mouse model, it was confirmed that re-vascularization of repair tissue in a uni-cortical bone window defect was delayed and dis-organised and associated with alterations in osteoclast and macrophage activity. Osteoclast activity in the mast cell deficient mice was initially stunted, with less activity and TRAP positive cells in the fibrous tissue of the wound, while at later stages it was significantly increased. Macrophage numbers in these group were lower compared to WT littermates. Furthermore, no direct impact on osteoblast activity was identified, however more osteoid was observed in the mast cell deficient mice, suggesting that mast cell activity in required for appropriate mineralization of the repair tissue. The outcome at eight weeks postoperative was also fibrous mal-union of most of the bone defects and pitting of the surrounding uninjured bone due to excessive osteoclast activity (Ramirez-GarciaLuna et al., 2017).

A recent report by Kroner et al., which used *Mcpt5-Cre+* R-DTA mice, demonstrated that mast cells do not affect the physiological bone formation or turnover (Kroner et al., 2017). Similar to our own findings in *Cpa3*$^{Cre/+}$ mice, the bone phenotype of *Mcpt5-Cre+* R-DTA mice was not different from WT mice. However, after generating a full tibial fracture, the authors found that while cytokine-release kinetics was similar between mast cell deficient and control mice, the levels of the pro-inflammatory cytokines IFN-γ, IL-1β, IL-6, TNF-α, IL-12p70 and IL-18 were reduced 6 to 24 hours after the fracture both in the fracture hematoma and systemically. The lowering of these key cytokines, particularly IL-6 and TNF-α, could potentially explain the stunting effect initial osteoclast activity seen by other authors, as these cytokines promote osteoclastogenesis. Additionally, concentrations of the chemotactic mediators G-CSF, keratinocyte-derived chemokine, MCP-1 and MIP-2 were reduced similarly. Taken together, these results suggest that MC deficiency does not modify the physiological bone phenotype or remodeling process, but the systemic immune response following trauma is impaired in the absence of mast cells. Consistent with our previous findings, a few MC were found 3 days after the fracture at some distance from the fracture gap, but at 7 days after the injury, they were found adjacent to the endosteal callus. In the later phases of fracture healing, MC number peaked, and they accumulated in the peripheral fracture callus near newly formed trabecular structures and at sites of bone resorption, in close proximity to osteoclasts. Also, less inflammatory cells were observed in in MC deficient mice compared with controls (Kroner et al., 2017).

Independently of the mast cell deficiency model and surgical technique used, the authors of these papers found several consistent results. First, fewer macrophages were found in the fracture hematoma, repair tissue and in systemic reserves of mast cell deficient mice, thus

confirming that mast cells are involved in the recruitment of macrophages during bone healing. Second, at 14 days of healing, no significant differences in the formation of bone could be identified between mast cell deficient and control mice, thus strongly suggesting that mast cells do not affect the activity of osteoblasts or the recruitment of pre-osteoblasts. The influence of mast cells on osteoblasts is not entirely understood, and experimental results are inconsistent. In other studies, mast cell chymase has been shown to affect osteoblast by modifying the ECM in vitro. The partially degraded ECM led to osteoblast detachment and decreased spreading on different ECM proteins without affecting the viability of osteoblasts (Banovac et al., 1995). In contrast, experiments assessing the bone remodelling in vivo in mast cell deficient $Kit^{W/W\text{-}v}$ mice showed a reduction in local osteoblast population and decreased osteoblastic matrix production (Silberstein, Melnick, Greenberg, & Minkin, 1991). However, in mast cell deficient $Kit^{W\text{-}sh}$ and $Cpa3^{Cre/+}$ mice, the osteoblast activity in a cortical bone defect was found to be similar to wild type mice (Behrends et al., 2014; Ramirez-GarciaLuna et al., 2017). Instead, mast cells seem to have more influence on osteoclasts and bone resorption. The third consistent finding was an intense periosteal reaction in the surrounding un-injured bone, particularly on the opposite side to the uni-cortical defects, leading to the deposition of and excess of bone in this area was observed in mast cell deficient mice compared to *WT* controls. Finally, the authors found that mast cell number exhibit a specific temporal distribution pattern, where cells are not present in the bone marrow before injury, start to accumulate during the inflammatory phase of bone healing, peak at the start of the remodelling phase, and gradually diminish their numbers as time progresses and the bone is remodelled. A spatial distribution pattern was also observed since mast cells were mostly found in close proximity to blood vessels and osteoclasts in the three models (Behrends et al., 2014; Kroner et al., 2017; Ramirez-GarciaLuna et al., 2017).

Nonetheless, when compared the three mice models of bone repair in MC deficient mice, there are also significant discrepancies concerning osteoclast activity. TRAP activity and cathepsin-K immunostaining were increased in $Kit^{W\text{-}sh}$ mice after 14 days of healing, but no differences were found at later time points (Behrends et al., 2014). In $Cpa3^{Cre/+}$ mice, TRAP activity before two weeks of healing was not different from *WT* littermates, but afterwards, it was significantly increased. This increase was matched by intense remodelling of the cortical bone adjacent to the bone defect, which led to the creation of large channels and sequestrae (Ramirez-GarciaLuna et al., 2017). Both studies concluded that MC deficiency is deleterious to bone healing and that excessive osteoclast activity may contribute to this process. In contrast, Kroner et al. reported that in *Mcpt5-Cre*$^{+}$ R-DTA mice, at 14 days after the fracture, there is reduced osteoclast activity and callus remodelling, as assessed by micro-CT and qPCR. Furthermore, they also show that mast cell deficiency is protective against trabecular bone loss due to inhibition of osteoclast activity in ovariectomized mice (Kroner et al., 2017). Thus, the authors concluded that MC deficiency is protective against bone loss. The differences in the cellular activity observed in the studies may be explained in part by the type of bone injury created (stable window bone defect that heals through endomembranous ossification vs. full fracture healing by endochondral ossification), the choice of MC deficient mouse model used, and the time frame when bone repair was evaluated (early repair at 5 to 14 days vs. remodeling phases >28 days after injury).

Arguably, the most critical process for achieving adequate bone healing is neo-angiogenesis (Stegen et al., 2015). Mast cells have demonstrated to release several molecules in their granules, such as VEGF and renin, that alter vascular homeostasis (Reid, Silver, & Levi, 2007; Shaik-Dasthagirisaheb et al., 2013). Furthermore, mast cell proteases also promote the

paracrine expression of VEGF (Arai et al., 2017; Correia et al., 2016) and endothelin-1 (D'Orléans-Juste et al., 2008; Hültner & Ehrenreich, 2005) from surrounding cells. Both in *KitW-sh* and *Cpa3*$^{Cre/+}$ mast cell deficient mice, endothelial vascular cell activity was greatly diminished at 1 and 2-weeks post-injury, compared to *WT* controls (Behrends et al., 2014; Ramirez-GarciaLuna et al., 2017). Furthermore, in *Cpa3*$^{Cre/+}$ mice, we found that mast cell deficiency also leads to increased endothelial cell activity at 4 and 8-weeks post-injury (Ramirez-GarciaLuna et al., 2017). For both studies, the conclusion was that the defects in the neo-vascularization of the bone defects of MC deficient mice probably also contributed to the non-unions observed in both models. In addition, Zhang et al. found that intermittent rPTH treatment enhances bone allograft healing of calvarial defects by promoting anabolic effects on new bone formation via small vessel angiogenesis, and inhibition of angiopoietin-2–mediated arteriogenesis (L. Zhang et al., 2017). Because arteriogenesis is associated with fibrotic bone non-unions and accumulation of mast cells around large blood vessels (Antebi et al., 2016), the authors suggest that promotion of bone healing was achieved in part by limiting mast cell access via arterioles to the site of tissue repair, which in turn decreased fibrosis around and between the fractured ends of the bone. To confirm a direct role of mast cells on osteogenesis and neo-angiogenesis, the authors demonstrated that MC deficient *Mcpt5-Cre-iDTR* mice exhibited similar healing patterns as rPTH treated *WT* mice (L. Zhang et al., 2017). Taken together, the findings suggest that mast cells are involved in promoting the early neo-vascularization of the wound and in the pruning of the excess of blood vessels during the remodelling phase. Nevertheless, the balance between the degree of fibrosis deposition may be controlled by the number of mast cells that reach the defect, where too many or too few cells may shift the balance into a pro-fibrotic environment.

Finally, the effect of external factors on mast cell activity during bone repair has also been analyzed. Aging was associated to downregulation of mast cell gene activity and upregulation of pro-inflammatory genes in a murine long-bone fracture model, which may explain the delayed healing phenotype observed in elderly patients (Hebb et al., 2017). Similarly, human skin samples of healthy elder individuals show increased number of mast cells but less reactivity and functionality (Pilkington, Barron, Watson, Griffiths, & Bulfone-Paus, 2019), suggesting that the former finding could be explained in a similar way. The inflammatory milieu during bone healing has also been shown to affect mast cell number and distribution pattern in uni-cortical stable bone window fracture models. In mice treated with lipopolysaccharide (LPS) to promote systemic inflammation, the number of granulated mast cells was significantly reduced at one week of healing compared to controls, but no differences were found after two or six weeks of healing. This mast cell reduction was matched by significant increases in macrophage number at one and two weeks of healing in LPS treated animals, which led to non-union of the defects after 6-weeks of healing (Behrends et al., 2017). In contrast, chronic treatment with NSAID produced mast cell hyperplasia in bone defects, as mentioned before (Ramirez-Garcia-Luna et al., 2019). These findings highlight the plasticity of mast cell behaviour and activity under different inflammatory circumstances and can also partially explain the discrepancies observed in the different models of bone repair.

## 7.2 Effect of mast cells in muscle repair

For skeletal muscle, the involvement of mast cells in pathophysiological responses and tissue repair is still obscure. Skeletal muscle has several unique properties, such as the syncytial nature of the cell's cytoplasm extending from tendon to tendon, the requirement for satellite cells to achieve healing, and the paucity of connective tissue around the myocytes, which have specific bearing on wound healing (Frontera & Ochala, 2015). Muscle healing is, therefore, based on activation of satellite cells and their differentiation into myoblasts, which proliferate, fuse, and differentiate into myotubes to form new myofibers or fuse with the existing ones (Zammit, Partridge, & Yablonka-Reuveni, 2006).

Mast cells have been linked to pathogenesis and destruction of skeletal muscle tissue. Both patients and animal models of dystrophinopathies are characterized by an increase in mast cell content in the affected tissue, especially in muscles involved in the postural activity (Gorospe, Tharp, Hinckley, Kornegay, & Hoffman, 1994). It has been shown that mast cell infiltration to skeletal muscle after injury also correlates with neutrophil and monocyte recruitment, and that dramatic increases in mast cell density and mast cell infiltration of the muscle layer of large arteries near the injury site strongly suggest that the cells recruited to these areas are bloodborne (Gorospe, Nishikawa, & Hoffman, 1996). In this same paper, Gorospe et al. report that dystrophin-deficient *mdx* mice exhibit significantly higher levels of mast cell infiltration into the gastrocnemius compared to wild type littermates, even after *WT* muscle has been injured.

In autoimmune myositis, mast cell accumulation has also been documented, along with less CD8+ T lymphocytes, which are involved in the pathogenesis of polymyositis, and macrophages in mast cell deficient c-*kit* $W/W^V$ mice compared to *WT* littermates. These findings correlated with a less severe phenotype of the disease and lower vascular permeability in the affected tissue in the mast cell deficient animals, that became similar to the observed in the *WT* animals after mast cell reconstitution, thereby indicating a negative role of mast cells in the pathogenesis of this disease (Yokota et al., 2014). However, another report from Lefaucheur et al. (Lefaucheur, Gjata, & Sebille, 1996) showed that the disruption of myofibrils by either ischemia or the phospholipase notexin, promote mast cell recruitment and accumulation into the tissue that mimics what is observed in *mdx* mice. Furthermore, there seems to be a gradient where younger animals that undergo more successful tissue repair have more mast cell density in the muscle, thus contending the notion that mast cells have a pathogenic role in dystrophies.

In line with the latter finding, other reports show a positive role of mast cells in skeletal tissue repair. Mast cell tryptase has been shown to directly stimulate myoblast differentiation both *in vitro* and *in* vivo from precursor cells through activation of the protease-activated receptor-2 (PAR-2), cyclooxygenase-2 (COX-2) activity, and, prostaglandin J2 production (Arnold et al., 2007; Duchesne, Bouchard, Roussel, & Côté, 2013; Duchesne, Tremblay, & Côté, 2011). Additionally, mast cell accumulation in injured muscle results in increased proliferation an accumulation of macrophages, which also stimulate myogenesis and myofibrillar repair (Arnold et al., 2007; Duchesne et al., 2013). Finally, mast cells have been found to exert positive trophic influences on myocytes. After muscular denervation, the mast cell distribution pattern is modified from high density in tendons and low density in muscle tissue, to the opposite; as well as an

increase in the density of mast cells in all connective tissue compartments (endomysium, perimysium and epimysium), and increase in the H1 histamine receptors (Sánchez-Mejorada & Alonso-deFlorida, 1992). Taken together, the findings suggest that there is a neural regulatory aspect in the distribution of mast cells in muscular tissue, giving another layer of complexity to mast cell involvement in muscle repair.

## 7.3 Mast cells and biomaterials

Non-activated mast cells are attracted by very few agents, such as monocyte chemotactic protein-1 and CCL-5, but when they are activated, mast cells exhibit chemotactic responses to a wider variety of chemokines (Taub et al., 1995) and artificial molecules (Lu, Parmar, Kulka, Kwan, & Unsworth, 2018). In the context of bone healing, this could potentially be translated to differences in the recruitment and activity of mast cells induced by the biomaterials used in implants. Shortly after implantation, biomaterials induce a local inflammatory response that leads to the coating of the implant with fibrinogen, which in turn activates and attracts more inflammatory cells to the implant surface (L. Tang & Eaton, 1993). In fact, the accumulation of fibrin around implants has been found to induce mast cell chemotaxis and activation in as short as 15 minutes after implantation (Tsai et al., 2014). In a recent paper, we demonstrated that coating ceramic implants with either collagenous or non-collagenous bone protein extracts induced better integration of the implant to native rat tibial bone and elicited different immunogenic responses, compared to control non-coated implants. Moreover, non-coated implants showed a higher number of MC infiltration along with macrophages and lymphocytes that induced higher inflammation and fibrosis of the wound (Mansour et al., 2019). These responses are attributed to MC degranulation, as β-hexosaminidase release has been detected in response to implants (Farrugia et al., 2014).

MC mediators such as histamine, have been associated with fibrosis and potentially to rejection of implants (Li, 2010; L. Tang et al., 1998). In turn, this has been associated with the lower performance of the implants and changes in their lifetime (Anderson, Rodriguez, & Chang, 2008). Inhibition of tissue site MC activation and degranulation through the local delivery of

masitinib, a tyrosine kinase inhibitor, reduced the thickness of the foreign body capsule formed around implants after 28 days post-implantation (Avula, Rao, McGill, Grainger, & Solzbacher, 2013). A similar study using polypropylene or polyester mesh implants demonstrated that blocking MC degranulation with cromolyn treatment also reduced inflammation and fibrosis around the subcutaneous implant site (Orenstein, Saberski, Klueh, Kreutzer, & Novitsky, 2010). Lastly, in a murine model of continuous glucose monitoring, glucose sensing was erratic after seven days of implantation in *WT* mice, while mast cell deficient *B6.Cg-Kit(W-sh)/HNihrJaeBsmJ* (Sash) mice exhibited excellent sensor function for up to 28 days after implantation. Histopathological assessment of the glucose sensor implant sites showed significantly more inflammatory response in the *WT* mice, consistent MC accumulation in the margins of the sensor and significantly more fibrotic tissue deposition that interfered with the glucose sensor readings (Klueh, Kaur, Qiao, & Kreutzer, 2010). MC have demonstrated to recruit CD45(+)/Collagen 1(+) fibrocytes and promote myofibroblast differentiation to form a fibrotic capsule around implants. Interestingly, the recruitment of these cells is either enhanced or reduced by the localized release of compound 48/80 and cromolyn, respectively, and is stunted in *WBB6F1/J-Kit(W)/Kit(W-v)/J MC* deficient mice (Thevenot, Baker, Weng, Sun, & Tang, 2011). Taken together, all of these findings demonstrate the key role of mast cells in controlling inflammation, tissue response to injury and fibrosis of implants. It also highlights how MC deficiency, either congenital or drug-induced, provides potentially modulatory responses to implanted biomaterials, which in turn can be used to tailor biomaterial response and integration.

## 7.4 Perspectives and conclusion

The development of Kit-independent murine models of MC deficiency has challenged some of the imputed roles of MC in some diseases, such as in experimental autoimmune encephalomyelitis (Feyerabend et al., 2011). Because most of the experiments that show a role of MC in tissue repair and implant response have been done using Kit mutants, confirmation of these putative roles in Kit-independent mice is still needed. Additionally, MC reconstitution has traditionally been the confirmatory hallmark to identify a specific role of the cells in disease. However, the numbers, distribution, and functional responses of reconstituted mast cells may not reproduce their physiological roles to a full extent (Ebmeyer et al., 2010; Grimbaldeston et al., 2005). Absolute proof that engrafted mast cells behaves immunologically like normal endogenous mast cells are lacking. Furthermore, in the case of tissues such as bone, where MC normally do not reside, homing the engraftment of the cells may be close to impossible.

In any case, due to the observed roles of MC in tissue repair and in the fact that they already have pre-formed inflammatory mediators, cytokines, growth factors and vasoactive substances, investigation on their pharmacological manipulation to achieve better healing outcomes is warranted. However, the specific response and its timing still need to be elucidated. For example, our results on bone healing suggest that MC exhibit distinct temporal and spatial distribution patterns that very likely are matched by the release of distinct mediators (Behrends et al., 2014; Ramirez-GarciaLuna et al., 2017). Priming of mast cells to recognize injured tissue receptors may be a way to target their homing and activity to specific tissues. A recent report by Lu et al. showed how an IgE independent peptide was able to form self-assembling nanoscaffolds and activate

tissue-resident mast cells (Lu et al., 2018). This strategy paves the way for targeted activation of MC to either promote their activation or inhibition under specific circumstances.

MC has consistently demonstrated to be involved in angiogenesis in the cancer context (Y. Chen et al., 2017; de Souza Junior, Santana, da Silva, Oliver, & Jamur, 2015), in tissue repair (Mansour et al., 2019; Ramirez-GarciaLuna et al., 2017), and a direct interaction and positive-feedback loop with endothelial cells has been demonstrated *in vitro* (de Souza Junior, Mazucato, Santana, Oliver, & Jamur, 2017). Whether this angiogenic response can be tailored to promote better implant integration or promote tissue repair is yet to be investigated as there are very few reports delving on this. *In vitro* culture of MC on bioresorbable vascular grafts demonstrated that activation by IgE and dinitrophenol-conjugated albumin significantly enhanced MC adhesion, proliferation, migration, and secretion of several cytokines (Garg et al., 2011). Similarly, another report showed that polymer architecture influences mast cell response and elicit distinct cytokine and VEGF secretion, thus helping tailor implants to achieve better integration profiles (Abebayehu et al., 2019).

In conclusion, beyond their traditional roles in allergy, asthma and immunity, MC have been found to be key effectors of tissue homeostasis. Throughout several different reports, MC have demonstrated to be versatile cells whose response to injury, repair, tissue remodelling, and biomaterial implantation differ depending on the inflammatory context and type of tissue. MC normally only reside in tissues and organs that have close contact to the body's exterior, but after an injury, their precursors can mobilize and appear in tissues where normally they do not reside, such as bone. MC are armed with a wide array of pre-formed compounds that include proteases,

vasoactive factors, cytokines, and growth factors, and retain throughout their life the ability to synthesize compounds *de novo* and to re-granulate. These cells have demonstrated regulatory roles in maintaining homeostasis and tissue repair. However, their specific effect has not been fully elucidated and is obscured by their plasticity. Research has demonstrated both positive and negative roles for mast cells in tissue healing, particularly in musculoskeletal tissues. For this reason, and paraphrasing the Spaghetti Western film by Sergio Leone, we find MC to be the good, the bad and the ugly.

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**Author roles**

Project design and execution: DB, JRGL, JEH, PAM. Data collection and analysis: DB, JRGL, DH, JEH, PAM. Manuscript draft: DB, JRGL, DH, JEH, PAM. All authors share responsibility for the integrity of the data analysis.

## 7.5 Future directions

There are several questions that could be addressed to continue where the work in this thesis ended:

1. *What molecular targets are affected by mast cell activity during bone repair?*

   All the experiments presented in this thesis are based on phenotypic differences. Identification of the exact molecular pathways that are affected by mast cell activity would pave the way for the development of pharmacological targets to modify bone repair either by targeting mast cell activity or their effectors.

2. *Can pharmacological modification of mast cell activity promote better bone healing?*

   There is evidence that antihistamines modify bone density (Kinjo, Setoguchi, & Solomon, 2008) and therefore, that pharmacological modification of mast cell degranulation affects bone homeostasis. However, the impact of drugs that inhibit or promote mast cell degranulation has not been tested in the context of bone repair.

3. *How do mast cell precursors affect bone healing?*

   Our research shows differences in the neo-vascularization pattern and TRAP positive (osteal macrophage) and macrophage content of healing bones after 5 days post-operative, even in the absence of granulated mast cells. This strongly suggests that mast cell precursors are active from the beginning of the healing phase, thus identifying their role can help identify targets that can be used to promote better healing.

4. *Can mast cell precursors be used to promote better bone repair?*

Our research demonstrates that mature mast cells do not appear in healing bone and that they are not attracted to it after their administration after a fracture. However, this may be different for precursor cells, as in other diseases they are attracted to inflamed tissue. Transplant of precursor cells could be used as a cellular therapy to treat broken bone if they are attracted to the injury site.

5. *Can the bone repair phenotype be rescued in mast cell deficient mice through a full bone marrow transplant?*

A full bone marrow transplant is probably needed to rescue the bone healing phenotype of mast cell deficient mice.

6. *How do other immune cells, such as T-lymphocytes, interact with mast cells during bone healing?*

T-lymphocytes have demonstrated to play major roles in wound healing, especially by modulating the inflammatory response and maintaining immune tolerance against auto-epitopes. Discovering if there is an interaction between mast cells and lymphocytes in the context of bone repair may also serve to identify amenable targets for pharmacological interaction. Moreover, there is a big likelihood that these cells, which are also memory cells, play a role in the effect observed in the fracture priming experiment.

7. *For how long does the effect of a subsequent fracture on bone remodelling last?*

Confirmation of this effect and determining the temporality of the observed effect is needed for clinical translation of the results.

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# IX. APPENDICES

## 10.1 Related works

During my Ph.D. I contributed as a co-author to eight papers related to my thesis work on bone repair:

Drager J, **Ramírez Garcia-Luna JL**, Kumar A, Gburek U, Harvey E, Barralet J. Hypoxia Biomimicry to Enhance Monetite Bone Defect Repair. *Tissue Eng Part A*. 2017 Dec;23(23-24):1372-1381.

*Contribution:* Data acquisition and analysis, statistical analysis, manuscript writing.

Castano D, Comeau-Gauthier M, **Ramirez-Garcia Luna JL**, Drager J, Harvey E, Merle G. Noninvasive Localized Cold Therapy: A New Mode of Bone Repair Enhancement. *Tissue Eng Part A.* 2019;25(7-8):554-562.

*Contribution:* Data acquisition and analysis, manuscript writing.

Fairag R, Rosenzweig DH, **Ramirez-Garcialuna JL**, Weber MH, Haglund L. Three-Dimensional Printed Polylactic Acid Scaffolds Promote Bone-like Matrix Deposition in Vitro. *ACS Appl Mater Interfaces*. 2019;11(17):15306-15315.

*Contribution:* Data acquisition and analysis, manuscript writing.

Mansour A, Abu-Nada L, Al-Waeli H, Mezour MA, Abdallah MN, Kinsella JM, Kort-Mascort J, Henderson JE, **Ramirez-Garcialuna JL**, Tran SD, Elkashty OA, Mousa A, El-Hadad AA, Taqi D, Al-Hamad F, Alageel O, Kaartinen MT, Tamimi F. Bone extracts immunomodulate and enhance the regenerative performance of dicalcium phosphates bioceramics. *Acta Biomater.* 2019 Mar 8. pii: S1742-7061(19)30178-3.

*Contribution:* Data acquisition and analysis, artificial intelligence development, manuscript writing.

Alshahrani NS, Abu Nada L, **Ramirez-GarciaLuna JL**, Alamri A, Makhoul NM, Tamimi F. Ranitidine Impairs Bone Healing and Implant Osseointegration in Rats' Tibiae. *Under peer review*.

*Contribution:* data acquisition and analysis, statistical analysis, manuscript writing.

Comeau-Gauthier M, Tarchala M, **Ramirez-GarciaLuna JL**, Harvey E, Merle G. Unleashing β-catenin with a new anti-Alzheimer drug for bone defect repair. *Under peer review*.

*Contribution:* Data acquisition and analysis, manuscript writing.

Hadidi L, Ge S, Comeau-Gauthier M, **Ramirez-GarciaLuna JL**, Harvey E, Merle GE. Local delivery of therapeutic boron for bone healing enhancement. *Under peer review*.

*Contribution:* Data acquisition and manuscript writing.

Rangel-Berridi K, **Ramirez-Garcialuna JL**, Jabbour Z, Olasubulumi OO, Jalali Dil EE, Favis BD, Hoemann CD, Makhoul N, Henderson JE. PCL scaffolds fail to promote repair of mandibular defects in rats with bisphosphonate-related osteonecrosis of the jaw. *Under peer review*.

*Contribution:* Data acquisition and analysis, statistical analysis, manuscript writing.

## 10.2 Non-related works

During my Ph.D. I also contributed as the first author to four papers and as a co-author to ten papers related to other topics in biomedicine. These contributions represent collaborations with research groups in Montreal, Toronto, Mexico, and Denmark.

Kolosovas-Machuca ES, Martínez-Jiménez MA, **Ramírez-GarcíaLuna JL**, González FJ, Pozos-Guillen AJ, Campos-Lara NP, Pierdant-Perez M. Pain measurement through temperature changes in children undergoing dental extractions. *Pain Res Manag.* 2016;2016:4372617.

*Contribution:* Study design and statistical analysis.

Shiguetomi-Medina JM, Rahbek O, Abood AA, Stødkilde-Jørgensen H, **Ramírez Garcia-Luna JL**, Møller-Madsen B. Does radiofrequency ablation (RFA) epiphysiodesis affect adjacent joint cartilage? (2016). J Child Orthop. 2016;10(4):359-64.

*Contribution:* Surgical procedures and manuscript draft.

**Ramírez Garcia-Luna JL**, Araiza-Alba P, Martínez-Aguiñaga S, Rojas-Calderón H, Pérez-Betancourt MM. Correlation and agreement between depressive symptoms in children and their parent's perception *Salud Mental.* 2016;39(5):243-248.

*Contribution:* Design and supervision of the study, statistical analysis, manuscript writing.

Shiguetomi-Medina JM, **Ramirez-GarciaLuna JL**, Stødkilde-Jørgensen H, Møller-Madsen B. Systematized water content calculation in cartilage using T1-mapping MR estimations: design and validation of a mathematical model. *J Orthop Traumatol.* 2017;18(3):217-220.
*Contribution:* Mathematical and statistical model development and analysis, manuscript writing.

Martinez-Jimenez MA, Valadez-Castillo FJ, **Ramirez-GarciaLuna JL**, Gaitan-Gaona FJ, Pierdant-Perez M, Valdes-Rodriguez R, Sanchez-Aguilar JM. Effects of local use of insulin on wound healing in non-diabetic patients. *Plastic Surgery.* 2017;26(2):75-79.
*Contribution:* Data analysis, statistical analysis, manuscript writing.

**Ramirez-Garcialuna JL**, Dominguez-Paulin F, Ramirez-Martinez J, Sanmiguel-Delgadillo LF. Comparison and agreement of outcome scores through nine months after acetabular fracture fixation. *J Clin Orthop Trauma.* 2018;9(2):181-185.
*Contribution:* Project planning, data analysis, statistical analysis, manuscript writing.

Hernandez-Sanchez PG, Guerra-Palomares SE, **Ramirez-GarciaLuna JL**, Arguello JR, Noyola DE, Garcia-Sepulveda CA. Prevalence of Drug Resistance Mutations in Protease, Reverse Transcriptase and Integrase genes of North-Central Mexico HIV Isolates. *AIDS Research and Human Retroviruses.* 2018 Jun;34(6):498-506.
*Contribution:* Data acquisition and analysis.

Burnett AS, Mouhanna J, **Ramirez-Garcialuna JL**, Lee E, Breau J, Diovisalvi M, Alcindor T, Asselh J, Vanhuyse M, Alfieri J, David M, Mueller CL, Spicer JD, Cools-Lartigue J, Ferri L. Enrollment of esophago-gastric cancer patients in a clinical fast-track program and its effect on time to treatment and quality of life. *J Clin Oncol* 2018;36(4):S180.

*Contribution:* Data analysis and manuscript writing.

Boulila CA, Renaud S, Al-Lawati Y, Hasbini K, Abou-Malhab J, **Ramirez-GarciaLuna JL**, Lee E, Mueller CL, Spicer JD, Molina JC, Cools-Lartigue J, Ferri L. A Novel Endoscopic Pyloromyotomy Technique for Minimally Invasive Esophagectomy. *Gastroenterology* 2018;154(6):S-1261.

*Contribution:* Data analysis and manuscript writing.

Martinez-Jimenez MA*, **Ramirez-GarciaLuna JL*,** Kolosovas-Machuca S, Drager J, Gonzalez FJ. Development and validation of an algorithm to predict the treatment modality of burn wounds using thermographic scans: prospective cohort study. *PLoS ONE* 2018;13(11):e0206477. * *Equal contributions*

*Contribution:* Design of the study, data acquisition and analysis, artificial intelligence development, manuscript writing.

Zuniga-Villanueva G, **Ramirez-GarciaLuna JL**, Weingarten K. Factors Associated with Knowledge and Comfort Providing Palliative Care: A Survey of Pediatricians in Mexico. *J Palliative Care*. 2019;34(2):132-138.

*Contribution:* Data analysis and manuscript writing.

Sanchez-Espino LF, Zuniga-Villanueva G, **Ramirez-GarciaLuna JL**. An educational intervention to implement skin-to-skin contact and early breastfeeding in a rural hospital in Mexico. *Int Breastfeed J* 2019; 14:8.

*Contribution:* Project supervision, data analysis and manuscript writing.

Al Lawati Y, Cools-Lartigue J, **Ramirez-GarciaLuna JL**, Molina-Franjola JC, Pham D, Skothos E, Mueller C, Spicer J, Ferri L. Dynamic alteration of neutrophil-to-lymphocyte ratio over the treatment trajectory is associated with survival in esophageal adenocarcinoma. *Under peer review.*

*Contribution:* statistical analysis and manuscript writing.

**Ramirez-GarciaLuna JL**, Vera-Bañuelos LR, Guevara-Torres L, Martinez-Jimenez MA, Ortiz-Dosal A, Kolosovas-Machuca ES, Gonzalez FJ. Infrared thermography of abdominal wall in acute appendicitis: proof of concept study. *Under peer review*.

*Contribution:* data acquisition and analysis, statistical analysis, manuscript writing.

## 10.3 Peer review activities

During my Ph.D. I contributed as a peer reviewer for the following journals:

1. *Tissue Engineering*. Impact factor: 3.892. Number of papers reviewed: 11
2. *Nanoscale Research Letters*. Impact factor: 2.726. Number of papers reviewed: 2
3. *Acta Tropica*. Impact factor: 2.46. Number of papers reviewed: 2
4. *Sage Open*: Impact factor: 0.67. Number of papers reviewed: 1
5. *Adolescent Psychiatry*. Impact factor: 0.38. Number of papers reviewed: 1

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