

— THE TWO FACES OF —
INFLAMMATION
DURING FRACTURE HEALING



OKAN BASTIAN

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DURING FRACTURE HEALING

The two faces of inflammation during fracture healing

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PhD thesis, University of Utrecht, the Netherlands

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THE TWO FACES OF INFLAMMATION DURING FRACTURE HEALING

De twee gezichten van
ontsteking tijdens de botgenezing
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties in het openbaar te verdedigen
op dinsdag 30 oktober 2018 des middags te 2.30 uur

door

Okan William Bastian
geboren op 23 augustus 1985 te Utrecht

Promotoren: Prof. dr. L.P.H. Leenen
Prof. dr. L. Koenderman

Copromotor: Dr. T.J. Blokhuis

“*Life is not about
how hard you hit, it's
about how hard you
can get hit and keep
moving forward*”

Rocky Balboa

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The background features several abstract shapes in shades of purple and blue. There are large, soft-edged purple shapes in the top-left and bottom-left corners. Scattered throughout the white space are various sized circles and dots in shades of purple and blue. Some circles are solid, while others have a slight gradient or shadow, giving them a three-dimensional appearance.

CHAPTER 1

Introduction and thesis outline

INTRODUCTION AND OUTLINE OF THIS THESIS

Inflammation is the body's attempt to remove harmful stimuli and begin the healing process. However, inflammation, which means "set on fire" in Latin, can also inflict collateral damage to a variety of processes within our body and thereby disturb their function¹. Inflammation therefore has two faces: it is designed to limit further damage and induce healing, but it is also a major driver of complications and fatal outcomes.

Bone injury is one of the most common injuries that humans experience and fracture healing starts with an inflammatory response². Several animal studies have shown that inflammation is essential for adequate bone regeneration³. However, hyper-inflammatory conditions after major trauma have been associated with impaired fracture healing³.

We believe that clarifying the role of inflammation during bone repair may contribute to development of therapies that augment tissue regeneration and prevent impaired fracture healing after major trauma.

In **Chapter 2**, we tried to clarify how inflammatory cells contribute to human fracture healing during the inflammatory phase of bone repair. We hypothesized that leukocytes rapidly infiltrate the fracture hematoma (FH) and start to synthesize a type of extracellular matrix (ECM) that is different from the ECM that becomes synthesized by stromal cells during the regenerative phase of bone healing.

FHs were isolated from trauma patients at different time points after injury during an Open Reduction Internal Fixation (ORIF) procedure, ranging from day 0 until day 23 after trauma. The FHs were placed into a single tissue microarray and stained using (immuno)histochemistry. We determined whether ECM was present within the early FHs that were isolated before stromal cells could be identified within the FHs. In addition, we tried to elucidate the composition and origin of this early leukocyte-derived ECM.

At the beginning of this thesis, trauma-induced systemic inflammation was not considered a risk factor of impaired bone healing after major trauma. We hypothesized that the trauma-induced systemic inflammatory response contributes to the high incidence of nonunion in multitrauma patients⁴.

In **Chapter 3**, we performed a review of the literature in order to determine whether any circumferential evidence exists that supports this hypothesis. One animal study showed that intraperitoneal injection of lipopolysaccharides (LPS) impairs fracture healing in rats⁵. LPS, which is part of the outer membrane of bacteria, can induce a strong systemic inflammatory response once it is recognized by LPS receptors on cells of the immune system. It is therefore tempting to speculate that not only LPS-induced systemic inflammation, but also trauma-induced systemic inflammation can impair fracture healing.

In **Chapter 4**, we retrospectively compared the trauma-induced systemic inflammatory response during the first two weeks after injury between multitrauma patients with normal and impaired fracture healing of the tibia. Peripheral blood concentrations of leukocyte subsets, but also of erythrocytes and thrombocytes reflect the systemic inflammatory response to tissue injury. The Utrecht Patient Oriented Database (UPOD) stores a variety of clinical and hematological parameters, including peripheral blood cell counts, even when clinicians did not request these parameters. By using the UPOD data, we were able to determine whether the trauma-induced systemic inflammatory response correlates with the outcome of fracture healing in multitrauma patients with tibial fractures.

After finding several significant differences between the systemic inflammatory response of multitrauma patients with normal and impaired fracture healing of the tibia, we performed a similar study in multitrauma patients with fractures of the femur, as described in **Chapter 5**.

In **Chapter 4** and **Chapter 5**, we demonstrate that peripheral blood neutrophil counts are significantly lower during the first two weeks after injury in multitrauma patients with impaired fracture healing of the tibia and the femur. A possible explanation for this difference is increased extravasation of neutrophils from the peripheral circulation towards sites of injury such as the FH, in patients that develop impaired fracture healing. A recent animal study also showed that systemic inflammation induced by blunt chest injury in rats induces increased influx of neutrophils into the FH and impairs fracture healing⁶. Based on these findings, we believe that increased influx of (alternatively activated) neutrophils into the FH may negatively affect the outcome of fracture healing in multitrauma patients.

In order to clarify how high neutrophil concentrations within the FH could negatively affect bone healing, we performed a co-culture of human neutrophils and multipotent stromal cells (MSCs) and determined how high neutrophil concentrations affect ECM synthesis by MSCs, as well as alkaline phosphatase activity, which is a marker of osteogenic differentiation and cell counts of MSCs in vitro. The results of these co-cultures are described in **Chapter 6**.

We found that high neutrophil concentrations negatively affect synthesis of mineralized ECM by human MSCs in vitro. Two animal studies have shown that systemic depletion of neutrophils improves bone healing^{7,8}. Based on these findings, we believe that modulating influx of neutrophils into the FH may be a target of future therapies that aim to prevent impaired fracture healing in multitrauma patients. However, systemic neutrophil depletion may carry an increased risk of developing infectious complications and is therefore not a realistic therapeutic strategy.

Clarifying the pathways involved in neutrophil chemotaxis towards the human FH may provide a target that allows selective inhibition of neutrophil chemotaxis towards sites of injury,

without affecting neutrophil chemotaxis towards sites of infection. We therefore developed an assay that allows analysis of neutrophil chemotaxis towards the human FH in vitro, which is described in **Chapter 7**. We subsequently determined whether chemotaxis of neutrophils towards the human FH was mediated by the CXCR1, CXCR2, FPR and C5aR receptors.

In **Chapter 8**, we summarize and discuss the results of the abovementioned studies and describe potential future studies.

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CHAPTER 2

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

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ABSTRACT

The role of inflammatory cells in bone regeneration remains unclear. We hypothesize that leukocytes contribute to fracture healing by rapidly synthesizing an “emergency extracellular matrix (ECM)” before stromal cells infiltrate the fracture hematoma (FH) and synthesize the eventual collagenous bone tissue.

53 human FHs were isolated at different time points after injury, ranging from day 0 until day 23 after trauma and stained using (immuno)histochemistry.

FHs isolated within 48 hours after injury contained fibronectin+ ECM, which increased over time. Neutrophils within the early FHs stained positive for cellular fibronectin and neutrophil derived particles were visible within the fibronectin+ ECM. Stromal cells appeared at day 5 after injury or later and collagen type I birefringent fibrils could be identified during the second week after injury.

Our study suggests that neutrophils contribute to bone regeneration by synthesizing an “emergency ECM” before stromal cells infiltrate the FH and synthesize the eventual bone tissue.

INTRODUCTION

Leukocytes are well known for their role in innate immune responses to invading microorganisms, but only little is known about their role in bone tissue regeneration. After bone injury, a blood collection forms around the fracture site, which is generally referred to as fracture hematoma (FH). Leukocytes rapidly infiltrate this FH, which marks the inflammatory phase of fracture healing¹. The current literature suggests that the inflammatory phase of fracture healing affects downstream processes of bone repair^{1,2}. This is illustrated by the finding that removal³ or repetitive irrigation⁴ of the early FH impairs bone healing in animals. Moreover, transplantation of the early FH into muscle tissue induces ectopic bone formation within muscle tissue⁵.

These findings imply that inflammatory processes, which occur during early bone repair, are essential for adequate bone regeneration. However, other studies suggest that certain inflammatory conditions can negatively affect the outcome of fracture healing^{1,2}. For instance, open fractures, severe soft-tissue injury and presence of multiple injuries, which are considered to induce local and systemic inflammation, have been associated with impaired fracture healing⁶. Moreover, local injection of beta glucan into the FH⁷ or intraperitoneal injection of lipopolysaccharides⁸, which are experimental models of local and systemic inflammation, negatively affect the outcome of bone regeneration in rats.

In summary, the currently available evidence implies that inflammation has a significant impact on the outcome of bone tissue regeneration. Clarifying the role of inflammatory cells during bone healing may contribute to the development of therapies that augment tissue regeneration and/or prevent impairment of bone healing after local and systemic hyper-inflammatory conditions.

We hypothesize that inflammatory cells contribute to bone healing by synthesizing some sort of emergency extracellular matrix (ECM) before stromal cells infiltrate the FH and synthesize the eventual bone ECM, which consists mainly of mineralized collagen type 1 fibrils^{9,10}.

To test this hypothesis, we analyzed the temporal changes in the composition of the human fracture hematoma during early bone healing. We determined whether ECM was present within FHs that were isolated before stromal cells infiltrated the FH and collagen type I positive fibrils could be identified.

MATERIALS AND METHODS

ISOLATION OF FRACTURE HEMATOMAS

53 fracture hematomas (FH) of closed fractures were isolated from trauma patients during an Open Reduction Internal Fixation procedure between 01-03-2011 and 01-04-2013. Four time-groups were defined based on the time between injury and isolation of the FH: 1) within 2 days, 2) 3-5 days, 3) 6-10 days and 4) >10 days. As a control, peripheral blood was drawn from healthy donors into a coagulation tube using a Vacutainer® system. After 1 hour, the coagulated blood was removed from the coagulation tube and treated similar to the freshly isolated fracture hematomas.

All FHs were fixed directly after isolation in 3.7% buffered formaldehyde solution (pH 4.0) for at least one week. The FHs were then dehydrated and embedded in paraffin with a Leica Embedding Center (EG1160, Leica Microsystems). Sections of 4 µm were cut with a Microm microtome (HM 335E, Thermo Scientific) and incubated at 54 °C overnight to allow firm adherence of the tissue to the microscopy slides (Superfrost Ultra Plus, Thermo Scientific). Fracture hematomas were residual samples and therefore collected without informed consent, unless the patient refused explicitly (opt-out method). Our local medical-ethical review committee has approved this study.

TISSUE MICROARRAY

A tissue microarray (TMA) was built to allow simultaneous and thereby comparable staining of all fracture hematomas in a single procedure. Two 1 mm cylindrical biopsy cores of each fracture hematoma were transferred to a single TMA paraffin block with a TMA GRAND Master microarrayer (3DHISTECH). The biopsy points were identified based on a hematoxylin and eosin (H&E) stained section of the entire fracture hematoma. A Dako CoverStainer was used to stain the sections with H&E. Two criteria were used to determine the biopsy locations: 1) when cells with a fibroblast-like morphology^{11,12} could be identified, that area was biopsied and 2) when these cells could not be identified within the H&E sections, a representative area of the FH was biopsied. Subsequently, multiple sections of 4 µm were cut and incubated at 54 °C overnight. The slides were deparaffinized, rehydrated and stained as follows 1) Hematoxylin and Eosin, 2) Picrosirius Red, 3) immunohistochemistry of CD45 (leukocytes), CD66b (neutrophils), CD68 (monocytes/macrophages), collagen type I and insoluble cell-derived fibronectin (the antibodies used do not recognize soluble plasma derived fibronectin).

ANALYSIS OF COLLAGEN FIBRES

Collagen fibers are anisotropic and therefore birefringent. Picrosirius Red binds to collagen and enhances its natural birefringence. Placing the FH section stained with PicroSirius Red between two polarization filters with 90 degrees of rotational difference between both filters allows clear identification of birefringent fibrils. In brief, TMA sections were deparaffinized, washed in dH₂O and stained with PicroSirius Red solution (Sirius red F3B (80115, Klinipath) diluted 1 gr/L in saturated aqueous picric acid (36011, Riedel-deHaën) 1 hour at room temperature. The sections were rinsed twice with 0.01 M HCl. The cell nuclei were stained with Hoechst 33258 (861405-100MG, Sigma-Aldrich) 10 µg/ml in dH₂O for 10 minutes in the dark at room temperature and rinsed in 3 changes with dH₂O. The sections were dehydrated, embedded with a ClearVue coverslipper (Thermo Scientific) and stored in the dark until further analysis.

IMMUNOHISTOCHEMISTRY

Five TMA sections were stained using immunohistochemistry.

In the first section, leukocytes were stained red and collagen type I was stained blue. In the second section, neutrophils were stained red and insoluble cell-derived fibronectin was stained blue (the antibodies used do not recognize soluble plasma derived fibronectin). In the third section, macrophages were stained red and insoluble cell-derived fibronectin was stained blue. In the fourth section, neutrophils were stained red and monocytes/macrophages were stained blue. In the fifth section, two isotype-matched control antibodies were applied. The nuclei of all cells were fluorescently labeled with Hoechst.

TMA sections were deparaffinized and rinsed in dH₂O. The sequential alkaline phosphatase (ALP) double immunostaining was carried out as described previously^{13,14} with Liquid Permanent Red Substrate Chromagen (K0640, Dako) and the Vector Blue Alkaline Phosphatase Substrate Kit III (SK-5300, Vector Laboratories).

As first primary antibodies, we used mouse anti-human Leukocyte Common Antigen CD45 (Clones 2B11+PD7/26, Dako, 3,75 µg/mL), mouse anti-human CD66b (MCA216, AbD Serotec, 10 µg/mL), mouse anti-human CD68 (NCL-CD68-KP1, Novocastra, Leica Biosystems, stock diluted 1:100) and mouse IgG1 as negative isotype control (X0931, Dako, 10 µg/mL). The second primary antibodies were mouse anti-human CD68 (NCL-CD68-KP1, Novocastra, Leica Biosystems, stock diluted 1:100), mouse anti-human cellular fibronectin (IST-9/ab6328, Abcam, 2 µg/mL), mouse anti-human collagen type I (COL-1/ab90395, Abcam, stock diluted 1:100) and mouse IgG1 as second negative isotype control (X0931, Dako, 10 µg/mL). Secondary antibodies were BrightVision polyclonal ALP-Anti-Mouse IgG (DPVM110AP, ImmunoLogic, undiluted).

IMAGING AND ANALYSIS

Each stained TMA core was imaged with a Leica DFC425C camera (Leica Microsystems) mounted to a Leica microscope (Leitz DMRXE, Leica Microsystems), using a point revisiting automated stage. A custom built algorithm was used to count cell numbers and the amount of Vector Blue Stained ECM or birefringent fibrils. The number of cells and the amount of extracellular matrix was normalized for the amount of tissue that was present on the image by subtracting all white pixels from the image. Representative images were taken with an Olympus DP70 camera, connected to an Olympus BX51 microscope using Cell[^]F software version 3.4 (Olympus Soft Imaging Solutions). All bright field images were exported as TIFF from Cell[^]F. Each bright field images was imported into Adobe Photoshop CS6 version 13 and arranged without manipulation of the original images. Fluorescent images of each filter were exported from Cell[^]F as TIFF files and also imported into Adobe Photoshop. In Photoshop, the blue channel of Hoechst images were merged with the red channel of Liquid Permanent Red (LPR) images without further modifying the images. The scale bar of the merged Hoechst/LPR image was not white, since it only contained a blue and red channel image of the scale bar. The white scale bar from the original Hoechst was therefore copied and pasted onto the merged Hoechst/LPR image. The merged images were subsequently arranged in Photoshop and exported as TIFF images. Graphs were made using GraphPad Prism version 5.03 (GraphPad Software, Inc.). Fracture hematoma characteristics were analyzed using SPSS version 20.0.0 (IBM Corporation).

STATISTICAL ANALYSIS

GraphPad Prism version 5.03 (GraphPad Software, Inc.) was used to determine whether the differences in cell count, leukocyte count, neutrophil count, monocyte count, fibronectin, collagen type 1 and the amount of birefringent fibrils was statistically significant between groups. First, a Kolmogorov-Smirnov test was used to determine whether the data was normally distributed. The differences between peripheral blood and fracture hematomas isolated within 48 hours after injury were tested with an Independent T-test when data were normally distributed or with a Mann-Whitney U test when data required non-parametric testing. Fracture hematomas that were isolated within 48 hours after injury already had a significantly different composition compared to peripheral blood. We therefore did not statistically compare peripheral blood with older fracture hematomas.

In addition to the abovementioned analysis, all four fracture hematoma groups were compared with an ANOVA and Newmann-Keuls post-hoc analysis or a Kruskal-Wallis ANOVA with a Dunn's post-hoc analysis depending on whether the data were normally distributed or not. The post-hoc analysis tested all possible combinations of fracture hematoma groups and corrected the p-values for multiple testing accordingly. A p-value of <0.05 was considered statistically significant.

RESULTS

FRACTURE HEMATOMA CHARACTERISTICS

53 fracture hematomas (FHs) were isolated from 53 different patients during an open reduction internal fixation procedure. The time of FH isolation ranged between 5 hours and 23 days after injury. 15 FHs were isolated within 2 days after injury, 10 FHs between 3 and 5 days, 15 FHs between 6 and 10 days and 13 FHs were isolated between day 10 and 23. 18 FHs were isolated from the upper extremities, 22 from the lower extremities, 10 from the pelvis and 3 from the thorax. 26 of the patients were male and the mean age \pm standard deviation of the patients was 45 ± 19 years.

FRACTURE HEMATOMAS ISOLATED WITHIN 48 HOURS AFTER INJURY

This group of FHs contained FHs that were isolated ranging from 6 hours after injury until 48 hours after injury. Practically all of the nucleated cells within fracture hematomas (FHs) isolated within 48 hours after injury were leukocytes characterized by CD45 positive staining using immunohistochemistry (*Figure 2B*). The majority of leukocytes were neutrophils as identified by a positive staining for CD66b (*Figure 1C and 2D*) and monocytes/macrophages, identified by CD68+ staining (*Figure 1D and 2E*).

Stromal cells could not be identified within FHs isolated within 48 hours after trauma.

There were significantly more neutrophils and monocytes within fracture hematomas that were isolated within 48 hours after injury compared to coagulated peripheral blood of healthy donors (*Figure 1C, 1D*).

H&E staining showed a significant amount of ECM (*Figure 2A*), which stained positive for insoluble cell-derived fibronectin (*Figure 2D and 2E*). The amount of fibronectin within FHs that were isolated within 48 hours after injury was significantly higher compared to the amount of fibronectin within coagulated peripheral blood (*Figure 1E*).

Some parts of the ECM stained mildly positive for collagen type 1. Birefringent fibrils could not yet be identified within these FHs, as demonstrated in *Figure 2C*.

An evident organization of leukocyte subsets became apparent, as seen in *Figure 2D, 2E and 3C*. Macrophages (CD68+ cells) were mainly localized within the fibronectin-positive ECM, while neutrophils (CD66b+ cells) were mainly localized adjacent to the ECM (*Figure 2D, 2E and 3C*). Neutrophils within these early FHs stained positive for cell-derived/cellular fibronectin, in contrast to neutrophils within coagulated peripheral blood (*Figure 3A and 3B*). We were unable to clearly identify macrophages outside of the ECM that stained double positive for CD68 and fibronectin.

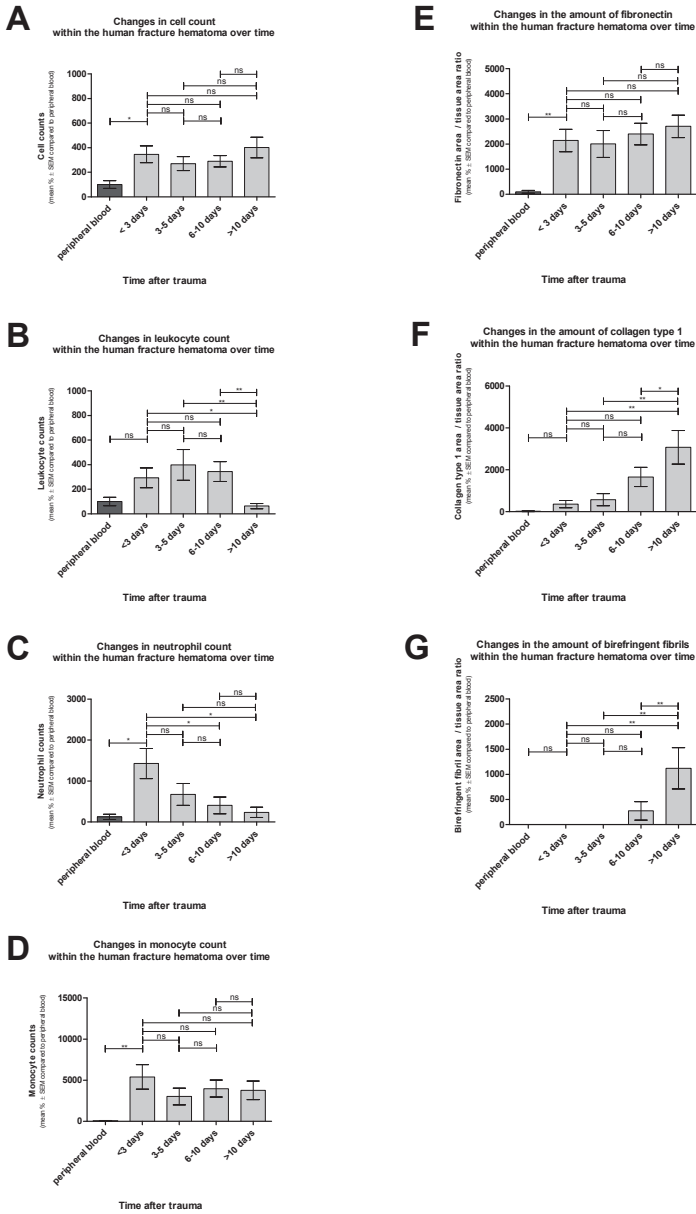


Figure 1 Changes in (A) Cell count, (B) Leukocyte count, (C) Neutrophil counts, (D) Monocyte count, (E) Fibronectin / tissue area ratio, (F) Collagen type 1 / tissue area ratio, (G) Birefringent fibrils / tissue area ratio within human fracture hematomas (FH) over time.

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 hours after injury. In addition, all FH groups were compared to each other. A p-value <math>< 0.05</math> is indicated by *, p < 0.01 by **, p < 0.001 by *** and p > 0.05 as ns (not significant).

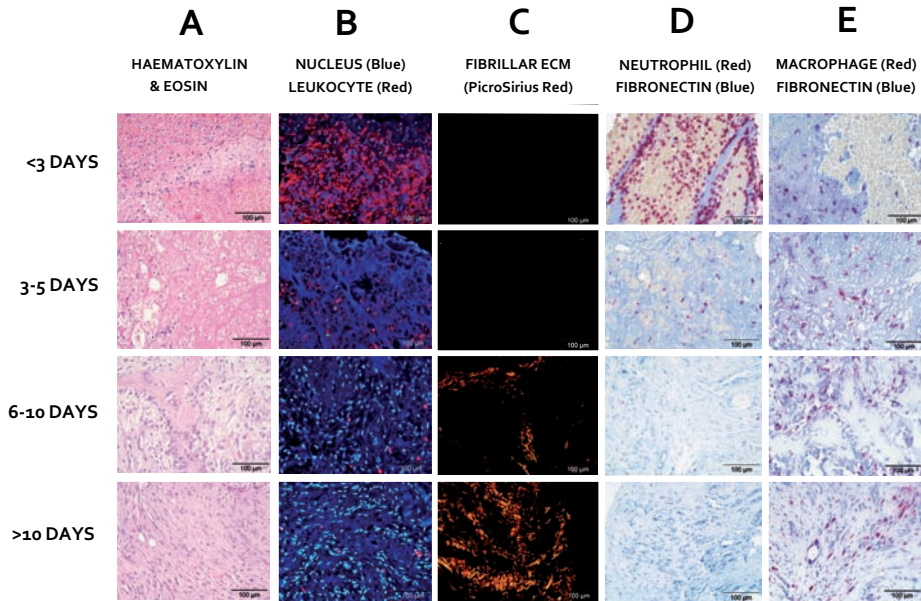


Figure 2 Changes in the composition of the human fracture hematoma over time.

Representative images of fracture hematomas (FHs) that were isolated at different time points after injury. Extracellular matrix (ECM) was evident in the early FHs (A) when practically all nucleated cells were leukocytes ($CD45^+$ cells, (B). Extracellular matrix (ECM) within FHs that were isolated 48 hours after injury or later stained positive for cell-derived insoluble fibronectin (D and E). Macrophages ($CD68^+$ cells) were mainly localized within the ECM (2E) and neutrophils ($CD66b^+$ cells) were mainly localized adjacent to the ECM (D).

During the second week after injury, $CD45^+$ cells with a fibroblast-like morphology could be identified within the FH (A/B). After influx of these $CD45^+$ cells, birefringent fibrils (C) became visible within the ECM.

Since macrophages were almost exclusively localized within the ECM, we could not determine whether cell-derived insoluble fibronectin that co-localized with macrophages was either intra- or extracellular fibronectin.

We could identify certain particles within the fibronectin-positive that stained positive for $CD66b$ (Figure 3D).

FRACTURE HEMATOMAS ISOLATED 3-5 DAYS AFTER TRAUMA

At day 5 after injury, the first stromal cells could be identified, which were $CD45$ -negative and had a fibroblast-like morphology (Figure 2A and 2B). These stromal cells started to form colonies (Figure 2A-B). At this time-point macrophages started to become the most prevalent leukocyte, instead of neutrophils that were the most prevalent leukocyte in FHs isolated within 48 hours after injury (Figure 1C, 2D and 2E). The majority of the ECM stained positive for fibronectin (Figure 2D and 2E). In addition, the ECM stained increasingly positive for collagen type 1 (Figure 1F). However, the presence of birefringent fibrils within collagen type I positive areas could not yet be demonstrated (Figure 1G and 2C).

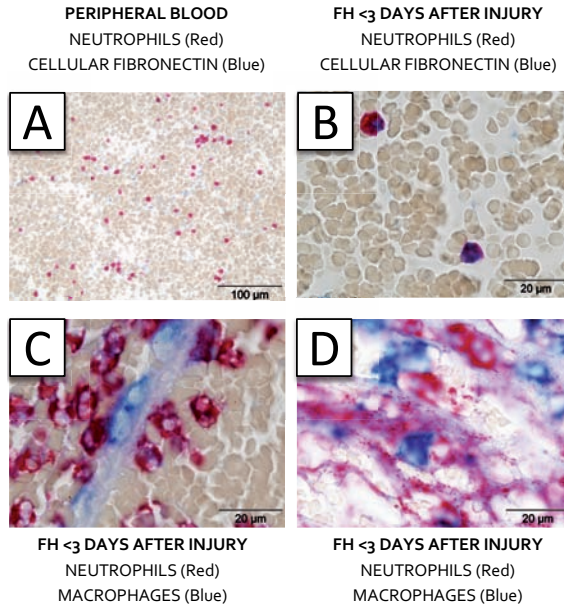


Figure 3 Localization of innate immune cells in relation to fibronectin+ extracellular matrix (ECM) during the first week after injury.

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

FRACTURE HEMATOMAS ISOLATED DURING THE SECOND AND THIRD WEEK AFTER TRAUMA

During the second and third week after trauma, the vast majority of nucleated cells are stromal cells (*Figure 2A and 2B*). At this time point, alignment and organization of stromal cells became increasingly evident, which is illustrated in *Figure 2A*. The number of neutrophils significantly decreased over time (*Figure 1C*) and neutrophils were almost completely absent during the third week after injury (*Figure 1C and 2D*).

Macrophages remained present within the FHs that were isolated during the second and third week after injury (*Figure 1D and 2E*). Macrophages were the predominant leukocyte during the second week after injury. The ECM within the FH evolved from a loose configuration to a more dense aspect (*Figure 2A*). This newly formed ECM stained strongly positive for collagen type I (*Figure 1F*). Well-defined areas of the ECM still stained positive for cellular fibronectin (*Figure 2D and 2E*). Most of the ECM now clearly contained birefringent fibrils when analyzed with polarized light microscopy of Picrosirius Red stained sections (*Figure 2C*), which significantly increased over time (*Figure 1G*). Most birefringent fibrils appeared aligned in a woven configuration.

DISCUSSION

Our data suggests that neutrophils contribute to fracture healing by synthesizing fibronectin+ extracellular matrix (ECM) within 48 hours after injury.

Fracture hematomas (FHs) that were isolated within two days after trauma mainly contained leukocytes (*Figure 2B*), and the first stromal cells only became apparent within the FH at day 5 or later (*Figure 2A and 2B*). Organization of collagen type I indicated by the presence of birefringent fibrils became evident during the second week after injury (*Figure 1G and 2C*). Our finding that stromal cells were not yet present in the early phase of fracture healing implies that leukocytes were the source of the newly synthesized fibronectin. The concept that leukocytes start to synthesize fibronectin during early fracture healing is supported by the finding that neutrophils within the early FHs stained positive for cellular fibronectin (*Figure 2D and 3B*), while neutrophils within coagulated peripheral blood stained negative for fibronectin (*Figure 3A*). Others have described fibronectin mRNA expression within 3 days after a mandibular fracture in rabbits¹⁵. Moreover, fibronectin expression within the fracture gap during various stages of fracture healing has been demonstrated previously¹⁶. However, the concept that neutrophils synthesize fibronectin during early fracture healing before stromal cells appear has not been described previously.

In FHs isolated within 48 hours after injury, the ECM diffusely stained positive for fibronectin and macrophages were mainly localized within this ECM (*Figure 2E and 3C*). It can therefore not be ruled out that not only neutrophils, but also macrophages start to synthesize fibronectin during the early phase of bone repair.

Synthesis of fibronectin by neutrophils has been shown previously *in vitro*¹⁷. Moreover, neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis (RA) synthesized up to 20 times the amount of fibronectin *in vitro* compared to neutrophils isolated from peripheral blood¹⁷. The finding that fibronectin synthesis was increased in neutrophils isolated from synovial fluid from RA patients compared to neutrophils isolated from peripheral blood suggests that inflammatory stimuli released by tissue injury can up-regulate fibronectin synthesis in neutrophils. This hypothesis is consistent with our findings that neutrophils within the FH stained positive for fibronectin, while practically all neutrophils within coagulated peripheral blood did not stain positive for fibronectin (*Figure 3A and 3B*). In our study CD66b⁺ particles were found within the fibronectin positive ECM (*Figure 3D*). Although the content and biological function of these CD66b⁺ particles within the early FH has not yet been elucidated, their presence may indicate an additional mechanism through which inflammatory cells contribute to tissue regeneration. The current literature describes that neutrophils can secrete CD66b⁺ microparticles upon activation *in vitro* and *in vivo* at the site of inflammation under various conditions¹⁸. CD66b is expressed on the cell membrane of neutrophils, in specific granules and on neutrophils derived ectosomes, but not on secretory vesicles, gelatinase granules or azurophilic granules¹⁸. These CD66b⁺ granules

affect the inflammatory phenotype of macrophages *in vitro*, as CD66b+ ectosome binding to macrophages can induce a decreased inflammatory response of macrophages, but also an increased TGF- β 1 secretion *in vitro*¹⁹. It has previously been shown that local injection of TGF- β 1 into the fracture hematoma of rats improved the outcome of fracture healing²⁰, indicating that TGF- β 1 might play a significant role during bone repair. Based on these findings, it is tempting to speculate that neutrophils not only contribute to fracture healing by rapidly synthesizing fibronectin, but also by inducing a regenerative phenotype in macrophages.

Although our findings suggest that neutrophils contribute to adequate fracture healing, several animal studies have shown that systemic depletion of neutrophils improved the outcome of bone regeneration^{21,22}, which implies that neutrophils have a negative effect on fracture repair. Additional research is required to clarify the role of neutrophils during normal fracture healing, as well as their role in impairment of fracture healing after local and systemic hyper-inflammatory conditions, such as open fractures and trauma-induced systemic inflammation². Relevant in this respect is our finding that multiple functional phenotypes of neutrophils are found during systemic inflammation, which may have different functions in tissue repair^{23,24}.

Neutrophil subsets can be identified in uncoagulated peripheral blood of trauma patients by flow-cytometry using antibodies against CD11b (α M component of the β 2-integrin MAC-1) and CD62L (L-Selectin)²⁴. We have attempted to identify neutrophil subsets in FHs at different time points after injury (data not shown). Unfortunately, we found that practically all neutrophils within the FH were CD62L-low and CD11b-high. We also found that neutrophils isolated from coagulated peripheral blood of healthy donors were CD62L-low and CD11b-high, in contrast to neutrophils isolated from uncoagulated peripheral blood from the same healthy donors. These findings imply that blood coagulation significantly affects CD62L and CD11b expression and therefore renders these markers unsuitable to identify neutrophil subsets within FHs. Future studies should focus on identifying which neutrophil subsets synthesize fibronectin within the FH. If these regenerative neutrophils are different from neutrophil subsets that battle pathogens it is relevant to assess where in the body neutrophils acquire their regenerative potential. Theoretically, differentiated regenerative neutrophils may home towards the FH. Alternatively, naïve neutrophils may infiltrate the FH and subsequently differentiate into regenerative neutrophils. Clarifying this underlying mechanism would be of great importance in the development of therapies that augment tissue regeneration.

A limitation of our study is that we were unable to link FH samples to patient characteristics. FHs were residual samples and therefore collected anonymized without informed consent, unless the patient refused explicitly (opt-out method). We could therefore not test whether conditions that affect the composition of the FH were equally distributed in our patient

population. These conditions include the amount of systemic inflammation, the condition of surrounding soft tissue and patient comorbidities.

These factors could affect the composition of the FH and thereby the function of leukocytes and the outcome of fracture healing². However, if these factors were unequally distributed in our patient population, this would not affect our proof of concept that leukocytes contribute to fracture healing by synthesizing fibronectin+ ECM. Future studies should focus on whether the amount of fibronectin within the FH becomes altered after severe systemic inflammation, open fractures or poor condition of the surround soft tissues. These studies may contribute to the development of therapies that prevent impairment of fracture healing by hyper-inflammatory conditions after severe trauma.

In conclusion, our study indicates that fibronectin becomes synthesized within the fracture hematoma within 48 hours after injury. Our finding that stromal cells were not yet present during this time point implies that leukocytes are the source of fibronectin. This indicates that leukocytes do not only battle pathogens, but also contribute to tissue regeneration by synthesizing ECM.

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OWB designed the study, performed sectioning and staining of all FHs and TMA slides, collected data and wrote the manuscript. LK and JA contributed to the design of the study, discussed the results and commented on the manuscript. LPHL and TJB isolated FHs from trauma patients, contributed to the design of the study, discussed the results and commented on the manuscript.

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CHAPTER 3

Systemic inflammation and fracture healing

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ABSTRACT

Apart from their pivotal role in the host defense against pathogens, leukocytes are also essential for bone repair, as fracture healing is initiated and directed by a physiological inflammatory response. Leukocytes infiltrate the fracture hematoma and produce several growth and differentiation factors that regulate essential downstream processes of fracture healing. Systemic inflammation alters the numbers and properties of circulating leukocytes and we hypothesize that these changes are maintained in tissue leukocytes and will lead to impairment of fracture healing after major trauma. The underlying mechanisms will be discussed in this review.

INTRODUCTION

Fractures in severely injured patients require a significantly longer healing time compared to isolated fractures and carry an increased risk of developing *nonunion*¹, which is failure of the bone healing process to unite the bone fragments within 9 months after injury². This impairment of fracture healing not only has a detrimental effect on quality of life of the patient but also carries a substantial cost to society. The direct costs of treating nonunions have been estimated between £15,566 and £17,200 per nonunion in the UK with considerable additional costs due to the loss of productivity of patients during the period of post-injury disability³.

Several local changes after major trauma have been well recognized as factors that contribute to impairment of fracture healing, such as poor condition of the surrounding soft tissue and open fracture sites¹. Systemic changes after major trauma, however, have received far less attention as possible risk factors that contribute to impairment of fracture healing. Systemic inflammation, which is characterized by leukocytosis, priming of leukocytes and systemic release of cytokines, is typically induced in trauma patients within 24 hours after admission⁴. These patients are also at risk for prolonged systemic inflammation as surgical ICU patients maintain a systemic inflammatory response during their unit stay⁵. Factors that contribute to prolonged systemic inflammation after major trauma include (I) the high incidence of sepsis after major trauma⁶ and (II) extended trauma by multiple additional surgical interventions⁷.

NORMAL FRACTURE HEALING

Bone tissue can either heal through direct (primary) cortical healing or indirect (secondary) healing by formation of callus, which is the newly developed mass of fibrocartilaginous and osseous tissue surrounding the fracture site. Primary bone healing is rare and does not involve major influx of inflammatory cells into the fracture hematoma⁸. Due to the absence of a major inflammatory component, primary bone healing is less likely to be affected by the cellular changes that are caused by systemic inflammation and, therefore, will not be discussed in this review.

In most fractures, a certain amount of mechanical instability leads to interfragmentary movement and thus to secondary fracture healing. This involves a local inflammatory reaction followed by a proliferative and remodeling phase that leads to reconstruction of bone. The inflammatory phase is initiated when disruption of the vasculature leads to a hematoma around the fracture site that becomes infiltrated by inflammatory cells.

The concentration of leukocytes within the fracture hematoma remains similar to their concentration in the peripheral circulation for several hours after injury⁹. Within 24 hours, however, there is a major influx of neutrophils into the fracture hematoma, making neutro-

phils the predominate leukocyte within the fracture hematoma¹⁰. Hereafter they become replaced by macrophages¹¹. Recruited neutrophils are thought to mediate this switch by producing several macrophage chemo-attractants, such as monocyte chemotactic protein-1 (MCP-1), also known as chemokine ligand 2 (CCL2) and IL-6(12;13). After this macrophage phase, T-lymphocytes are selectively recruited into the fracture hematoma and subsequent granulation tissue, whereas practically no B-lymphocytes are found at any stage of fracture healing¹¹. An extended overview of the current literature that addresses the composition and potential of the early fracture hematoma has recently been reviewed by Kolar and colleagues¹⁴.

Within several days, the fracture hematoma becomes intrinsically osteogenic and angiogenic, which is demonstrated by the fact that transplantation of the 4 day old fracture hematoma into muscle tissue induces extraskelatal bone formation¹⁵ and angiogenesis¹⁶. The composition of the fracture hematoma is predominantly established by factors that are produced by infiltrated inflammatory cells, illustrating the importance of local controlled inflammation for adequate bone repair. Disturbance of the inflammatory phase of bone repair is known to impair fracture healing as removal of the fracture hematoma at 30 minutes, 2 or 4 days after injury in a rat femur fracture model leads to a significant decrease in bone mechanical characteristics¹⁷. Moreover, irrigation and debridement of the fracture hematoma on the first and second day after osteotomy in a rabbit model consistently leads to atrophic fracture nonunion¹⁸.

Studies that have compared early mRNA expression of genes that regulate cartilage, bone and vessel formation between standard bone healing and mechanically induced delayed bone healing in sheep, found that at day 7 after osteotomy, expression of Sox-9, VEGF receptor 2 and Tie-2 was significantly higher in the delayed healing group and expression of TGF- β 1 and VEGF was significantly lower compared to the standard healing group(19;20). This suggests that the composition of the fracture hematoma within days after injury correlates with the eventual outcome of the bone healing process.

The inflammatory phase is followed by the proliferative phase which involves a combination of intramembranous and endochondral ossification. During intramembranous ossification, bone tissue is formed directly by committed osteoprogenitor cells that reside in the periosteum without first forming cartilage. Endochondral ossification, in contrast, involves the recruitment, proliferation and differentiation of undifferentiated multipotent stromal cells (MSCs) into chondroblasts that form cartilage, which eventually becomes replaced by bone²¹. The initially avascular cartilage provides mechanical support to the fracture and is converted to bone when hypertrophy of chondrocytes progresses, which causes calcification of cartilage matrix and concurrent invasion of vasculature²². As vasculature begins to invade, the calcifying hypertrophic chondrocytes are removed by chondroclasts after which undifferentiated MSCs that differentiate into osteogenic cells form woven bone.

It remains unclear which cells play an essential role in directing MSCs towards the osteogenic lineage *in vivo*. However, it has been shown that both macrophages²³ and activated T-lymphocytes²⁴ stimulate osteogenic differentiation of MSCs *in vitro*.

Osteogenic differentiation of MSCs is stimulated by several factors of which bone morphogenetic proteins (BMPs) are the most powerful known²⁵. BMPs are members of the transforming growth factor (TGF- β) family that bind to Type I and Type II serine-threonine kinase containing receptors²⁶. Binding of these receptors leads to phosphorylation of specific downstream effector proteins called Smads²⁷.

Inflammatory cytokines also have the potential to stimulate osteogenic differentiation of MSCs. A mixture of TNF- α , TGF- β , INF- γ and IL-17 stimulates osteogenic differentiation of MSCs *in vitro*²⁴, whereas either individually, or in combinations of up to three, these inflammatory cytokines fail to stimulate osteogenic differentiation. These data imply that osteogenic differentiation of MSCs is modulated by a complex ratio of cytokines and that alteration of inflammatory cytokine concentrations can affect differentiation of MSCs.

The final stage of fracture repair encompasses the remodeling of the woven bone into the original laminar bone configuration by osteoclasts that first create erosive pits on the bone surface on which new bone is then laid down by osteoblasts. This process eventually restores the laminar configuration and strength of the original bone tissue²⁸.

In summary, the hypothesis that inflammation is essential for fracture healing is supported by the findings that removal of the early fracture hematoma leads to a significant decrease in bone mechanical characteristics¹⁷ and repetitive irrigation of the early fracture hematoma leads to atrophic fracture nonunion¹⁸.

SYSTEMIC INFLAMMATION AND FRACTURE HEALING

Although local controlled inflammation seems essential for bone repair(15;18), previous studies have shown that increased or prolonged inflammation within the fracture hematoma impairs fracture healing. For instance, local application of semisoluble aminated glucan at the fracture site, which leads to release of inflammatory cytokines by local inflammatory cells, has been shown to result in immature hypertrophic callus formation with reduced bone mechanical properties²⁹. Other studies have shown that fractures that are accompanied by severe overlying muscle crush injury in rats demonstrate delayed healing, which is consistent with a detrimental effect of local hyper-inflammation on fracture healing³⁰.

Systemic inflammation, induced by intraperitoneal injection of lipopolysaccharides (LPS) into rats with femur fractures has shown to induce formation of hypertrophic and immature callus with reduced bone mechanical characteristics³¹. However, the mechanism through which systemic inflammation affects the local inflammatory phase of bone repair remains to be addressed adequately.

To speculate on a mechanism through which systemic inflammation impairs bone healing, it is relevant to first address the inflammatory changes that occur during systemic inflammation.

Tissue injury leads to the release of endogenous damage-associated molecular patterns (DAMPs) that activate innate immune cells³². Activation of the immune system is associated with an increased heart rate, respiratory rate, an increased or decreased body temperature and leukocyte count⁴. The presence of at least two of these symptoms is termed systemic inflammatory response syndrome (SIRS) and the severity of systemic inflammation positively correlates with the extent of injury³³.

The increased leukocyte count is predominantly established by the rapid release of neutrophils from the bone marrow into the peripheral circulation^(34;35), which leads to a functionally heterogeneous neutrophil compartment that consists of young banded neutrophils with a refractory phenotype and segmented neutrophils with a primed phenotype³⁴. Neutrophils form the first natural immunological defense against pathogens and are involved in debridement of injured tissue. These cells are relatively short-lived and circulate for several days³⁶. Their concentration rapidly increases in the peripheral circulation during systemic inflammation and peaks within several hours³⁴ until 3 days after injury³⁵. Neutrophils become pre-activated by several factors that are up-regulated after severe trauma³⁷ and the neutrophil pool can remain primed until 13 days after trauma³⁸. These priming factors include tumor necrosis factor- α (TNF- α), interleukin-8 (IL-8) and lipopolysaccharides (LPS)³⁵. Primed neutrophils are prone to respond to inflammatory stimuli. The importance of this process is illustrated by the fact that neutrophils isolated from trauma patients show a primed phenotype characterized by enhanced chemotaxis towards IL-8 *in vitro*³⁹. Moreover, it has been shown that systemic administration of β -glucan in rats, which induces a primed state of neutrophils⁴⁰, results in a more than 150% increase of neutrophil infiltration into subcutaneously implanted polyvinyl alcohol sponges⁴¹. In addition, systemic β -glucan treatment resulted in a more than 150% increase in neutrophil number in the bronchoalveolar lavage fluid of *Escherichia coli* pneumonic animals⁴¹, which also illustrates the enhanced migratory function of primed neutrophils.

It is, therefore, tempting to speculate that neutrophilia and neutrophil priming, leading to an increased influx of neutrophils into the fracture hematoma, plays an important role in the pathogenesis of impaired fracture healing after systemic inflammation (*Figure 1*). This is based on the finding that systemic neutrophil reduction with anti-neutrophil serum in rats with femur fractures induces enhanced fracture healing⁴². Moreover, systemic neutrophil reduction in rats with growth plate injury lead to a 60% reduction of neutrophils within the fracture hematoma and also improved bone repair. Rats treated with anti-neutrophil serum showed decreased cartilaginous tissue, chondrogenic transcription factor Sox-9 and cartilage matrix collagen II, along with an increased bone matrix protein osteocalcin and osteogenic transcription factor cbfa-1¹⁰. This implies that neutrophils may stimulate chon-

drogenesis and inhibit osteogenesis. An increased or prolonged influx of neutrophils into the fracture hematoma during systemic inflammation may therefore impair fracture healing through overstimulation of chondrogenesis and inhibition of osteogenesis.

In addition to this hypothesis, several other mechanisms through which systemic inflammation may impair fracture healing should be considered.

It is known, for instance, that monocytes can differentiate towards several subtypes of macrophages depending on environmental circumstances. IFN- γ and LPS exposure induces a "classically activated" pro-inflammatory phenotype of macrophages, while IL-4 or IL-13 induces an "alternatively activated" regenerative phenotype⁴³. It has been shown that monocyte subsets of trauma patients exhibit preferential differentiation towards pro-inflammatory phenotypes⁴⁴. The finding that macrophages which were exposed to LPS lose their ability to stimulate osteogenic differentiation of MSCs in vitro²³ raises the question whether altered differentiation of monocytes during systemic inflammation has a negative effect on fracture healing.

It remains unknown, however, whether macrophages within the fracture hematoma also exhibit enhanced differentiation towards a pro-inflammatory phenotype after systemic inflammation and whether this has a significant effect on bone repair.

In addition, it has been shown that severe injury induces unresponsiveness of T-lymphocytes, characterized by simultaneous diminished proliferation and IL-2 production^(45;46). The finding that unactivated T-lymphocytes fail to stimulate osteogenic differentiation of MSCs in vitro while activation of T-lymphocytes leads to stimulation of osteogenic differentiation²⁴ implies that unresponsiveness of T-lymphocytes during systemic inflammation may also be involved in reduced osteogenesis after systemic inflammation. The assumption that T-lymphocyte activation is essential for fracture healing, however, is contradicted by a recent study⁴⁷ that showed accelerated fracture healing in recombination activating gene 1 knockout (RAG1^{-/-}) mice, which lack functional B and T lymphocytes⁴⁸. Although this study implies a negative effect of the adaptive immune system on fracture healing, it is difficult to determine which lymphocyte subsets predominantly mediate this negative effect on bone repair. Absence of RAG-1 proteins, for instance, may predominantly improve fracture healing by disturbing the function of γ/δ T-lymphocytes. This assumption is supported by the finding that δ T-cell receptor (TCR) knockout mice, which lack γ/δ T-lymphocytes, also exhibit improved fracture healing⁴⁹. In addition, it is difficult to assess whether bone repair is predominantly improved by disturbance of either the regulatory or effector function of γ/δ T-cells. It has been shown that γ/δ T-cells play an important role in the recruitment of inflammatory cells towards inflammatory sites, which is illustrated by the fact that γ/δ T-lymphocyte deficient mice showed a 6-fold reduction in cellular infiltrate into polyvinyl alcohol sponges that were subcutaneously implanted beneath sites of thermal injury compared to wildtype mice⁵⁰.

Several studies also suggest an important role of γ/δ T-lymphocytes in neutrophil mediated tissue damage after systemic inflammation. This is illustrated by the fact that wildtype mice subjected to thermal injury show neutrophil accumulation in the lung and small intestines, while γ/δ T-lymphocyte deficient mice did not show a similar increase in neutrophil tissue content⁵¹. It is therefore possible that improved fracture healing in both RAG-1 knockout mice and δ TCR knockout mice is mediated by a decreased influx of neutrophils into the fracture hematoma. The finding that γ/δ T-cells exhibit increased expression of activation markers during systemic inflammation⁵², additionally raises the question whether increased activity of γ/δ T-cells during systemic inflammation induces an increased influx of neutrophils into the fracture hematoma and therefore impairs fracture healing.

In summary, systemic inflammation induces several changes in leukocyte characteristics such as neutrophil priming(37;38), altered monocyte differentiation⁴⁴, T-lymphocyte unresponsiveness(45;46) and increased γ/δ T-cell activation⁵². The effect of these changes on fracture healing have not been investigated and therefore it is difficult to determine which mechanism mediates impairment of fracture healing after systemic inflammation.

It is tempting, however, to speculate that neutrophilia and neutrophil priming during systemic inflammation induces an increased influx of primed neutrophils into the fracture hematoma, which leads to an aberrant concentration of growth- and differentiation factors and therefore disturbs downstream processes of fracture healing (*Figure 1*). This hypothesis is supported by the findings that primed neutrophils exhibit enhanced migration towards inflammatory sites *in vivo*⁴¹ and systemic neutrophil reduction improves fracture healing⁴², stimulates osteogenesis and inhibits chondrogenesis¹⁰.

ANGIOGENESIS-OSTEOGENESIS COUPLING

Disruption of the inflammatory phase of fracture healing may not only affect osteogenesis but also angiogenesis, as these two processes are linked⁵³. Whereas cartilage is an avascular and hypoxic mesenchymal tissue, bone is highly vascularized⁵⁴, which suggests that angiogenesis is essential for endochondral ossification. It has been shown that osteoblasts can be stimulated to secrete VEGF in culture by several BMPs⁵⁵. The action of BMPs on osteoblasts establishes a positive feedback loop where the BMP-induced VEGF release causes vessel in-growth, leading to the delivery of osteogenic precursor cells on which BMPs will act to further increase VEGF concentrations at the fracture site⁵⁶.

Apart from osteoblasts, macrophages⁵⁷ and neutrophils⁵⁸ also produce VEGF. Release of VEGF by neutrophils can be stimulated by PMA, fMLP and TNF- α ⁵⁸. It has been shown that VEGF not only regulates recruitment, survival and activity of endothelial cells, osteoblasts and osteoclasts but also regulates cartilage maturation and resorption(59;60). As osteogenesis and angiogenesis are linked, failure of leukocytes to adequately stimulate osteogenesis after systemic inflammation may therefore further impair fracture healing by also affecting

angiogenesis. The fact that pro-inflammatory macrophages fail to stimulate autologous BMP production of MSCs²³ may therefore also affect BMP-induced VEGF production of MSCs and negatively affect angiogenesis.

HYPOTHESIS

SYSTEMIC INFLAMMATION IMPAIRS FRACTURE HEALING BY INAPPROPRIATE HOMING OF PRIMED NEUTROPHILS TOWARDS THE FRACTURE HEMATOMA

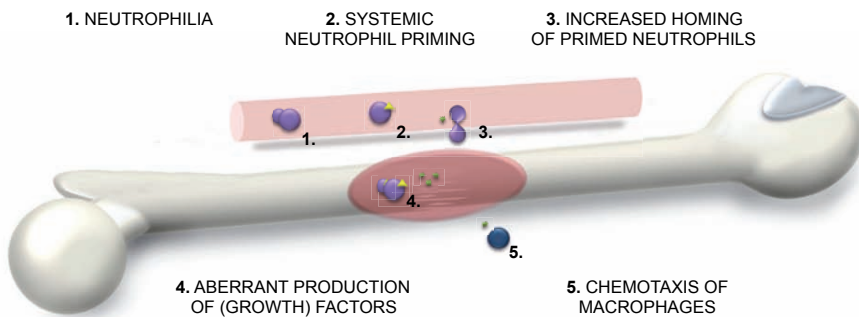


Figure 1 hypothetical model of the early inflammatory phase of fracture healing during systemic inflammation

Neutrophilia and neutrophil priming during systemic inflammation leads to an increased influx of neutrophils into the fracture hematoma, which alters the local concentrations of growth and differentiation factors and thereby disturbs downstream processes of bone repair.

CONCLUSION

The inflammatory phase of fracture healing not only initiates but also directs downstream processes of bone repair and disruption of this phase, either by systemic inflammation or by local hyper-inflammation has been shown to impair fracture healing. The mechanism through which systemic inflammation impairs fracture healing, however, remains unknown. As delineated in *Figure 1*, we hypothesize that an increased influx of primed neutrophils into the fracture hematoma during systemic inflammation alters the composition of the fracture hematoma and disturbs downstream processes of fracture healing. This hypothesis is based on the fact that neutrophils form the majority of leukocytes, not only in the peripheral circulation but also within the early fracture hematoma and neutrophil numbers increase dramatically during systemic inflammation. Moreover, neutrophils become primed during systemic inflammation and primed neutrophils are prone to home towards local sites of inflammation. We believe that an increased influx of neutrophil impairs fracture healing

due to the fact that reduction of systemic neutrophils improves fracture healing, stimulates osteogenesis and inhibits chondrogenesis.

Although the potency of the fracture hematoma has gained increasing attention in the literature, the vast complexity of processes that occur during the inflammatory phase of fracture healing still remains poorly understood. Additional research that addresses these processes will not only provide tools to augment artificial cartilage and bone tissue generation, but may also contribute to the development of preventive therapies against risk factors of impaired fracture healing.

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CHAPTER 4

Impaired bone healing in multitrauma patients is associated with altered leukocyte kinetics after major trauma

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ABSTRACT

Animal studies have shown that the systemic inflammatory response to major injury impairs bone regeneration. It remains unclear whether the systemic immune response contributes to impairment of fracture healing in multitrauma patients. It is well known that systemic inflammatory changes after major trauma affect leukocyte kinetics. We therefore retrospectively compared the cellular composition of peripheral blood during the first two weeks after injury between multitrauma patients with normal (n=48) and impaired (n=32) fracture healing of the tibia.

The peripheral blood count curves of leukocytes, neutrophils, monocytes and thrombocytes differed significantly between patients with normal and impaired fracture healing during the first two weeks after trauma (p-values were 0.0122, 0.0083, 0.0204 and <0.0001 respectively). The mean myeloid cell counts were above reference values during the second week after injury.

Our data indicate that leukocyte kinetics differ significantly between patients with normal and impaired fracture healing during the first two weeks after major injury. This finding suggests that the systemic immune response to major trauma can disturb tissue regeneration.

INTRODUCTION

In developed countries, each year approximately 1 in 100 inhabitants suffers a fracture.¹ In 5-10% of all cases, fractures fail to heal within 9 months after injury, which is referred to as nonunion.² Impaired bone healing has a detrimental effect on quality of life and carries a substantial cost to society.³ The direct costs of treating nonunions of the tibia have been estimated between \$31.144 and \$34.413 per nonunion in the United Kingdom with considerable additional costs due to the loss of productivity of patients during the period of post-injury disability.³

The incidence of nonunion is significantly higher in trauma patients with multiple injuries than in patients with isolated injuries.⁴ Impaired bone regeneration in multitrauma patients may be caused by several local changes that occur after high-energy impact, such as open fractures, poor condition of the surrounding soft tissue and large bone defects.⁴ However, animal studies suggested that not only local, but also systemic changes after multitrauma could disturb fracture healing.⁵⁻⁷ A recent animal study showed that experimental blunt chest injury altered the cellular composition of the fracture hematoma in rats and negatively affected the outcome of bone repair by inducing hypertrophic callus formation.⁸ Also, intraperitoneal injection of lipopolysaccharides (LPS), a frequently used model that mimics a trauma-induced systemic immune response, disturbed fracture healing in rats by inducing hypertrophic callus formation.⁹ The mechanism through which these systemic changes impaired bone regeneration remains unclear.

Leukocytes play an important role in fracture healing, as leukocytes not only initiate¹⁰ but also direct¹¹ bone repair. Changes in the early inflammatory phase of bone repair may therefore disturb downstream processes of fracture healing.¹² Cytokines released systemically after severe trauma affect leukocyte kinetics such as leukocyte mobilization from the bone marrow and leukocyte migration toward injured tissue, as well as the phenotype of peripheral blood leukocytes and hematopoiesis.^{5,13,14} Peripheral blood concentrations of leukocyte subsets, but also of erythrocytes and thrombocytes therefore reflect the systemic immune response to tissue injury.^{15,16}

We hypothesized that these systemic changes after severe injury can impair fracture healing by disturbing the inflammatory phase of bone regeneration. This impairment could be the result of either a changed number or phenotype of inflammatory cells within the fracture hematoma.⁵ To test whether the systemic immune response to trauma is associated with the outcome of fracture healing, we compared the peripheral blood count curves of leukocytes, neutrophils, monocytes, lymphocytes, thrombocytes and hemoglobin during the first two weeks after injury between multitrauma patients with normal and impaired fracture healing of the tibia.

PATIENTS AND METHODS

The peripheral blood count curves of several hematological parameters during the first two weeks after injury were compared between multitrauma patients with normal and impaired fracture healing of the tibia. The primary focus of our analysis was comparing the peripheral blood count curves of leukocytes between both healing groups. In addition to this analysis, peripheral blood count curves of neutrophils, monocytes, lymphocytes, thrombocytes and hemoglobin were compared between both healing groups in the context of an explorative sub-analysis. The p-values of these explorative sub-analyses were therefore not corrected for multiple testing.

PATIENT POPULATION

From a prospectively collected trauma register, all severely injured trauma patients with tibia fractures who were aged 18 years or older and required clinical admission to the University Medical Center of Utrecht (UMC Utrecht) between the 1st of January 2005 and the 1st of May 2012 were evaluated. Severe trauma was defined as an Injury Severity Score (ISS) of 16 or higher.^{17,18} The following clinical data were obtained: age, gender, trauma mechanism, ISS, associated injuries (abbreviated injury score), characterization of the tibia fracture according to the AO classification, soft tissue injury according to the Gustilo classification,¹⁹ duration from injury until definitive fracture fixation, type of fracture fixation, number and date of additional surgical interventions, total intensive care stay, total hospital stay, complications and the outcome of fracture healing. Impaired fracture healing was defined as lack of clinical or radiological evidence of union at the fracture site at least 16 weeks after the index injury, or at the most recent intervention.²⁰ Delayed healing was defined as lack of clinical or radiological evidence of union between 16-36 weeks after trauma. Nonunion was defined as lack of clinical or radiological evidence of union 36 weeks after trauma, or when the patient was subjected to secondary procedures to promote healing. Missing data were retrieved from the hospital's central electronic medical record if possible.

Our study is a retrospective database study with anonymized data and therefore does not need a formal review by an institutional review board.

HEMATOLOGICAL PARAMETERS

The abovementioned hematological parameters were obtained from the UPOD database Utrecht Patient Oriented Database (UPOD). The technical details of UPOD are described elsewhere.²¹ In short, UPOD is an infrastructure of relational databases that allows (semi-) automated transfer, processing and storage of data including administrative information, medical and surgical procedures, medication orders and laboratory test results for all

clinically admitted patients and patients attending the outpatient clinic of the UMC Utrecht since 2004. The process and storage of data are in accordance with privacy and ethics regulations. Routine hematological analysis was performed by using the Cell-Dyn Sapphire hematology analyzer (Abbott Diagnostics, Santa Clara, CA, USA).^{22,23} The reliability and validity of the laboratory results are monitored through routine quality control. The percentages of patients that required blood testing on each day during the first two weeks after injury are depicted in *Figure 2C*.

STATISTICAL ANALYSIS

Categorical variables were compared between both healing groups with a Chi-square test. Based on whether continuous data were normally distributed, an independent T-test or Mann-Whitney-U test was used. The equality of variances was assessed with a Levene's test. The mean hematological parameters (leukocytes, leukocyte subsets, thrombocytes and hemoglobin) are considered repeated measurements and the values of each patient on different time-points are therefore not completely independent. We analyzed the course of hematological parameters over time using linear mixed models because these models can adequately compare repeated measurements between outcome groups, they allow correction for possible confounders and they work well in the presence of missing data in repeated measurements.²⁴ This analysis only indicates whether the course of hematological parameters differs between outcome groups during the first two weeks after injury, but does not allow determination at which days exactly the outcome groups differ. We could not use the same linear mixed model technique to perform a post-hoc sub-analysis on the first and second week separately to determine whether the difference in hematological parameters occurs early or late after injury. Such analyses should have either been defined as primary analysis (not post-hoc on the same dataset) or should be performed on a different dataset than on which the original analysis was performed.

However, in order to speculate on which days the differences between outcome groups is most evident, we additionally compared all hematological parameters between outcome groups with an independent T-test or nonparametric equivalent for each time point (*Figure 1 and 2*). A p-value <0.05 is indicated by *, p < 0.01 by ** and p < 0.001 by ***. The results of the independent T-tests and nonparametric equivalent are therefore mainly illustrative and we base our conclusions on the results of the linear mixed models.

We first determined whether the trends of hematological parameters over time were best described by a linear, quadratic or cubic function. To test whether the trends of hematological parameters differed between outcome groups, we fitted two models for each hematological parameter.

The first model allows the outcome groups to differ both on average and in trend over time and therefore included fixed effects for the appropriate polynomial time trends, an

indicator for “outcome group” (normal versus impaired fracture healing), and the interaction between “outcome group” and time trends. The second model assumes that the outcome groups have the same average and trend over time and therefore only had fixed effects for time trends. We corrected for possible confounding by adding clinical parameters to both of these models that significantly differed between outcome groups. The percentage of patients that were treated non-operatively and the percentage of patients that had open fractures (Gustilo grade I and higher¹⁹) significantly differed between outcome groups and therefore, these parameters were added to both models. The given p-values therefore represent differences between outcome groups that cannot solely be explained by differences in type of management or presence of open fractures. The two models were compared using a likelihood ratio test (LRT); when the first model significantly fits the observed data better than the second model (which assumes that both outcome groups have the same average and trend over time), it was concluded that the curve of that hematological parameter significantly differed between outcome groups after correcting for possible confounders. In order to minimize multicollinearity of the polynomial terms for time, orthogonal polynomials were used.²⁴ For each outcome, random effects per patient for the intercept and time trends were used in the models to account for the correlation of repeated measurements within patients. A p-value < 0.05 was considered to be statistically significant. Mixed model analysis was performed using R Software version 2.10.0.²⁵ All other statistical analyses were performed with IBM® SPSS® Statistics version 20.

RESULTS

PATIENT CHARACTERISTIC

123 multitrauma patients with a tibia fracture were treated in the UMC Utrecht between the 1st of January 2005 and the 1st of May 2012. 16 patients died during their hospital stay and 13 were lost to follow up. Another 14 patients were excluded due to bone disease (n=2), a history of malignancy (n=4), paraplegia (n=1) or amputation of the affected leg (n=7). Of the remaining 80 patients, 13 (16.3%) developed delayed union and 19 patients (23.8%) developed nonunion which required intervention, leading to a total of 32 patients (40%) with impaired fracture healing. Clinical parameters of separate fracture healing groups (normal versus impaired) are shown in *Table 1*. There was no significant difference in the age, gender, extent of injuries based on the Injury Severity Score (ISS), New Injury Severity Score (NISS), distribution or severity of associated injuries (data not shown), the localization of the tibia fracture (proximal, shaft, distal or intra-articular), the complexity of the fracture (AO-classification), or the incidence of (infectious) complications between both healing groups. There were significantly more open fractures (56% versus 31%, p=0,037) and

	All patients n=80	Normal healing n=48 (60%)	Impaired healing n=32 (40%)	p-value
age	40 [24-55]	37 [24-58]	42 [25-54]	ns
gender	58% male	54% male	63% male	ns
Injury Severity Score (ISS)	25 [19-34]	25 [18-34]	24 [19-33]	ns
New Injury Severity Score	27 [22-41]	31 [22-34]	27 [22-41]	ns
number of fractures	4 [2-5]	3 [2-5]	4 [2-6]	ns
tibia fracture localization				
proximal	20%	20%	19%	ns
shaft	49%	48%	52%	ns
distal	31%	32%	29%	ns
type of tibia fracture (AO)				
multi-fragmentary / complex	37%	32%	45%	ns
intra-articular	31%	32%	29%	ns
soft tissue injury (Gustilo)				
0. closed fracture	59%	69%	44%	0,037
I. wound < 1 cm	14%	13%	16%	ns
II. wound > 1 cm with moderate soft tissue damage	15%	10%	22%	ns
III. wound > 1 cm with				
IIIa. adequate soft tissue cover	6%	6%	6%	ns
IIIb. inadequate soft tissue cover	5%	2%	9%	ns
IIIc. associated arterial injury	1%	0%	3%	ns
time until tibia fixation (days)	0 [0-5]	0 [0-5]	1 [0-6]	ns
type of fixation				
non-operative	11%	19%	0%	0,010
ORIF	43%	44%	41%	ns
nail fixation (ETN, UTN or CTN)	44%	38%	53%	ns
external fixation	3%	0%	6%	ns
number of operations	2 [1-4]	2 [1-3]	2 [1-5]	ns
ICU stay (days)	0 [0-8]	1 [0-9]	0 [0-7]	ns
	5.1 ± (8.3)	4.9 ± (7.5)	5.4 ± (9.4)	
hospital stay (days)	27 [14-50]	27 [14-50]	28 [12-46]	ns
complications	56%	56%	56%	ns
infectious complications	41%	44%	38%	ns
sepsis	9%	8%	9%	ns
non-infectious complications	31%	27%	38%	ns
delayed union	16%	-	41%	-
nonunion	24%	-	59%	-
atrophic	-	-	- 47%	-
hypertrophic	-	-	- 53%	-

Table 1 Overview of clinical parameters of patients with normal and impaired fracture healing of the tibia.

Note: Data shown as median ± [interquartile range] or mean ± (standard deviation).

Abbreviations: ns, not significant; AO, Arbeitsgemeinschaft für Osteosynthesefragen (association for study of internal fixation); ORIF, open reduction internal fixation; ETN, expert tibial nail; UTN, unreamed tibial nail; CTN, cannulated tibial nail; ICU, intensive care unit.

significantly more operatively treated fractures (19% versus 0%, $p=0.010$) in the impaired healing group compared to patients with normal fracture healing. Non-operative treatment and open fractures were therefore both considered as potential confounders and added to the statistical model used to test whether the curves of hematological parameters differed significantly between healing groups.

HEMATOLOGICAL PARAMETERS

Figure 1A depicts the mean leukocyte counts in peripheral blood during the first two weeks after injury for patients with normal and impaired fracture healing of the tibia. The two leukocyte count curves differed significantly between both healing groups when above-mentioned confounders were included into the statistical model ($p=0.0122$). The average leukocyte counts were above reference values (indicated by the gray shading) at admittance to the emergency department and there was no significant difference in leukocyte counts at arrival between the healing groups. After day 1, mean leukocyte counts decreased to reference values. From day 5 on, leukocyte numbers increased in both healing group and rose above reference values after day 7 in both groups. Leukocyte counts increased further and peaked at day 12 in the normal healing group, whereas leukocyte numbers peaked at day 10 in the impaired healing group. When each time point was analyzed separately, the mean leukocyte counts differed significantly between outcome groups on day 2,3,4,5, 11, 12,13 and 14 (*Figure 1A*).

Mean neutrophil counts, monocyte counts and thrombocyte counts rose above reference values in the second week after trauma (*Figure 1B-1D*). In contrast, lymphocyte numbers remained within the normal boundaries and hemoglobin values remained below reference values during the entire two weeks after trauma (*Figure 2A-2B*). Neutrophil, monocyte and thrombocyte count curves were significantly different for both healing groups (p -values were 0.0083, 0.0204 and <0.0001 respectively). The curves of lymphocyte count and hemoglobin values did not significantly differ between healing groups (p -values were 0.0688 and 0.9275 respectively). When each time point was analyzed separately, the mean neutrophil counts differed significantly between outcome groups on day 2,3,4,11,12,13 and 14 (*Figure 1B*).

The mean monocyte counts differed significantly on day 3,10,11,13 and 14 (*Figure 1C*) and the mean thrombocyte counts were significantly different between outcome groups on day 0 and day 14 (*Figure 1D*).

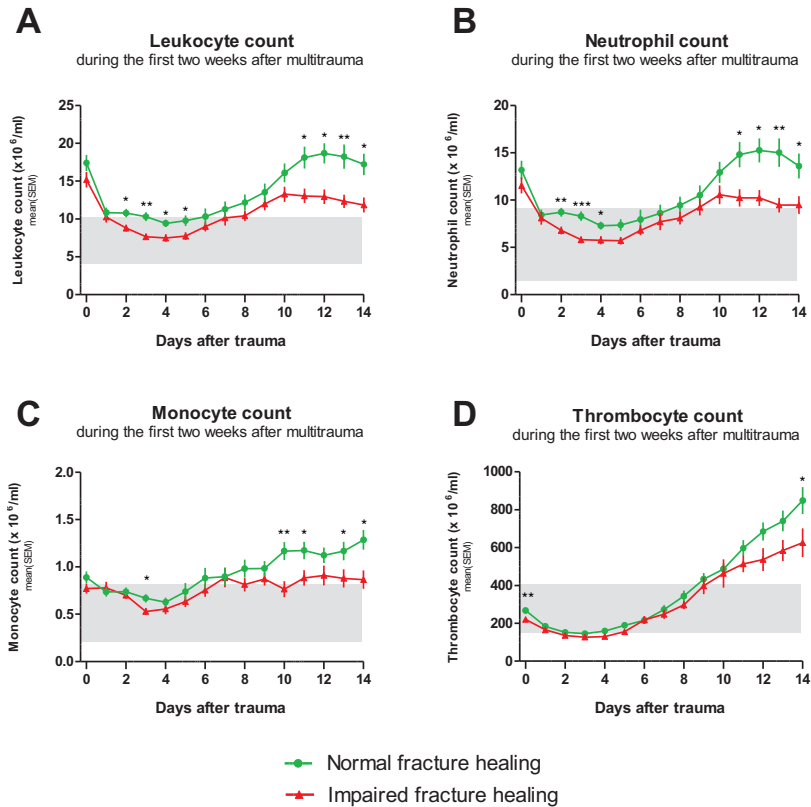


Figure 1 Peripheral blood counts of leukocytes, neutrophils, monocytes and thrombocytes during the first two weeks after major trauma for patients with normal (green) and impaired (red) fracture healing of the tibia.

The peripheral blood count curves of leukocytes, neutrophils, monocytes and thrombocytes were analyzed with mixed linear models and differed significantly between healing groups during the first two weeks after trauma (p-values were 0.0122, 0.0083, 0.0204 and <0.0001 respectively). In addition, each separate time point was compared between outcome groups using an independent T-test or nonparametric equivalent. A p-value of these sub-analyses <0.05 is indicated by *, p < 0.01 by ** and p < 0.001 by ***.

The gray bars represent reference values.

COMPLICATIONS

45 patients (56%) developed 69 complications: 33 (41%) patients developed 41 infectious complications and 25 patients (31%) developed 28 non-infectious complications. Infectious complications included 14 remote wound infections, 2 wound infections at the tibia fracture site, 9 pneumonias, 8 sepsis, 6 urinary tract infections and 2 other infectious complications. There was no significant difference between the normal and impaired healing groups in the percentage of patients who developed either infectious or non-infectious complications (Table 1).

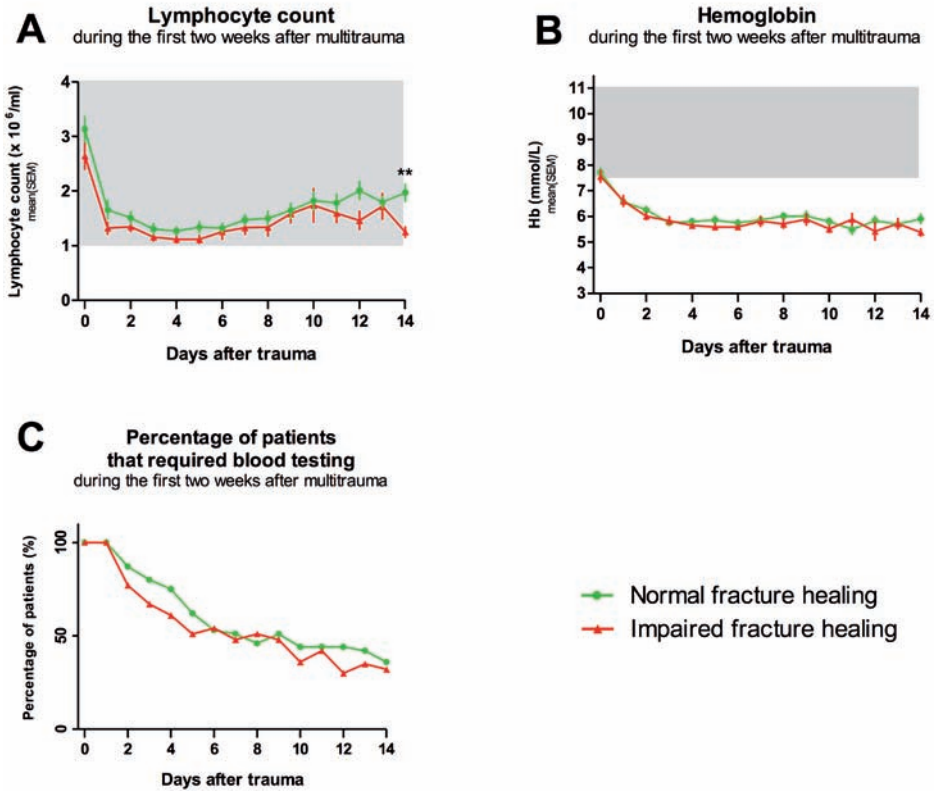


Figure 2 Peripheral blood lymphocyte counts (A) and hemoglobin values (B) during the first two weeks after major trauma for patients with normal (green) and impaired (red) fracture healing of the tibia.

Figure 2C illustrates the percentage of patients that required blood testing on each day during the first two weeks after injury. The peripheral blood lymphocyte counts and hemoglobin values were analyzed with mixed linear models and these analyses showed no significant differences between both healing groups (p -values were 0.0688 and 0.9275 respectively). In addition to the analyses with mixed linear models, each separate time point was also compared between outcome groups using an independent T-test or nonparametric equivalent. A p -value of these sub-analyses <0.05 is indicated by *, $p < 0.01$ by ** and $p < 0.001$ by ***. The gray bars represent reference values.

DISCUSSION

This study is the first clinical study to investigate the relation between the systemic immune response to severe injury and outcome of bone regeneration. We demonstrated that peripheral blood leukocyte kinetics differed significantly between multitrauma patients with normal and impaired fracture healing of the tibia during the first two weeks after injury (Figure 1A). The difference in leukocyte count curves between both groups may either reflect

increased extravasation of leukocytes towards injured tissue or a blunted trauma-induced bone marrow response. It is well known that the systemic inflammatory response after major trauma affects leukocyte kinetics and increased migratory function of leukocytes^{8,26} as well as bone marrow failure²⁷⁻²⁹ after trauma have both been described previously in the literature.

Several animal studies have illustrated the importance of local controlled inflammation for adequate bone healing. For instance, transplantation of the early fracture hematoma, which predominantly contains inflammatory cells, into muscle tissue of rats induces ectopic bone formation within muscle tissue.¹¹ These experiments suggest that inflammatory cells can initiate downstream processes of bone healing. Moreover, removal or repetitive irrigation of the early fracture hematoma impairs fracture healing in rats.¹⁰

Although these studies illustrate the importance of local controlled inflammation for adequate bone healing, other studies have shown that local or systemic "hyper-inflammatory" conditions can impair fracture healing. For instance, injection of beta-glucan into the fracture site induces local hyper-inflammation and impairs fracture healing in rats.³⁰ In addition, intraperitoneal injection of lipopolysaccharides in rats, which induces systemic inflammation, negatively affects the outcome of bone healing.⁹ Moreover, blunt chest injury, which is a model of trauma-induced systemic inflammation also impairs fracture healing in rats.^{31,32}

It is well known that multitrauma patients have an increased risk of developing delayed union and nonunion.⁴ Based on the abovementioned animal studies, we hypothesized that systemic inflammatory changes after major trauma contribute to this high incidence of impaired bone healing in severely injured individuals.⁵ We now show a correlation between leukocyte kinetics early after injury and the eventual outcome of bone healing in multitrauma patients, which supports this hypothesis.

The primary focus of our analysis was comparing the peripheral blood count curves of leukocytes between both healing groups. However, the UPOD-database also stores the number of leukocyte subsets in peripheral blood, even when clinicians did not request these values. Analysis of these subsets as a secondary outcome contributes to the understanding of the mechanism behind the difference in systemic immune response between outcome groups. However, we did not power our study to include multiple leukocyte subsets as our research population is too small. Therefore, we did not correct for multiple testing and only analyzed subsets in the context of an explorative sub-analysis.

These explorative sub-analyses showed that neutrophil, monocyte and thrombocyte counts are above reference values during the second week after injury in both healing groups, in contrast to lymphocyte counts and hemoglobin values (*Figure 1 and 2*). These findings suggest that trauma induces an increased concentration of myeloid cell within peripheral blood during the second week after trauma, potentially by stimulation of myelopoiesis.

When comparing outcome groups, we found that peripheral blood neutrophil-, monocyte- and thrombocyte- counts were lower (*Figure 1B-1D*) in the impaired fracture healing group.

These findings may be explained by relative inhibition of trauma-induced myelopoiesis in the impaired healing group. It remains unclear whether there is a causal relation between inhibition of trauma-induced myelopoiesis and poor bone regeneration or whether these two phenomena are separate consequences of an aberrant systemic immune response without a causal relation between them. We hypothesize that systemic inflammatory changes after major trauma affect the concentration or phenotype of inflammatory cells within the fracture hematoma and thereby disturb fracture healing (Figure 3).⁸

HYPOTHESIS OF THE MECHANISM THROUGH WHICH AN ABERRANT SYSTEMIC IMMUNE RESPONSE IMPAIRS FRACTURE HEALING

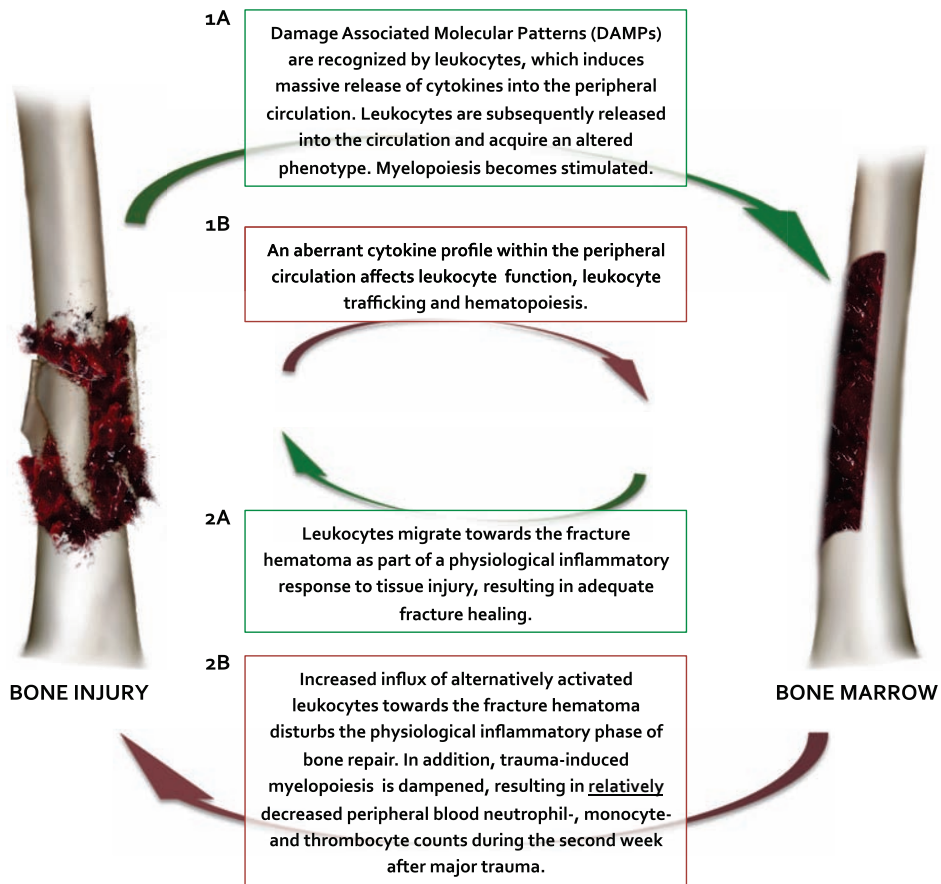


Figure 3 Our hypothesis of the mechanism through which an aberrant systemic immune response to trauma impairs fracture healing.

The green boxes describe a physiological systemic immune response to major trauma and the red boxes describe a different detrimental systemic immune response.

Factors that may contribute to a different systemic immune response include the type and extent of injury, the time between injury and resuscitation, the amount of ischemia/reperfusion damage or host-factors such as smoking and genetic background, infectious complications and the type, timing and number of operative procedures.⁵

We found no significant difference in the incidence of infectious complications, the total amount of tissue damage, or the severity and localization of injuries. However, our study did not have enough power to state that all abovementioned parameters were equally distributed between outcome groups. Moreover, we were only able to compare the amount of tissue injury based on a clinical scale of severity (ISS and NISS). This clinical scale of severity may not be sensitive enough to detect biological differences in the amount of tissue injury between both groups. The only differences between the two groups were that the impaired healing group had a significantly higher percentage of open fractures and a higher percentage of operatively treated fractures (*Table 1*). Open fractures and open surgical treatment have previously been described as risk factors of impaired fracture healing.⁴ It remains unclear whether these parameters can significantly affect the systemic immune response rapidly after injury. Therefore, we considered these factors as possible confounders and added these parameters to all statistical analysis. The difference in systemic immune response remained statistically significant even after correcting for these possible confounders.

The strength of our study lies predominantly in the fact that the UPOD (Utrecht Patient Oriented Database) database allowed us to retrospectively analyze hematological parameters of multitrauma patients and to correlate these values with the outcome of fracture healing, even when clinicians did not request these parameters. Potential limitations of our study are that it is retrospective, it comprises a relatively small cohort, and blood sampling was not performed daily in all patients.

Future research should focus on strategies that enable early identification of multitrauma patients who will mount an undesirable systemic immune response to trauma and may therefore require interventions that prevent development of impaired fracture healing. Moreover, the mechanism through which an altered systemic immune response can impair bone regeneration needs to be clarified in order to develop therapies that prevent non-union after an undesirable systemic immune response to severe injury.

In conclusion, our data indicate that leukocyte kinetics differ significantly between patients with normal and impaired fracture healing during the first two weeks after major injury. This finding supports the hypothesis that certain systemic inflammatory changes after extensive tissue injury can disturb tissue regeneration.

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OWB mainly designed the study, performed statistical analysis and wrote the article, AK acquired data and contributed to drafting of the manuscript, RS performed statistical analysis, contributed to the design of the study and revised the manuscript, LK, WvS, LL and TB contributed to the design of the study and revised the manuscript.

The results of this study have been presented as oral presentation at the 14th European Congress of Trauma and Emergency Surgery, Lyon, France, May 4-7, 2013 and its abstract will therefore be published online in a supplement of the European Journal of Trauma. The authors would like to thank Hanneke den Breeijen, Leon Stijvers for retrieving data from the UPOD, as well as Bob Surie for retrieving data from the trauma register.

For this study, data from the UPOD database (Utrecht Patient Oriented Database) were used. UPOD is an infrastructure of relational databases comprising data on patient characteristics, hospital discharge diagnoses, medical procedures, medication orders and laboratory tests for all patients treated at the University Medical Center Utrecht (UMC Utrecht) since 2004. The UMC Utrecht is a 1 042-bed academic teaching hospital in the center of the Netherlands, with annually about 28 000 clinical and 15 000 day-care hospitalizations and 334 000 outpatient visits. UPOD data acquisition and management is in accordance with current regulations concerning privacy and ethics. The structure and content of UPOD have been described in more detail elsewhere.³³

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CHAPTER 5

An increase in myeloid cells after severe injury
is associated with better fracture healing

*A retrospective cohort study of 62 patients
with a femoral fracture*

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ABSTRACT

Background and purpose: Nonunion is common in patients with femoral fractures. Previous studies suggested that the systemic immune response after trauma can interfere with fracture healing. Therefore, we investigated if there is a relation between peripheral blood cell counts and healing of femur fractures.

Methods: Sixty-two multitrauma patients with a femoral fracture presenting at the University Medical Centre Utrecht between 2007 and 2013 were retrospectively included. Peripheral blood cell counts from hematological analyzers were recorded from the first through the 14th day of the hospital stay. Generalized Estimating Equations were used to compare outcome groups.

Results: Twelve of the 62 patients developed nonunion of the femoral fracture. The peripheral blood-count curves of total leukocytes, neutrophils, monocytes, lymphocytes, and platelets were all statistically significantly lower in patients with nonunion, coinciding with significantly higher CRP levels during the first 2 weeks after trauma in these patients.

Interpretation: Patients who developed femoral nonunion after major trauma demonstrated lower numbers of myeloid cells in the peripheral blood than patients with normal fracture healing. This absent rise of myeloid cells seems to be related to a more severe post-traumatic immune response.

INTRODUCTION

Nonunion has been reported in one tenth of patients with femoral fractures. This risk further increases in cases of multiple fractures and open fractures, as frequently seen in multitrauma¹.

Local factors, such as severe soft tissue injury and reduced weight-bearing on the affected extremity, can impair bone healing¹⁻³. In addition, an increasing body of evidence suggests that the systemic immune response can also influence bone healing⁴. For instance, blunt chest injury in an experimental setting or intraperitoneal injection of lipopolysaccharides, which are both models for systemic inflammation, impaired fracture healing in animal models⁵⁻⁷. However, the exact mechanism underlying the fracture healing impairment after systemic inflammation remains unknown.

Secondary bone healing consists of at least 4 different stages: the inflammatory phase, soft callus formation, hard callus formation, and tissue remodeling. During the inflammatory phase, neutrophils and macrophages are recruited to the fracture hematoma within days up to a week after injury^{8,9}. The inflammatory phase normally ends within a week, after which the formation of callus starts^{9,10}. Disruption of the inflammatory process, for example by sustained inflammation, may interfere with the consecutive stages of bone healing and, thereby, increase the risk of nonunion⁹.

It is unclear if a correlation exists between the cellular systemic immune response after trauma and femoral fracture healing. Hence, we investigated if peripheral blood cell counts differ between multitrauma patients with normal and impaired fracture healing of the femur.

PATIENTS AND METHODS

STUDY DESIGN AND SETTING

Patients were selected from the trauma registry database of the University Medical Center (UMC) Utrecht which collected data of all patients who were admitted to the trauma department. Patients admitted between the 1st of January 2007 and the 31st of December 2013, were included. Total number of leukocytes, neutrophils, monocytes, platelets, erythrocytes, lymphocytes and hemoglobin concentrations were compared during the first 2 weeks after injury between multitrauma patients with nonunion and multitrauma patients with normal healing of the femoral fracture. In addition, the acute phase protein C-reactive protein (CRP) and reticulocyte counts were obtained. Outcome data concerning healing of the femoral fracture were obtained from the electronic medical record system 1 year after trauma.

PARTICIPANTS

Multitrauma patients ≥ 16 years of age with a femoral shaft or distal femoral fracture presenting in the emergency department (ED) of the UMC Utrecht were included. Exclusion criteria were: 1. transfer to another hospital and 2. non-weight bearing of the affected extremity, for instance due to paresis, amputation or severe head injury. Also, patients were excluded if the healing outcome could not be determined due to death or loss to follow-up. Data concerning patient characteristics, trauma mechanism, injuries and treatment were obtained from the trauma registry database and supplemented with information from the electronic medical record system.

PROCEDURES

Hematological parameters were obtained from the Utrecht Patient Oriented Database (UPOD). Data were collected from the day patients arrived in the Emergency Department (ED) through the 14th day of their hospital stay. The technical details of the UPOD are described elsewhere ¹¹. In short, this database is an infrastructure of relational databases that allows (semi)automated transfer, processing and storage of data, including administrative information, medical and surgical procedures, medication orders, and laboratory test results for all clinically admitted patients and patients attending the outpatient clinic of the UMC Utrecht since 2004. The process and storage of data are in accordance with privacy and ethics regulations. UPOD data acquisition and data management is in line with current Dutch regulations concerning privacy and ethics and is approved by the institution's medical ethics committee. Because no extra material, such as blood samples, was taken from patients, there was no requirement to obtain informed consent from individual patients. The data were analyzed anonymously. Routine hematological analysis was performed using the Cell-Dyn Sapphire hematology analyzer (Abbott Diagnostics, Santa Clara, USA) ¹¹. The reliability and validity of the laboratory results were monitored through routine quality control.

VARIABLES AND OUTCOME MEASURES

The study outcome was femoral fracture healing. Union was defined as pain-free mobilization (clinical union) or bridging of 3 of the 4 cortices (radiological union) within 12 months after injury. Nonunion was defined as lack of radiological and clinical union within 12 months after trauma or a fracture which required a re-intervention to achieve union. Peripheral blood cell counts of multitrauma patients with union were compared to peripheral blood cell counts of multitrauma patients with nonunion. Soft tissue injury was scored according to the Gustilo classification ¹².

STATISTICS

Data were analyzed with IBM SPSS version 23 (IBM Corporation, NY, United States). Descriptive statistics are presented as median (range) for non-normally distributed variables and mean (SD) for normally distributed variables. Comparison of baseline variables between outcome groups was performed with a Fisher's exact test for categorical variables or a Mann-Whitney U test for the continuous data, that were not normally distributed. Statistical significance was defined as a p-value < 0.05. Since the design of the study is longitudinal with repeated measurements, we chose linear generalized estimating equations (GEE) to compare the development over time of hematological parameters between outcome groups. This linear analysis was performed to analyze whether the course of hematological parameters differed between the outcome groups during the first two weeks after trauma. The GEE was used to account for within-subject correlation between repeated measurements. Based on spaghetti plots we chose the autoregressive working correlation structure for platelets and the exchangeable working correlation structure for the other hematological parameters.

ETHICS, FUNDING AND POTENTIAL CONFLICTS OF INTEREST

A waiver was provided by the institutional medical ethics committee for this study. In addition, in line with the academic hospital policy, an opt-out procedure is in place for use of patient data for research purposes. The process and storage of data are in accordance with privacy and ethics regulations. The authors did not receive financial support for the research, authorship and publication of this article. All authors declare to have no potential conflicts of interests.

PATIENTS

A total of 100 multitrauma patients with a distal or shaft femoral fracture were enrolled in the study (*Figure 1*). Multitrauma was defined as an Injury Severity Score (ISS) ≥ 16 ¹³. Eleven patients were lost to follow-up, of which 8 were transferred to another hospital, 2 did not visit the outpatient clinic and in one case the information in the patient record system was insufficient to determine healing outcome. Twenty-one patients died. Causes of death were severe traumatic injuries (n = 18), inflammatory complications (n = 2) and unknown (n = 1). Six patients were excluded because of the disability to bear weight on the affected leg due to severe ipsilateral injuries, an amputation, severe head trauma or spinal cord injury. Of the remaining 62 patients, union of the femoral fracture was seen in 50 patients (50/62) and nonunion was seen in 12 patients (12/62). Clinical parameters of patients with union and patients with nonunion are shown in *Table 1*.

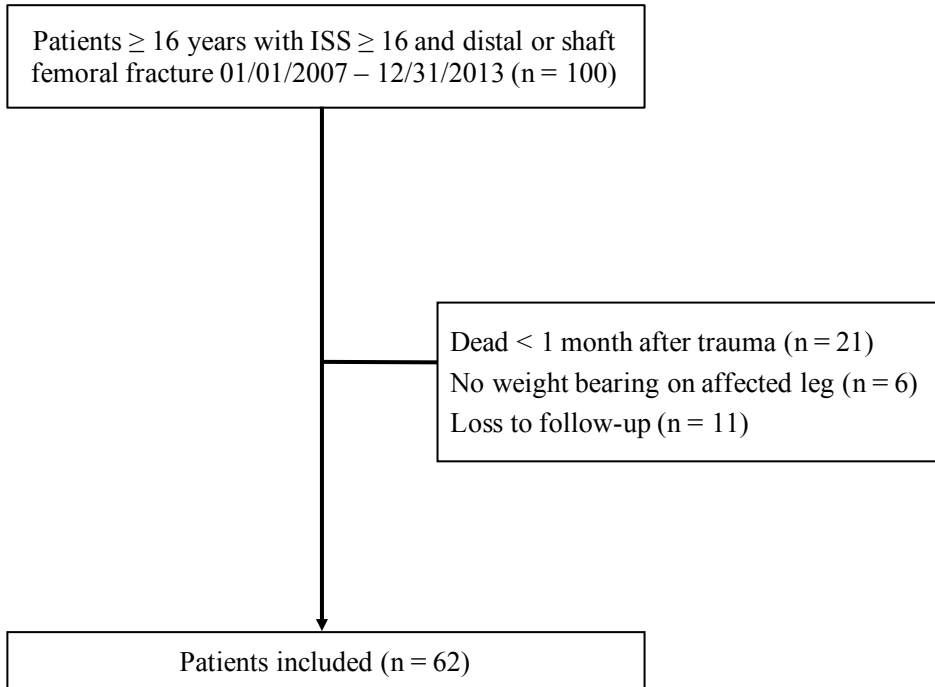


Figure 1 Flowchart of patients who met inclusion/exclusion criteria for the study population. AIS = Abbreviated Injury Scale. ISS = injury severity score.

RESULTS

DEMOGRAPHICS

There were no statistically significant differences in sex, age, ISS, new injury severity score (NISS), fracture localization, type of fracture, soft tissue injury, type of fixation, number of surgical procedures, inflammatory complications, length of stay in Intensive Care Unit (ICU) and total hospital stay between patients with normal and impaired fracture healing of the femur.

PERIPHERAL BLOOD CELL COUNTS (FIGURE 2)

Neutrophil and leukocyte counts were similarly elevated in both outcome groups upon arrival in ED and decreased to normal values within 1 day. In patients with union, mean leukocyte, neutrophil, monocyte and platelet counts rose above reference values in the second week after trauma. In contrast, leukocyte, neutrophil, monocyte and platelet counts of patients with nonunion remained within reference values. Lymphocyte counts remained

	Total (n=62)		Union (n=50)		Nonunion (n=12)		P-value
Gender, male	46	(74)	38	(76)	8	(67)	0.5
Age	32	[16-85]	30	[16-85]	40	[11.7]	0.2
Injury Severity Score	25	[17-48]	26	[17-48]	24	[4.9]	0.3
New Injury Severity Score	27	[17-50]	27	[17-50]	28	[6.2]	0.6
Femur fracture localization							
Shaft	43	(69)	36	(72)	7	(58)	0.5
Distal	19	(31)	14	(28)	5	(42)	0.5
Type of femur fractures (AO)							
Simple extra-articular	27	(44)	29	(60)	6	(50)	0.8
Complex extra-articular	21	(34)	10	(20)	3	(25)	0.7
Intra-articular	14	(23)	11	(22)	3	(25)	1.000
Soft tissue injury (Gustilo)							
0: closed fracture	35	(57)	29	(58)	6	(50)	0.5
1: wound < 1 cm	8	(13)	5	(10)	3	(25)	0.2
2: wound > 1 cm	6	(10)	5	(10)	1	(8)	1.000
3a: adequate soft tissue cover	4	(7)	3	(6)	1	(8)	1.000
3b: inadequate soft tissue cover	2	(3)	2	(4)	0		1.000
3c: associated arterial injury	0		0		0		
Unknown	7	(11)	6	(12)	1	(8)	
Type of fixation							
External fixation + IMN	6	(10)	5	(10)	1	(8)	1.000
External fixation + plates	9	(15)	7	(14)	2	(17)	1.000
External fixation + screws	1	(2)	1	(2)	0		1.000
IMN	35	(57)	30	(60)	5	(42)	0.4
Plates	10	(16)	6	(12)	4	(33)	0.1
Screws	1	(2)	1	(2)	0		1.000
Number of surgical procedures	3	[1-18]	3	[1-13]	3	[1-18]	0.4
Inflammatory complications							
Urinary tract infection	6	(10)	5	(10)	1	(8)	1.000
Surgical site infection	3	(5)	2	(4)	1	(8)	0.5
Pneumonia	10	(16)	9	(18)	1	(8)	0.7
MODS	3	(5)	2	(4)	1	(8)	0.5
Nonunion							
Atrophic	11	(18)			11	(92)	
Hypertrophic	1	(2)			1	(8)	
Infected nonunion	2	(3)			2	(17)	
ICU stay (days)	2	[0-68]	2	[0-46]	0	[0-68]	0.2
Hospital stay (days)	20	[4-154]	20	[4-95]	16	[4-154]	0.3

Table 1. Baseline characteristics.

Data are shown as number (percentage), median [range] or mean [standard deviation]. Baseline variables of patients with nonunion are compared to baseline variables of patients with union with the use of a Fisher's exact test or a Mann-Whitney U test as indicated. IMN = intramedullary nailing, ICU = intensive care unit, MODS = multiple organ dysfunction syndrome.

within reference values in patients with union and decreased to just below reference values in patients with nonunion. When compared to the union group, in the nonunion group there was an average change in leukocytes of $-0.33/\text{day}$ ($p = 0.03$), neutrophils of $-0.39/\text{day}$ ($p = 0.03$), monocytes of $-0.03/\text{day}$ ($p = 0.03$), platelets of $-21.2/\text{day}$ ($p = 0.001$) and lymphocytes of $-0.04/\text{day}$ ($p = 0.02$). Hemoglobin and erythrocytes decreased after trauma and remained below reference values for both outcome groups.

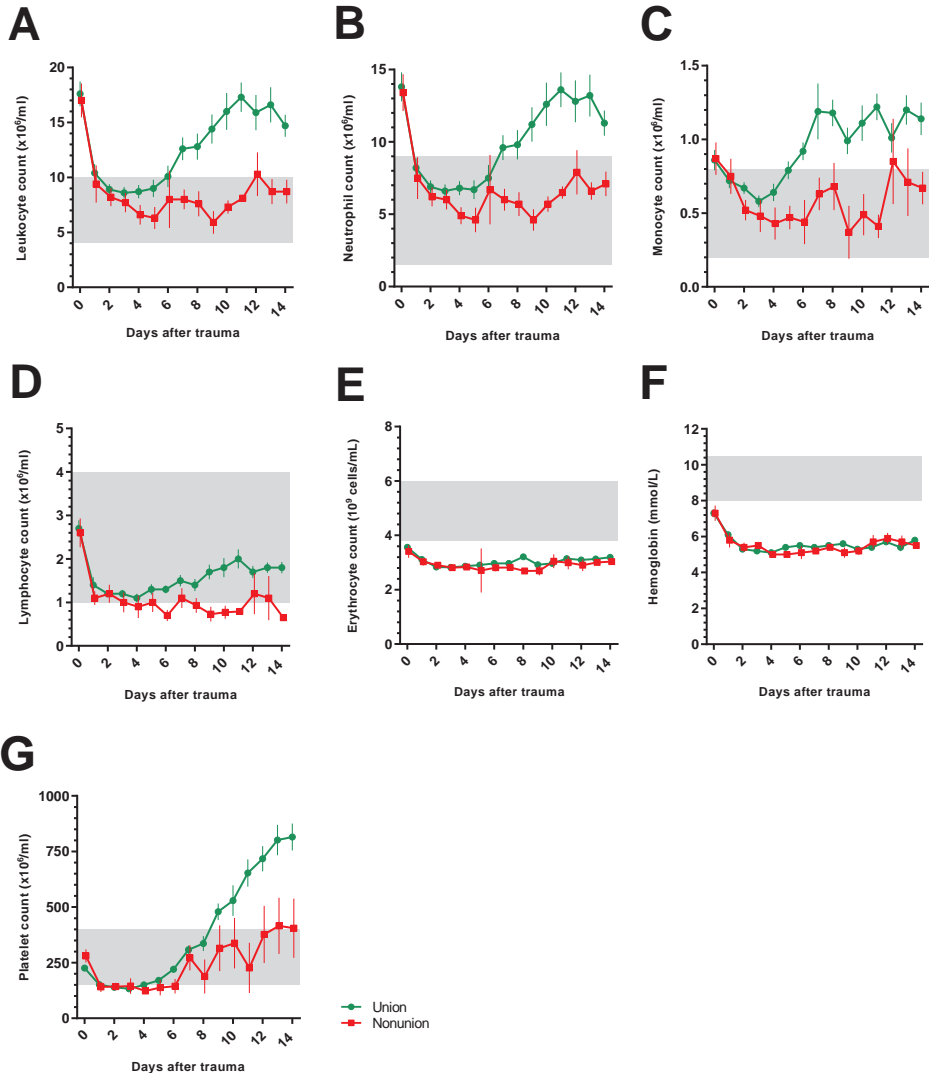


Figure 2 Leukocyte count (A), neutrophil count (B), monocyte count (C), lymphocyte count (D), erythrocyte count (E), hemoglobin (F) and platelet count (G) during the first two weeks after major trauma.

Patients with union are depicted in green and patients with nonunion in red. Data are presented as mean with standard error of the mean. Grey bars represent reference values. SEM = standard error of mean.

RETICULOCYTE COUNT AND CRP LEVEL (FIGURE 3)

Figure 3A and Figure 3B show reticulocyte counts and CRP levels during the first 2 weeks after trauma for patients with union and nonunion of the femoral fracture. In both outcome groups, CRP levels rose to 200 – 300 within 3 days, and gradually decreased thereafter. After day 2, higher CRP levels were observed in patients who later developed nonunion than in patients with union. The average change in CRP levels in patients with nonunion was 7.42/day ($p = 0.01$) compared to CRP levels of patients with union. No statistically significant differences were observed between outcome groups in the number of infections and the number of severe infections leading to multiple organ dysfunction syndrome. Reticulocyte count rose in both outcome groups after trauma, similar in patients with union and patients with nonunion.

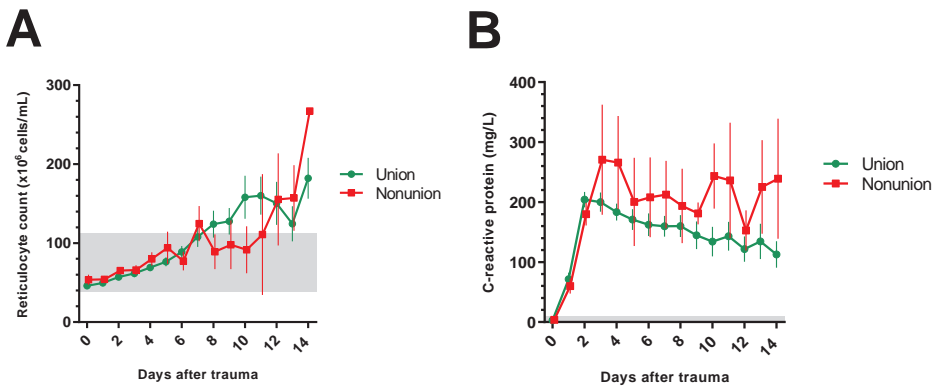


Figure 3 Reticulocyte count (A) and C-reactive protein (B) during the first two weeks after major trauma.

Patients with union are depicted in green and patients with nonunion in red. Data are presented as mean with standard error of the mean. Grey bars represent reference values. SEM = standard error of mean.

DISCUSSION

Leukocytes, neutrophils, monocytes and platelets were above reference values in patients with normal fracture healing during the second week after severe injury. Patients with nonunion did not exhibit such an increase in myeloid blood cells and exhibited a statistically significant, but minor, decrease in lymphocytes. Although CRP levels were elevated in both outcome groups, there was a small but statistically significant increase in CRP in the nonunion group compared to the union group.

An increase of myeloid cells after trauma, as seen in patients with union, has been described before^{14–16}. Moreover, a previous study found similar trends in peripheral blood cell counts in patients with and without union of their tibia fracture¹⁴. This study additionally investigated CRP and reticulocyte count. CRP provided information about inflammation and reticulocyte

count reflected the production of immature red blood cells from the bone marrow, and can thus be used as an indicator of bone marrow function^{17,18}. There are different hypotheses that can explain the lack of leukocytosis and thrombocytosis in the nonunion group. Firstly, persistent inflammation might suppress the bone marrow response¹⁸. However, the increase found in reticulocytes that did not differ between outcome groups, suggests an adequate bone marrow response and makes this hypothesis less likely. Secondly, the lack of leukocytosis and thrombocytosis might be caused by persistent extravasation of myeloid cells to the tissues, a process associated with inflammatory conditions^{19,20}.

Both hypotheses regarding the lack of leukocytosis and thrombocytosis in nonunion patients without bone marrow suppression, are based on sustained inflammation. This is supported by the finding that nonunion patients had slightly higher CRP levels, while there was no statistically significant difference in clinically evident infections between outcome groups. Previous studies have demonstrated that a local controlled inflammatory reaction is key to successful bone healing²¹ and that sustained systemic inflammation after trauma can impair this process^{6,22}. The influx of leukocytes in the fracture hematoma is an essential step during the inflammatory phase in the first week after trauma^{10,23}. However, termination of this phase to prevent persistent inflammation, seems to be at least as important^{9,24}. Decreased numbers of myeloid cells in the blood of nonunion patients during the second week after trauma supports the hypothesis of enhanced extravasation of these cells after the inflammatory phase and thus persistent local inflammation. This is in line with previous studies showing a relation between impaired fracture healing and increased numbers of pro-inflammatory leukocytes in the fracture hematoma²⁴ and decreased numbers of leukocytes in the peripheral blood during the second week after trauma¹⁴. Furthermore, previous studies showed that both the reduction of neutrophils in the fracture hematoma, and the inhibition of extravasation by blocking the anaphylatoxin C5a, improved fracture healing in rats²⁵⁻²⁷. Taken together, it is tempting to speculate that increased extravasation of myeloid cells can disturb fracture healing and that this is reflected by decreased numbers of myeloid cells in the peripheral blood.

It is not surprising that conditions that further enhance the post-traumatic immune response, such as open fractures and multiple injuries, are additional risk factors for nonunion. However, we did not find statistically significant differences in soft tissue injury and injury severity between both outcome groups. Other factors that can influence peripheral blood cell counts, such as infectious complications and the number of surgical procedures, were also not significantly different.

An important limitation of this study is that blood values were retrospectively obtained and were, therefore, not available for each patient at each time point. It is possible that blood was more frequently drawn from patients who were more severely injured or from patients who developed complications during hospital stay. Yet, we did not find a relation between ISS or complications and healing outcome, precluding a substantial bias.

In summary, multitrauma patients who developed femoral nonunion after major trauma demonstrated lower numbers of myeloid cells in the peripheral blood than patients with normal fracture healing. Patients with union demonstrated leukocyte numbers above reference values in the second week after trauma, reflecting a normal physiological response. These findings support the hypothesis that persistent systemic inflammation after major injury can affect physiological processes necessary for bone healing.

ACKNOWLEDGEMENTS

Contribution of authors: OWB, FH, LPHL, KJPW, LK, MH and LH developed the original study design. AH, MB, IEH, WWS and LH contributed to the data acquisition. OWB, FH and LH contributed to the analysis and drafted the paper.

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CHAPTER 6

Neutrophils inhibit synthesis of mineralized extracellular matrix by human bone marrow derived stromal cells in vitro

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ABSTRACT

Although controlled local inflammation is essential for adequate bone regeneration, several studies have shown that hyper-inflammatory conditions after major trauma are associated with impaired fracture healing. These hyper-inflammatory conditions include the trauma-induced systemic inflammatory response to major injury, open fractures and significant injury to the surrounding soft tissues. The current literature suggests that increased or prolonged influx of neutrophils into the fracture hematoma may mediate impairment of bone regeneration after hyper-inflammatory conditions. The underlying mechanism remains unclear. We hypothesize that high neutrophil numbers inhibit synthesis of mineralized extracellular matrix (ECM) by bone marrow stromal cells (BMSCs). We therefore studied the effect of increasing concentrations of neutrophils on ECM synthesis by human BMSCs in vitro. Moreover, we determined how high neutrophil concentrations affect BMSC cell counts, as well as BMSC osteogenic activity determined by alkaline phosphatase (ALP) expression and ALP activity.

Co-culture of BMSCs with neutrophils induced a 52% decrease in BMSC cell count ($p < 0.01$), a 64% decrease in the percentage of ALP+ cells ($p < 0.001$), a 28% decrease in total ALP activity ($p < 0.01$) and a significant decrease in the amount of mineralized ECM (38% decrease after 4 weeks ($p < 0.05$)). Co-cultures with peripheral blood mononuclear cells and neutrophils within transwells did not induce a significant decrease in ALP activity.

In conclusion, our data shows that a decreased amount of mineralized ECM became synthesized by BMSCs, when they were co-cultured with high neutrophil concentrations.

Moreover, high neutrophil concentrations induced a decrease in BMSC cell counts and decreased ALP activity. Clarifying the underlying mechanism may contribute to development of therapies that augment bone regeneration or prevent impaired fracture healing after hyper-inflammatory conditions.

INTRODUCTION

Fracture healing starts with a controlled local inflammatory response, during which inflammatory cells infiltrate the fracture hematoma (FH) that surrounds the fracture.¹ It is commonly accepted that inflammatory cells not only initiate bone regeneration, but are also involved in the downstream processes of fracture healing.^{1,2} This is illustrated by the finding that transplantation of the early FH into muscle tissue induces ectopic bone formation.³ Moreover, removal or repeated irrigation of the early FH impairs bone healing.^{4,5}

Although a controlled local inflammatory reaction seems essential for bone repair, several studies show that local and systemic hyper-inflammatory conditions are associated with impaired bone healing.^{1,2} These conditions include the trauma-induced⁶ systemic immune response to major injury^{7,8}, open fractures⁹ and significant injury to the surrounding soft tissues.¹⁰ The balance between the benefits of a controlled local inflammatory reaction on one hand and the detrimental effects of hyper-inflammation on the other hand, suggests an optimum in the local inflammatory activity at the fracture site. In order to develop therapies that augment bone regeneration and/or prevent impairment of bone healing after hyper-inflammatory conditions, it is essential to understand how inflammatory cells influence the outcome of bone repair.

It has been shown previously that macrophages play an essential role during fracture healing.¹¹ The numbers of anti-inflammatory M2 macrophages have been associated with increased proliferation and osteogenic differentiation of multipotent stromal cells (MSCs) in vitro¹² and increased bone formation in a recent animal study¹¹. In contrast to macrophages, only little is known about the role of neutrophils in bone healing. Our previous study showed that neutrophils contribute to fracture healing by rapidly synthesizing fibronectin+ extracellular matrix within the human fracture hematoma.¹³ However, animal studies suggest that high neutrophil counts within the FH are associated with impairment of fracture healing. For instance, experimental blunt chest injury, which is a model for trauma-induced DAMP-mediated systemic inflammation, induced an increased influx of neutrophils into the FH which was associated with impaired fracture healing in rats.^{7,14,15} Also, systemic depletion of neutrophils has been shown to improve the outcome of bone repair in rats.^{16,17} These studies imply that high neutrophil concentrations within the FH during hyper-inflammatory conditions may negatively affect bone healing. However, the mechanism by which neutrophils affect bone regeneration remains unclear.

The inflammatory phase of fracture healing is followed by a regenerative phase, during which bone marrow stromal cells (BMSCs) and their differentiated progeny synthesize new bone tissue.¹⁸ The extracellular matrix (ECM) of newly formed bone tissue mainly consists of collagen type I fibrils that become mineralized later on.¹⁸ Alkaline phosphatase (ALP) plays a crucial role in bone matrix mineralization and has therefore been consistently used as marker of osteogenic activity in vivo and in vitro.¹⁹

We hypothesize that high neutrophil counts negatively affect synthesis of mineralized ECM by BMSCs. To test this hypothesis, we co-cultured human neutrophils with BMSCs and studied the effect of increasing neutrophil concentrations on extracellular matrix mineralization by BMSCs in vitro, as well as their effect on BMSC cell count and BMSC osteogenic activity reflected by ALP expression and ALP activity

MATERIALS AND METHODS

HARVESTING AND ISOLATION OF BONE MARROW STROMAL CELLS

Bone marrow stromal cells (BMSCs) were isolated from different origins of separate donors: from patients undergoing elective orthopedic surgery of the talus (n=2), patients undergoing hip arthroplasty (n=3), from a 7 day old human fracture hematoma (FH) that was isolated during an Open Reduction Internal Fixation (ORIF) procedure (n=1) and from femoral shaft reaming residues (n=2), as has been described previously^{20–22} Briefly, the femur was reamed using a drill-like instrument (the Reamer/Irrigator/Aspirator (RIA), DePuy Synthes) under constant irrigation using sterile saline. The bone marrow, reaming residue and FH were transferred to a culture facility and treated under sterile conditions. Approximately 1 gram of fracture hematoma and 1 gram of reaming residue was divided into small fragments, re-suspended in 50 ml of expansion medium (EM, *Table 1*) and cultured at 37 °C and 5% CO₂ in a humidified incubator. Details of all media that were used are shown in *Table 1*. Ficoll-Paque was used for the isolation of mononuclear cells from bone marrow aspirate of the talus and hip arthroplasty, which were subsequently seeded at a density of 0.5x10⁶ /cm².

BM	Basic Medium	α-MEM, FCS, ASAP, P/S, BGP
EM	Expansion Medium	[α-MEM, FCS, ASAP, P/S, BGP] + bFGF
OM	Osteogenic Medium	[α-MEM, FCS, ASAP, P/S, BGP] + BMP2

Table 1 Different media used for this study.

Basic medium (BM) consisted of α-MEM supplemented with 10% (v/v) fetal calf serum (FCS), 0.2 mM L-ascorbic acid-2-phosphate (ASAP), 100 U/ml penicillin and 100 µg/ml streptomycin (P/S), 10 mM β-glycerophosphate (BGP). Expansion medium (EM) was basic medium supplemented with 1 ng/ml basic fibroblast growth factor (bFGF). Osteogenic medium was basic medium supplemented with 750 ng/ml rhBMP-2 (BMP2).

After 3 days of culture, bone particles and non-adherent cells were washed off the reaming residue, FH and bone marrow derived adherent cell population with phosphate buffered saline (PBS) twice. All subsequent washing steps were performed with PBS twice, unless mentioned otherwise. The expansion medium was refreshed every 5 days and the adherent cells were passaged at 70-80% confluency. Subsequently, BMSCs were detached with

2 ml 0.25% trypsin-EDTA, washed in medium and re-suspended at 1.0×10^6 cells/ml of FCS containing 5 % (v/v) DMSO (Sigma-Aldrich) for cryopreservation at -80°C until further use. For the different assays, one cryovial containing 1.8×10^6 BMSCs was rapidly thawed, diluted in basic medium, centrifuged and the cell pellet was re-suspended in basic medium until further use. The multipotency of BMSCs isolated from the talus of one donor was established previously by standard differentiation assays along osteogenic, adipogenic and chondrogenic lineages²³. To confirm their phenotype, these BMSCs were characterized for the expression of specific surface antigens defining human MSCs, according to the Mesenchymal and Tissue Stem Cell Committee of the ISCT.^{24,25} Cells incubated for 30 min at 4°C with human FcR blocking reagent (Miltenyi, Leiden, NL) and the following antibodies: CD45-PE (#560975 BD Pharmigen, Breda, NL), CD14 (#R0864, Dako, Heverlee, BE), CD19 (130-091-328, Miltenyi, Bergisch Gladbach, DE), CD34 (BD #555821), CD73 (BD #550257), CD90 (#B113673 Biologend, Fell, DE), CD105-Fitc (FAB 10971F, R&D, Minneapolis, MN) and CD140b (BD #558821). After staining, cells were washed with PBS and cell fluorescence was measured in 10 000 viable cells using a BD FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). SytoxBlue (Molecular Probes/Invitrogen, Eugene, OR) was used for exclusion of dead cells. $>95\%$ of cells were negative for CD45 and CD14, and $>99\%$ of cells were negative for CD19 and CD34. In addition, $>95\%$ were positive for CD73, CD90, CD105 and CD140b (*Figure 1A*). Previous authors have validated the plastic adherence technique used to isolate BMSCs extensively. We have therefore characterized only one bone marrow donor using expression of surface antigens defined by the Mesenchymal and Tissue Stem Cell Committee and differentiation assays along osteogenic, adipogenic and chondrogenic lineages instead of characterizing all BMSC donors^{24,25}. The effect of neutrophils on BMSCs was similar, regardless of BMSC source or BMSC donor.

ISOLATION OF LEUKOCYTES FROM PERIPHERAL BLOOD

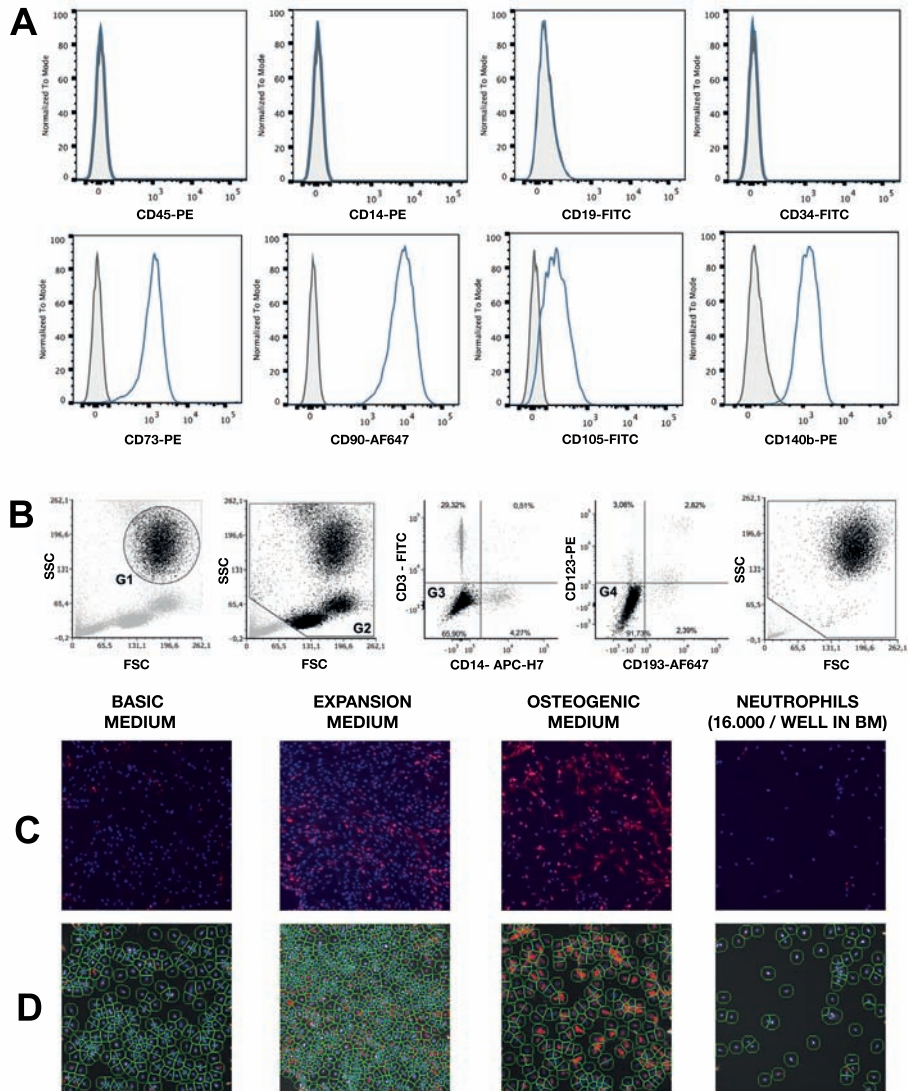
Neutrophils were isolated from 12 healthy donors using two different techniques as described by us before with small modifications.²⁶ Leukocytes were first isolated from peripheral blood of healthy volunteers by lysing erythrocytes using isotonic ice-cold ammonium chloride solution containing 155mM NH_4Cl , 10mM KHCO_3 , 0.1mM EDTA (pH 7.2). After centrifugation, leukocytes were resuspended in ice-cold HEPES3+ buffer until further use. HEPES 3+ contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM Mg_2SO_4 , 1, 2 mM KH_2PO_4 , 1 mM CaCl_2 , 0.5% (wt/vol) human serum albumin and 5 mM glucose. Neutrophils were either isolated from unlabeled leukocytes with fluorescence-activated cell sorting (FACS) using the granulocyte gate in the forward-sideward scatter (*Figure 1B*, gate 1 (G1)). Alternatively, leukocytes were stained using CD14 – APC-H7 (BD #560180), CD3-FITC (Sony Biotechnology, #2176530), CD193 (CCR3)-AF647 (Biologend, #310710) and CD123-PE (eBioscience, 12-1239-42). Neutrophils were defined as CD14-CD3-CD193-CD123- cells using the

gating-strategy as depicted in *Figure 1B*. Neutrophils may become activated when antibodies bind to neutrophil receptors during a positive selection technique. We therefore used a negative selection technique to isolate neutrophils with FACS.

Peripheral blood mononuclear cells were isolated from peripheral blood of healthy volunteers using Ficoll-Paque PLUS centrifugation (GE Healthcare) for 20 minutes at 900 *g*. The mononuclear cell layer (ring fraction) was aspirated, centrifugated, resuspended and stored in ice-cold HEPES 3+ until further use.

BONE MARROW STROMAL CELL/NEUTROPHIL CO-CULTURES

BMSC/neutrophil co-cultures were performed in either 96-wells plates with 200 μ l of medium in each well or 24-wells plate with 2000 μ l of medium. Neutrophils and BMSCs were isolated from different donors (non-autologous co-cultures). In 96-well experiments, 2000 BMSCs from the FH and remaining residues were sorted into each well (final BMSC concentration of 10.000 BMSCs/ml) using a fluorescence activated cell sorter (MoFlo Astrios, Beckman-Coulter) after excluding doublets and debris based on forward-scatter and sideward-scatter signals. Subsequently, unlabeled granulocytes were isolated from the entire leukocyte population based on the granulocyte specific forward-scatter and sideward-scatter signals (gate 1, *Figure 1B*).²⁷ Either 4.000, 8.000 or 16.000 granulocytes were sorted directly into each well containing 2.000 BMSCs in 200 μ l of basic medium (BM). This resulted in neutrophil concentrations of 20.000, 40.000 and 80.000 neutrophils/mL, respectively. In 24-wells plate experiments BMSCs derived from the bone marrow were counted and manually added to the well without the use of a sorter. Neutrophils were isolated using the FACS strategy depicted in *Figure 1B* (CD3-CD14-CD123-CD193- cells) and manually added to the well 160.000 neutrophils / well in 2 mL of medium (concentration of 80.000 neutrophils / mL). The cells were cultured at 37 °C and 5% CO₂ in a humidified incubator. After 3 days of culture, all media were refreshed. Since BMSCs are adherent cells and neutrophils are not, practically all neutrophils were removed from the co-culture at this time point. Subsequently, no new neutrophils were added to the monolayer of BMSCs and the media were refreshed approximately every 3-4 days. After 7 days BMSC cell counts and osteogenic activity of BMSCs was quantified as described below. After 4 weeks of culture, mineralization of extra-cellular matrix was quantified. Each condition was established in duplicates. The experiments were repeated with neutrophils and BMSCs from different donors and the experiments were set up at different dates.

**Figure 1**

(A) Surface antigen expression of BMSCs isolated from the talus bone marrow using flow cytometry. >95% of cells were negative for CD45 and CD14, and >99% of cells were negative for CD19 and CD34. In addition, >95% were positive for CD73, CD90, CD105 and CD140b. Since plastic adherence is a well-established and validated technique to isolate MSCs, we have only characterized one BMSC donor using flow cytometry instead of all donors. The blue lines are stained cells and the grey lines are negative (unstained) controls.

(B) FACS gating strategy used to isolate granulocytes / neutrophils from peripheral blood leukocytes. Granulocytes were either isolated from unlabeled leukocytes using gate 1 (G1) within the forward / side-scatter (FSC/SSC). Alternatively, leukocytes were stained using CD3, CD14, CD 193 and CD123. Within the FSC/SSC of these labeled cells, debris was first excluded (gate 2 (G2)). Subsequently, CD3+ cells (lymphocytes) and CD14+ (monocytes) were excluded (gate 3 (G3)). In addition, CD193+ cells (eosinophils) and CD123+ cells (basophils) were excluded (gate 4 (G4)).

Figure 1 (continued)

The remaining CD3-CD14-CD193-CD123- cells were defined FACS-sorted neutrophils (G2+,G3+,G4+ sorted neutrophils). Re-analysis of FACS-sorted neutrophils shows adequate exclusion of lymphocytes and monocytes based on their forward / sideward scatter.

(C) Images of BMSCs obtained by array scanning after 7 days of culture.

Bone marrow stromal cells (2,000 BMSCs / well) were seeded and imaged after 7 days of culture in basic medium (BM), expansion medium (EM), osteogenic medium (OM) and after co-culture with neutrophils (16,000 neutrophils/well) in basic medium. Nuclei were stained with Hoechst (blue) and alkaline phosphatase was stained (red) using Vector Red, which is a marker of osteogenic activity. All images within each experiment had similar exposure times and were not manipulated after capture with the array-scanner.

(D) Quantification algorithm used to quantify cell count and the percentage of alkaline phosphatase positive cells in the adherent BMSC population after 7 days of culture.

The blue rings within the algorithm images show identification of nuclei, the green rings are the area of interest around each nucleus in which Vector Red fluorescence was measured and each red pixel reflects Vector Red fluorescence above the threshold used to determine whether cells are ALP positive. The same protocol was used to quantify ALP+ cells in all experiments.

TRANS-WELL EXPERIMENTS

To determine whether an effect of neutrophils on BMSC osteogenic activity was dependent on soluble factors, transwell experiments were performed. The highest neutrophil concentration (80,000 neutrophils/ml) was compared to BMSC monocultures. The use of transwell-inserts required usage of 24-wells plates instead of 96-wells. Since the total volume of a 24-wells plate well is approximately 10 times the volume of a 96-wells plate well, the number of BMSCs and neutrophils per well were adjusted accordingly without altering the ratio between neutrophils and BMSCs and the concentration of neutrophils/ml. Each well within the 24-wells plate contained 2 ml of medium, seeded with 20,000 BMSCs and 160,000 neutrophils.

ANALYSIS OF ALKALINE PHOSPHATASE (ALP) ACTIVITY

For quantitative ALP determination, cells were lysed in 0.2% (v/v) Triton X-100 in PBS for 30 min. ALP activity was measured by conversion of the p-nitrophenyl phosphate Liquid Substrate System (Sigma Aldrich). The absorbance was measured at 405 nm and corrected at 655 nm on a multi-well plate reader (Bio-rad, Hercules, CA). Values were normalized to ALP activity in BMSCs cultured in basic medium.

ANALYSIS OF BMSC CELL COUNT AND ALKALINE PHOSPHATASE (ALP) EXPRESSION

To determine whether a decreased ALP activity was caused by a decreased number of ALP+ cells or a decreased expression of ALP on ALP+ cells, the adherent cell population was stained and imaged using an array-scanner. After 7 days of co-culture, adherent cells were washed, fixed with 3.7% neutral buffered formaldehyde solution for 10 minutes at room temperature, washed and incubated with Vector Red alkaline phosphatase substrate kit (Vector Labs) for

1 hour at room temperature in the dark. The Vector Red solution was prepared according to the manufacturer's protocol. Subsequently, adherent cells were washed and stained with Hoechst 33258 (Sigma-Aldrich) 10 µg/ml in PBS for 30 minutes at room temperature in the dark, washed and stored in PBS until further analysis. BMSCs were counted by quantifying the number of nuclei within 6 microscopy fields with an array-scanner (ArrayScan VTI HCS Reader, Thermo Scientific). The nuclei were identified based on Hoechst staining, size and shape of the nucleus (*Figure 1C and 1D*). All nuclei at the image borders were excluded from the analysis. The first microscopy field was the standardized center of each well and every following microscopy field followed a standardized automated spiral track toward the periphery of the well. Osteogenic activity was measured by placing a standardized ring-shaped area of interest around each nucleus and measuring fluorescence of Vector Red within this area of interest using the manufacturer's Spot Detection protocol (http://www.med.cam.ac.uk/wp-content/uploads/2016/02/SpotDetector_V4_LC06210800.pdf, *Figure 1D*). This technique allowed measurement of ALP expression for each individual BMSC. In brief, the Spot Detection protocol places a grid of 512 x 512 pixels over each grayscale array-scanner image. Each pixel within the image has a brightness-value ranging from black to white. The brightness of each pixel therefore corresponds with the amount of fluorescence. After setting a threshold, each pixel within the grid becomes either positive or negative (positive pixels become stained red in *Figure 1D*). The Spot Detection protocol counts the number of positive pixels within each area of interest around each nucleus (green circles in *Figure 1D*). The determination of the area of interest was standardized as described in the manufacturer's Spot Detection protocol (http://www.med.cam.ac.uk/wp-content/uploads/2016/02/SpotDetector_V4_LC06210800.pdf). An additional threshold makes each cell either positive or negative based on the number of positive spots within the area of interest. The exposure time was set, based on the first well containing BMSCs in basic medium without neutrophils. The exposure time remained identical for all conditions within an experiment. The threshold remained identical within and between experiments. The exposure time may vary between experiments, for instance based on the time between staining of BMSCs and the eventual time of imaging. ALP is crucial for ECM mineralization in vivo and in vitro and is, therefore, a well established marker of osteogenic activity.^{28,29}

ANALYSIS OF EXTRACELLULAR MATRIX (ECM) MINERALIZATION USING ALIZARIN RED

After 4 weeks of culture in osteogenic medium, the adherent cell population was washed with PBS and fixed in 4% (w/v) paraformaldehyde, stained for 10 minutes with 2% (w/v) Alizarin Red solution (pH 4.2, Sigma Aldrich) and examined by light microscopy. In addition, Alizarin Red was extracted from the monolayer by incubating the adherent cells in 1,0 mL 10% cetylpyridinium chloride (CPC) buffer for 30 minutes. The dye was dissolved in the well and 200 µL aliquots were transferred to a 96 well plate prior to reading at 595 nm. The data were corrected by subtraction of a background reading at 655 nm.

CFSE LABELING OF NEUTROPHILS

It has been shown previously that phagocytosis of apoptotic cells influences BMSC osteogenic differentiation.³⁰ We have therefore stained neutrophils with CFSE to determine whether BMSCs phagocytize (apoptotic) neutrophils. The membrane permeable CFDA-SE is converted to the fluorescent membrane-impermeable CFSE by intracellular esterases after which the fluorescent CFSE covalently couples to intracellular molecules.

Neutrophils were washed with serum-free RPMI medium (Gibco) and resuspended in RPMI at a concentration of 1×10^6 neutrophils/mL. Subsequently, neutrophils were labeled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Sigma-Aldrich) by diluting the 5mM stock 1:200 and incubate neutrophils for 5 minutes at room temperature. Subsequently, neutrophils were washed in HEPES 3+ twice and resuspended in basic medium. The fluorescently labeled neutrophils were imaged at day 0, day 1 and day 2 after co-culture with BMSCs using an Olympus XI53 fluorescent microscope (*Figure 3A*).

BMSC VIABILITY ASSAY AND BMSC CELL COUNTS

The effect of neutrophil co-culture on BMSC viability was tested using the Molecular Probes™ LIVE/DEAD™ assay (ThermoFisher Scientific) according to the manufacturers protocol (<https://www.thermofisher.com/nl/en/home/brands/molecular-probes/key-molecular-probes-products/live-dead-viability-brand-page.html#>). Viable cells were fluorescently labeled green and non-viable cells were fluorescently labeled red. Triton-X killed BMSCs were used as a positive control. BMSCs were imaged on day 0, day 1 and day 2 with and without neutrophils using an Olympus XI53 fluorescent microscope (*Figure 3B*). Total BMSC counts and percentage viable cells were manually counted and depicted in *Figure 3C*.

NEUTROPHIL VIABILITY ASSAY

The effect of BMSC co-culture on neutrophil viability was assessed using the PE Annexin V Apoptosis Detection Kit which also contains 7-AAD staining (BD Pharmingen) according the manufacturers protocol (<http://wwwbdbiosciences.com/ds/pm/tds/559763.pdf>). Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD. Annexin V staining precedes the loss of membrane integrity, which allows identification of early apoptotic cells (7-AAD negative, PE Annexin V positive). Neutrophil viability was assessed before co-culture at day 0, after 1 and 2 days of culture with and without BMSCs. *Figure 3D* shows the percentage of viable neutrophils (Annexin and 7-AAD double negative cells), the percentage apoptotic neutrophils (Annexin positive, 7-AAD negative) and the percentage of permeable neutrophils (Annexin and 7-AAD double positive cells) for abovementioned conditions.

EXPRESSION OF SURFACE MARKERS ON NEUTROPHILS

The effect of neutrophil culture with and without BMSC on expression of neutrophil surface markers associated with an activated phenotype³¹⁻³⁴ was assessed using multicolor flowcytometry. Neutrophils were washed with PBS containing sodium citrate 0.32% and albumin 4g/L (PBS²⁺) and resuspended in a solution containing CD35-FITC (E11), CD66b-PerCP-Cy5.5 (G10F5), CD49d-PeCy7 (9F10), CD64-AF647, CBRM1/5-AF700 and CD11b – AF750 from Biolegend, CD62L-BV650 from BD Biosciences, CD14- e450 from ThermoFisher Scientific and CD16-Krome Orange (3G8) from Beckman Coulter in PBS²⁺. After incubation with the antibody solution for 30 minutes on ice, neutrophils were washed with PBS²⁺ and analyzed using a BD LSRFortessa™ cell analyzer (Becton Dickinson, Mountain View, CA, USA). Neutrophils were identified according to their specific forward-/side- scatter patterns. Flow cytometry data were analyzed with FlowJo® v10 software (FlowJo, LLC, Ashland, OR, USA). The median fluorescence of each marker before and after culture with and without BMSCs after 24 hours is depicted in *Figure 4*.

STATISTICAL ANALYSIS

Based on whether the data was normally distributed, an ANOVA or Kruskal-Wallis analysis was used to compare groups. Each experimental condition was compared to the control condition (medium alone) with a Dunnet post-hoc test or Mann Whitney-U using Bonferoni's correction for multiple testing (two-tailed). All data are presented as mean ± standard error of the mean (SEM) compared to the mean of all control conditions together (BMSC monocultures in medium, unless indicated otherwise). The mean of all control conditions together was therefore 100%. The statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, Inc.). A p-value <0.05 was considered to be statistically significant. The data generated during this study are available from the corresponding author on reasonable request. BMSCs were isolated from residual samples and therefore collected without informed consent, unless the patient refused explicitly (opt-out method). All samples were acquired in accordance with relevant guidelines and regulations.

ETHICS STATEMENT

The medical-ethical committee of the University Medical Center of Utrecht has approved isolation and use of residual samples after anonymization without informed consent. Leukocytes from peripheral blood of healthy donors were acquired after informed consent. The medical-ethical committee of the University Medical Center of Utrecht also approved isolation and use of peripheral blood of healthy donors after informed consent and anonymization.

RESULTS

THE EFFECT OF NEUTROPHILS ON BMSC CELL COUNT IN VITRO

As illustrated in *Figure 2A*, co-culture of BMSCs with high neutrophil concentrations induced a 54% decrease in the number of BMSCs after 7 days of culture compared to monocultures of BMSCs in basic medium (100%, n=9, p<0.01). Culture of BMSCs in 200 μ l of expansion medium for 7 days induced a 100% increase in BMSCs cell counts compared to BMSCs that were cultured in basic medium for 1 week (100%, n=9, p<0.001).

THE EFFECT OF NEUTROPHILS ON OSTEOGENIC ACTIVITY OF BMSCS IN VITRO

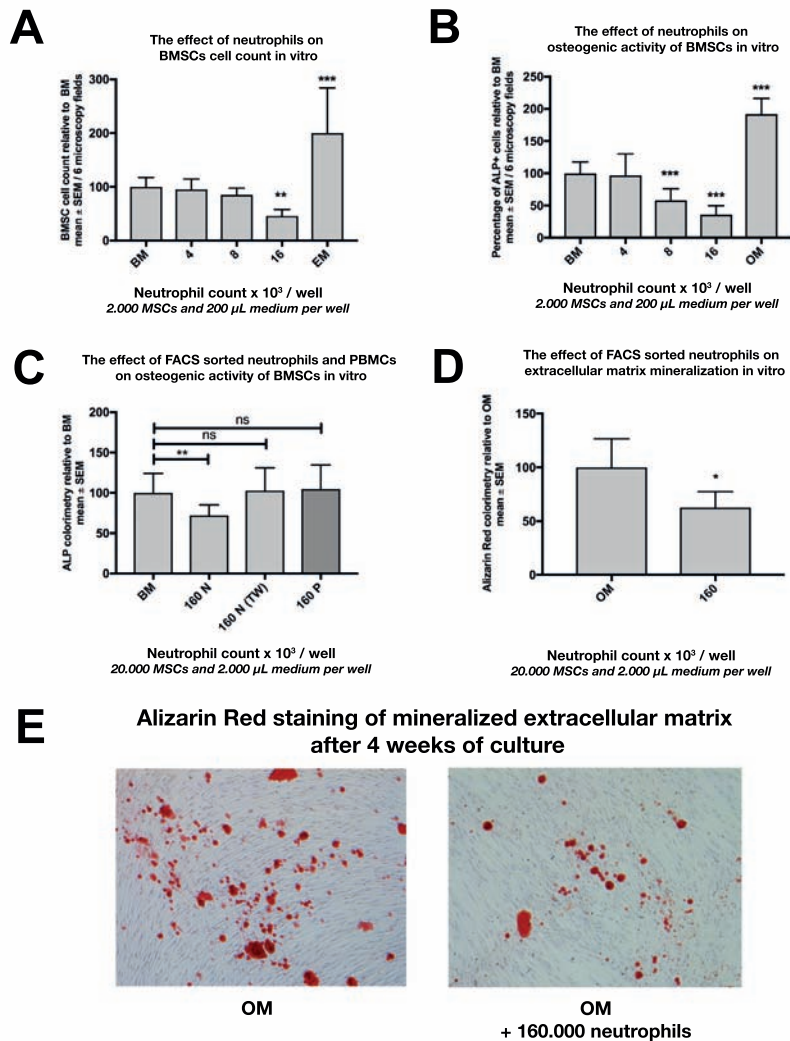
Subsequently the effect of neutrophil co-culture was studied on the percentage of ALP+ BMSCs as well as total ALP activity within the entire BMSC population. Co-culture of BMSCs with high neutrophil concentrations caused a 64% decrease in the percentage of ALP+ cells within the entire BMSC population after 7 days of culture compared to monocultures of BMSCs (100%, n=9, p<0.001, *Figure 2B*). Culture of BMSCs in osteogenic medium for 1 week induced a 92% increase in the percentage of ALP+ cells compared to BMSCs cultured in basic medium for 1 week (100%, n=9, p<0.001). FACS sorted CD3-CD14-CD123-CD193- neutrophils (*Figure 1B*) were co-cultured with bone marrow derived BMSCs (*Figure 1A*) for 1 week and induced a 28% decrease in ALP activity (p<0.01, n=8, *Figure 2C*). Co-culture of these BMSCs with Ficoll isolated peripheral blood mononuclear cells (PBMCs) did not induce a significant decrease in ALP activity (n=8, *Figure 2C*). Moreover, FACS sorted neutrophils co-cultured with transwell also did not induce a significant decrease in ALP activity (n=6, *Figure 2C*).

THE EFFECT OF NEUTROPHILS ON EXTRACELLULAR MATRIX MINERALIZATION IN VITRO

FACS sorted CD3-CD14-CD123-CD193- neutrophils (*Figure 1B*) were co-cultured with bone marrow derived BMSCs (*Figure 1A*) and induced a 38% decrease in mineralized extracellular matrix after 4 weeks of culture in osteogenic medium analyzed by Alizarin Red staining (P<0.05, n=4, *Figure 2D and 2E*).

BMSC CO-CULTURED WITH CFSE LABELED NEUTROPHILS

It has been shown previously that phagocytosis of apoptotic cells influences BMSC osteogenic differentiation.³⁰ We have therefore stained neutrophils with CFSE to determine whether BMSCs phagocytize (apoptotic) neutrophils. As shown in *Figure 3A*, BMSCs did not become CFSE positive after co-culture with CFSE labeled neutrophils. This finding suggests that phagocytosis of (apoptotic) neutrophils by BMSCs in vitro is not likely.

**Figure 2****(A) The effect of neutrophils on BMSCs cell count in vitro (mean ± SEM / 6 microscopy fields).**

Co-culture of BMSCs with different neutrophil concentrations resulted in decreased BMSC counts after 7 days of culture. Neutrophils were isolated from unlabeled leukocytes based on granulocyte specific FSC/SSC (Figure 1B) from three donors and cultured with 3 different BMSC donors (RIA (n=2) and FH (n=1)) in a 96 wells plate *** = $p < 0.001$, ** = $p < 0.01$ compared to BMSCs cultured in BM without neutrophils. BMSCs cultured without neutrophils in EM are illustrated by the dark grey bar.

(B) The effect of neutrophils on osteogenic activity of BMSCs in vitro (mean ± SEM / 6 microscopy fields).

Co-culture with different neutrophil concentrations induced a decreased percentage of alkaline phosphatase (ALP) positive cells after 7 days of culture. The same cells and number of donors were used as described in Figure 2A. The percentage of ALP+ FH and RIA derived BMSC was 32% and 29% respectively (cultured without neutrophils). FH and RIA derived BMSCs cultured without neutrophils were pooled (BM). All other conditions are depicted relative to BM. Therefore, the mean percentage of ALP+ of BM was set to 100%.

Figure 2 (continued)

BMSCs cultured without neutrophils in OM are illustrated by the dark grey bar.

*** = $p < 0.001$ compared to BMSCs cultured in BM without neutrophils.

(C) The effect of FACS sorted neutrophils, PBMCs and neutrophil transwell co-culture on osteogenic activity of BMSCs in vitro after 1 week of culture (mean \pm SEM).

FACS sorted CD3-CD14-CD123-CD193- neutrophils (3 donors, Figure 1B) were co-cultured with bone marrow derived BMSCs (2 donors) in a 24 wells plate containing basic medium, which induced a significant decrease in osteogenic activity (160N = 160.000 neutrophils / well). In contrast, Ficoll isolated PBMCs did not induce a significant decrease in ALP activity (160P = 160.000 PBMCs / well in BM). Moreover, transwell experiments in which neutrophils and BMSCs did not have cell-cell contact also did not significantly inhibit osteogenic activity (160N (TW) = 160.000 neutrophils / transwell insert in BM).

(D) The effect of FACS sorted neutrophils on ECM in vitro after 4 weeks of culture (mean \pm SEM). FACS sorted neutrophil co-culture with BMSCs in osteogenic medium induced a significant decrease in ECM mineralization after 4 weeks of culture as analyzed by Alizarin Red staining compared to BMSCs that were cultured in osteogenic medium alone. * = $p < 0.05$.

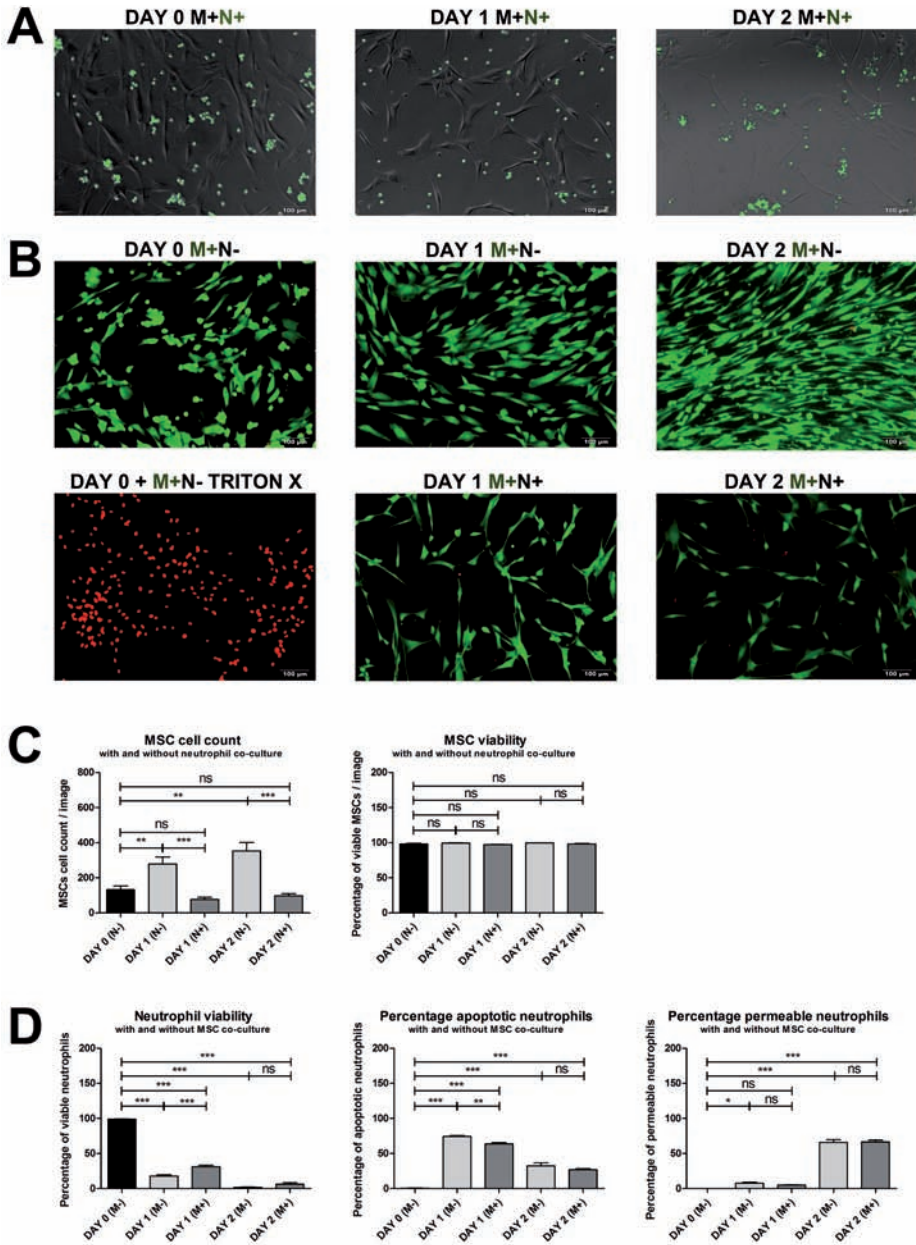
Representative images of Alizarin Red stained monolayers of BMSCs after 4 weeks of culture with and without neutrophils.

BMSCS VIABILITY AFTER CULTURE WITH AND WITHOUT NEUTROPHILS

We determined whether a decreased BMSC counts after neutrophil co-culture (*Figure 2A*) may be caused by neutrophil induced cell death of BMSC. As depicted in *Figure 3B and 3C*, BMSC counts increased during the first two days of culture without neutrophils and practically all cells were viable (green). In contrast, the number of BMSCs decreased during the first two days after co-culture with neutrophils. The percentage of viable BMSCs did not significantly differ at day 1 and day 2 when cultures with and without neutrophils were compared (*Figure 3C*).

NEUTROPHIL VIABILITY AFTER CULTURE WITH AND WITHOUT BMSCS

It has been shown previously that BMSCs increase neutrophil survival in vitro.³⁵ We determined neutrophil viability before and after culture with and without BMSCs. *Figure 3D* shows that the percentage of viable cells (Annexin and 7-AAD double negative cells) significantly decreases during the first two days of culture. As has been described previously, we found that BMSCs induced a significant increase in the percentage of viable neutrophils at day 1 of culture. However, after two days of culture, practically all neutrophils were non-viable and there was no significant difference between neutrophils cultured with and without BMSCs at day 2.

**Figure 3****(A) BMSC co-cultured with CFSE labeled neutrophils.**

BMSCs were co-cultured with CFSE labeled neutrophils (green) and imaged using fluorescence microscopy at day 0 and after one and two days of culture with BMSCs to determine whether BMSCs phagocytose neutrophils. BMSCs did not become CFSE positive after co-culture with CFSE labeled neutrophils. This finding suggests that phagocytosis of (apoptotic) neutrophils by BMSCs in vitro is not likely.

Figure 3 (continued)**(B) BMSCs LIVE/DEAD™ assay**

Representative images of BMSCs stained with a LIVE/DEAD™ kit before and after culture with and without neutrophils. Viable BMSCs are green and non-viable BMSCs are red. Triton-X killed BMSCs were used as positive control. BMSCs (M+) were imaged on DAY 0, DAY 1 and DAY 2, cultured with (N+) or without (N-) neutrophils.

(C) BMSCs viability and BMSC cell count (mean ± SEM).

BMSC were stained with the LIVE/DEAD™ kit before and after one and two days of culture with and without neutrophils. Three neutrophil donors and two BMSC donors (2 arthroplasty) were used (n=6 conditions). The total BMSC count per microscopy image and the percentage of viable (green) BMSCs is depicted. BMSC counts increased during the first two days of culture without neutrophils and practically all cells remained viable (green). In contrast, the number of BMSCs decreased during the first two days after co-culture with neutrophils. The percentage of viable BMSCs did not significantly differ at day 1 and day 2 when cultures with and without neutrophils were compared. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, ns = not significant.

(D) Neutrophil viability assay.

Neutrophils were stained with Annexin and 7-AAD before and after one and two days of culture with and without BMSCs. Three neutrophil donors and four BMSCs donors (1 talus and 3 arthroplasty) were used (n= 12 conditions). The graphs depict the percentage of viable cells (Annexin and 7-AAD double negative cells), apoptotic cells (Annexin positive, 7-AAD negative) and permeable cells (Annexin and 7-AAD double positive cells). As has been described previously, we found that BMSCs induced a significant increase in the percentage of viable neutrophils at day 1 of culture. However, after two days of culture, practically all neutrophils were non-viable and there was no significant difference between neutrophils cultured with and without BMSCs at day 2. The median fluorescence of Annexin and 7-AAD was pooled and depicted as mean ± SEM. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, ns = not significant.

EXPRESSION OF SURFACE MARKERS ON NEUTROPHILS

To determine whether BMSCs affect expression of surface markers on neutrophils associated with an activated phenotype, we used multicolor flow-cytometry to quantify expression of CD62L, CD64, CBRM1/5, CD16, CD14, CD35, CD11b, CD49d and CD 66b on neutrophils. We found that uncultured neutrophils had significantly higher expression of CD62L, CD64, CBRM 1/5, CD16 and lower expression of CD14 compared to neutrophils that were cultured with or without BMSCs. There was no significant difference in expression of CD11b, CD49d and CD66b between uncultured and cultured neutrophils. We did not find a significant difference in expression of surface markers between neutrophils that were cultured with and without BMSCs after 24 hours of culture.

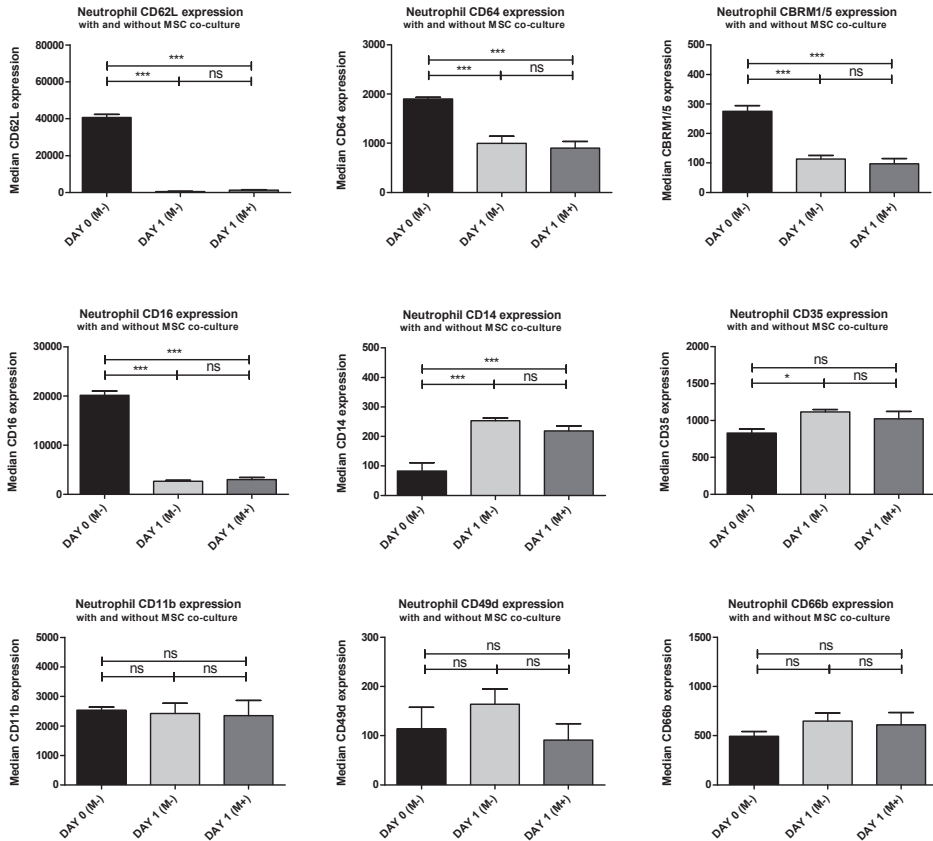


Figure 4 Expression of surface markers on neutrophils

Multicolor flow-cytometry was used to quantify expression of CD62L, CD64, CBRM1/5, CD16, CD14, CD35, CD11b, CD49d and CD 66b on neutrophils before and after 24-hour culture with and without BMSCs in BM. Neutrophils were isolated from 3 donors and cultured with BMSCs isolated from 4 donors (1 talus and 3 arthroplasty) (n=12 combinations). The black bars represent uncultured neutrophils and therefore without BMSCs (DAY 0 (M-)). The grey bars represent neutrophils cultured for 24 hours with (DAY 1 (M+), dark grey) or without (DAY 1 (M-), light grey) BMSCs in BM. The median fluorescence of each surface marker was pooled and depicted as mean \pm SEM. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, ns = not significant.

DISCUSSION

Our study shows that human neutrophils can induce a significant decrease in the amount of extracellular matrix (ECM) that becomes synthesized by bone marrow stromal cells (BMSCs) in vitro (Figure 2D and eC). Moreover, our data indicate that neutrophils induce a significant decrease in BMSC cell count, percentage of alkaline phosphatase (ALP) positive cells and total ALP activity, which is a marker of osteogenic activity (Figure 2A, 2B and 2C). Neutrophils that were co-cultured in transwells, which prevented cell-cell contact between neutrophils

and BMSCs, did not induce a significant decrease in ALP activity (*Figure 2C*). Moreover, peripheral blood mononuclear cells (PBMCs) also did not induce a significant decrease in ALP activity (*Figure 2C*). Our human data are in line with animal studies which have shown that systemic depletion of neutrophils improves bone healing and induces increased expression of osteogenic transcription factors at the site of bone injury.^{17,16}

It has been shown previously that peripheral blood leukocytes can inhibit growth of bone marrow derived stromal cells *in vitro*.³⁶ Our finding that neutrophils inhibit synthesis of mineralized ECM by BMSCs *in vitro* may be part of a physiological regenerative process. The early fracture hematoma (FH) contains a significant amount of debris that needs to become degraded and phagocytized by inflammatory cells, which are known to be capable of releasing reactive oxygen species (ROS) and ECM degrading enzymes.^{1,2,37,38} Moreover, the FH may be contaminated by pathogens in case of open fractures that also need to become neutralized by inflammatory cells. It is plausible that these processes make the early FH a suboptimal environment for BMSCs to start synthesizing new bone ECM. Our previous study showed that neutrophils infiltrate the human FH within 12 hours after injury and contribute to fracture healing by synthesizing fibronectin+ ECM.¹³ A recent animal study also suggested a beneficial effect of neutrophils during early bone regeneration.³⁹ At day 3 to 5 after injury, neutrophil concentrations decrease after which the first BMSCs become apparent within the human FH and BMSCs numbers start to increase.¹³ We hypothesize that neutrophils keep BMSCs in “stand-by mode” by inhibiting their proliferation and differentiation until debridement of the FH, neutralization of pathogens and synthesis of fibronectin+ ECM has sufficiently been accomplished. Although this effect of neutrophils on BMSCs may be physiological, we speculate that this effect can negatively affect the outcome of bone healing during or after hyper-inflammatory conditions. Based on the abovementioned study¹³, we believe that the ratios between neutrophils and BMSCs used in our *in vitro* co-cultures may be similar to the *in vivo* ratios.

Our recent study showed that multitrauma patients that develop impaired bone healing have decreased peripheral blood neutrophil concentrations during the first two weeks after injury, which may be explained by increased extravasation of neutrophils.⁸ It is known that severe injury causes release of Damage Associated Molecular Patterns (DAMPs)⁶ into the peripheral circulation, which induces a systemic inflammatory response⁴⁰. Neutrophils can become primed or pre-activated during trauma-induced systemic inflammation and exhibit enhanced migration towards inflammatory stimuli, such as the fracture hematoma.⁷ Our finding that neutrophils inhibit synthesis of mineralized ECM by BMSCs may explain how increased or prolonged influx of neutrophils into the FH disturbs the regenerative phase of fracture healing after major trauma.

Neutrophils are short lived, especially on plastic surfaces *in vitro*⁴¹, and it has been shown previously that BMSCs induce prolonged survival of neutrophil *in vitro*.³⁵ In our co-culture practically all neutrophils were non-viable after 2 days of culture, either with or without BM-

SCs (Figure 3C). These neutrophils were washed off the monolayer of adherent BMSCs at day 3, when the culture medium is refreshed. It is, therefore, likely that neutrophils exert their effect on BMSCs within the first three days of co-culture. It is well known that neutrophils are equipped with an extensive cytotoxic armamentarium, consisting of ECM degrading enzymes such as collagenase, elastase and proteases, as well as the capacity to form reactive oxygen species (ROS).³⁷ When activated by appropriate stimuli, neutrophils induce a respiratory burst, which is marked by an increased consumption of oxygen and generation of superoxide anions, hydrogen peroxide and hypochlorous acid.³⁷ These free radicals are highly cytotoxic and can induce tissue injury.⁴² It remains unclear whether neutrophils release their cytotoxic content upon contact with BMSCs. Future studies may investigate whether inhibition of neutrophil degranulation and ROS release prevents a negative effect on the osteogenic potential of BMSC. In addition to ECM degrading enzymes and ROS, neutrophils synthesize several cytokines, such as TNF- α ⁴³ and IL-17⁴⁴, that have been shown to inhibit osteogenic differentiation of BMSCs.⁴⁵

Our finding that PBMCs and neutrophil transwell experiments do not induce a significant decrease in ALP activity suggests that the negative effect of neutrophils on BMSCs is not caused by depletion of nutrients from the culture medium. Figure 3C shows that BMSC numbers increase during the first two days when cultured in basic medium (BM) without neutrophils, which suggests proliferation of BMSCs. The number of BMSCs significantly decreased during the first two days of co-culture with neutrophils. This may be caused by inhibition of proliferation of BMSCs, neutrophil induced cell death of BMSCs or a combination of both. However, we were unable to demonstrate a decrease in BMSCs viability (Figure 3C), which makes neutrophil induced BMSC cell death in vitro unlikely.

Our finding that neutrophil transwell experiments did not induce a significant decrease in ALP+ cells, suggests that neutrophils need to be in proximity of BMSCs to exert their effect. It remains unclear whether neutrophils mediate their effect by binding of their cell surface receptors to BMSCs, by local release of cytokines⁴⁶ or other factors (e.g. ROS) within an immunological synapse⁴⁷. The mechanism through which neutrophils exert their effect on BMSCs may be the focus of future research.

Moreover, only little is known about whether basic medium, osteogenic medium or expansion medium best represents the concentrations of growth factors present within the fracture hematoma in vivo. We used basic medium in most of the co-cultures in order to minimize the amount of growth factors that could affect either neutrophils or BMSCs. It is possible that the effect of neutrophils on BMSCs disappears when fibroblast growth factor (bFGF used in expansion medium) or bone morphogenetic protein -2 (BMP-2 used in osteogenic medium) are added to the culture medium.

It has been shown previously that mononuclear cells can stimulate osteogenic differentiation of BMSCs in vitro and stimulate bone regeneration.⁴⁸⁻⁵¹ We have previously shown that –in contrast to neutrophils–, monocytes/macrophages remain present within the FH

during the second week of fracture healing, when BMSCs are the most prevalent cell type within the human FH.¹³ A recent animal study showed that induction of the regenerative M2 macrophage phenotype by interleukin 4 and 13 significantly enhanced bone formation in mice.¹¹ M2 macrophages secrete high levels of anti-inflammatory cytokines, fibrogenic and angiogenic factors that serve to resolve inflammation and stimulate tissue regeneration.^{54,55} In contrast, M1 macrophages have a pro-inflammatory phenotype, exhibiting increased phagocytic activity and secretion of pro-inflammatory cytokines that aid in the removal of pathogens and injured tissue. Severe trauma induces release of different neutrophil subsets into the peripheral circulation, which includes young banded neutrophils and hypersegmented neutrophils.⁵² It is tempting to speculate that neutrophils can also acquire an inflammatory or regenerative phenotype that affect fracture healing differently. Future studies may focus on the role of different neutrophil subsets in fracture healing and whether trauma-induced systemic inflammation disturbs the balance between regenerative (M2) macrophages and inflammatory (M1) macrophage within the fracture hematoma. In conclusion, our data shows that human neutrophils negatively affect synthesis of mineralized ECM by BMSCs *in vitro*. Prolonged or increased influx of neutrophils into the fracture hematoma after hyper-inflammatory conditions may impair fracture healing by negatively affecting ECM synthesis by BMSCs. Clarifying the underlying mechanism may contribute to development of therapies that augment bone regeneration or prevent impaired fracture healing after hyper-inflammatory conditions.

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OB designed the study, performed the main experiments and statistical analysis and wrote the main manuscript text. MC aided in designing the study and performed experiments. JA, LK LL and TB aided in designing the study. All authors reviewed the manuscript.

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CHAPTER 7

Serum from the human fracture hematoma
contains a potent inducer of
neutrophil chemotaxis

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ABSTRACT

A controlled local inflammatory response is essential for adequate fracture healing. However, the current literature suggests that local and systemic hyper-inflammatory conditions after major trauma induce increased influx of neutrophils into the fracture hematoma (FH) and impair bone regeneration. Inhibiting neutrophil chemotaxis towards the FH without compromising the hosts' defense may therefore be a target of future therapies that prevent impairment of fracture healing after major trauma. We investigated whether chemotaxis of neutrophils towards the FH could be studied *in vitro*. Moreover, we determined whether chemotaxis of neutrophils towards the FH was mediated by the CXCR1, CXCR2, FPR and C5aR receptors.

Human FHs were isolated during an Open Reduction Internal Fixation (ORIF) procedure within 3 days after trauma and spun down to obtain the fracture hematoma serum. Neutrophil migration towards the FH was studied using Ibidi™ Chemotaxis^{3D} μ-Slides and image analysis of individual neutrophil tracks was performed. Our study showed that the human FH induces significant neutrophil chemotaxis, which was not affected by blocking CXCR1 and CXCR2. In contrast, neutrophil chemotaxis towards the FH was significantly inhibited by Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS), which blocks FPR and C5aR. Blocking only C5aR with CHIPSΔ1F also significantly inhibited neutrophil chemotaxis towards the FH.

Our finding that neutrophil chemotaxis towards the human FH can be blocked *in vitro* using CHIPS may aid the development of therapies that prevent impairment of fracture healing after major trauma.

INTRODUCTION

Fracture healing starts with an inflammatory phase during which leukocytes infiltrate the blood collection surrounding the fracture site^{1, 2}. Animal studies suggest that this blood collection, which is generally referred to as fracture hematoma (FH), forms a reservoir of essential factors and cells that regulate downstream processes of bone repair. This is illustrated by the finding that transplantation of the FH into muscle tissue induced ectopic bone formation and angiogenesis in animal models^{3, 4}. Moreover, removal or repetitive irrigation of the FH impaired fracture healing in rats^{5, 6}.

Although controlled local inflammation is essential for adequate fracture healing⁷, several animal studies have also shown that both local and systemic “hyper-inflammatory” conditions impair bone regeneration. For instance, injection of beta-glucan into the fracture site induces local hyper-inflammation and impairs fracture healing in rats⁸. Moreover, intraperitoneal injection of lipopolysaccharides in rats induces systemic inflammation and negatively affects the outcome of bone repair⁹. In addition, blunt chest injury, which is a model of trauma-induced systemic inflammation, also impairs fracture healing in rats¹⁰.

It is well known that severely injured patients have an increased risk of developing impaired fracture healing^{11, 12}. This not only has a significant impact on quality of life, but also carries a substantial economical burden to society¹³. Based on the abovementioned animal studies, we hypothesized that the systemic immune response after major trauma contributes to the high incidence of impaired fracture healing in multitrauma patients^{1, 11}. The underlying mechanism remains unclear. However, experimental studies suggest that major trauma pre-activates neutrophils and induces increased influx of neutrophils towards sites of inflammation, such as the fracture hematoma[14–16], and impairs bone healing.

Such a pathological role of neutrophils was supported by the finding that depletion of neutrophils improved the outcome of bone repair in rats^{17, 18}. However, systemic depletion of neutrophils would significantly compromise the hosts’ defense against pathogens.

Therefore, we tried to identify neutrophil chemoattractants within the sterile FH that may be blocked in the future without affecting chemotaxis of neutrophils towards sites of infection. As a first step, we tested whether neutrophil chemotaxis towards the human FH could be studied *in vitro*. Furthermore, we explored whether neutrophil chemotaxis towards the FH is mediated by IL-8 receptors CXCR1 and CXCR2, formylated peptide receptors (FPR) and complement receptor C5aR.

MATERIALS AND METHODS

ISOLATION OF NEUTROPHILS

Blood from anonymous healthy donors was acquired from the bloodbank “Mini Donor Dienst” of the University Medical Center Utrecht after written informed consent was obtained. Neutrophils were isolated from peripheral blood, as has been described previously¹⁹ and is summarized here. Briefly, 9 ml of blood was drawn into a sterile vacuum container with sodium citrate as anti-coagulant. The blood was diluted 1:1 in phosphate buffered saline (PBS) at room temperature. The diluted peripheral blood was pipetted onto 15 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged for 20 min at 900 g. After centrifugation, the plasma, Ficoll and mononuclear fraction were removed. The remaining erythrocytes and granulocytes were resuspended in 50 ml isotonic ice-cold ammonium chloride solution (NH₄Cl) containing 155mM NH₄Cl, 10mM KHCO₃, 0.1mM EDTA (pH 7.2) and incubated on ice for 20 min. Subsequently, the cell suspension was centrifuged, the supernatant was removed and the cell pellet was resuspended in 20 ml of NH₄Cl. After centrifugation, the cell pellet was resuspended in HEPES3+ (20 mM HEPES, 132 mM NaCl, 6.0 mM KCl, 1.0 mM MgSO₄, 1.2 mM KH₂PO₄, supplemented with 5.0 mM glucose, 1.0 mM CaCl₂, and 0.5% (w/v) human serum albumin) and centrifuged again. The supernatant was removed and the cell pellet was resuspended in HEPES3+. Cells were counted using the Cell-Dyn® 1800 (Abbott Laboratories, Abbot Park, Illinois, USA) and diluted in HEPES3+ to concentrations needed during the experimental conditions (3.2×10^6 cells/ml) and stored on ice until further use.

ISOLATION OF HUMAN FRACTURE HEMATOMA SERUM

Human fracture hematomas (FHs) were isolated during Open Reduction Internal Fixation (ORIF) procedures within 3 days after trauma from patients with closed fractures and without relevant comorbidity and collected in sterile plastic containers. The blood clot was isolated from the fracture site, which is generally required during an ORIF procedure in order to allow adequate reduction of the fracture and placement of fixation materials. FH was deemed residual tissue, and could therefore be collected without obtaining informed consent, unless the patient explicitly refused (opt-out method). This procedure is formalized in our hospital and therefore approval by our local ethics committee was not required. All samples were stripped of identifiers and fully anonymized. The serum of the FHs was obtained by centrifugation (5 min, 2300 rcf) of the FH within one hour after isolation. The serum was aliquoted and stored at -20°C until further use. The FH sera of different donors were used for each experimental condition and these sera were not pooled. 30 µL of FH was used for each experiment. There was no significant difference in chemotaxis towards fresh or frozen FH. The variation in neutrophil response towards the FH of different donors is depicted in Fig.2C.

CHEMOTAXIS ASSAY

The Ibidi™ Chemotaxis^{3D} μ -Slide was used to analyze neutrophil chemotaxis towards the FH serum in a three-dimensional, porous in vitro environment (IBIDI, Martinsried, Germany). Set-up and data analysis of the Ibidi™ Chemotaxis^{3D} μ -Slide has been described previously by other authors²⁰. The Ibidi™ Chemotaxis^{3D} μ -Slide is a chemotaxis chamber that enables the investigator to create time-lapse images and videos of cell migration.

3.0 μ l of ultra-pure human fibrinogen (25 mg/ml FIB3 obtained from Kordia, Leiden, the Netherlands) and 3.75 μ l of thrombin (20 U/ml in PBS, purchased from Sigma, St. Louis, Missouri, USA) were added to the 30 μ l of neutrophil suspension (final concentrations: fibrinogen 2.04 mg/ml; thrombin 2.04 U/ml; neutrophils 2.45×10^6 cells/ml). 6 μ l of this neutrophil/fibrinogen/thrombin suspension, containing approximately 1.5×10^4 neutrophils, was pipetted into each center channel of Ibidi™ Chemotaxis^{3D} μ -Slide (observation area) using round tips according to the manufacturer's protocol (Fig.1A). This fibrin gel was allowed to solidify for 10 min at room temperature. HEPES3+ was pipetted into the right (C0) chamber and each experimental condition was pipetted into the left (C100) chamber (Fig.1A).

A gradient of chemoattractants was rapidly established over the center channel (observation area). The slides were immediately placed in a pre-warmed microscopy chamber (37°C, Heidolph instruments inkubator 1000) onto an automated stage (Märzhäuser Wetzlar GmbH & Co., Wetzlar-Steindorf, Germany).

Time-lapse point revisiting microscopy (Quantimet 570C, DXMRE microscope, PL fluotar 5x low power objective lens, Leica, Heidelberg, Germany) was used to track the movement of neutrophils through the fibrin gel. Sequences consisted of 100 images per spot with a maximum of 3 revisited spots. The time-lapse interval was typically 15–25 seconds. Consecutive images were converted into a movie using ImageJ (version 1.46r, Public Domain). OPTIMAS software (version 6.51, Media Cybernetics, Inc) was used to derive trajectory plots and to quantify various parameters that describe chemotactic or chemokinetic responses which have been described previously²⁰. Neutrophil chemotaxis was measured using mean vector speed, which is the Euclidean distance between starting point and end point of all neutrophils that were analyzed (Fig. 1B) divided by imaging time.

EXPERIMENTAL CONDITIONS

N-formyl-methionyl-leucyl-phenylalanine (fMLF) (Sigma-Aldrich, St. Louis, MO, USA), and recombinant human IL-8 (PeproTech EC Ltd., Rocky Hill, NJ, USA) were diluted in HEPES3+ (10^{-7} M and 50 ng/ml, respectively) and used as positive controls, since these factors are well known chemoattractants for neutrophils^{21,22}. HEPES3+ was used as a negative control. CXCR1 and CXCR2 were simultaneously blocked on neutrophils using blocking antibodies α CXCR1 (Monoclonal Mouse IgG2A Clone # 42705, 500 μ g/ml, R&D Systems®, Abingdon, UK) and α CXCR2 (Monoclonal Mouse IgG2A Clone # 48311 500 μ g/ml, R&D Systems®, Abingdon,

UK). Additionally, the C5aR and FPRs were simultaneously blocked using Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS) which was donated and manufactured by the Department of Medical Microbiology, University Medical Center Utrecht, the Netherlands as described by de Haas et al.^{23,24}. In addition, a CHIPS mutant lacking the first N-terminal amino acid was used (CHIPSΔ1F), which has impaired or absent FPR but still intact C5aR-blocking activity²⁵. The isolated neutrophils were incubated with αCXCR1 and αCXCR2 or CHIPS and CHIPS Δ1F for 30 min on ice in 30 μl of solution (final concentrations: neutrophils 3.0×10^6 cells/ml; αCXCR1/2 20 μg/ml; CHIPS and CHIPSΔ 1F 10 μg/ml). After blocking the CXCR1 and CXCR2 receptors, neutrophil chemotaxis towards IL-8 and the FH was studied. After blocking

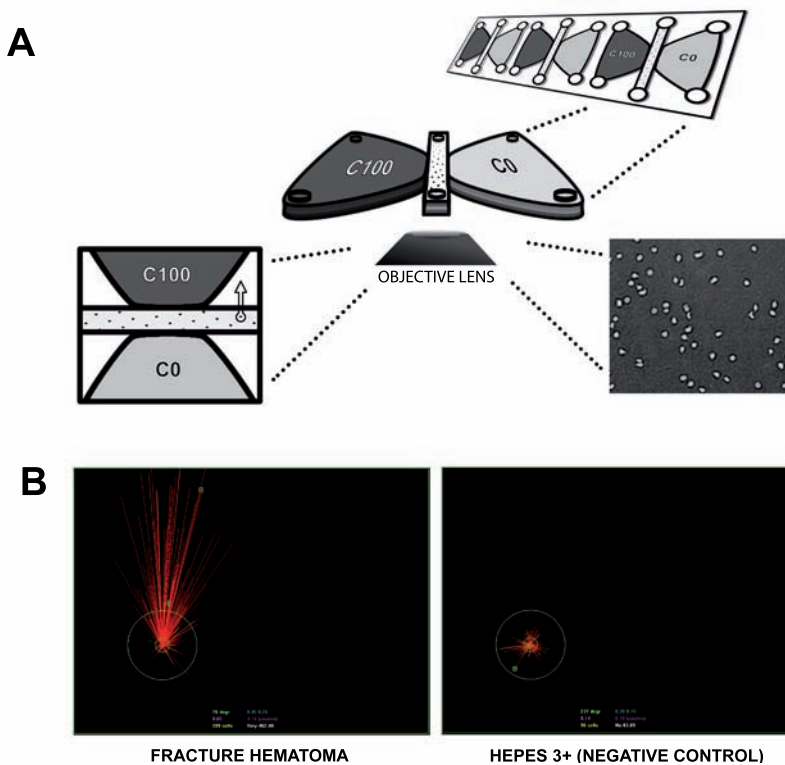


Figure 1
(A) Analysis of neutrophil chemotaxis towards the fracture hematoma using the Ibidi™ Chemotaxis^{3D} μ-Slide. A neutrophil/fibrinogen/thrombin suspension was injected into the observation area of the slide. After the fibrin gel solidified, HEPES3+ was injected into the C0 chamber. All experimental conditions were injected into the C100 chamber after which neutrophil chemotaxis was analyzed with time-lapse microscopy and cell tracking software.
(B) Representative example of neutrophil migration towards the fracture hematoma and towards HEPES3+. The red lines are Euclidean distances, which are the shortest distances between each beginning and endpoint of all neutrophils that were analyzed. Vector Speed was defined as the mean Euclidean distances of all neutrophils that were analyzed divided by imaging time.

the C5aR and FPR receptors with CHIPS, neutrophil chemotaxis towards fMLF and the FH was studied. Subsequently, neutrophil chemotaxis towards the FH was studied after blocking C5aR with CHIPSΔ 1F. We did not use technical duplicates or triplicates with the same FH/neutrophil donor combinations analyzed at the same time-point. Fig.2 therefore depicts the pooled data of single experiments with different FH/neutrophil donor combinations analyzed at different time-points.

STATISTICAL ANALYSIS

GraphPad Prism version 5.00 was used for all statistical analyses. All experimental conditions were compared using an ANOVA with a Bonferroni Multiple Comparison post-hoc test. Multiple dilutions of the FH were compared to HEPES3+ using an ANOVA with Dunnett's Multiple Comparison Test. A p-value <0.05 was considered statistically significant. P values are described in Fig.2 as * (<0.05), ** (<0.01), *** (<0.001).

RESULTS

CHEMOTAXIS OF HUMAN NEUTROPHILS TOWARDS FMLF AND IL-8

As a control study, we first determined whether neutrophil chemotaxis towards interleukin -8 (IL-8) and *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) could be studied with the Ibidi™ Chemotaxis^{3D} μ-Slides, since these two factors are well known neutrophil chemoattractants. Neutrophil chemotaxis towards HEPES3+ (negative control), IL-8 and fMLF is depicted in Fig.2A and 2B, respectively. When compared to HEPES3+ there was a significant increase in migration towards IL-8 (mean: 0.4 vs 6.1 μm/min, n = 21 vs 8, p<0.001) and fMLF (mean: 0.4 vs 3.6 μm/min, n = 21 vs 14, p<0.001).

BLOCKING CHEMOTAXIS OF HUMAN NEUTROPHILS TOWARDS IL-8 AND FMLF

Chemotaxis of neutrophils towards IL-8 is dependent on the CXCR1 and CXCR2 receptors. Blocking these two receptors induced a significant decrease in vector speed (mean: 6.1 vs 2.6 μm/min, n= 8 vs 4, p <0.001 without and with blocking the CXCR1 and CXCR2 receptors) as depicted in Fig.2A. Chemotaxis Inhibitory Protein of Staphylococcus aureus (CHIPS) specifically binds to the formylated peptide receptors (FPR) and C5a receptor (C5aR). Chemotaxis of neutrophils towards fMLF was significantly inhibited by CHIPS (mean: 3.6 vs 0.4 μm/min, n= 14 vs 12, p<0.001, without and with CHIPS), as depicted in Fig.2B.

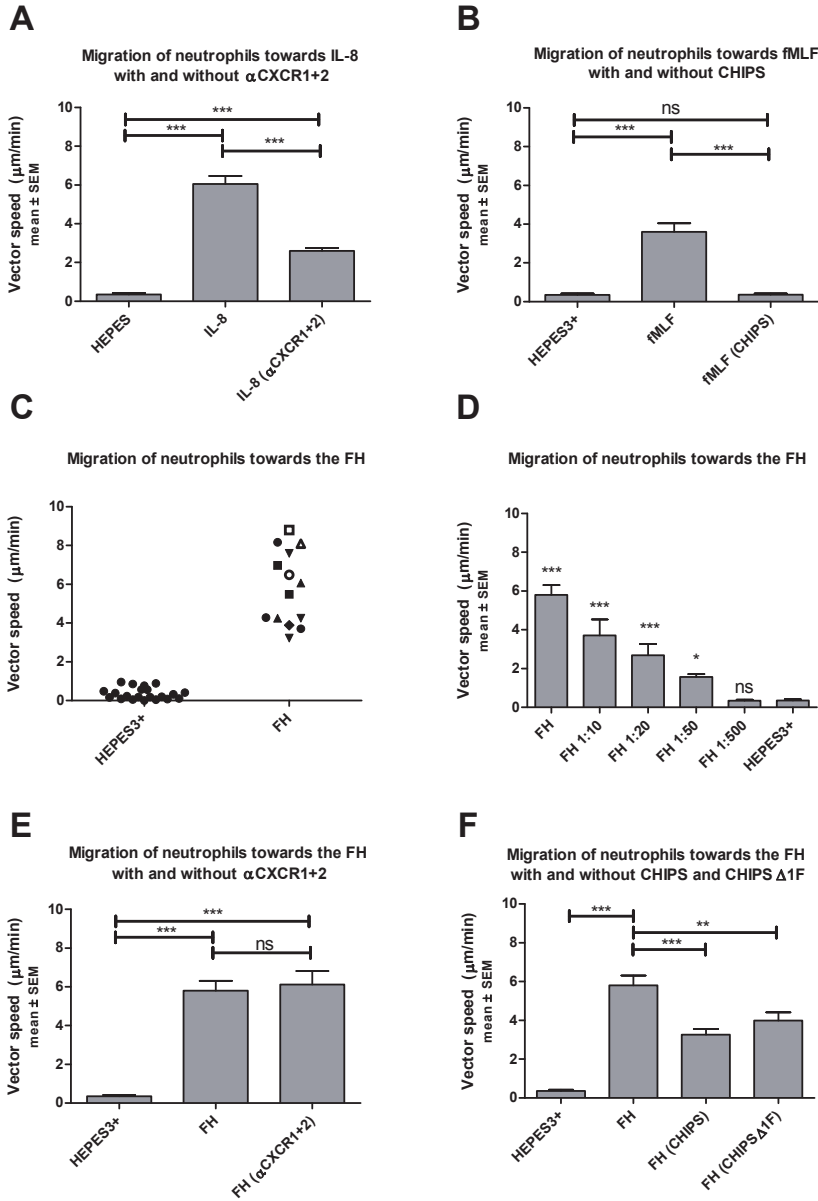


Figure 2

(A) Neutrophil chemotaxis towards IL-8 with and without blocking CXCR1 and CXCR2. IL-8 induced significant neutrophil chemotaxis compared to HEPES3+. Blocking CXCR1 and CXCR2 significantly inhibited migration towards IL-8. *** = $p < 0.001$

(B) Migration of neutrophils through a 3D fibrin gel towards fMLF with and without blocking FPR with CHIPS. There was significant chemotaxis of neutrophils towards fMLF compared to the negative control HEPES3+. Blocking the FPR receptors with CHIPS significantly inhibited migration towards fMLF. *** = $p < 0.001$.

(C) Migration of neutrophils through a 3D fibrin gel towards the fracture hematoma (donor variation).

Figure 2 (continued)

Neutrophil migration towards the FH is depicted for each neutrophil/FH donor combination. 14 neutrophil donors were combined with 8 FH donors (14 neutrophil/FH donor combinations). A distinct icon is used to plot each FH donor. We were unable to find a significant difference in neutrophil migration towards the FH between different FH donors.

(D) Migration of neutrophils through a 3D fibrin gel towards the fracture hematoma (dose response).

Neutrophils significantly migrated towards the FH, even after diluting the FH 1:10, 1:20 and 1:50 in HEPES 3+. The 1:500 diluted FH did not induce significant neutrophil chemotaxis. *** = $p < 0.001$ and * = $p < 0.05$ compared to HEPES3+.

(E) Neutrophil chemotaxis towards the fracture hematoma serum with and without blocking CXCR1 and CXCR2. Chemotaxis towards the FH was not significantly inhibited by blocking CXCR1 and CXCR2. *** = $p < 0.001$

(F) Neutrophil chemotaxis towards the fracture hematoma with and without blocking C5aR and FPR with CHIPS and blocking C5aR with CHIPSΔ1F. CHIPS and CHIPSΔ1F significantly inhibited neutrophil chemotaxis towards the FH. *** = $p < 0.001$, ** = $p < 0.01$.

CHEMOTAXIS OF NEUTROPHILS TOWARDS THE HUMAN FRACTURE HEMATOMA

Neutrophils exhibited very potent chemotaxis towards the human fracture hematoma (FH) serum *in vitro*. The variation in neutrophil migration towards the FH for each neutrophil/FH donor combinations ($n=14$) is depicted in Fig.2C. A dose response is depicted in Fig.2D. The vector speed of neutrophils towards the FH was significantly greater when compared to migration towards HEPES3+ (mean: 5.8 vs 0.4 $\mu\text{m}/\text{min}$, $n= 14$ vs 21, $p < 0.001$, respectively). Neutrophil chemotaxis towards the FH remained significant when the FH was diluted in HEPES3+ 1:10 (mean: 3.7 vs 0.4 $\mu\text{m}/\text{min}$, $n=4$ vs $n= 21$, $p < 0.001$), 1:20 (mean: 2.7 vs 0.4 $\mu\text{m}/\text{min}$, $n= 5$ vs 21, $p < 0.001$) and 1:50 (mean: 1.6 vs 0.4 $\mu\text{m}/\text{min}$, $n= 8$ vs 21, $p < 0.05$). When the FH was diluted 1:500, no significant chemotaxis could be observed (mean: 0.4 vs 0.4 $\mu\text{m}/\text{min}$, $n=6$ vs 21).

CHEMOTAXIS OF NEUTROPHILS TOWARDS THE FRACTURE HEMATOMA AFTER BLOCKING THE CXCR1, CXCR2, FPR AND C5aR RECEPTORS

Blocking the CXCR1 and CXCR2 receptors did not significantly affect neutrophil chemotaxis towards the FH (mean: 5.8 vs 6.1 $\mu\text{m}/\text{min}$, $n= 14$ vs 8, without and with blocking the CXCR1 and CXCR2 receptors). There was still significant chemotaxis towards the FH after blocking these receptors compared to HEPES3+ (mean: 6.1 vs 0.4 $\mu\text{m}/\text{min}$, $p < 0.001$) as depicted in Fig.2E. CHIPS, which blocks FPR and C5aR, significantly inhibited neutrophil chemotaxis towards the FH (mean: 5.8 vs 3.3 $\mu\text{m}/\text{min}$, $n= 14$ vs 4, $p < 0.001$ without and with CHIPS, Fig.2F). In addition, CHIPSΔ 1F, which only blocks C5aR, also induced a significant decrease in neutrophil chemotaxis towards the FH (mean: 5.8 vs 4.0 $\mu\text{m}/\text{min}$, $n= 14$ vs 5, $p < 0.01$ without and with CHIPSΔ 1F, Fig.2F).

DISCUSSION

The current literature suggests that increased influx of neutrophils into the fracture hematoma (FH) during hyper-inflammatory conditions impairs fracture healing after major trauma^{1,26}. Future therapies that inhibit influx of neutrophils into the FH without compromising the hosts' defense against pathogens may therefore prevent impairment of bone healing in multitrauma patients. Our study shows that chemotaxis of neutrophils towards the FH can be studied in vitro with Ibidi™ Chemotaxis^{3D} μ-Slides. We found that serum from the human FH significantly induces neutrophil chemotaxis, which was not affected by blocking the CXCR1 and CXCR2 receptors (Fig.2E). In contrast, Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS) induced a significant decrease in neutrophil chemotaxis towards the human FH in vitro (Fig.2F). CHIPS is an exoprotein produced by several strains of *S. aureus*, and is a potent inhibitor of neutrophil and monocyte chemotaxis toward C5a and formylated peptides like fMLF²⁴. It is known that tissue injury induces complement activation and release of C5a^{27,28}, as well as release of formylated peptides from mitochondria into the circulation²⁹. CHIPS exclusively binds directly to the C5aR and FPR1 and FPR2 receptors, thereby preventing their natural ligands from activating these receptor^{24,30}. We additionally used a CHIPS mutant lacking the first N-terminal amino acid (CHIPSΔ 1F), which has impaired or absent FPR but still intact C5aR-blocking activity²⁵. Our data shows that blocking C5aR with CHIPSΔ 1F also significantly inhibits neutrophil chemotaxis towards the FH (Fig.2F). Previous studies have shown that systemic antagonism of the C5aR improves fracture healing after major trauma in rats¹⁶. It is tempting to speculate that systemic C5aR antagonism prevents increased influx of neutrophils into the FH and thereby reduces the deleterious effect of major trauma on fracture healing.

In our in vitro experiments, we were unable to completely block neutrophil chemotaxis towards the FH using CHIPS or CHIPSΔ 1F. One possible explanation for this effect is that the concentrations of blocking antibodies were insufficient to completely block all receptors. Also, several additional neutrophil chemoattractants may be present within the FH that do not exert their effect through CXCR1/2, FPR or C5aR. Neutrophils possess several receptors that detect chemoattractants, such as chemokines, complement components and several other chemotactic lipids and peptides³¹. Nineteen chemokine receptors have been identified so far, which include seven CXC receptors (CXCR1-7), ten CCR (CCR1-10), one CX₃CR (CX₃CR1), and one CR (XCR1) receptor³². Neutrophils are traditionally known to express only a very limited number of chemokine receptors and mainly express CXCR1 and CXCR2 in healthy individuals³³. CXCR1 and CXCR2 are used by neutrophils to recognize N-terminal ELR (glutamic acid-leucine-arginine) motif-containing CXC chemokines. Human CXCR1 binds to CXCL8 (interleukin-8/IL-8) and CXCL6 (granulocyte chemotactic protein-2)^{21,31}, as well as the ECM breakdown product N-acetyl PGP³⁴. These three factors can also bind to CXCR2. However, CXCR2 is more promiscuous and binds different additional CXC chemokines, in-

cluding CXCL1 (growth regulated oncogene-alpha/GRO- α), CXCL2 (GRO- β), CXCL3 (GRO- γ), CXCL5 (epithelial cell-derived neutrophil activating peptide-78/ ENA-78) and CXCL7 (neutrophil activating protein-2/GCP-2)³¹. Our study implies that these CXCR1 and CXCR2 ligands are not relevant in migration of neutrophils towards the FH in vitro. However, although neutrophils in healthy individuals mainly express CXCR1 and CXCR2³³, it has been shown that infiltrated neutrophils from patients with chronic inflammatory lung diseases and rheumatoid arthritis express additional chemokine receptors on their surface, i.e., CCR1, CCR2, CCR3, CCR5, CXCR3, and CXCR4³³. Moreover, major trauma induces the release of several neutrophil subsets into the peripheral circulation, including young banded neutrophils and hyper-segmented neutrophils, which exhibit different properties and receptor expressions compared to mature neutrophils from healthy individuals³⁵. Future studies may focus on the role of these neutrophil subsets in fracture healing and determine whether neutrophils within the FH express other chemokine receptors compared to neutrophils isolated from peripheral blood of healthy donors.

Another chemotactic factor for neutrophils is leukotriene B4 (LTB4), which is recognized by a high-affinity receptor (BLT1) and a low-affinity receptor (BLT2)³⁶. Animal studies have shown that LTB4 mediates neutrophil influx after experimental spinal cord injury³⁷. It is tempting to speculate that LTB4 also mediates neutrophil influx into other types of sterile tissue injury, such as bone injury. An additional chemoattractant for neutrophils is platelet activating factor (PAF), which is a phospholipid that is bound by the PAF-receptor (PAFR)³⁸. Little is known about the role of PAF in tissue injury although animal studies did show that inactivation of PAF by PAF-acetylhydrolase significantly decreased neutrophil influx in a rabbit model of myocardial ischemia/reperfusion injury³⁹. Future studies should investigate to which extent the abovementioned factors are also relevant in chemotaxis of neutrophils towards the FH. In summary, our study shows that chemotaxis of neutrophils towards the FH can be studied in vitro with Ibidi™ Chemotaxis^{3D} μ -Slides. We found that serum from the human FH significantly induces chemotaxis, which was not affected by blocking CXCR1 and CXCR2. In contrast, CHIPS and CHIPS Δ 1F, which blocks C5aR, induced a significant decrease in chemotaxis of neutrophils towards the FH. These findings may aid the development of therapies that prevent impairment of fracture healing after major trauma.

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The background features several abstract shapes in shades of purple and blue. There are large, soft-edged purple shapes in the top-left and bottom-left corners. Scattered throughout the white space are various circles and dots in different sizes and colors, including purple, blue, and a small dark blue dot. The overall aesthetic is clean and modern.

CHAPTER 8

Summary and General Discussion

TWO FACES OF INFLAMMATION

Calor (increased heat), *rubor* (redness), *tumor* (swelling) and *dolor* (pain) are the four cardinal signs of inflammation, which were described by Celsus during the first century¹. Later, a fifth sign was added to Celsus tetrad, which is *functio laesa* (disturbance or loss of function). It is well known that inflammation, which means “set on fire” in Latin, can inflict collateral damage to a variety of processes important for the homeostasis within our body and thereby disturb their function¹. Inflammation therefore has two faces: it is designed to limit further damage and induce healing, but it is also a major driver of complications and fatal outcomes².

Modulating the inflammatory response may be a powerful tool to augment tissue regeneration and prevent complications, especially in high-risk individuals, such as patients with multiple injuries. In order to modulate inflammation, it is essential to first understand both of its faces. Bone injury is one of the most common injuries that humans experience³. We, therefore, aimed to clarify how inflammation contributes to bone repair, how systemic inflammation can impair fracture healing and finally, we identified a receptor on neutrophils which is involved in chemotaxis of neutrophils towards the human fracture hematoma (FH) in vitro. This receptor may be a target for future therapies that aim to prevent impairment of fracture healing after major trauma.

THE INFLAMMATORY PHASE OF FRACTURE HEALING

Fracture healing is typically divided into three overlapping phases: the inflammatory phase, the regenerative phase and the remodeling phase.^{4,5} Two of these phases can be further subdivided to make a total of five phases, which were already described by Dupuytren in 1811⁶: First, “blood ceases to escape and a mild inflammation is set up” (fracture hematoma formation). Subsequently, “a stringy and viscid matter...likewise a reddish substance...is poured out between the ends of the bone” (granulation tissue formation). Over weeks “... it forms a distinct tumor...its color white, its consistence firm and its resistance analogous to that of fibrocartilages” (immature callus formation). Later “the provisional callus becomes condensed...and ossified” (mature callus formation). Finally the callus “disappears...the internal deposit of bone disappears and the intermedullary canal is insensibly re-established... the process of consolidation is completed” (remodeling)⁶ (*Figure 1*).

In order to determine how inflammation contributes to fracture healing it essential to first define “inflammatory cells” and “inflammatory phase of bone healing”. It is generally accepted that leukocytes form the predominant cell type in the affected tissue during a variety of pathologies that exhibit signs of inflammation. Consequently, leukocytes and inflammatory cells are frequently used interchangeably. Leukocytes are characterized by their

expression of the common leukocyte antigen CD45⁷. However, it has been shown that bone marrow-derived hematopoietic precursors are attracted to sites of skin injury where they differentiate into fibrocytes, which mediate tissue repair and fibrosis⁸. These fibrocytes are known to express markers of both stromal cells and hematopoietic cells, such as CD45, but can also lose their CD45-expression⁸. Another recent study in mice, showed that 7 days after experimental cutaneous wounding approximately two-thirds of all fibroblasts within granulation tissue are derived from myeloid cells⁹. These findings illustrate that it is challenging to adequately define inflammatory cells. We would like to define inflammatory cells as all cells that are derived from the hematopoietic stem cell (HSC) which normally participate in the body's response to an infection or to sterile injury of the tissue. This definition distinguishes these cells from non-hematopoietic stromal cells, which include multipotent stromal cells (MSCs), osteoblasts and chondroblasts⁴.

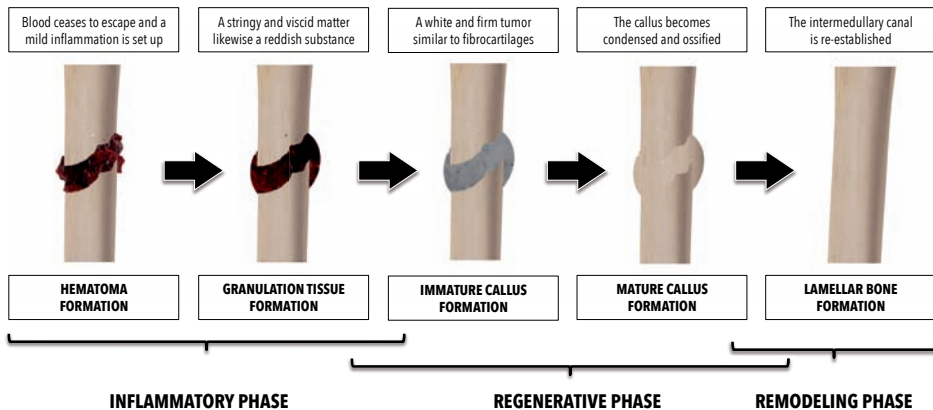


Figure 1 The different phases of fracture healing

It is generally believed that stromal cells synthesize bone tissue during fracture healing by both endochondral and intramembranous ossification. The concept of endochondral and intramembranous ossification was first described during the 19th century. Staining animals with madder and its derivatives, which were previously used as cloth stains^{10,11}, showed two types of processes leading to new bone formation around the fracture site that resembled that of bone growth during fetal development. Long bones primarily grow in length by converting cartilage into bone tissue (endochondral ossification) and grow in width through direct bone formation without a provisional cartilage scaffold (intramembranous ossification)^{12,13}. This concept of bone formation with and without synthesis of a provisional cartilage extracellular matrix (ECM) was later confirmed using immunohistochemistry¹⁴ by staining different types of collagen¹⁵. The beginning of the regenerative phase of fracture healing is marked by the presence of stromal cells within the fracture hematoma (FH). This

eventually leads to proliferation and synthesis of bone tissue through either endochondral or intramembranous ossification. The processes that precede this phase can be considered to occur during the inflammatory phase of fracture healing.

GRANULATION TISSUE

It is believed that granulation tissue is formed during early fracture healing and is characterized by the formation of a provisional ECM that is populated by numerous inflammatory cells and is associated with a marked proliferation of fibroblasts^{4,16}. In *wound* healing, it is believed that fibrin and plasma derived fibronectin form a provisional matrix within the blood clot, that is later converted to a cell-derived fibronectin and collagen-containing ECM produced by fibroblasts¹⁷. It is unclear whether granulation tissue in *fracture* healing has a similar composition and origin. We determined whether ECM becomes synthesized within the human FH during the inflammatory phase of fracture healing, before CD45 negative stromal cells such as fibroblasts, osteoblasts, chondroblasts and multipotent stromal cells (MSCs)¹⁸ are identified within the FH.

We isolated FHs from trauma patients at different time points after injury during an Open Reduction Internal Fixation (ORIF) procedure, ranging from day 0 until day 23 after trauma and stained the FHs using (immuno)histochemistry. A representative part of all FHs was biopsied and placed in chronological order into a tissue micro-array (TMA), *Figure 2*. This technique allowed rapid and equal staining, as well as high-throughput analysis of all FHs, since all samples were present on a single microscopy slide. The nuclei of all cells within the FH were fluorescently stained with Hoechst and all leukocytes were stained using antibodies against the common leukocyte antigen CD45. The first CD45- cells were identified at day 5 after injury and these cells had a different morphology compared to leukocytes when stained with Hematoxylin and Eosin (*Figure 3A vs 3B*).

It is unclear whether these CD45 negative cells are fibroblasts, fibrocytes, osteoblasts, chondroblast, multipotent stromal cells or (a combination of) other types of stromal cells. It is also unknown whether these stromal cells extravasated from peripheral blood or migrated from adjacent tissues such as the bone marrow or periosteum. A limitation of using human samples is that we were unable to analyze the entire FH for ethical reasons as the complete removal of the FH is not an objective during an ORIF procedure. We can, therefore, not exclude the possibility that CD45- cells infiltrated the human FH earlier than day 5 after injury. After CD45- cells infiltrated the FH, a collagen type I positive birefringent extracellular matrix (ECM) could be identified within the FH (*Figure 3D*). These birefringent fibers ran in various directions and were not identified before CD45- cells infiltrated the FH (*Figure 3C*). It is unclear whether these fibers represent collagen fibrils within granulation tissue, newly

formed cartilage or woven bone tissue. Future studies may stain collagen type II and type X and clarify to some extent the nature of this birefringent ECM.

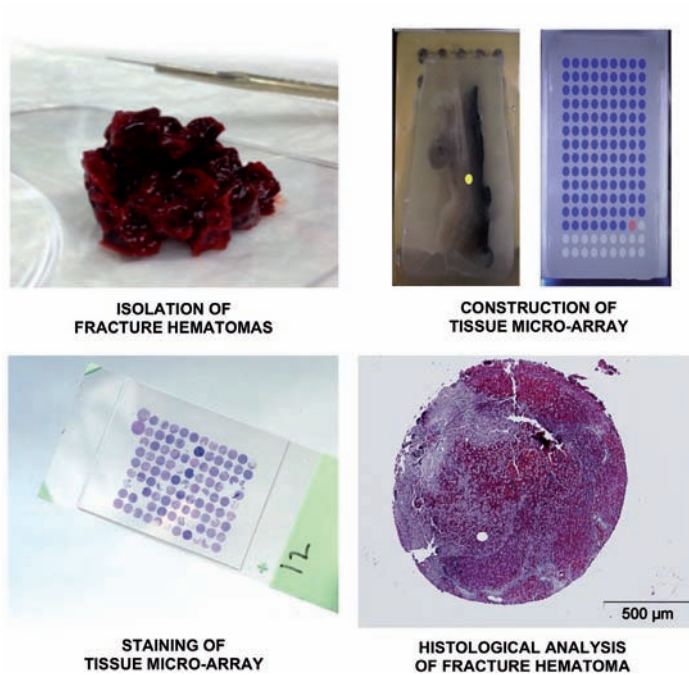


Figure 2 Human fracture hematomas were isolated during an ORIF procedure at various stages of fracture healing and placed in chronological order into a tissue micro-array, which allows rapid and equal staining, as well as high-throughput analysis of all FHs.

A non-birefringent ECM was identified in FHs that were isolated as early as 12 hours after injury. This ECM stained positive for cellular fibronectin (*Figure 3E*) and increased over time, well before the first CD45- cells could be identified within the FH. This finding suggests that leukocytes contribute to fracture healing by synthesizing a fibronectin+ ECM rapidly after injury. Macrophages (CD68+ cells) were mainly localized within this ECM and neutrophils (CD66b+) adjacent to the ECM (*Figure 3F*). It is therefore possible that neutrophils and macrophages play a distinct role in synthesis of ECM. Although formation of granulation tissue during early fracture healing is frequently mentioned throughout the literature, only little is known about its composition and origin^{4,16}. We believe that leukocytes contribute to formation of granulation tissue by synthesizing fibronectin.

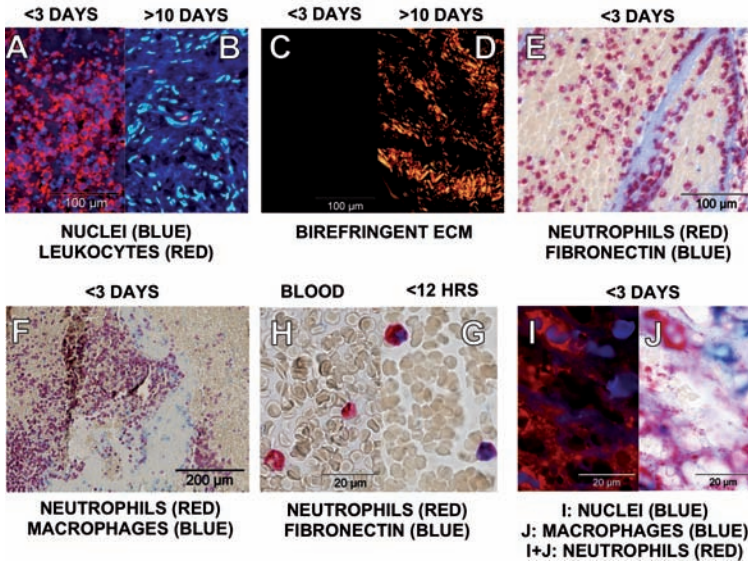


Figure 3 The time between injury and isolation of the FH is mentioned above each image.

Nuclei are fluorescently stained using Hoechst. Leukocytes, neutrophils and macrophages are stained using antibodies against CD45, CD66b and CD68 respectively. Birefringent fibrils are identified after staining the FH with Picrosirius Red.

REGENERATIVE NEUTROPHILS

Only little is known about the role of neutrophils in tissue regeneration. Two studies have recently suggested a beneficial role of neutrophils during fracture healing. Systemic reduction of neutrophil counts using antibodies against Ly-6G in mice with femoral¹⁹ and tibial²⁰ fractures induced a significant decrease of neutrophil counts within the FH and impaired fracture healing. Although these studies showed contradicting effects on the number of macrophages present within the early FH after systemic neutrophil depletion, they both imply that neutrophils play an important role in bone regeneration. This beneficial role of neutrophils was not observed in earlier studies that used anti-neutrophil serum to deplete neutrophils in rats with femur fractures²¹ and growth plate injuries²². The studies in rats found improved bone healing after systemic neutrophil depletion. However, a crucial drawback of using unspecific antiserum is that it bears a high risk of influencing other immune cell populations.

We found that neutrophils within the 6 hours old FH stained positive for cellular fibronectin (Figure 3G), in contrast to neutrophils within coagulated peripheral blood of healthy donors (Figure 3H). This finding implies that neutrophils may be an important source of fibronectin within the ECM and, thereby, contribute to fracture healing.

It is unclear whether fibronectin+ neutrophils home towards the FH or whether naïve neutrophils within the FH differentiate or polarize into fibronectin+ neutrophils. There is accumulating evidence that neutrophils consist of a heterogeneous population with distinct functions under both homeostatic and pathological conditions²³. Multiple functional phenotypes of neutrophils are found during systemic inflammation, including banded and hypersegmented neutrophils^{24,25}. These subsets were previously identified with flow-cytometry using antibodies against CD11b (αM component of the β2-integrin MAC-1) and CD62L (L-Selectin)²⁵. Unfortunately, these markers were unsuitable to identify neutrophil subsets within the FHs. This was due to the fact that practically all neutrophils within the FH and within coagulated peripheral blood of healthy donors were CD62L-low and CD11b-high (data not shown). This phenotype has also been described for neutrophils in bronchoalveolar lavage fluid of healthy individuals²⁶. This finding implies that even in the absence of inflammatory or infectious pathologies, homing of neutrophils towards tissue is associated with an activated phenotype.

Recent literature provides evidence that neutrophils can become polarized in response to certain signals, synonymous to M1/M2 polarization of macrophages (inflammatory vs regenerative macrophages). These distinctive phenotypes induce very different effects on the immune system. Fridlender et al. have first introduced the concept of N1/N2 polarization of neutrophils in an effort to reconcile some of the opposing functions for tumor-associated neutrophils (TANs)²⁷. N1 neutrophils are anti-tumoral and are characterized by hypersegmented nuclei, increased TNF-α expression and reduced or absent VEGF and MMP-9 production. These mediators are key to angiogenesis, and therefore related to tumor progression. N2 neutrophils are pro-tumoral, and promote angiogenesis and tumor growth, and suppress the immune system²⁸. N2 polarized neutrophils have also recently been identified in the injured brain following stroke²⁹ and N1 to N2 polarization was demonstrated within the heart following myocardial infarction³⁰. It is tempting to speculate that “regenerative” N2 neutrophils also contribute to bone repair by synthesizing fibronectin. Moreover, polarization of neutrophils towards inflammatory N1 neutrophils, may be involved in impairment of fracture healing after hyper-inflammatory conditions, such as the trauma-induced systemic inflammatory response.

In addition to fibronectin synthesis, neutrophils may contribute to bone repair through other mechanisms as well. Neutrophil derived CD66b+ particles were present within the fibronectin+ ECM (*Figure 3I and 3J*), which may indicate an additional mechanism through which neutrophils contribute to tissue regeneration. The current literature describes that neutrophils can secrete CD66b+ microparticles upon activation *in vitro* and *in vivo* at the site of inflammation under various conditions³¹. CD66b is expressed on the cell membrane of neutrophils, in specific granules and on neutrophil derived ectosomes, but not on secretory vesicles, gelatinase granules or azurophilic granules³¹. These CD66b+ particles affect the inflammatory phenotype of macrophages *in vitro*, as CD66b+ ectosome binding to

macrophages leads to a decreased inflammatory response of macrophages, and also an increased TGF-beta1 secretion *in vitro*³². It has previously been shown that local injection of TGF-beta 1 into the FH of rats improved the outcome of fracture healing³³, indicating that TGF-beta 1 might play a significant role during bone repair.

It has also been demonstrated that granular content from neutrophils is present within Neutrophil Extracellular Traps (NETs), which are mesh-like DNA fibres that are cast out from neutrophils in response to certain stimuli³⁴. These NETs are covered with multiple cytotoxic proteins originating from the neutrophil cytosol as well as granules. NETs were initially discovered as a novel form of neutrophil-mediated immunity with antimicrobial properties³⁴⁻³⁶. Recently, NETs have been identified within healing wounds of diabetic mice³⁷ and within ischemic stroke thrombi³⁶. These NETs exhibited diffuse DNA staining and additional granular staining of neutrophil-derived (Ly6G+ and CD66b+) particles that closely resembled our FHs, as shown in *Figure 3I*. Although it is tempting to speculate that NETs are formed within the early FH, additional staining of the FH with antibodies against citrullinated histone H3 (H3Cit, which allows decondensation of nuclear chromatin³⁶), is required to determine whether NET formation indeed occurs within the FH. NETs are known to affect recruitment of ECM synthesizing cells like macrophages and stromal cells²⁸. Moreover, NET release has been associated with promotion of fibrosis³⁸ and impaired wound healing in diabetic mice³⁷. Future studies should clarify the role of neutrophil polarization, NET formation and neutrophil derived (CD66b+) particles in fracture healing.

OTHER PLAYERS

Although our study focused on the role of neutrophils in fracture healing, several other inflammatory cells may also play an important role during bone repair. We found that macrophages were mainly localized within the fibronectin+ ECM (*Figure 3F*), which made it difficult to assess whether single macrophages were fibronectin positive and, therefore, may also be considered a source of fibronectin. It is known that macrophages can synthesize fibronectin³⁹. Moreover, the fact that macrophages (CD68+ cells) were mainly localized within the ECM does suggest that they are involved in ECM synthesis. During the second week after injury, practically all of the neutrophils disappeared from the FH and CD45- stromal cells were the most prevalent type of nucleated cells. In contrast to neutrophils, macrophages did not disappear from the FH at this time. It is, therefore, tempting to speculate that macrophages not only contribute to fracture healing by synthesizing or modulating ECM during the inflammatory phase, but also direct stromal cells or synthesize ECM during the regenerative phase of bone healing. Animal studies have also shown that macrophages remain present within the callus during the regenerative phase of bone healing⁴⁰. Ablation of macrophages led to a significant reduction in the number of stromal progenitors and

decreased the ability of these cells to differentiate into osteoblasts⁴¹. In addition, several animal studies have shown that depletion of macrophages during different time points after injury impairs bone healing⁴⁰⁻⁴². It is suggested that depletion of macrophages impairs bone healing by negatively affecting endochondral ossification, but not intramembranous ossification⁴⁰. Depending on exogenous or endogenous stimuli, macrophages can differentiate into inflammatory (M1), anti-inflammatory / regenerative (M2) and angiogenic (M2-like) phenotypes⁴⁰. Although M1 macrophages are indispensable for the initiation of the regeneration process, chronic inflammation and unsuccessful healing are associated with a prolonged infiltration of pro-inflammatory macrophages. A prolonged pro-inflammatory reaction has already been shown to negatively influence bone healing⁴⁰. A recent study showed that macrophage phenotype within the FH of mice changed during the first 3 days of healing from predominantly M1 to M2. Moreover, experimental polarization of macrophages towards the M2 phenotype with interleukin (IL)-4 and IL-13 improved bone formation in mice⁴⁰. We did not analyze polarization of macrophages in our study, but this may well be a focus of future research.

In addition to macrophages, a growing number of studies support the concept that also lymphocytes contribute to bone tissue regeneration. We did not stain lymphocytes within human FHs during our study and the kinetics of lymphocytes during human fracture healing remains unclear. In mice, high lymphocyte counts within the FH have been observed on day 3 after injury. After the soft callus was formed, T and B cells disappeared from the fracture site. Thereafter, lymphocytes massively infiltrated the callus region during callus mineralization⁴³. Studies with recombination activating gene 1 knockout (RAG1^{-/-}) mice, which lack mature lymphocytes, initially suggested that bone healing was accelerated compared to wild-type mice⁴⁴. However, later studies showed that fractured bones of RAG^{-/-} mice mineralized faster but were much less capable to withstand deformation. A possible explanation for decreased resistance to deformation was the highly disordered collagen type 1 network that was observed in RAG^{-/-} mice compared to wild-type mice⁴⁵. A lack of an adaptive immune response therefore did not seem to lead to better, but rather deregulated and suboptimal bone healing. Another study showed that the initial accelerated mineralization seen in RAG1^{-/-} mice, occurred well before lymphocytes could be detected within the callus of wild-type animals. Bone marrow osteogenic cells from RAG1^{-/-} mice, taken in normal conditions, displayed abnormal mineralization capacity compared to wild-type controls upon cell culture. This suggests that lymphocytes control bone regeneration by programming the basal activity or capacity of osteoblast precursors to respond to damage⁴⁶.

In addition to abovementioned leukocyte subsets, platelets may also play an important role during fracture healing. It is known that platelets can release growth factors, cytokines and adhesive proteins like fibronectin, fibrinogen and vitronectin^{47,48}, platelet derived growth factor (PDGF), transforming growth factor (TGF-) beta, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiopoietin-1⁴⁹. It is known that

these factors can regulate angiogenesis, recruit stromal cells and affect their proliferation and differentiation. Moreover, they can induce recruitment, activation and polarization of leukocyte subsets⁴⁹. Therefore, platelets should also be considered as potentially important players during bone regeneration and may be the focus of future studies.

A RESERVOIR OF ESSENTIAL FACTORS

The current literature suggests that the FH serves as a reservoir of essential factors that initiate and direct downstream processes of fracture healing⁵⁰. The FH becomes inherently osteogenic, which is demonstrated by the finding that transplantation of the four-day old FH into muscle tissue or underneath the periosteum of the parietal bone of rats induces ectopic bone formation⁵¹. In contrast, the two-day old FH induced bone formation only at the subperiosteal site, but not when transplanted into muscle tissue. Transplantation of clotted peripheral blood into the subperiosteal site or into muscle tissue did not induce ectopic bone formation⁵¹. Clarifying which components of the FH make it inherently osteogenic may significantly contribute to development of therapies that induce or augment bone regeneration.

Based on our analysis of human FHs, it is tempting to speculate that the four-day old FH in the previously mentioned study induced bone formation in muscle tissue due to presence of CD45- stromal cells that were not present in the two-day old FH. A possible explanation why the two-day old FH induced bone formation at the subperiosteal site in contrast to coagulated peripheral blood may be due to the presence of fibronectin+ ECM or infiltrated and polarized leukocyte subsets. This theory should be scrutinized by future studies. If these components that make the FH inherently osteogenic are identified, a subsequent study may determine whether the components can be 3D printed and transplanted or injected into muscle tissue, the subperiosteal site and into the FH. Ideally, such a construct would not include patient-specific cellular components such as ex vivo polarized neutrophils or macrophages, since this would make it less feasible as a “off the shelf” form of therapy. A similar construct may subsequently be injected or transplanted into other types of injuries such as animals with myocardial infarction, surgical wounds, anastomosis of intestinal resections or burn injuries.

THE TRAUMA-INDUCED SYSTEMIC INFLAMMATORY RESPONSE

The high incidence of impaired bone healing in multitrauma patients has mainly been attributed to local changes that occur after severe injury, such as open fractures and poor condition of surrounding soft tissue⁵². During the initiation of this thesis, trauma-induced

systemic inflammation was not considered a risk factor for impairment of fracture healing after major trauma. Systemic inflammation is defined as the systemic body's response to an infectious or non-infectious insult and is the result of release of cytokines and activation of the immune system. The complex cascade of molecular and cellular events that follow, can lead to several clinical symptoms. The group of symptoms that frequently occur during systemic inflammation, were defined by the American College of Chest Physicians and the Society of Critical Care Medicine in 1992 as: Systemic Inflammatory Response Syndrome (SIRS). This syndrome is defined as, but is not limited to, having more than one of the following clinical manifestations: a) a body temperature of $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$, b) a heart rate of >90 beats/min c) tachypnea as manifested by a respiratory rate of >20 breaths/min or hyperventilation, as indicated by a PaCO_2 of <4.3 kPa, d) an alteration of the white blood cell counts of > 12.000 cells / mm^3 , <4.000 cells / mm^3 , or presence of $>10\%$ immature (banded) neutrophils⁵³.

We performed a literature review in search of circumferential evidence that would support our hypothesis that trauma-induced systemic inflammation can impair bone healing.

We found one study by Reikeras et al., which showed in 2005 that intraperitoneal injections with lipopolysaccharides (LPS), which is part of the outer membrane of bacteria, into rats with femur fractures impairs fracture healing⁵⁴. It is known that LPS also referred to as endotoxin, can induce a strong systemic inflammatory response once it is recognized by LPS receptors. These receptors include Toll-like receptor-4 (TLR4) and are expressed by multiple cells of the immune system⁵⁵. Reikeras described that they used the endotoxemia model since it was shown that trauma induces translocation of LPS from the gut into the peripheral circulation. Sepsis is the systemic inflammatory response to infection and by definition requires the presence of microorganisms⁵⁶. Endotoxemia mimics the presence of microorganisms and is therefore frequently used as a model of sepsis⁵⁷. Sterile severe tissue injury and endotoxemia are clearly different entities, however, both conditions are similar in the fact that they can induce SIRS.

After having found this study, we developed a hypothesis about how systemic inflammation can negatively affect bone regeneration. A significant amount of research has focused on development of Acute Respiratory Distress Syndrome (ARDS) and Multiple Organ Dysfunction Syndrome (MODS) after major trauma. It is a generally accepted concept that neutrophils play an important role in development of these severe complications seen after major trauma⁵⁸. Moreover, two animal studies have shown that depletion of neutrophils improves bone healing in animals^{21,22}. Therefore, we hypothesized that increased influx of alternatively activated neutrophils into the FH impairs fracture healing after major trauma⁵⁰. During the same month that our review was published, Recknagel et al. published a study, which showed that experimental blunt thoracic injury impairs fracture healing in rats. A later study showed that blunt chest injury induced increased influx of neutrophils into the FH at day 3 after injury, as well as decreased numbers of macrophages at day 3 and

day 7 after trauma in rats⁵⁹. These findings supported our hypothesis that increased influx of (alternatively activated) neutrophils into the FH mediates impairment of bone repair after severe injury. The authors mentioned that induction of a systemic inflammatory response as a possible underlying mechanism⁶⁰. Subsequent studies showed that adding soft-tissue injury to thoracic injury even further disturbs bone healing in rats^{61,62}. Intravenous administration of a C5aR-antagonist could abolish the deleterious effect of blunt chest injury on fracture healing in rats⁶³. This finding implies that C5a mediates the deleterious effect of trauma-induced systemic inflammation on the outcome of fracture healing. A recent study in mice showed that depletion of neutrophils did not abolish the negative effect of blunt chest injury on fracture healing¹⁹. The authors therefore propose that neutrophils may not play a crucial pathomechanistic role in compromised fracture healing induced by additional thoracic trauma. However, it is questionable whether this conclusion is justified. Blunt chest injury in mice did not induce increased influx of neutrophils and decreased influx of macrophages into the FH at day 3 post-injury¹⁹, which is in contrast to a similar experiment performed in rats⁵⁹. Moreover, the number of neutrophils / mm² within the FH at day 3 after an isolated femur fracture was 10-fold higher in mice compared to rats. There is extensive data which illustrates that humans, mice and rats react differently to injury, inflammation and infection^{64,65}. Recent articles have highlighted tradeoffs that species make to balance often opposing evolutionary strategies for resistance versus tolerance to inflammatory stimuli⁶⁴. **Resistance** is the ability to limit antigen burden and **tolerance** is a state of indifference or non-reactivity towards an antigen that would normally be expected to excite an immunological response⁶⁶. Relative to the human response, mice are highly tolerant to inflammatory challenge. For example, the lethal dose of endotoxin is 5–25 mg/kg for most strains of mice, whereas a dose that is 1,000,000-fold less (30 ng/kg) has been reported to cause shock in humans⁶⁷. The results of animal studies therefore need to be interpreted with caution. In my opinion, neutrophils may therefore still play an important role in impairment of fracture healing after major trauma.

UPOD

Our hypothesis that trauma-induced systemic inflammation could impair fracture healing was eventually supported by several animal studies, but human studies were still lacking. We, therefore, performed two retrospective studies in severely injured patients with either tibial or femoral fractures and determined whether the systemic inflammatory response to major trauma correlates with the outcome of fracture healing.

Cytokines released systemically after severe trauma affect leukocyte kinetics, such as leukocyte mobilization from the bone marrow and leukocyte migration towards injured tissue, as well as the phenotype of peripheral blood leukocytes and hematopoiesis⁶⁸.

Peripheral blood concentrations of leukocyte subsets, but also of erythrocytes and platelets, thus reflect the systemic response to tissue injury. We retrospectively compared the peripheral blood-count curves of leukocytes, neutrophils, monocytes, lymphocytes, thrombocytes, and hemoglobin during the first 2 weeks after injury between multitrauma patients with normal and impaired fracture healing of the tibia. We subsequently performed a similar study in multitrauma patients with femoral fractures. The abovementioned hematological parameters were retrieved from the Utrecht Patient Oriented Database (UPOD), which stores hematological parameters, even when these parameters were not requested by clinicians during the patients' admission.

Patients with normal fracture healing of the tibia and femur showed similar leukocyte kinetics during the first two weeks after injury (Figure 4). Leukocytes were above reference values at admittance to the emergency department.

It is believed that release of cytokines after major trauma induces early release of leukocytes from the marginated pool into the circulation⁶⁹, which may explain leukocytosis during admittance to the emergency department.

After day 1, mean leukocyte counts decreased to reference values and from day 5 onward, leukocyte numbers increased and rose above reference values after day 7. The average leukocyte count was above reference values during the second week after injury in patients

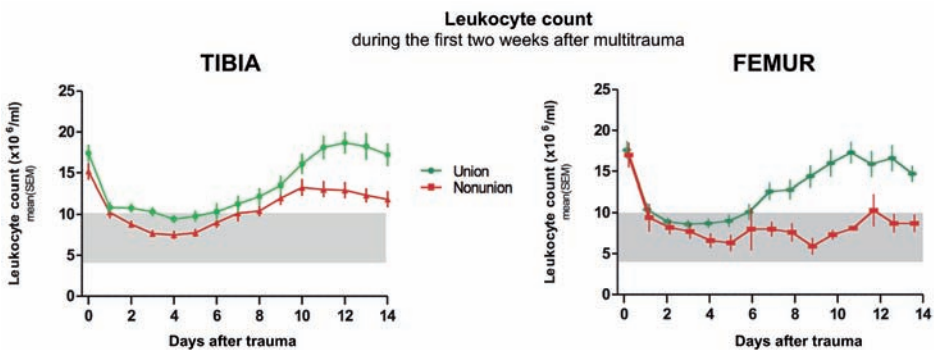


Figure 4 Peripheral blood leukocyte counts during the first two weeks after in multitrauma patients with normal and impaired fracture healing of the tibia and femur.

with normal fracture healing of the tibia and femur (Figure 4). Increased white blood cell counts or leukocytosis is considered a sign of systemic inflammation⁵³.

We found that during the second week after injury, patients also exhibited leukocytosis without clinically evident infections or recent surgical interventions. We, therefore, believe that leukocytosis during the second week after trauma may not necessarily represent systemic inflammation, but rather may be caused by a physiological bone marrow response to severe tissue injury. Even though such a bone marrow response follows a non-infectious

insult to the body during the preceding week, it is debatable whether this response should be termed “systemic inflammation” as well, or whether it is a bone marrow response caused by trauma-induced systemic inflammation. It has been shown previously that experimental tissue injury in animals stimulates myelopoiesis⁷⁰. We found that neutrophil, monocyte and thrombocyte counts, which are part of the myeloid lineage, rose above reference values during the second week after injury in multitrauma patients with normal fracture healing of the tibia and femur. In contrast, lymphocytes remained within reference values and hemoglobin remained below reference values during the first two weeks after injury in patients with normal fracture healing. Our finding that peripheral blood myeloid cells also increased during the second week after injury supports the idea that leukocytosis may be caused by trauma-induced stimulation of myelopoiesis. The correlation between stimulated myelopoiesis and adequate fracture healing remains unclear. Our data suggests that myeloid cells contribute to fracture healing by rapidly establishing fibronectin+ ECM within the FH (Chapter 2). Increased myeloid cell counts within peripheral blood of multitrauma patients may therefore represent adequate recruitment of these cells from the bone marrow, that subsequently home towards injured tissue and start to synthesize ECM. Another explanation for the correlation that we found between high myeloid cells counts and adequate fracture healing is that certain cytokines do not only stimulate myelopoiesis but also (in) directly stimulate stromal cells that contribute to synthesis of bone tissue.

In multitrauma patients with impaired healing of the tibia and femur we found that during the second week after injury peripheral myeloid cell counts were decreased compared to patients with normal fracture healing. The decreased myeloid cell counts may reflect increased extravasation of inflammatory cells toward injured tissue in the impaired healing-group, which would support the hypothesis that we described in our review⁷¹. An alternative explanation of decreased peripheral blood myeloid cells in the impaired healing group is inhibition of trauma-induced myelopoiesis. Hematopoietic failure has been observed in animals after experimental shock, hypoxia, soft tissue injury and thermal injury⁷²⁻⁷⁴. Bone marrow failure has also been described as one facet of the multiple organ dysfunction syndrome (MODS) and is commonly seen in patients recovering from severe trauma and hemorrhagic shock⁷². It remains unclear whether there is a causal relation between inhibition of trauma-induced myelopoiesis and poor bone regeneration or whether these two phenomena are separate consequences of an aberrant systemic response without a causal relation between them. In humans, hematopoietic failure after major trauma has been associated with inhibited growth of stromal cells⁷². Based on this finding, we believe that impaired function of stromal cells may also mediate impairment of fracture healing in multitrauma patients that exhibited decreased peripheral blood myeloid cells during the second week after injury.

Factors that may contribute to a different systemic inflammatory response include the type and extent of injury, the time between injury and resuscitation, the amount of ischemia/re-

perfusion damage, or host factors, such as smoking and genetic background, the extent of shock, infectious complications, and the type, timing, and number of operative procedures. We found no significant difference in the incidence of infectious complications, total amount of tissue damage, or severity and localization of injuries. However, our study did not have enough power to state that all aforementioned parameters were equally distributed between the outcome groups.

Future studies should try to clarify to which extent the difference in systemic response between outcome groups is caused by host factors or by differences in type and extent of injury. We were only able to compare the amount of tissue injury based on clinical scales of severity (ISS and New ISS). These scales may not be sensitive enough to detect biological differences in the amount of tissue injury between outcome groups. The Injury Severity Score (ISS) is an established medical score to assess trauma severity⁷⁵ and is used to define the term “major trauma” or “multitrauma victim” (ISS above 15). The ISS is based upon the Abbreviated Injury Scale (AIS), which classifies each injury to its relative severity on a six-point ordinal scale: 1. minor, 2. moderate, 3. serious, 4. severe, 5. critical and 6. currently untreatable (maximal). Subsequently, the body is divided into six ISS body regions (head and neck, face, chest, abdomen, extremities and external). The ISS is calculated by taking the highest AIS in each of the three most severely injured ISS body regions, squaring each AIS code and adding the three squared numbers ($ISS = A^2 + B^2 + C^2$). The New ISS (NISS) allows the three most severe injuries to be scored, irrespective of region affected. It is debatable whether moderate brain injury has a similar immunological impact as moderate injury of the extremities.

In a prospective study, blood may be drawn from trauma patients during admittance to the emergency department, which allows quantification of several Damage Associated Molecular Patterns (DAMPs), as well as pro- and anti-inflammatory cytokines. This together may better reflect the extent of tissue injury than a clinical scale such as the ISS and NISS. Moreover, host factors such as genetic variations can be determined in these blood samples and compared between outcome groups. Possible targets of genetic variations that can be compared between outcome groups include single nucleotide polymorphisms (SNPs)⁷⁶ of receptors that recognize different DAMPs such as Toll-like receptors (TLRs)⁷⁷ as well as pro- and anti-inflammatory cytokines and targets involved in the signaling cascade that follows receptor binding. Ideally, the FHs of these patients would also be isolated if ORIF is indicated and linked to the composition of peripheral blood, genetic factors and outcome of bone healing.

Clarifying to which extent host and injury related factors contribute to the difference in systemic response between outcome groups may allow early identification of patients that have an increased risk of developing impaired fracture healing.

Finally, we included only multitrauma patients ($ISS > 15$) into our study. Due to the extent of their injuries, blood is frequently drawn from these multitrauma patients during the first

two weeks after injury. It is difficult to perform a similar retrospective study in patients with isolated fractures using the UPOD, since blood is drawn far less frequently in patients with isolated injuries. This may prevent adequate comparison of the systemic response during the second week after injury between outcome groups. Future prospective studies should therefore also include patients with isolated injuries after low energy trauma.

INHIBITION OF STROMAL CELLS

Our finding that peripheral blood leukocyte counts are decreased during the first two weeks after injury may reflect increased extravasation of leukocytes towards sites of injury such as the FH in multitrauma patients with impaired fracture healing. We hypothesized that high neutrophil numbers within the FH inhibit synthesis of mineralized extracellular matrix (ECM) by bone marrow stromal cells (BMSCs) and thereby impair bone healing. We, therefore, studied the effect of increasing concentrations of neutrophils on ECM synthesis by human BMSCs *in vitro*. Moreover, we determined how high neutrophil concentrations affect BMSC cell counts, as well as BMSC osteogenic activity determined by alkaline phosphatase (ALP) expression and ALP activity. Co-culture of BMSCs with neutrophils induced a significant decrease in BMSC cell count, in the percentage of ALP+ cells and in total ALP activity. In addition, a significant decrease was found in the amount of mineralized ECM synthesized by BMSCs after 4 weeks of culture. These findings may explain how increased or prolonged influx of neutrophils into the FH disturbs fracture healing after major trauma.

Co-cultures with peripheral blood mononuclear cells and neutrophils within trans-wells did not induce a significant decrease in ALP activity, which makes depletion of nutrients within the medium an unlikely underlying mechanism. Our finding that trans-well experiments did not induce a significant decrease in ALP implies that cell-cell contact between neutrophils and BMSCs is required for neutrophils to exert their effect. However, it may well be that after cell-cell contact, neutrophils degranulate and finally exert their effect through one or multiple secreted agents. We were unable to demonstrate a decrease in BMSCs viability, which makes neutrophil-induced BMSC cell death *in vitro* unlikely. However, BMSCs are cells that adhere to the plastic surface of each well. Theoretically, neutrophils may cause apoptosis or destruction of BMSCs after which BMSCs become non-adherent and may dissolve or become phagocytized by neutrophils. Our vitality assay was performed on the adherent cell population and may miss the abovementioned theoretical scenario.

It is possible that inhibition of BMSCs function by neutrophils is part of a physiological regenerative process. The early FH contains a significant amount of debris that needs to become degraded and phagocytized by inflammatory cells. Neutrophils are known to be equipped with an extensive cytotoxic armamentarium, consisting of ECM degrading enzymes such as collagenase, elastase, and proteases, as well as the capacity to form reactive

oxygen species (ROS), which are highly cytotoxic and can induce tissue injury⁷⁸. In addition, the FH may be contaminated by pathogens in case of open fractures that also need to become neutralized by inflammatory cells. It is plausible that these processes make the early FH a suboptimal environment for BMSCs to start synthesizing new ECM. Moreover, our previous study showed that neutrophils synthesize fibronectin+ ECM within the early FH. Hypothetically, BMSCs may use this neutrophil derived fibronectin+ ECM as a scaffold on which they synthesize a collagenous ECM. Neutrophils may therefore keep BMSCs in “stand-by mode” until synthesis of a fibronectin+ ECM by neutrophils is completed and debris and pathogens are removed from the FH.

Although this effect of neutrophils on BMSCs may be part of a physiological process, we speculate that increased or prolonged inhibition of BMSCs by neutrophils may impair bone healing. Future studies should focus on the mechanism through which neutrophils exert their effect on BMSCs. The current literature provides several potential targets that may be blocked on neutrophils in order to prevent a negative effect of neutrophils on BMSCs. These include blocking membrane bound receptors on neutrophils that stimulate degranulation, several intracellular targets that inhibit one of the four steps of exocytosis (granule recruitment from the cytoplasm to the target membrane, vesicle tethering and docking, granule priming and granule fusion) or direct inhibitors of granule content⁷⁹. Neutrophils express several surface receptors of which some provide inhibitory and others provide activating signals. Key activating receptors such as proteinase-activated receptor (PAR)-2, Fcγ receptors, Toll-like receptors, integrin and chemokine receptors have the potential for their effects to be blocked by small-molecule antagonists or antibodies⁸⁰. In addition, neutrophils contain at least four different types of granules: (1) primary granules, also known as azurophilic granules; (2) secondary granules, also known as specific granules; (3) tertiary granules; and (4) secretory vesicles. The primary granules are the main storage site of the most toxic mediators, including elastase, myeloperoxidase, cathepsins, and defensins. The four secretory organelles present in neutrophils engage sequentially in exocytosis depending on stimuli strength⁸¹. Recent studies identified small molecule inhibitors of the interaction between the small GTPase Rab27a and its effector JFC1, two central regulators of neutrophil exocytosis. These compounds, named Nexinhibs (neutrophil exocytosis inhibitors), inhibit intracellular trafficking, docking, fusion, and exocytosis of specific and azurophilic granules in human neutrophils *in vitro* and *in vivo* without affecting other important innate immune responses, including phagocytosis and neutrophil extracellular trap (NET) production⁸¹. Future studies may incubate neutrophils with Nexinhibs before co-culture with BMSCs and determine whether a negative effect on BMSCs persists. Moreover, different neutrophil subsets may be co-cultures with BMSCs to determine whether they exhibit a similar effect as neutrophils isolated from peripheral blood of healthy donors. These subsets include banded and hypersegmented neutrophils from peripheral blood of trauma patients^{25,82}, N1 and N2 polarized neutrophils²⁷, as well as neutrophils isolated from the FH and adjacent bone marrow.

CHEMOTAXIS

In addition to the abovementioned targets that could prevent a negative effect of neutrophils on BMSCs, we believe that modulating chemotaxis of neutrophils towards the FH may also be a target to prevent impairment of fracture healing after major trauma. We therefore investigated whether chemotaxis of neutrophils towards the FH could be studied *in vitro*. Moreover, we determined whether chemotaxis of neutrophils towards the FH was mediated by the CXCR1, CXCR2, FPR, and C5aR receptors. Human FHs were isolated during an ORIF procedure within 3 days after trauma and spun down to obtain the FH serum. Neutrophil migration towards the FH was studied using Ibidi™ Chemotaxis3D μ -Slides and image analysis of individual neutrophil tracks was performed. Our study showed that the human FH induces significant neutrophil chemotaxis, which was not affected by blocking CXCR1 and CXCR2. In contrast, neutrophil chemotaxis towards the FH was significantly inhibited by chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS), which blocks FPR and C5aR⁸³. Blocking only C5aR with CHIPS Δ 1F also significantly inhibited neutrophil chemotaxis towards the FH.

Based on these results, we speculate that blocking C5aR *in vivo* may prevent increased influx of neutrophils into the FH after major trauma and thereby prevent impairment of bone healing. This assumption is supported by a recent study in rats, which showed that short-term inhibition of C5aR could abolish the deleterious effects of blunt chest injury on fracture healing⁶³. C5aR-antagonism did not significantly affect bone regeneration in an experimental model of isolated fracture healing in rats without blunt chest injury⁸⁴. It remains unclear whether the observed beneficial effect of C5aR antagonism after blunt chest injury in rats was mediated by inhibition of neutrophil influx into the FH.

It has been demonstrated that C5aR is abundantly expressed in the fracture callus of rats, not only by immune cells during the early inflammatory phase but also by osteoblasts, chondroblasts and osteoclasts throughout the entire healing period⁸⁵. Moreover, a recent study showed that fracture healing is impaired in C5- knockout mice, which implies that C5 may be important during multiple stages of fracture healing.⁸⁶

Future studies that aim to prevent impaired fracture healing after major trauma should therefore carefully consider patient selection as well as timing and duration of a therapeutic intervention. Theoretically, prolonged or delayed C5aR-antagonism may negatively affect bone repair, while short-term inhibition of C5aR may positively affect bone healing after major trauma. In addition, the mode of administering a therapeutic agent needs consideration. Systemic administration of C5aR antagonists at the emergency department or local injection of Nexinhibs into the FH several days after injury may both be considered as possible therapeutic strategies.

CONCLUSION

Our data suggests that neutrophils contribute to fracture healing by synthesizing a fibronectin+ extracellular matrix within the FH rapidly after injury. The current literature suggests that major trauma may induce an undesirable systemic inflammatory response that impairs fracture healing, potentially through an increased influx of neutrophils into the FH. We found that the systemic response to trauma differs between multitrauma patients with normal and impaired fracture healing of the tibia and femur. The difference in response that we found may represent increased extravasation of inflammatory cells towards sites of injury or trauma-induced inhibition of a myeloid bone marrow response. Our additional study showed that high neutrophil concentrations negatively affect synthesis of ECM by BMSCs in vitro. This finding could explain how increased influx of neutrophils into the FH impairs bone healing after severe injury. Blocking the C5a-receptor in neutrophils with CHIPSΔ 1F could inhibit chemotaxis of neutrophils towards the FH in vitro. Future studies should further clarify how inflammatory cells contribute to fracture healing. These studies may contribute to the development of therapies that augment tissue regeneration and prevent impairment of bone healing after major trauma.

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The page features several decorative purple circles and shapes of varying sizes and shades, scattered across the white background. Some are solid purple, while others are lighter or have a gradient effect. The shapes are positioned in the upper left, middle left, and lower right areas of the page.

APPENDICES

Lekensamenvatting

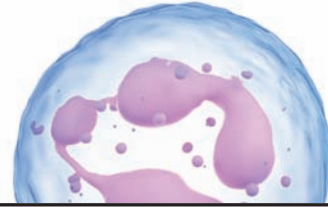
List of abbreviations

Dankwoord

List of publications

Curriculum Vitae

LEKENSAMENVATTING



HET PROBLEEM

Wanneer iemand tijdens het voetballen zijn scheenbeen breekt, is de kans dat die breuk niet geneest ongeveer 1-5%. Dit percentage stijgt naar 25% wanneer iemand tegelijkertijd meerdere letsels oploopt, zoals bij een ernstig auto-ongeval. Verstoorde botgenezing heeft een forse impact op het leven van patiënten en brengt aanzienlijke kosten met zich mee. Er



BOTBREUKEN GENEZEN SLECHTER WANNEER IEMAND MEERDERE LETSELS TEGELIJKERTIJD OPLOOPT.

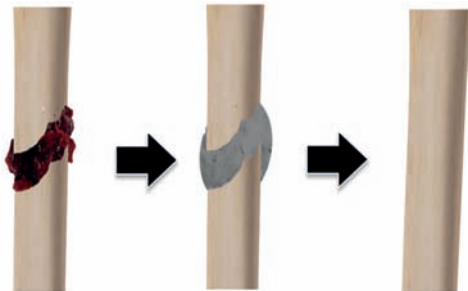
wordt daarom veel onderzoek gedaan naar stimulatie van het genezingsproces en het voorkomen van verstoorde botgenezing. De huidige literatuur suggereert dat ontsteking een belangrijke rol speelt tijdens de normale botgenezing, maar ook bij de verstoring van de botgenezing. Ons onderzoek probeert de rol van ontsteking tijdens zowel de normale botgenezing als de verstoring van de botgenezing op te helderen.

DE BOTGENEZING

Botten genezen vaak via drie overlappende fases: de ontstekingsfase, de genezingsfase en de remodeleringsfase. Direct na het letsel ontstaat een bloeditstorting, die fractuurhematoom genoemd wordt. Witte bloedcellen (leukocyten) infiltreren deze bloeditstorting. Witte bloedcellen staan met name bekend om hun capaciteit om ziekteverwekkers te doden en beschadigd weefsel op te ruimen. Hun rol tijdens het genezingsproces is daarentegen nog erg onduidelijk, maar lijkt wel essentieel te zijn voor adequate genezing. Zo leidt het verwijderen van het fractuurhematoom tot verstoorde botgenezing en leidt transplantatie van het fractuurhematoom naar spierweefsel tot botvorming in de spier.

Na de ontstekingsfase, wordt het fractuurhematoom geïnfiltreerd door cellen die bestaan uit stamcellen (multipotente stromale cellen of MSCs), botvormende cellen (osteoblasten), kraakbeenvormende cellen (chondroblasten) en bindweefselvormende cellen (fibroblasten). Tijdens deze genezingsfase wordt er steunweefsel (extracellulaire matrix of ECM) gemaakt dat kris-kras door elkaar is neergelegd en later verkalkt (mineraliseert).

Tijdens de remodeleringsfase wordt dit weefsel omgezet naar parallel georganiseerd bot (lamellair bot). In tegenstelling tot de meeste andere weefsels, geneest bot littekenloos en krijgt het zijn originele sterkte weer terug.

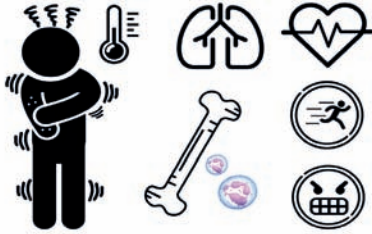


BOTGENEZING WORDT OPGEDEELD IN:
 1. DE ONTSTEKINGSFASE,
 2. DE GENEZINGSFASE
 3. DE REMODELLERINGSFASE

LEKENSAMENVATTING



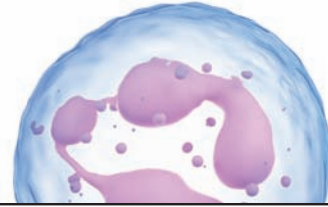
SYSTEMISCHE INFLAMMATIE



**TIJDENS SYSTEMISCHE INFLAMMATIE:
STIJGT DE LICHAAMSTEMPERATUUR, DE ADEMHALINGSFREQUENTIE,
EN DE HARTSLAG.
ER KOMEN EXTRA WITTE BLOEDCELLEN VRIJ UIT HET BEENMERG.
WITTE BLOEDCELLEN BEWEGEN SNELLER EN WORDEN AGRESSIEVER.**

Wanneer iemand meerdere letsels tegelijkertijd oploopt, kan het lichaam reageren met een afweerreactie die systemische inflammatie wordt genoemd. Deze reactie lijkt op de afweerreactie tegen een infectie, zoals de griep. De hartslag, lichaamstemperatuur en ademhalingsfrequentie nemen toe. Extra witte bloedcellen worden vrijgemaakt uit het beenmerg en komen in de bloedsomloop terecht. Tevens gaan de witte bloedcellen sneller bewegen en worden ze "agressiever". Wanneer een infectie uit de hand loopt draagt een groter aantal "agressieve" witte bloedcellen vaak bij aan het onder controle krijgen van de infectie. Deze afweerreactie kan echter ook doorschieten en forse schade aan meerdere organen veroorzaken, met soms zelfs een dodelijke afloop. Systemische inflammatie komt veel vaker voor na het oplopen van meerdere letsels dan na enkelvoudig letsel. Wij denken dat er tijdens systemische inflammatie te veel agressieve witte bloedcellen naar het fractuurhematoom bewegen en dat dit de ontstekingsfase van de botgenezing verstoort.

LEKENSAMENVATTING



HET PROMOTIEONDERZOEK

Dit promotieonderzoek wordt opgedeeld in vier onderzoeksvragen:

1. Wat is de functie van witte bloedcellen tijdens de ontstekingsfase van de botgenezing?
2. Hoe beïnvloeden witte bloedcellen de vorming van botweefsel door stamcellen?
3. Verschilt de afweerreactie tussen patiënten met normale en verstoorde botgenezing?
4. Kan beweging van witte bloedcellen richting de breuk geremd worden?

Om de functie van witte bloedcellen tijdens de ontstekingsfase van de botgenezing op te helderen, worden fractuurhematomen van patiënten, die een ongeval hebben doorgemaakt, in chronologische volgorde geplaatst en bekeken onder de microscoop. Op deze manier kan de ontwikkeling van bloeditstorting tot botweefsel bestudeerd worden. Vervolgens worden bepaalde witte bloedcellen (neutrofielen) in kweek gebracht met stamcellen (multipotente stromale cellen of MSCs) en bekeken hoe neutrofielen de celdeling van stamcellen, de uitrijping van stamcel tot botvormende cel en de productie van botweefsel door stamcellen beïnvloeden. Het derde onderzoek vergelijkt de afweerreactie tussen patiënten met normale en verstoorde botgenezing van een scheenbeenbreuk en dijbeenbreuk na een ernstig letsel. De concentratie van bloedcellen (verschillende typen witte bloedcellen, rode bloedcellen en bloedplaatjes) in bloed tijdens de eerste twee weken na het letsel wordt daarbij gezien als een afspiegeling van de afweerreactie. Het vierde onderzoek probeert de eerste stap te zetten richting een oplossing voor de verstoorde botgenezing na een ernstig letsel. Als onze hypothese klopt dat toegenomen beweging van witte bloedcellen (neutrofielen) richting het fractuurhematoom de botgenezing verstoort, zal het volgende doel zijn om deze toegenomen beweging te remmen. Receptoren zijn een soort zintuigen van cellen, waarmee ze kunnen "ruiken" waar bijvoorbeeld een infectie of letsel is. Door verschillende receptoren op neutrofielen te blokkeren, proberen we de beweging van neutrofielen naar het fractuurhematoom te remmen.

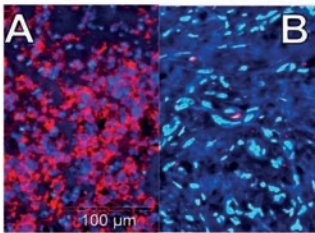


LEKENSAMENVATTING

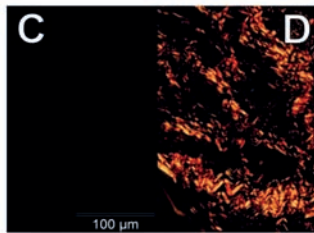


DE ROL VAN WITTE BLOEDCELLEN TIJDENS DE BOTGENEZING

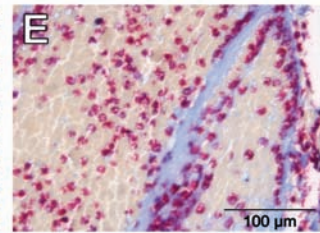
Fractuurhematomen zijn geïsoleerd uit patiënten op verschillende momenten na het letsel, variërend van enkele uren tot 3 weken na het ongeval. Door deze fractuurhematomen in chronologische volgorde te plaatsen konden we de rol van witte bloedcellen tijdens de botgenezing bestuderen. We vonden dat witte bloedcellen bijdragen aan de botgenezing door binnen enkele uren weefsel te maken in het fractuurhematoom. Dit "spoed-weefsel" werd tijdens de ontstekingsfase van de botgenezing gemaakt en had een andere samenstelling dan botweefsel dat tijdens de genezingsfase werd gemaakt.



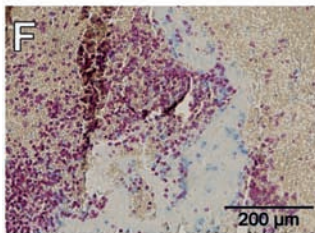
HET FRACTUURHEMATOOM ZIT TIJDENS DE EERSTE PAAR DAGEN NA HET LETSEL (A) VOL MET WITTE BLOEDCELLEN (ROOD). NA 3 WEKEN (B) ZIJN PRAKTISCH ALLE WITTE BLOEDCELLEN (ROOD) VERDWEENEN.



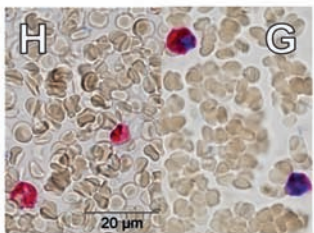
TIJDENS DE EERSTE PAAR DAGEN (C) IS ER PRAKTISCH GEEN BOTWEEFSEL ZICHTBAAR. NA 3 WEKEN IS ER DUIDELIJK BOTWEEFSEL (GEEL/ORANJE) ZICHTBAAR IN HET FRACTUURHEMATOOM DAT KRIS-KRAS IS NEERGELEGD.



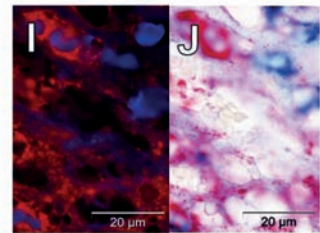
BINNEN DRIE DAGEN NA HET LETSEL BEGINNEN WITTE BLOEDCELLEN (ROOD) EEN SOORT SPOED-WEEFSEL AF TE ZETTEN (BLAUW) DAT EEN ANDERE SAMENSTELLING HEEFT DAN BOTWEEFSEL.



VERSCHILLENDE TYPEN WITTE BLOEDCELLEN (NEUTROFIELEN ROOD EN MACROFAGEN BLAUW) HEBBEN EEN VOORKEURSLLOCATIE IN HET FRACTUURHEMATOOM.

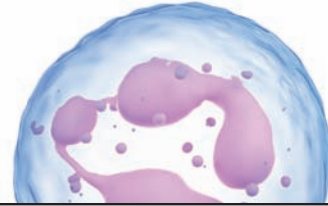


NEUTROFIELEN (ROOD) IN DE BLOEDSOMLOOP (H) MAKEN NOG GEEN SPOED-WEEFSEL (BLAUW) MAAR IN HET FRACTUURHEMATOOM (G) BEGINNEN ZE DIT WEL AAN TE MAKEN.



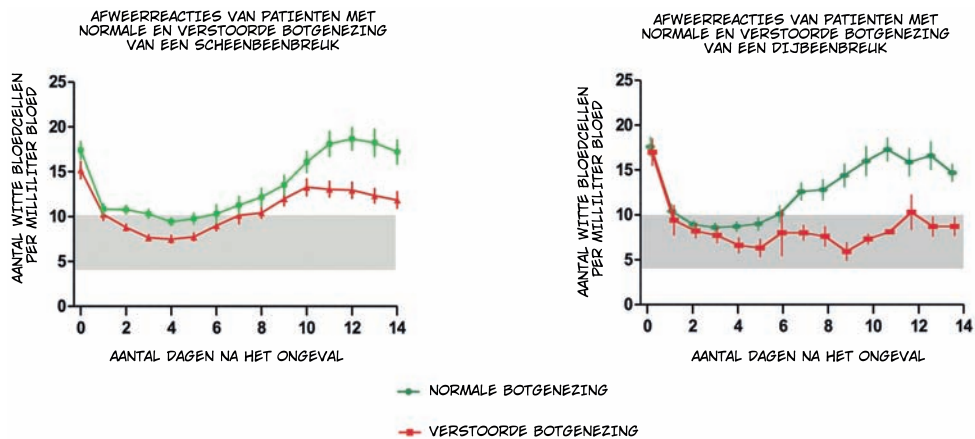
IN HET FRACTUURHEMATOOM ZIJN PARTIKELS ZICHTBAAR DIE AFKOMSTIG ZIJN VAN NEUTROFIELEN (ROOD). WELLIJCH BEVATTEN DEZE PARTIKELS BOLWISTENEN VOOR SPOED-WEEFSEL.

LEKENSAMENVATTING



DE RELATIE TUSSEN DE AFWEERREACTIE EN DE UITKOMST VAN DE BOTGENEZING

In het UMC Utrecht worden bloedwaarden van patiënten opgeslagen in een database, zelfs wanneer deze waarden niet door een arts zijn aangevraagd. Deze database heet de Utrecht Patient Oriented Database (UPOD). Door gebruik te maken van de UPOD, konden we de afweerreactie vergelijken tussen patiënten met normale en verstoorde botgenezing van scheenbeenbreuken en dijbeenbreuken. Het aantal witte bloedcellen in de bloedsomloop weerspiegelt de afweerreactie (systemische inflammatie). We vonden dat de concentratie witte bloedcellen in de bloedsomloop tijdens de eerste twee weken na het letsel significant lager was bij patiënten met verstoorde botgenezing van scheenbeen breuken en dijbeenbreuken. Een lager aantal witte bloedcellen in de bloedsomloop kan veroorzaakt worden doordat meer witte bloedcellen uittreden vanuit de bloedsomloop richting de letsels. Dit komt overeen met onze hypothese dat een toegenomen migratie van agressieve witte bloedcellen naar het fractuurhematoom bij patiënten na een ernstig ongeval de botgenezing verstoort.



LEKENSAMENVATTING



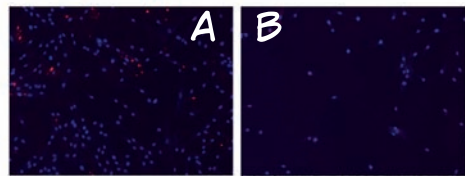
HET EFFECT VAN WITTE BLOEDCELLEN OP STAMCELLEN

Stamcellen zijn cellen, die kunnen delen en veranderen in andere celtypes (differentiëren). In het beenmerg bevinden zich stam cellen (MSCs), die niet tot elk type cel uitrijpen, maar wel tot botcel, kraakbeencel en vetcel. Wij hebben deze stamcellen in kweek gebracht met oplopende concentraties van een type witte bloedcel (neutrofielen). Vervolgens hebben we het effect van oplopende concentraties neutrofielen op de celdeling (proliferatie) van stamcellen onderzocht, alsmede op hun uitrijping tot botvormende cellen (osteoblasten) en de hoeveelheid botweefsel (ECM) die gevormd wordt.

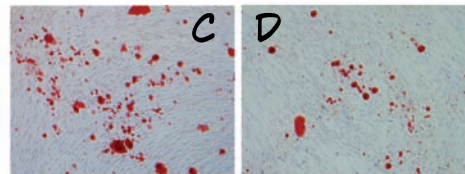
Nadat de stamcellen een week met neutrofielen samen werden gekweekt, waren er minder stamcellen, minder botvormende cellen en minder gemineraliseerd botweefsel dan wanneer de stamcellen zonder neutrofielen werden gekweekt. Dit suggereert, dat hoge concentraties neutrofielen een nadelig effect hebben op stamcellen.

Wellicht is het juist de bedoeling dat stamcellen nog even op "stand-by" gehouden worden tijdens de ontstekingsfase van de botgenezing zodat alle beschadigde cellen en bacteriën eerst opgeruimd kunnen worden. Voor deze processen gebruiken neutrofielen zeer agressieve stoffen, die weefsels afbreken, zodat ze opgeruimd kunnen worden. Dit lijkt geen goed milieu voor stamcellen om nieuw botweefsel te gaan produceren. Na een week zijn deze neutrofielen verdwenen uit het fractuurhematoom en is er veel spoedweefsel ontstaan, wat mogelijk een mal is voor stamcellen om het nieuwe botweefsel in af te zetten.

De vraag is of een toegenomen en langdurigere migratie van "agressieve" neutrofielen naar het fractuurhematoom wellicht een te sterke en langdurige onderdrukking van stamcellen geeft, waardoor de genezingsfase niet goed wordt opgestart en er onvoldoende botvorming optreedt.

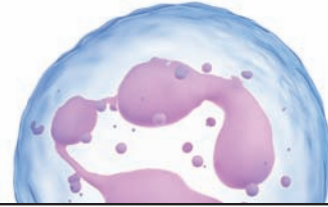


WANNEER STAMCELLEN MET HOGERE CONCENTRATIES NEUTROFIELEN (B) IN KWEEK GEBRACHT WORDEN DAALT HET AANTAL STAMCELLEN (BLAUW) EN BOTVORMENDE CELLEN (ROOD) T.O.V. EEN KWEEK ZONDER NEUTROFIELEN (A).



OOK DE HOEVEELHEID VERKALT BOTWEEFSEL (ROOD) NEEMT AF WANNEER STAMCELLEN IN KWEEK GEBRACHT WORDEN MET HOGERE CONCENTRATIES NEUTROFIELEN (D) T.O.V. EEN KWEEK ZONDER NEUTROFIELEN (C).

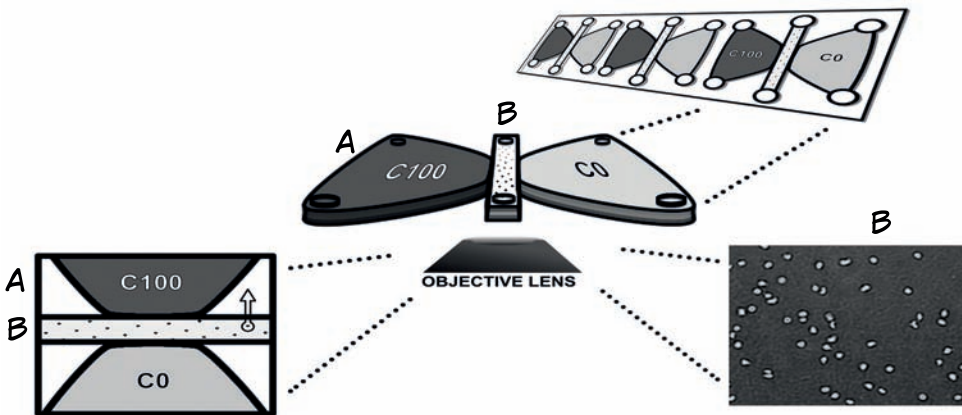
LEKENSAMENVATTING



REMMING VAN DE WITTE BLOEDCELMIGRATIE RICHTING HET FRACTUURHEMATOOM

Door het fractuurhematoom te centrifugeren, ontstaat er bovenop de afgedraaide cellen een vloeistof: het serum. Deze vloeistof hebben we in een klein kamertje geplaatst (A). Uit het bloed van gezonde donoren hebben we neutrofielen (een type witte bloedcel) geïsoleerd en in een tweede kamertje met een gel geplaatst (B), die grenst aan de kamer met het serum van het fractuurhematoom (A). Binnen enkele minuten begonnen de neutrofielen zeer duidelijk richting het serum van het fractuurhematoom te bewegen. Deze nieuwe studieopzet toont aan dat migratie van neutrofielen richting het fractuurhematoom buiten het lichaam bestudeerd kan worden.

Vervolgens hebben we verschillende receptoren (een soort zintuig van cellen, waarmee ze een infectie of letsel kunnen "ruiken") geblokkeerd en gekeken of de neutrofielen nog steeds richting het serum van het fractuurhematoom bewogen. Bij blokkade van een bepaalde receptor (de C5a receptor) was er significant minder beweging van neutrofielen richting het fractuurhematoom. Dit zou in de toekomst een mogelijk aangrijpingspunt van medicijnen kunnen worden, die de botgenezing proberen te verbeteren na een ernstig ongeval.



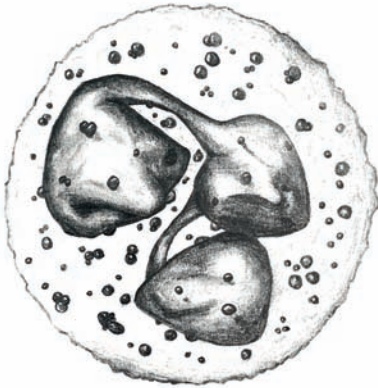
LEKENSAMENVATTING



SAMENVATTING

DE NEUTROFIEL

(EEN BEPAALD TYPE WITTE BLOEDCEL)



...DRAAGT BIJ AAN DE BOTGENEZING DOOR SNEL NA HET LETSEL SPOED-WEEFSEL TE PRODUCEREN IN HET FRACTUURHEMATOOM.

...KOMT IN LAGERE CONCENTRATIES VOOR IN DE BLOEDSOMLOOP VAN PATIENTEN DIE VERSTOORDE BOTGENEZING ONTWIKKELEN VAN EEN SCHEENBEEFBREUK EN DIJBEEFBREUK. DIT WORDT MOGELIJK VEROORZAAKT DOORDAT ER MEER NEUTROFIELEN VANUIT DE BLOEDSOMLOOP NAAR LETSELS MIGREREN BIJ PATIENTEN DIE EEN ERNSTIG ONGEVAL HEBBEN DOORGEMAAKT.

...REMT DE CELDELING VAN STAMCELLEN, DE WITRIJPING VAN STAMCEL TOT BOTVORMENDE CEL EN DE HOEVEELHEID GEMINERALISEERD BOTWEEFSEL DAT DOOR STAMCELLEN WORDT GEMAAKT.

...GEBRUIKT DE CSA-RECEPTOR OM RICHTING HET FRACTUURHEMATOOM TE BEWEGEN.

ONZE HYPOTHESE

Na een ernstig ongeval ontstaat systemische inflammatie, waarbij er meer agressieve witte bloedcellen (voornamelijk neutrofielen) vanuit de bloedsomloop naar het fractuurhematoom bewegen en daar de ontstekingsfase van de botgenezing verstoren. Een hoge concentratie neutrofielen in het fractuurhematoom na systemische inflammatie verstoort de botgenezing door een negatief effect op stamcellen. Deze verstoring kan mogelijk voorkomen worden door bij zorgvuldig geselecteerde patiënten de migratie van neutrofielen naar het fractuurhematoom te remmen.

LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
AO	Arbeitsgemeinschaft für Osteosynthesefragen
ARDS	Acute Respiratory Distress Syndrome
ASAP	L-Ascorbic Acid-2-Phosphate
bFGF	basic Fibroblast Growth Factor
BGP	β-Glycerophosphate
BM	Basic Medium
BMP	Bone Morphogenetic Protein
BMSC	Bone Marrow Stromal Cells
BSA	Bovine Serum Albumin
CBFA	Core Binding Factor Alpha
CCL	CC Chemokine Ligand
CFDA-SE	CarboxyFluorescein DiAcetate Succinimidyl Ester
CHIPS	CHemotaxis Inhibitory Protein of Staphylococcus aureus
CHIPS Δ1F	CHIPS mutant lacking the first N-terminal amino acid
CRP	C-reactive protein
CTN	Cannulated Tibial Nail
CXCR	CXC Chemokine Receptor
DAMP	Damage-Associated Molecular Patterns
ECM	Extracellular Matrix
EM	Expansion Medium
ETN	Expert Tibial Nail
FH	Fracture Hematoma
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FPR	Formylated Peptide Receptor
H&E	Hematoxylin and Eosin
ICU	Intensive Care Unit
IgG	Immunoglobulin G
IL	Interleukin
ISS	Injury Severity Score
INF	Interferon
IMN	Intramedullary Nailing
LPR	Liquid Permanent Red
LPS	Lipopolysaccharides
MCP	Monocyte Chemotactic Protein
MODS	Multiple Organ Dysfunction Syndrome
MSC	Multipotent Stromal Cell

NET	Neutrophil Extracellular Trap
NISS	New Injury Severity Score
OM	Osteogenic Medium
ORIF	Open Reduction Internal Fixation
PBS	Phosphate Buffered Saline
PMA	Phorbol Myristate Acetate
RA	Rheumatoid Arthritis
RAG	Recombination Activating Gene
ROS	Reactive Oxygen Species
SIRS	Systemic Inflammatory Response Syndrome
SNP	Single Nucleotide Polymorphisms
SOX	Sex determining region y-related high mobility group bOX
TAN	Tumor-Associated Neutrophils
TCR	T-Cell Receptor
TGF	Transforming Growth Factor
TIE	Tyrosine kinase with Immunoglobulin-like and EGF-like domains
TMA	Tissue Microarray
TNF	Tumor Necrosis Factor
UTN	Unreamed Tibial Nail
UMCU	University Medical Center Utrecht
UPOD	Utrecht Patient Oriented Database
VEGF	Vascular Endothelial Growth Factor

DANKWOORD

Dit proefschrift is het resultaat van 8 jaar onderzoek, waaraan veel mensen hebben bijgedragen. Een aantal personen wil ik in het bijzonder bedanken.

Beste professor Leenen,

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Beste professor Koenderman,

I fell with the door in the house, but there's something on the hand! Het is een speciaal gevoel om na 6 jaar geneeskunde als arts-onderzoeker plaatst te nemen in een research-meeting met biomedische wetenschappers. Ik hoorde Griekse letters, medeklinkers en getallen in vloeiend Dungleish voorbijkomen en had in het begin werkelijk geen enkel idee waar jullie het over hadden. Het zou je bijna onzeker maken. Deze onzekerheid verdween compleet toen ik hoorde dat een collega arts-onderzoeker er net achter was gekomen dat een *guinea pig* toch echt geen varken uit Guinea is.

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Geachte selectiecommissie van het Alexandre Suerman MD/PhD programma en Lucette Teurlings,

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CURRICULUM VITAE

Okan William Bastian was born on the 23rd of August 1985 in Utrecht. During his time at the Johan van Oldenbarnevelt gymnasium in Amersfoort, he followed an internship at the Department of Plastic Surgery in the University Medical Center of Utrecht (UMCU) by the age of 15. He observed professor M. Kon while he was performing a COMMANDO procedure and at that moment he realized he wanted to become a surgeon.



During his Medical study at the UMCU he participated in several extracurricular activities. Thanks to professor I.H.M. Borel Rinkes (UMCU), he was allowed to participate in basic research at the Shriners Hospital in Boston (USA), which focused on isolation of endothelial progenitor cells from children with thermal injuries using microfluidic cellular affinity chromatography under supervision of professor M. Toner (HMS/MIT) and professor R. Tompkins (HMS/MGH). At the American Hospital in Istanbul (Turkey) he followed an extracurricular internship at the department of pediatric cardiothoracic surgery and wrote an article that focused on complications after newborn circumcision under supervision of Dr. E. Eroglu. In the Wilhelmina Children's Hospital in Utrecht, he participated in research that focused on non-operative management of blunt hepatic injuries in children under supervision of Dr. W.L.M. Kramer and Dr. J.M. Hoogendoorn.

In Boston, he became fascinated by basic research and tissue regeneration and decided to write a PhD proposal focusing on skin regeneration after thermal injury, which he presented to several institutions in the Netherlands. Eventually, he met professor L.P.H. Leenen at the Department of Traumatology in the UMCU, who was setting up a research-line that would focus on the effect of systemic inflammation on fracture healing. He rewrote his research proposal from skin regeneration after thermal injury into bone regeneration after systemic inflammation, which became the foundation of this thesis.

After finishing Medical School, he wrote several research grant proposals and received 5 different research grants: the Alexandre Suerman MD/PhD stipend, the AO Foundation research grant, the OTC Foundation research grant, the Catharijne stichting research grant and the Annafonds – NOREF research grant. This helped him to start his PhD at the Department of Traumatology under supervision of professor L.P.H. Leenen (Traumatology), professor L. Koenderman (Pulmonary Diseases) and Dr. T.J. Blokhuis (Traumatology). During his PhD, he supervised over 20 students and presented the results of his research during several national and international congresses, including ESTES, ESSR, BBC, SEOHS and NVVI.

In 2013 he started as a resident (ANIOS) at the Department of Surgery at the Meander Medical Center in Amersfoort and after one year he started his surgical residency under supervision of professor M.R. Vriens (UMCU) and Dr. E.C.J. Consten (MeanderMC Amersfoort). During the past years, Okan has been a member of several committees including the SEOHS organizing committee (Symposium Experimenteel Onderzoek Heelkundige Specialismen), the Medical Dispute of his fraternity (Veritas, Utrecht) and the Association of Residents in the Meander Medical Center, during which he contributed to the development of E-learning modules and introduction videos for new residents. He now lives in Utrecht with his fiancée Marieke Sanders and their sons Jules and Quinten.

