

# Marrow compartment contribution to cortical defect healing

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**Background and purpose** — Healing of shaft fractures is commonly described as regards external callus. We wanted to clarify the role of the bone marrow compartment in the healing of stable shaft fractures.

**Patients and methods** — A longitudinal furrow was milled along the longitudinal axis of the femoral shaft in mice. The exposed bone marrow under the furrow was scooped out. The mice were then randomized to no further treatment, or to receiving 2 silicone plugs in the medullary canal distal and proximal to the defect. The plugs isolated the remaining marrow from contact with the defect. Results were studied with histology and flow cytometry.

**Results** — Without silicone plugs, the marrow defect was filled with new bone marrow-like tissue by day 5, and new bone was seen already on day 10. The new bone was seen only at the level of the cortical injury, where it seemed to form simultaneously in the entire region of the removed cortex. The new bone seemed not to invade the marrow compartment, and there was a sharp edge between new bone and marrow. The regenerated marrow was similar to uninjured marrow, but contained considerably more cells. In the specimens with plugs, the marrow compartment was either filled with loose scar tissue, or empty, and there was only minimal bone formation, mainly located around the edges of the cortical injury.

**Interpretation** — Marrow regeneration in the defect seemed to be a prerequisite for normal cortical healing. Shaft fracture treatment should perhaps pay more attention to the local bone marrow.

The healing of shaft fractures is often described in terms of external callus formation. Less interest has been directed towards the marrow compartment in the bone proximal or distal to the fracture. This compartment might be an important source of various cell types required for healing, and therefore of clinical importance (Masquelet 2003, Wenisch et al. 2005, Masquelet and Beuqe 2010, Kuehlfluck et al. 2015).

Mesenchymal cells contributing to external fracture healing may in large part be derived from periosteum, but also from surrounding muscle (Liu et al. 2011). It has been debated to what extent these cells can also derive from the circulation. Parabiosis experiments, where 2 different mice share circulation, seemed to show a considerable contribution of mesenchymal cells from circulating blood (Kumagai et al. 2008). However, later experiments with bone marrow transplantation suggest that the cells that had reached the callus from the circulation were exclusively of hematopoietic origin. These leukocytes play an important part in the regulation of bone formation. Osteoblasts seem to depend on stimuli from a type of macrophages, so-called osteomacs, which are not comprehensively characterized (Chang et al. 2008). However, bone healing seems not be governed by any single cell category, but a complex interplay with most parts of the immune system. Cells indicated in bone healing include granulocytes, macrophages, T-cells and B-cells, CD4+ T-cells, CD8+ T-cells, and regulatory T-cells (Sato et al. 2006, Zaiss et al. 2007, Chang et al. 2008, Reinke et al. 2013, Könnecke et al. 2014, Chan et al. 2015). This complexity might be best overviewed by using a broad method of study, to see how patterns rather than individual cell types change during the course of healing.

Much effort has been spent on describing callus and callus formation in shaft fracture healing. Less interest has been directed to the marrow compartment in the bone proximal or distal to the fracture, despite the fact that it might be an important source of various cell types required for healing (Siclari et al. 2013). It has been suggested that successful pseudarthrosis treatment with a cement spacer followed by bone transplantation (the Masquelet procedure) requires communication between the defect and the adjacent intact bone marrow (Gianoudis et al. 2011, Auregan and Béugé 2014). This suggests an important role for the intact bone marrow adjacent to the injury, which might previously have been somewhat overlooked.

We performed an experiment to try to estimate the importance of the adjacent, uninjured marrow compartment for

the healing of a stable cortical defect in mice. We hypothesized that the bone formation in the cortical defect would be impaired without the presence of marrow cells in the underlying marrow cavity.

## Methods

### Experimental overview

Mice had a portion of the diaphyseal femoral cortex milled away, creating a longitudinal furrow. The marrow was scooped out, while avoiding traumatizing the endosteum. Half of the mice also had 2 custom-made silicone plugs inserted, proximal and distal to the cortical defect, to block access from the medullary canal (Figure 1). The mice were killed after 5 (n = 6) or 10 days (n = 20), and the femurs prepared for histology.

As a control, a second experiment was carried out to explore the leukocyte composition of the regenerated bone marrow in this cortical defect model. The femoral cortex was milled and marrow scooped out in 7 mice. The mice were killed after 5 days and the regenerated tissue in the cortical defect was harvested and analyzed by flow cytometry. As control, 7 mice underwent sham surgery, leaving the femur intact.

### Animals

40 male C57BL/6 mice with a mean weight of 25 (SD 1.3) g were used. The animals were kept 4 per cage and given ad libitum access to food and water.

### Surgical procedure

The mice were anesthetized with isoflurane gas and received a subcutaneous injection of 0.2 mg/kg oxytetracycline as infection prophylaxis and 0.1 mg/kg buprenorphine as postoperative analgesia. The left hind leg was shaved and cleaned with chlorhexidine. A 7 mm longitudinal incision was made along the lateral thigh. Knee extensors and flexors were separated bluntly, exposing the lateral femur surface. A handheld electrical drill was used to mill away a roughly 1.6 mm long and 1 mm wide portion of the mid-diaphysis cortex. It corresponded to about a quarter of the femoral circumference. The periosteum over the defect was removed, but caution was taken not to damage the periosteum surrounding the defect. The exposed marrow was scooped out using a bent needle. Half of the mice received 2 custom-made silicone plugs (length 2 mm, diameter 1.1 mm) placed into the medullary cavity, distal and proximal to the cortical defect (Figure 1). The surgeon was unaware which animals would receive plugs until the defect was fully prepared and it only remained to insert the plugs. An assistant then stated whether the animal should receive plugs or not from a randomization protocol prepared prior to surgery. This prevented any systemic differences in surgical treatment. The skin was then sutured.

For the mice undergoing sham surgery, an incision was made in the skin and the thigh muscles were separated. Cau-

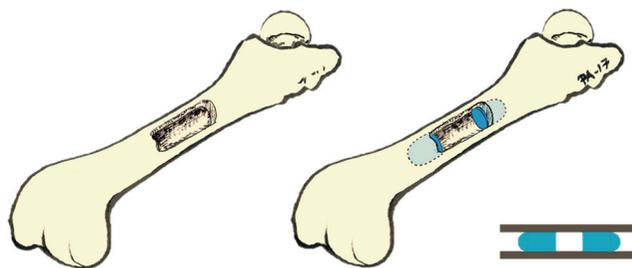


Figure 1. The cortical defect model. The mid-portion of the femoral cortex was milled away, and two silicone plugs were inserted to block entry to the emptied marrow compartment in the treatment group. Actual proportions shown below right.

tion was made not to harm bone or periosteum. Apart from that the mice were treated as above.

### Histology

The specimens were decalcified in 10% formic acid for 7 days, dehydrated in a series of increasing concentration of ethanol and embedded in paraffin for sectioning. The femoral shaft specimens were sectioned transversally in 4 µm sections, starting in intact bone, continuing until the cortical defect was fully visible in the sections. An additional 10 sections were then made and discarded before the following 6–10 sections were mounted on slides and stained with HE. 1 representative section of these 10 was then selected for scoring from each specimen.

The following histological parameters were evaluated: Cortical bridging: degree of bridging between the cortical ends in the cortical defect, marrow compartment regeneration: degree of new bone marrow-like tissue inside the marrow compartment, suspected preosteoblasts in cortical defect: amount of extra cellular matrix producing cells (not typically bone cells) located specifically between the cortical ends in the cortical defect, intramedullary bone formation: degree of woven bone formation in the marrow compartment, and periosteal reaction: degree of tissue formation adjacent to the periosteum.

Analysis of the slides was performed using a 3-point scoring system, where 0 was none, 1 was partial and 2 was full. The scoring was conducted by MB, who was unaware of specimen treatment and harvest time (blinded). Due to the differences between the healing phases, not all parameters could be estimated at both time points (e.g. with full bony defect healing there was no room for suspected preosteoblasts).

### Cell preparation for flow cytometry

The tissue was digested with collagenase IV 300 U/mL (Thermo Fisher Scientific, Waltham, MA, USA) and DNaseI 300 U/ml (Roche, Switzerland) in SB with 20 mM magnesium added for 20 min at 37°C, and then washed (600 g for 6 minutes at 4°C for all centrifuge steps) with SB and filtered through a 30 µm nylon strainer. The tissue suspension was then washed with staining buffer (Biolegend, CA, USA).

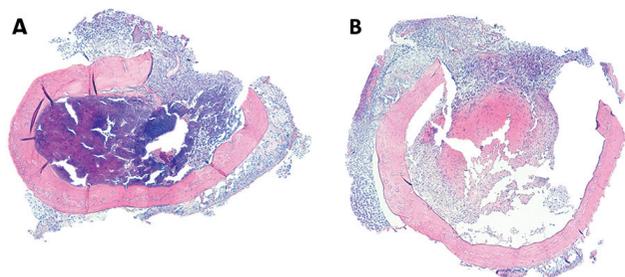


Figure 2. Histology images 5 days after cortical defect, 5X. New bone marrow-like tissue could be seen in the controls (A), whereas no marrow regeneration could be seen in the silicone plug group (B).

Zombie Red and anti-CD16/32 (Biolegend, CA, USA) were added to stain for dead cells and block unspecific Fc binding. The suspension was incubated in the dark on ice for 20 min. An aliquot of 1/10 (vol/vol) from each tissue suspension was used to form a pooled sample from the respective tissue of all animals in the group, to be used for “fluorescence minus one” (FMO) gating. The remaining 9/10 (vol/vol) of cells from each tissue suspension was divided equal to two staining tubes for immune phenotyping. Tissues for flow cytometric analysis were harvested and prepared on the same day.

### Cell staining

Staining was performed in 2 panels (Table 3, see Supplementary data), in the dark on ice for 30 min. Cells were then fixed in 2% paraformaldehyde (Biolegend, San Diego, CA) for 20 min at room temperature, followed by washing twice with staining buffer. Cells were stored at 8°C for 1 day before analysis by flow cytometry.

### Flow cytometry

Flow cytometry was performed on a FACS Aria III (BD Biosciences, Franklin Lakes, NJ, USA) equipped with a purple (405 nm), blue (488 nm), green (561 nm), and a red (633 nm) laser. A nozzle of 100 µm was used. Wavelength filters were used as recommended by the manufacturer. Cytometer Setup & Tracking Beads (BD Biosciences) were used to ensure stability of the flow cytometer. Compensation was performed with cellular controls from mice, as well as with VersaComp Beads (Beckman Coulter, Brea, CA, USA), depending on antigen. Antibodies were titrated for optimal resolution in case of commonly expressed antigens, otherwise the recommended titer from the manufacturer was used.

### Statistics

The cortical bridging parameter was considered the primary outcome variable and marrow compartment regeneration as secondary. The other parameters were seen as exploratory. We hypothesized that the score for the cortical bridging and marrow compartment regeneration parameters would be lower

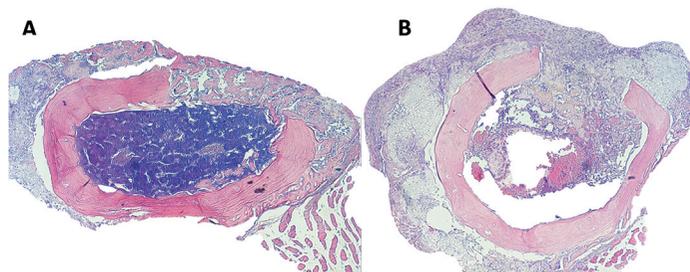


Figure 3. Histology images 10 days after cortical defect, 5X. Complete cortical bridging, with a distinct interface between the regenerated marrow-like tissue and newly formed bone, could be seen in the controls (A). No tendencies to cortical bridging could be seen in the silicone group (B), however, some animals showed newly formed bone in the marrow compartment.

in the silicone plugs groups, compared with the control group. The analysis was conducted using a contingency table exact test (SPSS® v. 24, IBM Corp, Armonk, NY, USA).

### Ethics, funding, and potential conflicts of interest

The study was approved by the Regional Ethics Committee for Animal Experiments and the animals were treated according to the institutional guidelines for care and treatment of laboratory animals (registration ID 04-15). Funding was received from the Swedish Research Council (2031-47-5), AFA insurance company, EU 7th framework program (FP7/2007-2013, grant 279239) and a specific grant from Linköping University. None of the authors had any conflict of interest.

## Results

### Exclusions

1 specimen in the day 3 plug group was excluded due to damage during sectioning for histology.

### Descriptive histology

In the controls (without plugs) after 5 days, newly regenerated bone marrow-like tissue filled the emptied marrow space, and cells producing extracellular matrix could be seen in the cortical defect (Figure 2A). After 10 days, complete cortical bridging could be seen with a distinct interface between the newly formed bone and the underlying regenerated marrow (Figure 2B).

In the animals with plugs, the marrow compartment was either empty (of tissue) or partially filled with scar tissue after 5 days (Figure 3A). After 10 days, no tendencies to cortical bridging could be seen, and there were no signs of functional marrow regeneration (Figure 3B). However, a few specimens showed newly formed woven bone within the marrow compartment, partially or completely filling it.

### Histological scoring

Cortical bridging after 10 days was less evident in the plug

**Table 1.** Histology at Day 5 in controls without plug (Ctr) and animals with silicone plugs (SPs)

	Suspected preosteoblasts in cortical defect		Marrow compartment regeneration		Intramedullary bone formation		Periosteal reaction	
	Ctr	SPs	Ctr	SPs	Ctr	SPs	Ctr	SPs
None	1	0	0	2	3	2	1	0
Partial	1	1	0	0	0	0	2	2
Complete	1	1	3	0	0	0	0	0

**Table 2.** Histology at Day 10 in controls without plug (Ctr) and animals with silicone plugs (SPs)

	Cortical bridging		Marrow compartment regeneration		Intramedullary bone formation		Periosteal reaction	
	Ctr	SPs	Ctr	SPs	Ctr	SPs	Ctr	SPs
None	0	9	0	7	5	5	0	0
Partial	4	1	0	3	5	4	3	6
Complete	6	0	10	0	0	1	7	4

group compared with the control group (Table 2;  $p < 0.001$ ). Marrow compartment regeneration was lower in the plug group compared with the control group both at day 5 (Table 1) and at day 10 (Table 2;  $p < 0.001$ ).

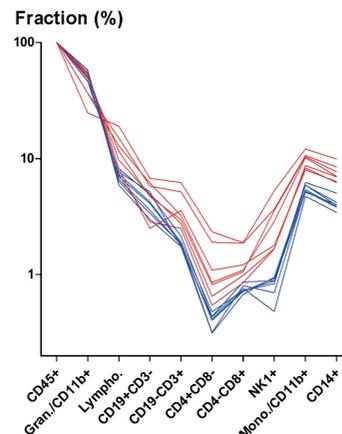
**Flow cytometry**

The regenerated bone marrow-like tissue showed a slightly higher number of lymphocytes and macrophages compared with non-traumatized bone marrow, but the leukocyte composition was otherwise remarkably similar (Figure 4).

**Discussion**

We found that restricting access for the adjacent marrow to the site of injury considerably reduced healing of a stable cortical defect. Although several explanations are possible, we argue that the most likely interpretation is that the regenerated bone marrow contributed some kind of necessary support signals for bone formation by external cells arriving from other sources. Presence of bone marrow has been shown to be necessary for periosteal growth and induction of cortical fracture repair (Ozaki et al. 2000). In other contexts, a supportive or regulatory function has been described for macrophages and cytotoxic t-cells (Chang et al. 2008, Reinke et al. 2013).

It is unlikely that the effect of the plugs on cortical healing has to do with vascular supply. Periosteal vessels are known to increase their blood flow when the intramedullary circulation is compromised (Greksa et al. 2012). Neovascularization from the surrounding periosteum would easily reach the



**Figure 4.** Flow cytometry data (logarithmic axis) illustrating the similarity between the newly regenerated tissue in the marrow compartment and normal bone marrow. Leukocyte subpopulations in regenerated bone marrow and non-traumatized bone marrow 3 days after surgery. Each line represents one mouse and connects points illustrating the proportion of cells carrying the surface marker labelled at the bottom. Red lines: regenerated marrow, Blue: intact marrow. A higher number of lymphocytes and macrophages could be seen in the regenerated bone marrow compared with non-traumatized bone marrow. However, the leukocyte composition, in both groups, was generally similar.

cortical defect, unhindered by the plugs. The distance from the proximal or distal marrow to the cortical defect is much longer, and it is unlikely that blocking intramedullary vessels would play a big role.

Yet another possibility would be that the marrow was the main source of bone-forming cells. Marrow ablation is indeed a well-known model for inducing bone formation in the marrow compartment (Bab 1995). However, the marrow ablation model includes traumatizing the endosteum, which is the probable source of the new bone cells. We tried to avoid such trauma, and in most cases new bone was completely absent in the former marrow cavity. It was seen only at the interface between the regenerated marrow and the extraosseous space. The new bone had already appeared in the cortical defect after 10 days, and we could see a separate type of tissue already forming in this region at day 5. This excludes the possibility that bone had first formed in the entire marrow cavity and then been resorbed to leave only the layer at the interface. Such a process would have taken more than a week. Thus, in most cases the bone formation was initiated at the interface, and only there.

We have noted in another model that a localized trauma to a small cancellous bone region leads to very fast bone regeneration. Interestingly, however, this regeneration is strictly confined to the injury site (Bernhardsson et al. 2015). There is no bone formation infiltrating as much as half a millimeter into the surrounding intact bone marrow, in spite of the extremely strong and fast regeneration at the trauma site. A sharp border between the osteoblastic region and the surrounding, seemingly undisturbed marrow is established in 3 days (manuscript

in preparation). Also in the current study, a sharp border was already established between the new marrow and the bone-forming region on day 10. The impression is that the marrow plays dual roles: it contributes support signals for bone formation, while at the same time keeping bone formation fenced out from the marrow space. If so, this would require 2 types of signaling from the marrow: long-distance signaling in support of bone formation, and short-distance signaling keeping it away from infiltrating the marrow.

Even though the regenerated marrow had similarities to uninjured marrow, there were some differences. We do not know how these differences might have influenced healing. However, most trauma situations involving bone marrow are likely to induce changes in marrow composition locally and systemically, and we therefore regard the cellular composition of the marrow regenerate in our model as relevant.

Our findings might explain, at least in part, why it seems important to allow marrow access at some stage during the procedure, when using the pseudo-membrane technique in the clinical treatment of skeletal defects (Giannoudis et al. 2011, Karger et al. 2012, Taylor et al. 2012). In pseudoarthroses, where the fracture defect contains mature scar tissue, it seems obvious that it is necessary to remove the scar and allow invasion from intact, nearby marrow. This might not always be appreciated in surgical treatment that concentrates on stimulating external callus.

In conclusion, the bone marrow seems to play an important regulatory role for cortical bone healing. Both in clinical fracture treatment and in research, this might be of further interest.

### Supplementary data

Details of antibodies used for flow cytometry (Table 3) and histology images of all specimens (Figures 5 and 6) are available in the online version of this article, <http://dx.doi.org/10.1080/17453674.2017.1382280>

PA, OS, and MB planned the study. OS and MB conducted the surgery. LT conducted the flow cytometry. PA, MB, and LT did the data analysis. PA and MB wrote the manuscript draft. All authors revised and approved the final manuscript.

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Auregan J-C, Bégué T. Induced membrane for treatment of critical sized bone defect: A review of experimental and clinical experiences. *Int Orthop* 2014; 38 (9): 1971-78.

Bab I A. Postablation bone marrow regeneration: An in vivo model to study differential regulation of bone formation and resorption. *Bone* 1995; 17 (4): 437-41.

Bernhardsson M, Sandberg O, Aspenberg P. Experimental models for cancellous bone healing in the rat. *Acta Orthop* 2015; 86 (6): 745-50.

Chan J K, Glass G E, Ersek A, Freidin A, Williams G A, Gowers K, Espirito Santo A I, Jeffery R, Otto W R, Poulsom R, Feldmann M, Rankin S M, Horwood N J, Nanchahal J. Low-dose TNF augments fracture healing in normal and osteoporotic bone by up-regulating the innate immune response. *EMBO Mol Med* 2015; 7: 547-61.

Chang M K, Raggatt L-J, Alexander K A, Kuliwaba J S, Fazzalari N L, Schroder K, Maylin E R, Ripoll V M, Hume D A, Pettit A R. Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function in vitro and in vivo. *J Immunol* 2008; 181 (2): 1232-44.

Giannoudis P V, Faour O, Goff T, Kanakaris N, Dimitriou R. Masquelet technique for the treatment of bone defects: tips-tricks and future directions. *Injury* 2011; 42 (6): 591-98.

Greksa F, Tóth K, Boros M, Szabó A. Periosteal microvascular reorganization after tibial reaming and intramedullary nailing in rats. *J Orthop Sci* 2012; 17 (4): 477-83.

Karger C, Kishi T, Schneider L, Fitoussi F, Masquelet A C, French Society of Orthopaedic Surgery and Traumatology (SoFCOT). Treatment of posttraumatic bone defects by the induced membrane technique. *Orthop Traumatol Surg Res* 2012; 98 (1): 97-102.

Kuehfluck P, Moghaddam A, Helbig L, Child C, Wildemann B, Schmidmaier G. RIA fractions contain mesenchymal stroma cells with high osteogenic potency. *Injury* 2015; 46 (Suppl 8): S23-32.

Kumagai K, Vasanji A, Drazba J A, Butler R S, Muschler G F. Circulating cells with osteogenic potential are physiologically mobilized into the fracture healing site in the parabiotic mice model. *J Orthop Res* 2008; 26 (2): 165-75.

Könnecke I, Serra A, El Khassawna T, Schlundt C, Schell H, Hauser A, Ellinghaus A, Volk H D, Radbruch A, Duda G N, Schmidt-Bleek K. T and B cells participate in bone repair by infiltrating the fracture callus in a two-wave fashion. *Bone* 2014; 64: 155-65.

Liu R, Birke O, Morse A, Peacock L, Mikulec K, Little D G, Schindeler A. Myogenic progenitors contribute to open but not closed fracture repair. *BMC Musculoskelet Disord* 2011; 12: 288.

Masquelet A C. Muscle reconstruction in reconstructive surgery: Soft tissue repair and long bone reconstruction. *Langenbecks Arch Surg* 2003; 388 (5): 344-46.

Masquelet A C, Beque T. The concept of induced membrane for reconstruction of long bone defects. *Orthop Clin North Am* 2010; 41 (1): 27-37.

Ozaki A, Tsunoda M, Kinoshita S, Saura R. Role of fracture hematoma and periosteum during fracture healing in rats: interaction of fracture hematoma and the periosteum in the initial step of the healing process. *J Orthop Sci* 2000; 5(1): 64-70.

Reinke S, Geissler S, Taylor W R, Schmidt-Bleek K, Juelke K, Schwachmeyer V, Dahne M, Hartwig T, Akyüz L, Meisel C, Unterwaller N, Singh N B, Reinke P, Haas N P, Volk H D, Duda G. Terminally differentiated CD8+ T cells negatively affect bone regeneration in humans. *Sci Transl Med* 2013; 5 (177): 177ra36.

Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, Tanaka S, Kodama T, Akira S, Iwakura Y, Cua D. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* 2006; 203 (12): 2673-82.

Siclari V A, Zhu J, Akiyama K, Liu F, Zhang X, Chandra A, Nah H D, Shi S, Qin L. Mesenchymal progenitors residing close to the bone surface are functionally distinct from those in the central bone marrow. *Bone* 2013; 53: 575-86.

Taylor B C, French B G, Fowler T T, Russell J, Poka A. Induced membrane technique for reconstruction to manage bone loss. *J Am Acad Orthop Surg* 2012; 20 (3): 142-50.

Wenisch S, Trinkaus K, Hild A, Hose D, Herde K, Heiss C, Kilian O, Alt V, Schnettler R. Human reaming debris: A source of multipotent stem cells. *Bone* 2005; 36 (1): 74-83.

Zaiss MM, Axmann R, Zwerina J, Polzer K, Gückel E, Skapenko A, Schulze-Koops H, Horwood N, Cope A, Schett G. Treg cells suppress osteoclast formation: A new link between the immune system and bone. *Arthritis Rheum* 2007; 56 (12): 4104-12.