T and B cell participate in bone repair by infiltrating the fracture callus in a two-wave fashion

Ireen Könnecke, Alessandro Serra, Thaquif El Khassawna, Claudia Schlundt, Hanna Schell, Anja Hauser, Agnes Ellinghaus, Hans-Dieter Volk, Andreas Radbruch

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Julia Lang
Introduction
• Bone:
  • scarless self-regeneration
  • 10-20% delayed healing or non-union

• Osteoblasts and Osteoclasts

https://www.easynotecards.com/notecard_set/7473
• Bone repair

Initial phase
• Fracture hematoma formation
• Inflammatory reaction triggering initiation of repair
• High expression of angiogenic factors

Soft callus formation
• About 7 days after fracture in mice
• Anti-inflammatory signaling
• Chondrocytes differentiate

Woven bone (hard callus) formation
• About 14 days after fracture in mice
• High expression of angiogenic factors

Remodelling
• Starting about 21 days after fracture in mice
• Fracture healing and callus formation

https://www.orthobullets.com/basic-science/9009/fracture-healing
• B- and T-cell interactions with skeletal cell system

• Activated T-cells and osteoclasts: RANKL -> Osteoclastogenesis
  Direct activation of osteoclasts via TNF-α
  • -> bone resorption
  • BUT: CD4+ T-cells promote Osteoprotegerin (OPG) secretion by T-cells and wild regulatory T-cells support development and healing

• B-cells and osteoblasts: OPG receptor -> blocks RANK-RANKL ligation -> inhibits osteoclastogenesis
  • -> bone apposition
  • B-cell deficient mice are osteoporotic (Li et al., 2007)
Osteoprotegerin: decoy receptor for RANKL -> neutralizes its function in osteoclastogenesis

https://clinicalgate.com/bone-modeling-and-remodeling/
Methods
• 65 Wild type mice
• 61 mice with unilateral closed fracture (4 control mice)
  • Opening knee joint with patella dislocation
  • To produce the fracture they placed a pin into bone marrow cavity and used a three point bending machine

• Sacrification after 3, 7, 14, 21 and 28 post fracture
  • N = 11 per time point
  • Contralateral bones analyzed 1, 2, 3, 7, 14 and 21 days after surgery N = 6
  • Control group without intervention at t=0 (N = 4)
• Flow cytometry
  • Of flushed out bone marrow
  • Staining of CD3, CD4, CD8 and LIVE/DEAD stain

• Histological analysis
  • Femurs with surrounding muscle

• Immunofluorescence analyses
  • Fractures bones with surrounding muscle tissue
  • Staining with antibodies for B220, IgM, IgD, IgG1, IgG2ab, CD3e, CD4, Laminin, cathepsin K, osteocalcin and OPG (osteoprotegerin)
  • For quantitative analysis: 14d and 21d bones stained for T- and B-cells were gritted for counting

• Gene expression
  • Callus tissue around intramedullary fixating pin
Results
Fig. 1: Flow cytometry

A: comparison of CD3+ T-cells in contralateral bone marrow at different time points

B: Comparison of CD4+ and CD8+ T-cells in contralateral bone marrow at different time points
Inflammatory phase still ongoing

Hematoma undergoes organization evolving into granulomatous tissue

T- and B-cells are visible throughout the complete area around fractured bone - also in surrounding muscle and fracture gap

Fig. 2
CD3+ T-cells: red
B220+ B-cells: gray
Cell nuclei: blue (DAPI)
Development of soft cartilaginous callus

Consisting mainly of chondrocytes

T- and B-cells were absent from areas of cartilage and mainly confined to endosteal tissue close to the fractured bone ends.

Fig. 2
CD3+ T-cells: red
B220+ B-cells: gray
Cell nuclei: blue (DAPI)
Hard callus formation

Chondrocytes become hypertrophic synthesizing mineralized matrix.

Avascular cartilage is vascularized and woven bone evolves.

Callus originates from periosteum and growing to fracture gap.

T- and B-cells reappear in large numbers at areas nearby woven bone but not in cartilage (S).

B-cells >> T-cells

Fig. 2

CD3+ T-cells: red
B220+ B-cells: gray
Cell nuclei: blue (DAPI)
Remodelling phase
No cartilage remaining
Bone marrow between new woven bone
High amount of B- and T-cells in callus
B-cells >> T-cells

Completion remodeling process
Bone regained pre-fracture form
CD3+ T-cells: red
B220+ B-cells: gray
Cell nuclei: blue (DAPI)
14 days after fracture
2nd wave of lymphocytes

Colocation of T-cells and vasculature-associated protein laminin (A)

T- and B-cells intraluminal

-> strongly suggesting
Infiltration of 2nd wave of lymphocytes via inner vasculature of callus

Fig. 3
Functional state of B-cells in callus

14 days:
- majority: naive B220+ IgM+ IgD+
- Fewer: B220+ IgM+ IgD-
  - memory or immature (arrows)

21 days:
- Naive B220+ IgM+ IgD+ AND B220- IgG1 and 2+ (arrows)
  - plasma cells
  - Most potent OPG secretion

Fig. 4
B220+ gray
IgD green
IgM red
IgG1 and 2 purple
Cell-cell contact interaction of osteoclasts and lymphocytes

14 and 21 days: Cathepsin K secreting osteoclasts (green) Several Lymphocytes directly contacting osteoclasts

Fig. 5
CD3+ red
B220+ gray
Cathepsin K (Osteoclasts) green
Cell-cell contact interaction of osteoblasts and lymphocytes

14 and 21 days:
- Osteocalcin positive osteoblasts (green)
  - Round shaped precursor cells
  - Paslisade shape activated osteoblasts
- B-cells have cell-cell contact with both types

Fig. 6
B220+ gray
Osteocalcin (Osteoblasts) green
Cell nucleii blue
Cells producing OPG

14 and 21 days:
B-cells produced OPG
Also expressed by CD3+, B220-cells – probably osteoblasts

T-cells produced OPG

**Fig. 7**
Balance change of OPG and RANKL

A: RANKL
B: OPG
C: OPG/RANKL ratio

- Increasing shift towards OPG

Fig. 8
• Summary
  • Lymphocytes enter the fracture callus in two migratory waves
  • In first wave T- and B-cells are equally distributed across the callus
  • Second wave of T- and B cells
    • accesses the callus probably via its inner vessels
    • Higher amount of B- than T-cells
    • OPG secreting capacity progressively increases at further stages of fracture healing
    • B- and T-cells also stay in remodelling phase
    • Direct cell-cell contact of lymphocytes and osteoclasts and osteoblasts

Fig. 9
T-cells (red)
B-cells (blue)
Discussion
• Bone injury alters bone marrow composition not only in the fracture bone but systematically (Fig.1)

• Physiologically higher percentage of CD8+ than CD4+ T-cells in bone marrow (Di Rosa and Pabst, 2005)

• Reversed in injury situation
• T- and B-cells might directly affect the bone healing process

• Through OPG and TNF-α
  • Mice with reduced or ablated TNF-α expression showed impaired healing (Aizawa et al., 2001)
  • OPG deficient mice show osteoporosis due to increased osteoclast activity (Kong et al., 1999)
  • Lymph node activation correlates with healing (Szczesny et al., 2007)

• Until remodelling phase

• Bone draining lymph nodes are affected by fracture (Szczesny et al., 2007)
• Further questions
  • Can also cell-cell contacts between lymphocytes, osteoblasts and osteoclasts lead to further differentiation of B- and T-cells?
  • Possibilities of using immune cells to further improve bone healing
    • Regulatory T-cells combined with bone marrow stroma cells enhance healing (Liu et al., 2011)
    • Cytokines as possible treatment (Mountziaris et al., 2011)

> Newly discovered role of B-cells – is stimulation helpful for bone regeneration?
• References
  • Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. Trends Immunol 2005; 26: 360-6