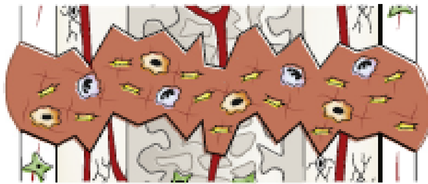
Neutrophils contribute to fracture healing by synthesizing fibronectin+ extracellular matrix rapidly after injury

Bastian OW, Koenderman L, Alblas J, Leenen LPH, Blokhuis TJ. Neutrophils contribute to fracture healing by synthesizing fibronectin+ extracellular matrix rapidly after injury. Clinical Immunology. 2016;164:78-84.

Lisa Michels
Journal Club presentation

Introduction: Phases of bone regeneration

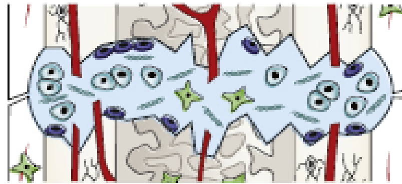
A Inflammatory phase



Inflammation
CCL-2 TGF-β
IL-1, -6 M-CSF

Recruitment of MSCs
PDGF-BB TNF-α
SDF-1
BMP-2, -4, -5, -6

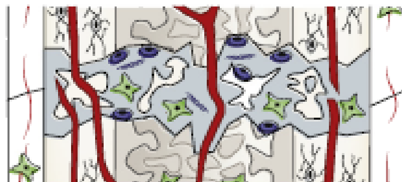
B Soft callus formation



Angiogenesis
Ang-1, -2 FGF-2
VEGF-A PlGFs
PDGFs BMPs

Chondrogenesis and matrix production
TGF-βs IGF-1
PDGF-BB FGFs
GDF-5 BMPs

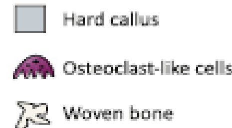
C Mineralization/resorption of the callus



Angiogenesis
VEGFs

Cartilage resorption
M-CSF RANKL
OPG TNF-α

MSCs differentiation and bone deposition
BMPs Wnt ligands



D Bone remodeling

Bone resorption
RANKL

New bone formation
BMP-2 PTH
IGF-1 Wnt ligands

→ Fracture hematoma (FH) formation: template for soft callus

→ First cells recruited: polymorphonuclear neutrophils

→ Those attract monocytes/macrophages

Martino et al. Extracellular matrix-inspired growth factor delivery systems for bone regeneration. Advanced Drug Delivery Reviews. 2015.

Role of inflammation in bone regeneration

positive	negative
Essential for early bone regeneration (inflammatory phase): - Removal or irrigation of FH → impaired bone healing - Transplantation of early FH into muscle tissue → ectopic bone formation	Negative effects of certain inflammatory conditions onto fracture healing: - Inducers of local or systemic inflammation: open fractures, severe soft tissue injury, multiple injuries - Experimental models of local/systemic inflammation: LPS-injection intraperitoneal, beta glucan-injection into FH

Bone histology

- 45% bone minerals, 30% organic matrix, 25% water
- Cells: osteoblasts, osteocytes and osteoclasts
- Mineralized extracellular matrix (ECM):
    - Collagen fibrils (covalent binding mainly of collagen type I fibres)
    - Hydroxyapatite (calcium and phosphate ions)
    - proteoglycans, glycoproteins like fibronectin

→ synthesised by stromal cells like fibroblasts

Lüllmann-Rauch. Taschenlehrbuch Histologie. 2003.

Fibronectin

- Is an adhesion molecule
- Synthesised by fibroblasts, endothelial cells, macrophages
- Connecting cells and ECM
  - Binding to the cell via integrins
  - Binding to collagen fibrils, fibrin, proteoglykans of the ECM

Lüllmann-Rauch. Taschenlehrbuch Histologie. 2003.

Hypothesis

- Contribution of inflammatory cells to bone healing by synthesis of an “emergency extracellular matrix (ECM)“

- Before the infiltration of stromal cells, which are synthesizing the actual bone ECM

  (consists mainly of mineralized collagen type I fibrils)

Material and Methods - Outline

- Analysis of temporal changes in the composition of human FH during early bone healing
- → Presence of ECM within FHs isolated before infiltration of stromal cells?
- → Identification of collagen type I + fibrils?

- Isolation of FHs – tissue microarray – analysis of collagen fibers – immunohistochemistry – imaging and analysis

Material and Methods – Isolation of FHs

- 53 FHs of closed fractures of trauma patients were isolated in Open Reduction Internal Fixation procedure
- 4 groups based on the time between injury and isolation

1	2	3	4
Within 2 d	3-5 d	6-10 d	>10 d
15 FHs	10 FHs	15 FHs	13 FHs

  - ➢ time ranging from 6h to 23 days
  - ➢ 18 FHs from upper extremity, 22 FHs from lower extremity, 10 FHs from pelvis, 3 FHs from thorax
  - ➢ 26 male, 27 female; mean age 45 +/- 19 years

- and 1 control group: coagulated peripheral blood from healthy donors → after 1 h: removed from coagulation tube and treated similar to the freshly isolated FHs

Material and Methods – Isolation of FHs

- Preparation of FHs:
- Directly fixed in 3.7% buffered formaldehyde solution (pH 4) for one week
- Dehydrated and embedded in paraffin (with a Leica Embedding Center)
- Cut into sections of 4µm thickness, incubated at 54˚C overnight to allow firm adherence to microscopy slides

Material and Methods – Tissue microarray (TMA)

- many small representative tissue samples assembled on a single histologic slide → allows simultaneous and comparable staining in one procedure
- Two 1mm cylindrical biopsy cores of each FH were transferred to one TMA paraffin block
- Identifaction of the biopsy points by staining the sections with hematoxylin and eosin (H&E) with a Dako CoverStainer
- Criteria of the biopsy locations:
  - 1) Area with cells with fibroblast-like morphology
  - 2) Where these cells could not be identified

Material and Methods - TMA

- Sections of 4µm were cut and incubated at 54°C overnight
- Deparaffinisation and rehydration
- Staining:
  - 1) Hematoxylin and eosin
  - 2) Picrosirius Red
  - 3) Immunohistochemistry

Material and Methods – Analysis of collagen fibers – Picrosirius Red

- Collagen fibers are anisotropic and birefringent
- Picrosirius Red enhances their natural birefringence
- TMA sections were deparaffinised, washed with dH2O, stained with PicroSirius Red solution 1h at room temperature (RT)
- Cell nuclei: stained with Hoechst for 10min in the dark at RT
- Dehydration of the sections, embedded with a ClearVue coverslipper and stored in the dark
- Identification of birefringent fibrils: placing the stained sections between two polarization filters with 90° of rotational difference between both filters

Materials and Methods - Immunohistochemistry

- 5 TMA sections
- Deparaffinisation, washing with dH2O
- Sequential alkaline phosphatase (ALP) double immunostaining with Liquid Permanent Red Substrate Chromagen and Vector Blue ALP Substrate Kit III
- mouse anti-human primary antibodies, secondary antibodies were BrightVision polyclonal ALP-Anti-Mouse IgG, cell nuclei staining with Hoechst
- Negative isotype control: mouse IgG1

Material and Methods - Immunohistochemistry

- Overview of the five sections and the primary antibodies (ab)

	1	2	3	4	5-control
1. prim. ab red	leucocytes CD45	neutrophils CD66b	macrophages CD68	neutrophils CD66b	IgG1
2. prim. ab blue	collagen type I	insoluble cell-derived fibronectin	insoluble cell-derived fibronectin	monocytes and macrophages CD68	IgG1

- Ab against insoluble cell-derived fibronectin did not recognize soluble plasma-derived fibronectin

Material and Methods - Imaging

- Imaging of each stained TMA core with a Leica DFC425C camera mounted to a Leica microscope
- Imaging of representative images with Olympus DP70 camera connected to Olympus BX51 microscope
- Algorithm to count cell numbers and amount of Vector Blue stained ECM or birefringent fibrils
- Photoshop: merging of blue channel Hoechst images with red channel Liquid Permanent Red images

Results - Group 1: isolation of FH within 2 d

- All nucleated cells were CD45+ leucocytes → majority built of CD66b+ neutrophils and CD68+monocytes/macrophages
  - No identification of stromal cells
- Significant amount of ECM stained positive for insoluble cell-derived fibronectin
  - Parts of ECM stained mildly positive for collagen type I
  - No identification of birefringent fibrils
- Organization: macrophages mainly localized within fibronectin+ ECM, neutrophils mainly adjacent to the ECM
- Identification of CD66b+ particles within fibronectin+ ECM

Results – Group 2: isolation after 3-5 d

- Day 5 after injury: first identification of stromal cells (CD45-, fibroblast morphology)
  - Macrophages were the most prevalent leukocytes
- ECM majorly positive for fibronectin and increasingly positive for collagen type I
  - No birefringent fibrils

Results – Group 3 & 4: 6-10 d & >10 d

- Majority of nucleated cells were stromal cells
  - Neutrophils decreased further, macrophages remained
- ECM: - strongly positive for collagen type I

  - cellular fibronectin positive in defined areas

  - clear identification of birefringent fibrils

Results - Summary

Group 1	Group 2	Group 3 & 4
neutrophils **monocytes/ macrophages**	**monocytes/ macrophages** neutrophils **stromal cells** (day 5)	**stromal cells** monocytes/ macrophages (neutrophils)
fibronectin (collagen type I)	**fibronectin** collagen type I	**birefringent fibrils** collagen type I fibronectin

Results – Changes in leukocyte counts

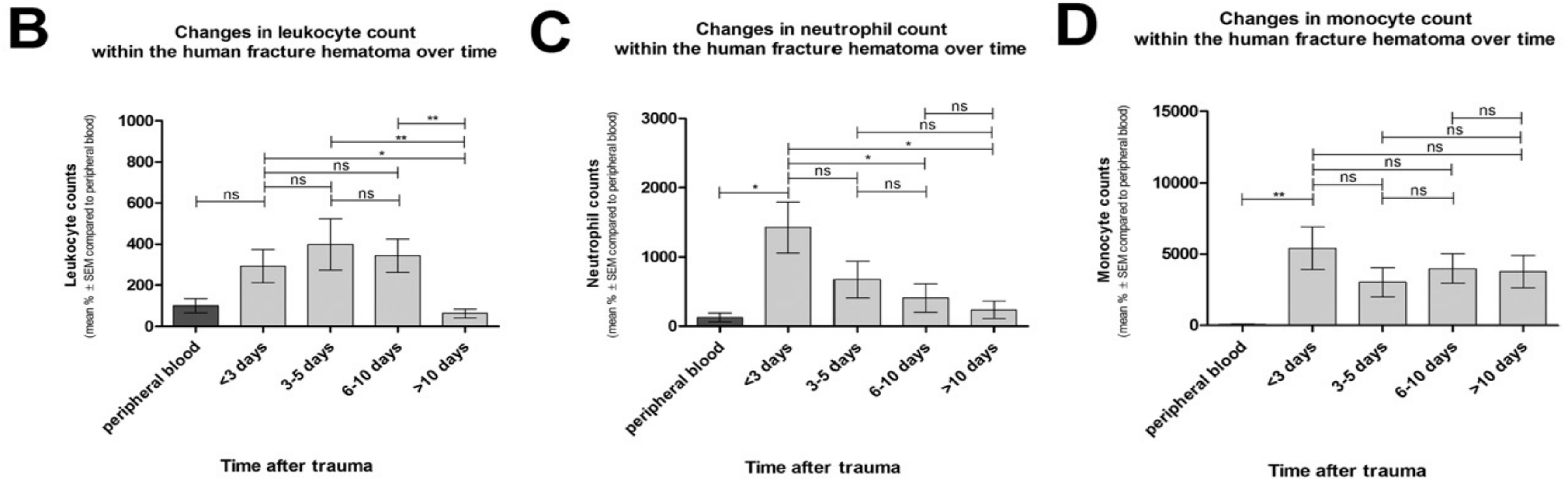
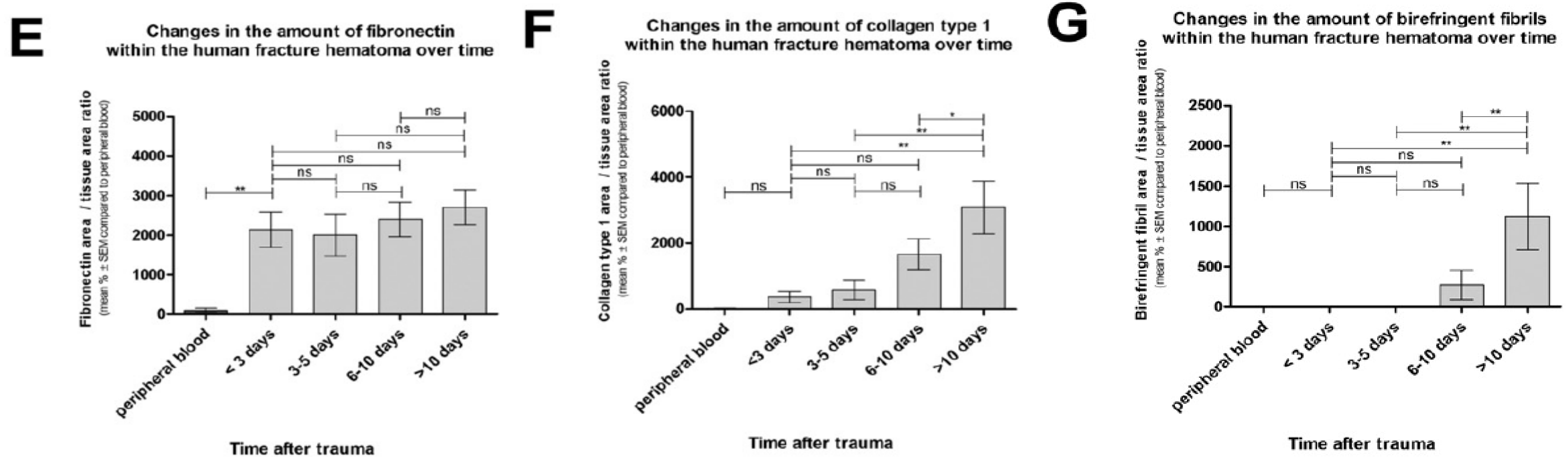


Fig. 1. Changes in A) cell count, B) leukocyte count, C) neutrophil counts, D) monocyte count, E) fibronectin/tissue area ratio, F) collagen type 1/tissue area ratio, G) birefringent fibrils/tissue area ratio within human fracture hematomas (FH) over time. The bars represent mean percentage compared to peripheral blood + standard error of the mean (SEM). The dark gray bars indicate coagulated peripheral blood and the light gray bars show groups of FHs that were isolated at different time-points after injury. Peripheral blood was compared to FHs that were isolated within 48 h after injury. In addition, all FH groups were compared to each other. A p-value < 0.05 is indicated by *, $p < 0.01$ by **, $p < 0.001$ by *** and $p > 0.05$ as ns (not significant).

Results – Changes in ECM composition



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Results – Changes in compostion of FH

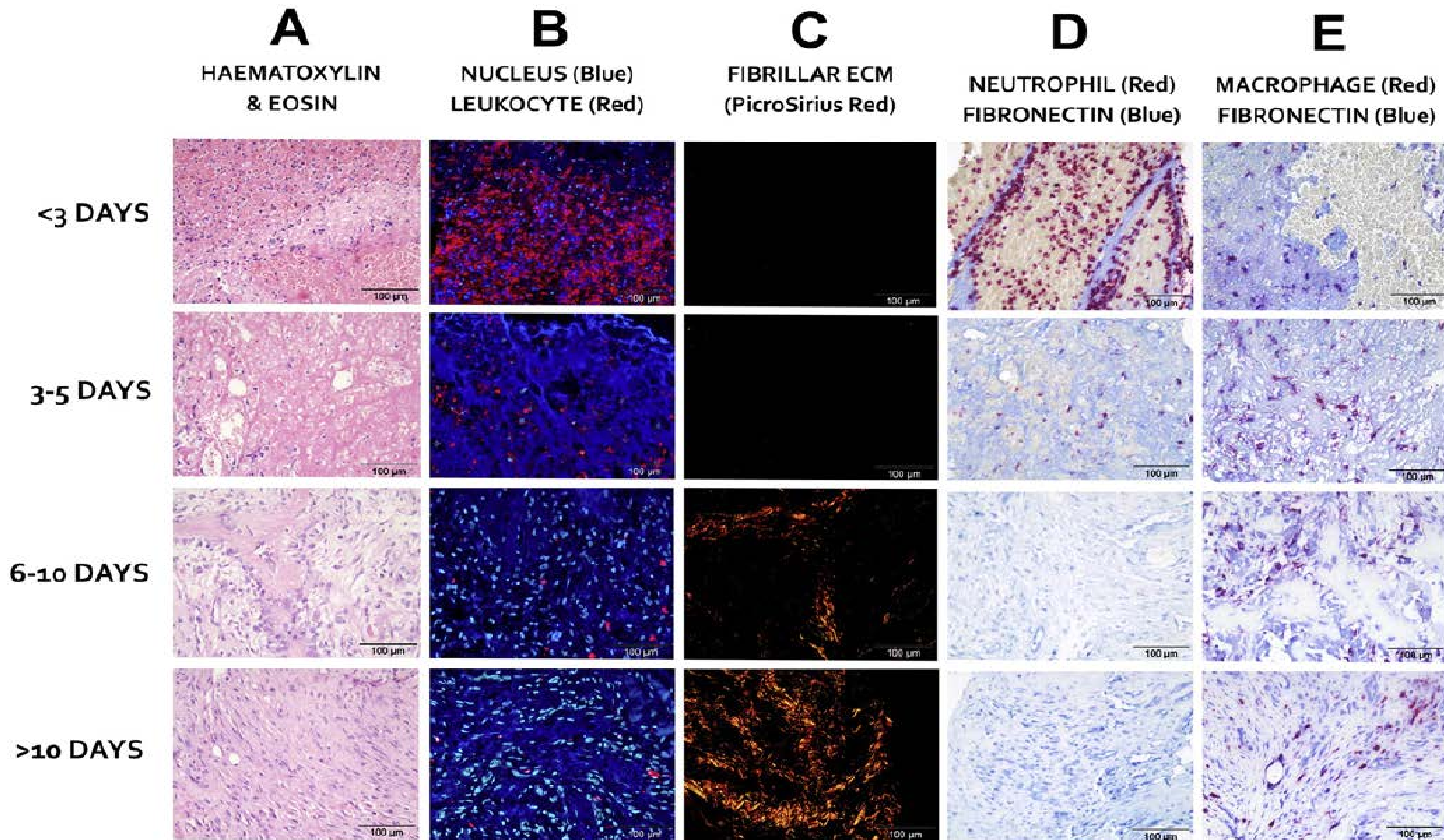
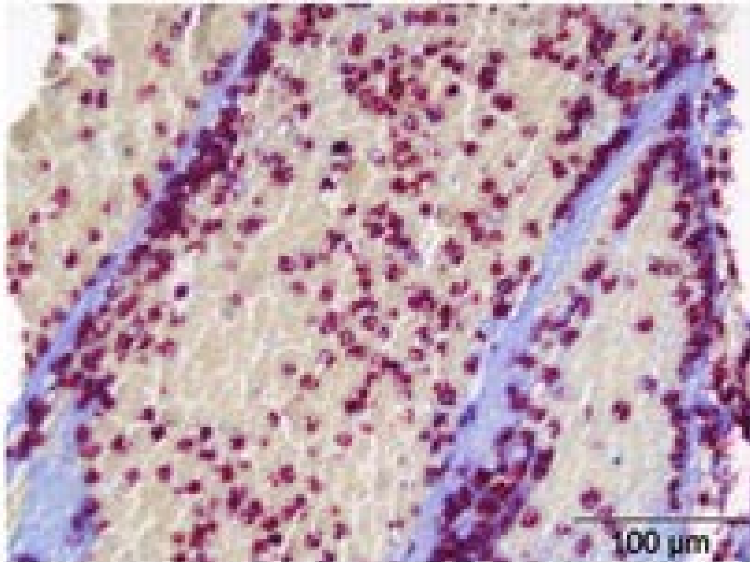


Fig.2. Changes in the composition of the human fracture hematoma over time. Representative images of fracture hematomas (FHs) that were isolated at different time points after injury. Extracellular matrix (ECM) was evident in the early FHs (2A) when practically all nucleated cells were leukocytes ($CD45^+$ cells, 2B). Extracellular matrix (ECM) within FHs that were isolated 48 h after injury or later stained positive for cell-derived insoluble fibronectin (2D and 2E). Macrophages ($CD68^+$ cells) were mainly localized within the ECM (2E) and neutrophils ($CD66b^+$ cells) were mainly localized adjacent to the ECM (2D). During the second week after injury, $CD45^-$ cells with a fibroblast-like morphology could be identified within the FH (2 A/2B). After influx of these $CD45^-$ cells, birefringent fibrils (2C) became visible within the ECM.

Results - Co-localization of leucocytes and fibronectin



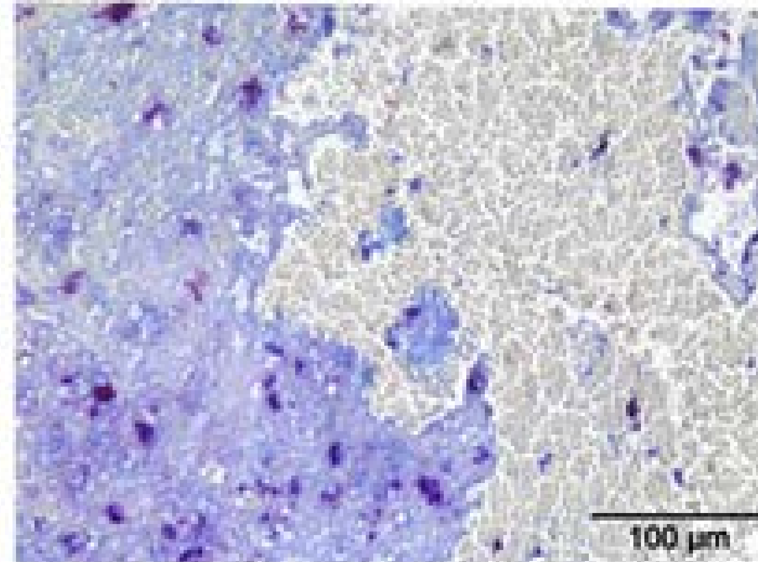


Fig. 2D and 2E: <3 days

Results – Leucocytes and fibronectin

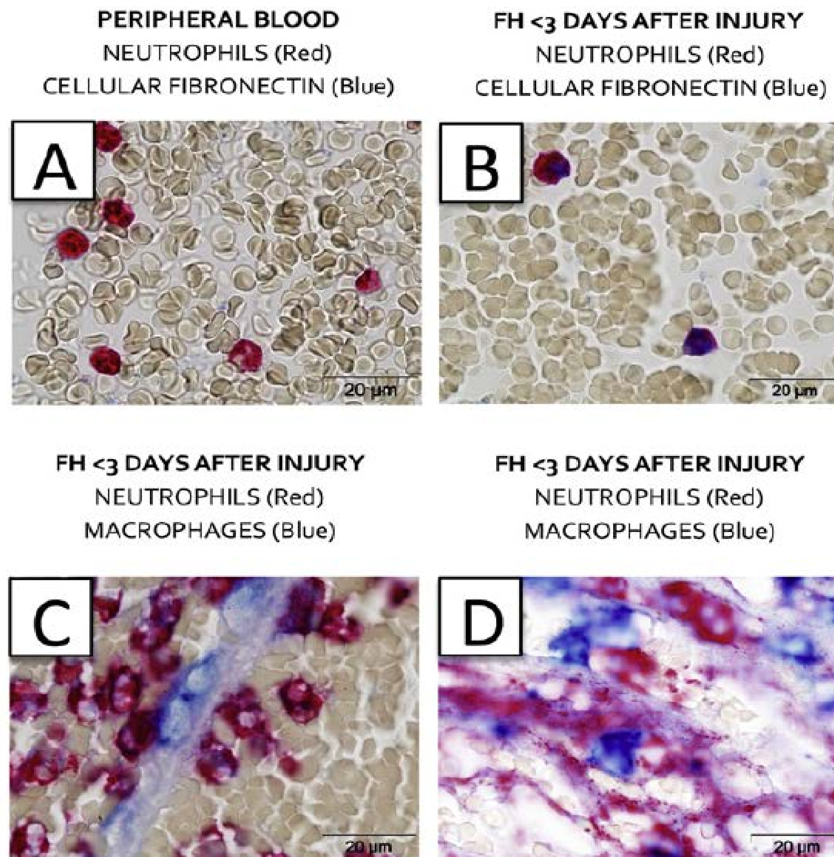


Fig. 3. Localization of innate immune cells in relation to fibronectin$^+$ extracellular matrix (ECM) during the first week after injury. Practically all neutrophils (CD66b$^+$ cells) within coagulated peripheral blood did not stain positive for cellular fibronectin (3A). In contrast, neutrophils stained positive for fibronectin$^+$ in fracture hematomas that were isolated within 48 h after injury and later (3B). Macrophages (CD68$^+$ cells) were predominantly localized within ECM and neutrophils were mainly localized adjacent to the ECM (2C). CD66b$^+$ fragments (2D) could be identified within the ECM during the first week after trauma.

Discussion

- 48h after injury: mainly leucocytes in the FH, fibronectin+ ECM
- Day 5: first appearance of stromal cells, presence of birefringent fibrils

→ Neutrophils as source of fibronectin+ ECM as they stained positive for cellular fibronectin in the FH

- Neutrophils in coagulated blood stained negative for fibronectin

→ Macrophages might also contribute to fibronectin synthesis

Discussion

- Fibronectin within the fracture gap:
  - EDA+ and EDB+ fibronectin, tenascin-C found in the initial fibrin matrix
  - EDA+ fibronectin and tenascin-C: fracture gap connective tissue
  - Osteofetal EDB+ fibronectin: in osteoblastic cells
- In rheumatoid arthritis patients: higher fibronectin synthesis by polymorphonuclear leucocytes (PMNL) from synovial fluid than by PMNL from peripheral blood

→ Inflammatory stimuli as possible regulators?

Kilian et al. mRNA expression and protein distribution of fibronectin splice variants and high-molecular weight tenascin-C in different phases of human fracture healing. Calcif. Tissue Int. 2008.
Menard et al. Studies on fibronectin in inflammatory vs non-inflammatory polymorphonuclear leucocytes of patients with rheumatoid arthritis. II. Synthesis and release of fibronectin in vitro. Clinical and Experimental Immunology. 1985.

Discussion

- CD66b+ particles: possibly secreted microparticles, role in FH remains unclear
  - In vitro: affection of macrophage phenotype: decrease in inflammatory response, increase in TGF-beta1 secretion → local injection of TGF-beta1 improved fracture healing outcome in rats

→ Neutrophils induce regenerative phenotype?

- Systemic depletion of neutrophils: improved outcome

→ Clarify role of neutrophils in (impairment of) fracture healing

- Different neutrophil subsets: CD62L-low and CD11b-high neutrophils both in FH and coagulated blood – coagulation might affect these markers

→ Different subtypes of regenerative and pathogen-battling neutrophils?