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UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Nina Lukač

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GRADUATE THESIS



Zagreb, 2016.

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This graduate thesis was made at the Department of Anatomy, University of Zagreb School of Medicine and at the Croatian Institute for Brain Research, Laboratory for Molecular Immunology, mentored by prof. dr. sc. Vedran Katavić and submitted for evaluation in the academic year 2015/16.

ABBREVIATIONS

α-MEM – minimum essential medium-α

ANOVA – analysis of variance

ALPS – autoimmune lymphoproliferative syndrome

B6 – C57BL/6J mice

$Ca_{10}(PO_4)_6(OH)_2$ – calcium hydroxyapatite

CD – cluster of differentiation

CO_2 – carbon dioxide

CSF1R – colony-stimulating factor-1 receptor

EDTA – ethylenediamine tetracetic acid

ERK – extracellular-signal regulated kinase

ES% – percent of eroded surface

gld – generalized lymphoproliferative disorder

GM-CSF – granulocyte-monocyte colony stimulating factor

FasL – Fas ligand (APO-1 ligand, CD95 ligand)

Fas -/- – Fas knockout mice

FasL -/- – Fas ligand knockout mice

FBS – fetal bovine serum

FR – fracture

IL-1 – interleukin 1

IL-3 – interleukin 3

JNK – c-Jun N-terminal kinase

lpr – lymphoproliferative syndrome

M-CSF – macrophage colony stimulating factor

NFATc1 – nuclear factor of activated T cells c1

NF-kB – nuclear factor κB

N.Oc – number of osteoclasts

OPG – osteoprotegerin

PBS – phosphate-buffered saline

PC – pin control

PKB – protein kinase B

RANK – receptor activator of nuclear factor κB

RANKL – receptor activator of nuclear factor κB ligand

rm – recombinant mouse

SD – standard deviation

TNF – tumor necrosis factor

TNF-α –tumor necrosis factor α

TRAP – tartrate-resistant acid phosphatase

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SUMMARY

Nina Lukač: *The role of the Fas/FasL apoptotic pathway on osteoclastogenesis in the mouse model of controlled tibial fracture*

Key words: bone, Fas, FasL, fracture, osteoclast

The Fas/FasL apoptotic system serves as a regulator of the immune system, but also has an influence on osteoclast survival and differentiation. The model of internally fixated controlled tibial fractures was used to evaluate its role on osteoclastogenesis in formation of new bone, as fracture healing serves as a model for embryonic skeletal development. The aim of this study was to evaluate the role of the apoptotic system on osteoclastogenesis *in vivo*, by evaluating the osteoclastogenic potential of different tissues; and *in vitro*, by analyzing the numbers of osteoclasts and eroded surface on histological sections of tibiae. The roles of Fas and FasL were investigated using knockout mice for the receptor and its ligand. We hypothesized that the number of osteoclasts would increase in the knockout strains because of a lacking of osteoclast apoptosis when Fas/FasL pathway is not functioning.

Observation of the histological sections of calluses in fractured tibiae confirmed that the healing of the fracture occurred by endochondral ossification. Our results showed that the loss of Fas or FasL caused an increase in the number of osteoclasts and the eroded surface in fracture calluses in the second week after the fracture, which was not observed in the B6 control. The analysis of osteoclasts on cartilage surfaces showed that the loss of Fas or its ligand did not cause a change in the number of osteoclasts on the cartilaginous part of the callus in the second week and did not cause a difference between the osteoclast numbers on the growth plate cartilage. The number of osteoclasts on those two cartilage surfaces did not differ between each other as well.

These results show that the Fas/FasL apoptotic system has a role in osteoclastogenesis during new bone formation, but further research is necessary to further elucidate its exact function.

PREFACE

1. Bone

Bone is the main component of the adult skeleton, which serves, among other roles, as the body's mechanical support. It also protects internal organs and the brain and provides attachment sites for muscles and leverage, both of which give body mobility. It stores approximately 99% of body's calcium and 85% of phosphate and thus serves as their main mineral reservoir. Bones contain a central medullary cavity, which harbors bone marrow, the main hematopoietic organ. It stores many cytokines and growth factors.

Based on their shape, bones can be classified as short, long and flat. Two types of bone can be differentiated based on gross observation: 1) compact (or cortical bone), the outer region located below periosteum; and 2) trabecular (also called cancellous or spongy) bone, the inner part which is made of interconnected trabeculae and lined by endosteum.

2. Osteoblasts, osteoclasts, and bone matrix

Histologically, bone is classified as connective tissue and is composed of the bone matrix and different cell types. Internal and external surfaces of the bone are lined by endosteum and periosteum. Both are made of dense connective tissue, containing collagen and fibroblasts and on places where the lining is in contact with underlying bone also of osteoblasts and osteoprogenitor cells.

Derived from mesenchymal progenitor cells, osteoblast are mononuclear cells that are responsible for synthesis of bone matrix (Ducy et al. 2000). As they secrete the organic components, collagen type 1, proteoglycans and glycoproteins, they are of cuboidal shape, but they flatten as their synthetic activity declines. As the deposited matrix progressively surrounds them, they become osteocytes, encased in lacunae, and acquire dendritic processes that are enclosed in canaliculi. Osteoblast are also responsible for control of function of osteoclasts, the other important cell found in the bone, which is described separately in the next paragraph. The bone matrix is composed of inorganic part, hydroxyapatite crystals ($Ca_{10}(PO_4)_6(OH)_2$) and organic part. The organic matter is made mostly of collagen type 1, but also contains

glycoproteins and proteoglycans. The matrix is a place harboring many cytokines and growth factors.

Osteoclasts are cells of hematopoietic origin, responsible for bone resorption. Their mononuclear marrow-derived progenitors are recruited to areas of bone resorption, where they acquire their characteristic multinuclear appearance by cell fusion. They reside in so-called Howship's lacunae or resorption bays in the bone, where they adhere to the bone to create an isolated space of resorptive environment between them and the bone matrix. A hallmark of an active osteoclast, the ruffled border, is responsible for secreting molecules needed for degradation and resorption of the bone.

Major regulators of osteoclast proliferation, survival and differentiation are RANKL (receptor activator of nuclear factor κB ligand)(Teitelbaum 2000) and M-CSF (macrophage-colony stimulating factor)(Felix et al. 1990). RANKL is a type 2 membrane protein belonging to TNF superfamily that is expressed on osteoblasts and acts through its receptor RANK (receptor activator of nuclear factor κB). Both osteoclast precursors and mature osteoclasts express RANK (Dougall et al. 1999). Ligand binding leads to activation of several signaling pathways: NFATc1, NF-kB, Akt/PKB, JNK, ERK, and p38 (Feng 2005). RANKL/RANK system has a negative regulator – OPG (osteoprotegerin) that is secreted from osteoblasts and acts as RANKL's decoy receptor (Lacey et al. 1998). M-CSF, the other critical molecule for osteoclast development, mediates proliferation and survival of osteoclast precursors by binding to the receptor CSF1R (colony-stimulating factor-1 receptor) (Stanley et al. 1997). Many other cytokines and growth factors have been linked to osteoclast regulation, including GM-CSF (Lee et al. 2009), IL-3 (Khapli et al. 2003), IL-1 (Kim et al. 2009), TNF-α (Kwan Tat et al. 2004) and other members of the TNF superfamily including FasL (Katavic et al. 2003, Wu et al. 2003).

Bone is highly metabolically active and undergoes constant changes by coordinated activity of osteoblasts and osteoclasts, which yearly replace approximately 10% of the adult skeleton (Kolar et al. 2010). This simultaneous activity of bone formation and resorption is called coupling (Kelava et al. 2014).

3. Bone development

Osteogenesis, the process of bone development, can occur by two mechanisms – endochondral or intramembranous ossification, depending on whether it occurs in long or flat bones, respectively. In both, bone tissue develops from mesenchyme; in intramembranous ossification this change occurs directly from mesenchymal progenitors into osteoblasts that start secreting bone matrix whereas in endochondral ossification, the mesenchyme first develops into hyaline cartilage, which is later invaded and replaced by osteoblasts. Long bones develop by endochondral ossification. Mesenchymal cells become committed to the cartilage lineage, condense and differentiate into chondrocytes. They proliferate and secrete extracellular matrix to make a model of the bone. Chondrocytes become hypertrophic, compress surrounding matrix and die by apoptosis, leaving empty space which is invaded by blood vessels that bring osteoprogenitor cells. In the diaphysis, this forms the primary ossification center, while secondary ossification centers appear later in epiphyses. (Gilbert 2000, Mescher 2013)

4. Fracture healing

Fractures are the most common injuries caused by trauma in humans (Einhorn & Gerstenfeld 2015) and their healing differs from wound healing, because it does not result in scar formation but usually restores tissue to its previous form (McKibbin 1978). Being relatively easy to reproduce, they are an ideal model for studying complex events occurring during bone tissue regeneration and embryological development of the skeleton (Hadjiargyrou & O'Keefe 2014).

Fracture healing proceeds through three phases: inflammatory, proliferative and remodeling phase. Hematoma formation due to tissue damage during fracture, which leads to inflammatory responses, is the starting point of the fracture healing cascade (Kolar et al. 2010). Cytokines and growth factors from the injury response are responsible for activation of osteoprogenitor cells that originate mainly from the periosteum (Hadjiargyrou & O'Keefe 2014). Healing in the center of fracture calluses will follow the steps of endochondral ossification, where progenitors will first develop into chondrocytes and form

the soft callus, but then hypertrophy and die and become replaced by osteoblasts (Claes et al. 2012). In the remodeling phase, osteoclast remove newly formed matrix, while osteoblast are continually laying down new bone, and this coupled processes leads to the reestablishment of previous bone architecture and continuity with the adjacent uninjured area (Einhorn & Gerstenfeld 2015).

5. Fas/FasL system

5.1. Role and expression

Fas (APO-1, CD95) and its ligand, FasL (APO-1L, CD95L) are both members of the TNF (tumor necrosis factor) family and are well known mediators of apoptosis in multiple cell types. They induce apoptosis via the extrinsic or the death receptor pathway (Wajant 2002). They are involved in regulation of the immune response – they reduce excess immune system activation, play a role in removal of autoreactive T lymphocytes and mediate some effects of cytotoxic T lymphocytes (Kavurma & Khachigian 2003). Fas is a type 1 transmembrane protein that is activated upon binding of its ligand (FasL), a type 2 membrane protein, to trigger a cascade of downstream signaling which ultimately leads to cell death. Even though it has mostly been seen as the mediator of apoptosis, non apoptotic functions of the death receptor have also been described (Peter et al. 2007, Park et al. 2005a). Its expression has been found on cells of the immune system, heart, liver, kidney, pancreas and brain, while FasL, is not found on as many cells – its expression is confined mostly to cells of the immune system, cells in the anterior chamber of the eye, Sertoli cells in testis and placenta, where it may contribute to maintenance of immune privilege (Kayagaki et al. 1997, Kovacic et al. 2010a).

The role of the Fas system has been studied on mice models with spontaneous Fas or FasL mutation (*lpr* and *gld* mice, respectively), on knockout mice (Fas-/- and FasL -/-) and cell specific or conditional knockout mice of both receptor and its ligand. *Lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) mice develop autoimmune manifestations and lymphadenopathy (Takahashi et al. 1994) and are mice

models for systemic lupus erythematosus and Sjögren syndrome. Incomplete penetrance of the Fas or FasL deficient phenotype has been suspected for both *gld* and *lpr* mutation and has been proven in *lpr* mice (Kovacic et al. 2010a, Kovacic et al. 2007, Mariani et al. 1994).

Fas mutation has also been described in human, leading to autoimmune lymphoproliferative syndrome (ALPS), which presents with chronic nonmalignant lymphadenopathy or splenomegaly, autoimmune disease and an increase in CD3+CD4-CD8- (double negative) T lymphocytes (Fisher et al. 1995, Sneller et al. 2003).

5.2. Role of Fas/FasL system in bone

Fas and FasL expression has also been found on bone cells. Both murine osteoblasts and osteoclasts have been found to express Fas and FasL, but the level of expression differs with the level of cell differentiation and between different studies (Kovacic et al. 2010a). While Fas expression was confirmed on osteoblasts, where it increases with their level of differentiation (Kovacic et al. 2007), reports about its expression on osteoclasts are contrasting – some data suggest weak expression (Kovacic et al. 2007), while others describe a strong expression (Wu et al. 2003).

The role of Fas and its ligand in osteoclast development, survival and apoptosis has been described in a number of studies. Some studies show that the Fas/FasL system is involved in mediating osteoclast apoptosis (Wu et al. 2003, Roux et al. 2005, Wang et al. 2015) and that a mutation in Fas or FasL will increase numbers of osteoclast cells on bone surfaces (Wu et al. 2003). Another study showed only a weak increase in apoptotic osteoclast when FasL was added to cultures (Kovacic et al. 2007). It has also been established that the Fas system is involved in prevention of development of osteoporotic phenotype after estrogen withdrawal in mice (Katavic et al. 2003, Kovacic et al. 2010b, Nakamura et al. 2007, Tang et al. 2014), and that loss of Fas (Kovacic et al. 2010b) or FasL (Katavic et al. 2003) protects mice from ovariectomy-induced bone loss. Estrogen has also been shown to increase FasL expression on osteoclasts (Nakamura et al. 2007). Park et al showed that interaction between Fas and FasL increases osteoclastogenesis (Park et al. 2005b). When bone mass was examined in mice with spontaneous

mutation for FasL and Fas, *gld* (generalized lymphoproliferative disorder) and *lpr* (lymphoproliferative syndrome) mice respectively, the results showed increased bone mass of *gld* mice compared to their wild type controls and decreased numbers of osteoclasts on bone surfaces (Katavic et al. 2003). Wu et al showed a significant decrease in *lpr* and an insignificant slight decrease in *gld* mice bone mass, with an increased osteoclast/bone surface number, however, sex and age of mice were different between the two experiments (Wu et al. 2003). Besides directly causing osteoclast apoptosis, the effects of Fas system on number of osteoclasts were also suggested to be mediated indirectly through osteoblasts, by influencing their survival and thus controlling the OPG/RANKL ratio which is dependent upon level of osteoblast development (Katavic et al. 2003).

When fracture healing was studied in *lpr* mice, Fas deficiency prolonged the cartilage resorption period, which was later followed by a decrease in mineralized tissue in callus of the Fas deficient (*lpr*) mice, when compared to wild type control. This was attributed to higher rates of bone turnover in *lpr* mice supported by the fact that *lpr* mice had higher number of osteoclasts per bone surface (Al-Sebaei et al. 2014).

HYPOTHESES

Loss of function of the Fas/FasL apoptotic system will cause an increased number of osteoclasts on bone surfaces *in vivo*, due to the lack of the apoptotic pathway and consequently longer survival of osteoclasts.

We hypothesized that *in vitro* the osteoclastogenic capacity of mononuclear progenitor cells would be increased in animals lacking the Fas/FasL pathway at all time points in comparison to wild type control.

OBJECTIVES

It has been previously described that the Fas/FasL apoptotic system, apart from acting as a regulator of the immune response, plays a role in regulation of bone formation, remodeling and resorption and has an influence on osteoclast life span and differentiation (Katavic et al. 2003, Wu et al. 2003, Roux et al. 2005, Wang et al. 2015, Park et al. 2005b).

Fracture healing is a recapitulation of many of the embryonic processes that occur in bone formation (Ferguson et al. 1999) and is an ideal model for studying the complex cellular and molecular interactions and temporal relations of events occurring during skeletogenesis.

The objective of this study were:

1) to evaluate the role of the Fas/FasL apoptotic system on osteoclastogenesis *in vivo* and
2) to evaluate the role of the Fas/FasL apoptotic system on osteoclastogenesis *in vitro*.

This was studied in a murine model of controlled tibial fracture, internally fixated by a stainless steel wire. Our goal was to analyze the influence of the Fas/FasL system on osteoclast numbers on bone surfaces in fractured and non-fractured tibiae (*in vivo*) and asses its influence on the osteoclastogenic potential of marrow and peripheral blood mononuclear progenitors (*in vitro*). All analyses were done at different time points, at 1, 2 ,and 3 weeks after the fracture procedure, to provide an insight into the chronological order of events.

MATERIALS AND METHODS

1. Experimental animals

Animals used in the experiment were male 12-14 week old Fas (Fas -/-) and FasL (FasL -/-) knockout mice and their age and sex matched wild type C57BL/6J controls (B6). Both knockout strains were on the C57BL/6J background. Animals were randomly allocated into three groups – a “fracture” group (described lower), a “pin control” group’ (no fracture performed, but a steel wire for internal fixation was introduced into the medullary cavity), and a “control” group (no fracture, no internal fixation), depending on the procedure that was performed.

Mice were bred and housed at the Croatian Institute for Brain Research, University of Zagreb Medical School. Experiments were approved by the Ethics Committee of the University of Zagreb School of Medicine.

2. Controlled tibial fracture

Bilateral transverse closed tibial fractures were performed according to a protocol originally described previously (Bonnarens & Einhorn 1984). Briefly, the fractures were done in 12-14 week old experimental mice anesthetized with AVERTIN (3-bromoethanol, Sigma Aldrich Corp.) after lubricating their eyes with bromine-vaselin ointment. The protocol was modified and approved by the *Animal Care and Use Committee, University of Conneticut Health Center (CT), USA*, where members of our laboratory were educated for the before mentioned procedure.

Before the fracture, a 25G bore needle was inserted into the medullary cavity through the tibial plateau. After retracting the needle, a stainless steel wire with 0,5 mm diameter was inserted into the same canal, to fixate bone fragments after the fracture. The fracture was done 1-2 mm proximal of the distal fibular attachment with minimal fragment movement, using a device composed of a fixation table and dull steel blade, according to the described protocol (Bonnarens & Einhorn 1984). After the procedure, mice were warmed on a warm electric mat. The animals were examined every 30 minutes after the operation (4 clinical examinations in 2 hours) and in case of appearance of signs of terminal illness (loss of body weight, loss of righting reflex, loss of

reaction to external stimuli), the animal was immediately euthanized by cervical dislocation.

Controlled tibial fracture was not done on mice of the pin control group (sham); those animals differed from the experimental group only by this part of the procedure. Mice of the pin control group were anesthetized, their eyes were lubricated by bromine-vaseline ointment, a stainless steel wire with 0,5 mm diameter was inserted into the tibial medullary canal, and after that they were warmed on a warm electric mat and examined every 30 minutes after anesthesia. In case of appearance of signs of terminal illness, the animal was immediately euthanized by cervical dislocation.

Mice were clinically evaluated every day and their behavior, changes in movement, existence of tremor, changes on the eyes and visible mucosa, general condition and posture, dyspnea/tachypnea, dehydration, loss of body mass and shape and emergence of stereotypies were recorded. Analgesic therapy was introduced when needed based on this assessments.

Mice were sacrificed 1, 2, and 3 weeks after the initial procedure with cervical dislocation after exposure to a lethal dose of the anesthetic. Fractured bones were put into fixative and the number of osteoclasts was analyzed on different bone and cartilage surfaces. Blood from the orbital plexus and bone marrow from femurs and tibiae were obtained from the animals for *in vitro* evaluation of the osteoclastogenic potential of those tissues.

3. Histological analysis

Changes in the number of osteoclasts per bone surface, percent of eroded surface, and number of osteoclasts on the surface of cartilage tissue in fracture callus and on epiphyseal hyaline cartilage were analyzed by histological analysis of tibiae 1, 2, and 3 weeks after the fracture or sham procedure.

Tibiae, fractured and those from the pin-control groups, were obtained from the animals after they were sacrificed. Attached soft tissue, as well as the intramedullary pin, were removed and the bones were then fixated in 4% paraformaldehyde for 24 hours. They were then demineralized in a 3% formalin in 14% EDTA solution for 2 weeks. Ascending concentrations of ethanol (70%, 96%, and 100%) were used to dehydrate the specimens which

were then embedded in paraffin. Sections 7 μm thick were made using a Leica RM 2135 rotary microtome (Leica, Nussloch, Germany). Tissues were histochemically stained for tartrate-resistant acid phosphatase (TRAP) enzyme activity for osteoclast identification and with Goldner-trichrome stain. TRAP positive cells adjacent to bone surfaces were considered as osteoclasts and counted on two cuts per bone. Results were expressed as number of osteoclasts per bone or cartilage surface, and percent of eroded bone surface. Osteoclasts were counted on the surfaces of trabecular bone, below cortical bone, and on areas adjacent to the hyaline cartilage in the epiphyseal plate, and on the cartilaginous fracture callus. Analysis was performed under 100× magnification with Zeiss Axio Imager microscope (Carl Zeiss, Oberkochen, Germany) connected to a computer with OsteoMeasure software (OsteoMetrics, Decatur, GA, USA).

4. Analysis of osteoclastogenic potential

Hematopoietic cells were obtained from bone marrow and peripheral blood of animals at 1, 2, and 3 weeks after the fracture or sham procedure, depending on whether the animal belonged to the ‘fracture’ or ‘pin control’ group, respectively. Cells were also obtained from the ‘control’ group. To analyze the osteoclastogenic potential of those tissues, osteoclast cell cultures were made from obtained cell suspensions, by inducing osteoclastogenesis with addition of RANKL and M-CSF.

4.1. Bone marrow

For obtaining bone marrow cell suspension, mice were sacrificed by cervical dislocation after being exposed to a lethal dose of anesthetic. Femurs and tibiae were removed and cleared of adherent soft tissues. Intramedullary steel wires were removed form tibiae. To obtain bone marrow cells, bone ends were cut and medullary cavities were flushed with minimum essential medium-α (α-MEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with a 23G needle. Cell suspensions were centrifuged at 1500RPM for 5 minutes at 4°C. Supernatant was discarded and remaining cell pellet resusupended in 3ml α-MEM with 10% fetal bovine serum (FBS) (Gibco,

Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin – streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 2mM GlutaMAX^{TM} (100×, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) for suspension form tibiae and 5ml for femur and filtered through a 40 μm nylon cell strainer. Cells were stained with Türk solution and counted in Bürker-Türk chamber, then cultured overnight in an incubator at 37°C and 5% CO_2 at density of 10×10^6 cells in 60x15mm tissue culture dish in 5ml α-MEM containing 10% FBS, 100 U/ml penicillin–streptomycin, 2mM GlutaMAX^{TM} and 5ng/ml recombinant mouse (rm) M-CSF (R&D Systems, Minneapolis, MN, USA). On the next day, nonadherent cells were collected and centrifuged like the day before. Medium was removed, new suspension was made in 1ml of medium supplemented as described above and cells were counted again. Cells were cultured in 96 well plate at density $0{,}25\times10^6$ cell in 0,2ml α-MEM with 10% FCS, 100 U/ml penicillin–streptomycin, 2mM GlutaMAX^{TM} per well. 20ng/ml rmM-CSF and 40ng/ml rmRANKL (R&D Systems, Minneapolis, MN, USA) were added to stimulate osteoclast differentiation.

4.2. Peripheral blood

Blood samples for cell suspension were obtained from orbital sinus, while mice were anesthetized with 3-bromoethanol (Avertin). The animal was placed on a flat surface, jugular vein was compressed right below the mandible and upper eyelid retracted to create mild exophthalmos. Glass capillary tube was introduced into medial angle of the eye and turned axially, to penetrate the wall of orbital sinus. Blood was put into test tube containing 0,5ml of 8mM ethylenediamine tetracetic acid (EDTA) solution in phosphate-buffered saline (PBS) at pH 8. 1,3 mL of 2% dextran solution in PBS was added to test tubes, to separate the blood cells. Suspension was incubated for 45min at 37°C and clear supernatant was collected until the border with erythrocyte sediment. Suspension was then made by adding α-MEM with 10% FBS, 100 U/ml penicillin–streptomycin, 2mM GlutaMAX^{TM} to the collected supernatant. It was centrifuged as described above, supernatant medium removed and 2ml of new medium were added. Cells were counted and cultured in 96 well plate at density $0{,}25\text{x}10^6$ cell in 0,2ml α-MEM with

10% FCS, 100 U/ml penicillin – streptomycin, 2mM GlutaMAX^{TM} per well with 20ng/ml rmM-CSF and 40ng/ml rmRANKL to stimulate osteoclast differentiation.

Medium was changed on day 3 and cells were fixed and stained day 5. Fixation was achieved by adding 2,5% glutaraldehyde in PBS for 30 minutes at room temperature and cells were stained using leukocyte acid phosphatase (TRAP) staining kit (Sigma Aldrich Corp., St Louis, MO, USA) according to manufacturer's protocol. Cells that stained positively for TRAP and had three or more nuclei were considered osteoclasts and counted per well with Zeiss Axiovert 200 microscope (Carl Zeiss, Oberkochen, Germany) under 40× magnification.

5. Statistical analysis

Results are presented as mean value +/- standard deviation (SD) and groups were compared by Student's t-test for two group comparison and one way ANOVA for three group comparison. Post hoc Tukey test was done to determine pairwise differences on the groups that showed significant differences with one way ANOVA. Differences between compared groups were considered statistically significant at p value lower than 0,05. Statistical analysis was done on MedCalc for Windows, version 14.0 (MedCalc Software, Ostend, Belgium).

RESULTS

1. Analysis of osteoclastogenic potential

In this part of the experiment, we analyzed the osteoclastogenic potential of mononuclear hematopoietic progenitors, derived from peripheral blood or bone marrow of femur or fractured tibia. Samples were obtained at 1, 2, or 3 weeks following the fracture procedure. Osteoclastogenic potential was evaluated *in vitro,* by examining the number of osteoclasts that differentiated from the progenitors in osteoclast cell cultures. Osteoclast differentiation was stimulated by rmM-CSF and rmRANKL addition into the culture medium and TRAP positive cells with three or more nuclei were considered osteoclasts and counted per well under light microscopy. 8 wells per each group were cultured and results were presented as mean number of osteoclasts per group +/- standard deviation.

Differences between osteoclastogenic potentials were examined at three different time points (week 1, week 2, and week 3) after the fracture procedure to evaluate how the loss of functional Fas receptor or its ligand, FasL changed the ability of progenitors to differentiate into mature osteoclasts from different target tissues (bone marrow of femur or fractured tibiae, and peripheral blood) when tibiae are fractured.

In the third week after the fracture, we also compared osteoclastogenic potential of tissues obtained from mice which had the fracture procedure performed to those from control groups and mice who underwent the sham procedure (‘pin control’ group), to further evaluate how the tibial fracture influenced osteoclast differentiation from the bone marrow of tibiae and femur and peripheral blood in the three different strains.

1.1. Effect of loss of functional Fas receptor or Fas ligand (FasL) on osteoclastogenic potential of bone marrow of tibiae

In the first week after the fracture, cultures from B6 and FasL -/- mice had a higher number of osteoclasts per well than those from Fas -/- mice. In the second week, osteoclastogenic potential of B6 control group was higher than in both Fas -/- and FasL -/- mice derived cell cultures. Bone marrow of tibiae from Fas -/- and FasL -/- had the same osteoclastogenic potential as B6 control 3 weeks after the fracture. (Figure 1)

Osteoclastogenic potential of bone marrow of fractured tibiae was also compared with potential of marrow from tibiae of the control and pin control groups. In all three different strains (B6, Fas-/-, FasL-/-), the cell cultures from mice that had the fracture procedure performed, had a significantly lower number of osteoclasts per well as their control groups. In B6 and Fas -/- cell cultures, the number of osteoclasts in pin control groups was higher than in control and fracture groups. In FasL -/-, there was no significant difference between control and pin control group. (Figure 2)
Results show that the fracture procedure caused a decrease in osteoclastogenic potential of the bone marrow of the fractured tibiae, when compared to the control group.

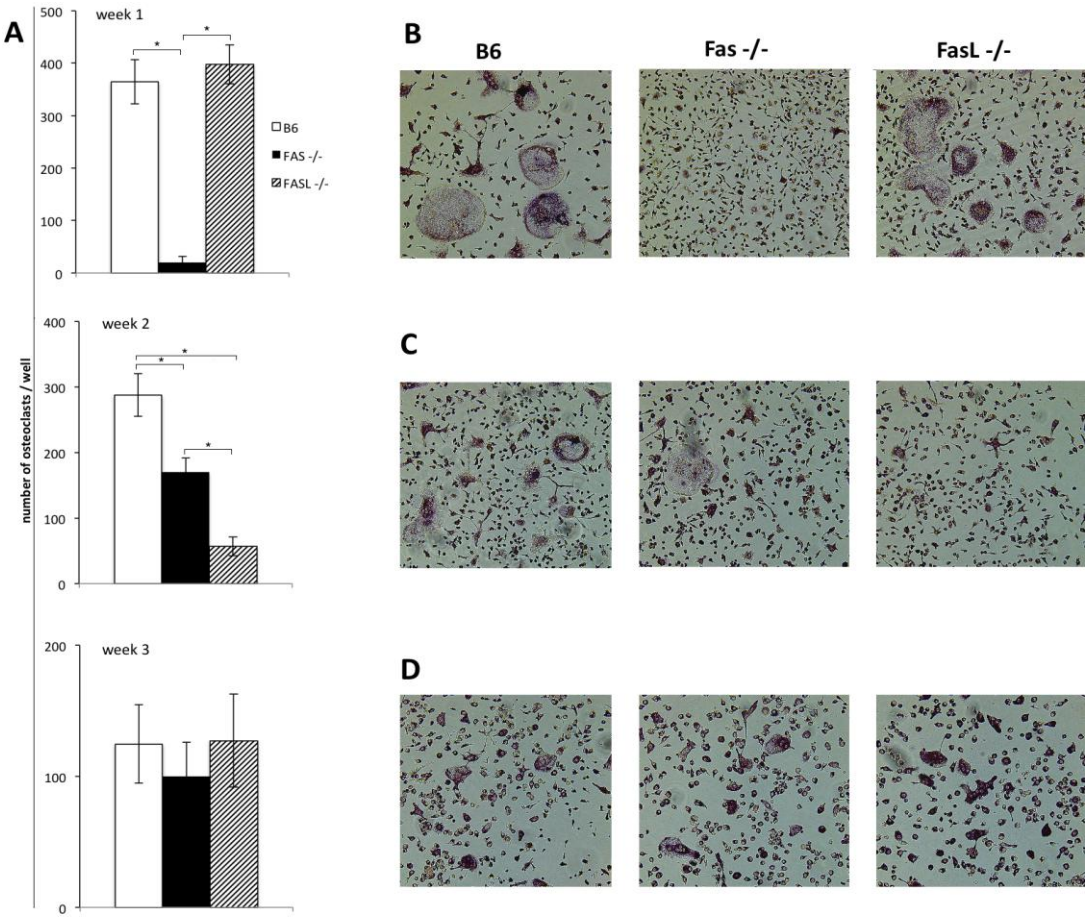


Figure 1. Osteoclastogenic potential of cells from bone marrow of fractured tibiae 1, 2, and 3 weeks after the fracture. Cell suspensions were made by combining bone marrow of tibiae of 2 mice per strain. Cells were cultured in 96 well plate at density $0{,}25\times10^6$ cell per well. 20ng/ml rmM-CSF and 40ng/ml rmRANKL were added to stimulate osteoclast differentiation. **A.** Number of osteoclasts per well is presented as mean +/- standard deviation **B, C & D.** Morphology of osteoclast cell cultures in the first (B), second (C), and third (D) week after the fracture under 100× magnification. Osteoclasts are TRAP positive (purple) cells with 3 or more nuclei. * denotes a statistically significant difference at $p < 0{,}05$.

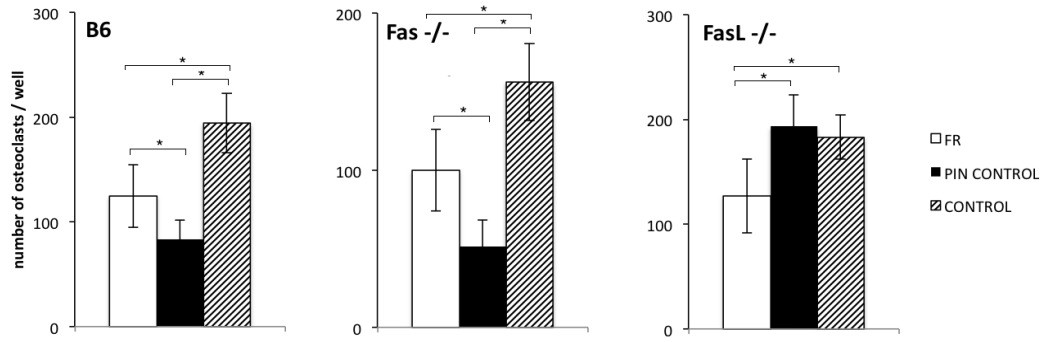


Figure 2. Osteoclastogenic potential of cells from bone marrow of tibiae three weeks after the fracture ('FR'), sham procedure ('PIN CONTROL') or in control group in B6, Fas -/-, and FasL -/- mice. Cell suspensions were made by combining bone marrow of tibiae of 2 mice per strain. Cells were cultured in 96 well plate at density $0{,}25\times10^6$ cell per well. 20ng/ml rmM-CSF and 40ng/ml rmRANKL were added to stimulate osteoclast differentiation. Number of osteoclasts per well is presented as mean +/- standard deviation. * denotes a statistically significant difference at $p < 0{,}05$.

1.2. Effect of loss of functional Fas receptor or Fas ligand (FasL) on osteoclastogenic potential of bone marrow of femur

In the first week after the tibial fracture, bone marrow cells from femur from B6 control group had higher osteoclastogenic potential than those from Fas-/- and FasL-/-. In the second week, Fas-/- still had lower osteoclastogenic potential than B6 control group but the number of osteoclasts per well in FasL-/- group was higher than in both of the previously mentioned. In the third week B6 control had a higher number of osteoclasts per well than FasL-/-. (Figure 3)

When the number of osteoclasts per well was compared between the animals with fractured tibiae and those from the control and pin control group, the number of osteoclasts that differentiated from the cells of bone marrow of femur in the mice that had their tibia fractured was lower than those in the control groups in tissue obtained from B6 control and FasL -/- mice. In bone marrow of femur of Fas -/- mice, there was no difference between the groups. Pin control group had a higher number of osteoclasts per well then the fracture group in FasL -/-, but the numbers did not differ in B6 control and Fas -/- (Figure 4).

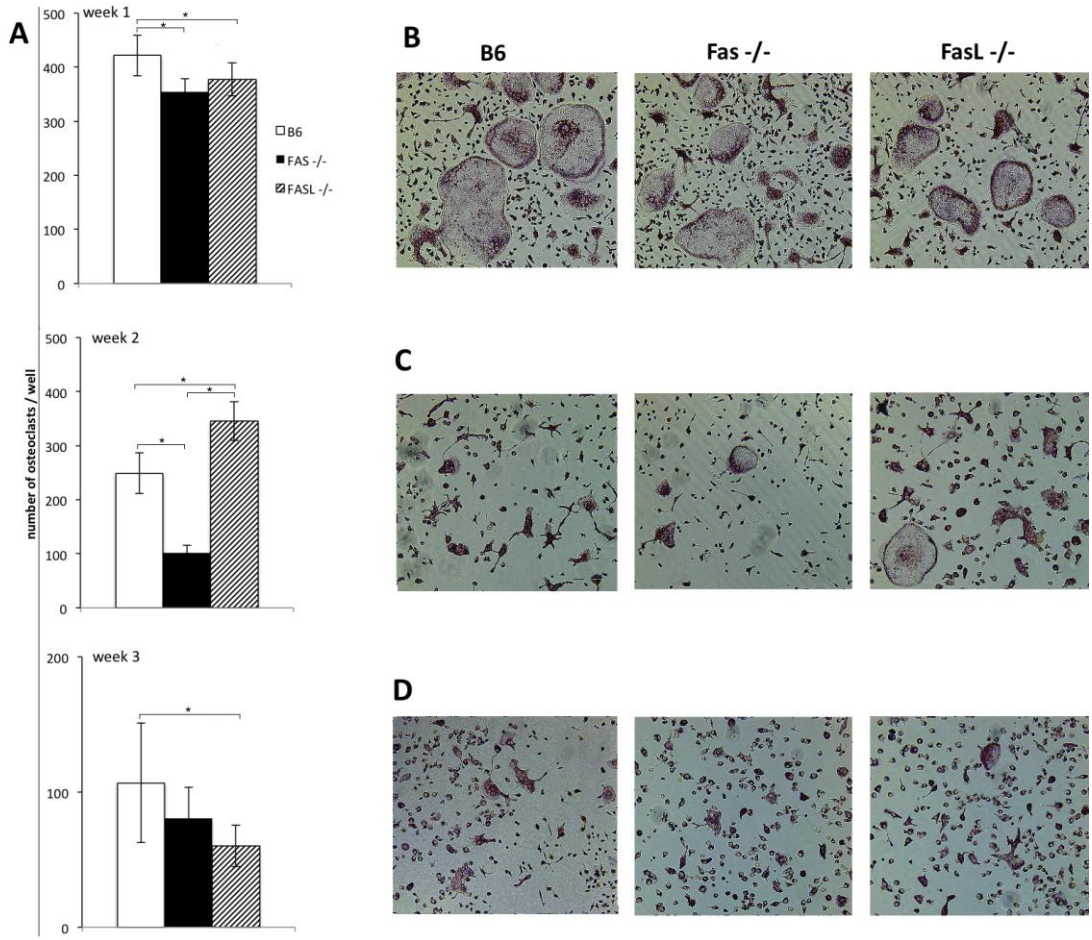


Figure 3. Osteoclastogenic potential of cells from bone marrow of femurs 1, 2, and 3 weeks after the fracture. Cell suspensions were made by combining bone marrow of femurs of 2 mice per strain. Cells were cultured in 96 well plate at density $0{,}25\times10^6$ cell per well. 20ng/ml rmM-CSF and 40ng/ml rmRANKL were added to stimulate osteoclast differentiation. **A.** Number of osteoclasts per well is presented as mean +/- standard deviation. **B, C & D.** Morphology of osteoclast cell cultures in the first (B), second (C) and third (D) week after the fracture under 100× magnification. Osteoclasts are TRAP positive (purple) cells with 3 or more nuclei. * denotes a statistically significant difference at $p < 0{,}05$.

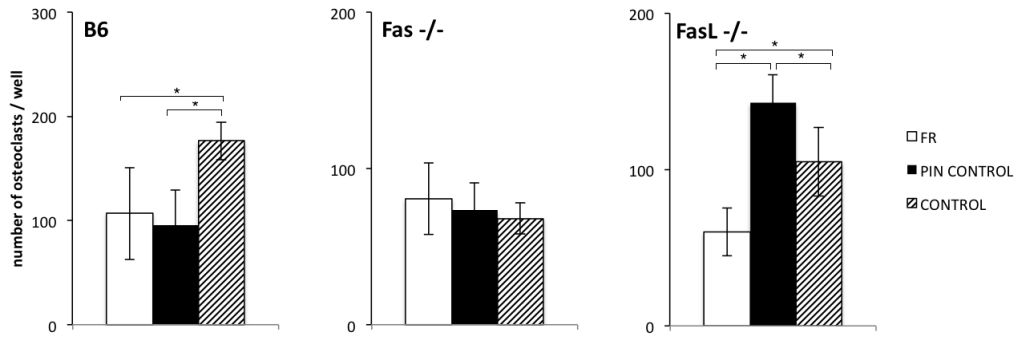


Figure 4. Osteoclastogenic potential of cells from bone marrow of femur three weeks after the tibial fracture ('FR'), sham procedure ('PIN CONTROL') or in control group in B6, Fas -/-, and FasL -/- mice. Cell suspensions were made by combining bone marrow of femurs of 2 mice per strain. Cells were cultured in 96 well plate at density $0{,}25\times10^6$ cell per well. 20ng/ml rmM-CSF and 40ng/ml rmRANKL were added to stimulate osteoclast differentiation. Number of osteoclasts per well is presented as mean +/- standard deviation. * denotes a statistically significant difference at $p < 0{,}05$.

1.3. Effect of loss of functional Fas receptor or Fas ligand (FasL) on osteoclastogenic potential of peripheral blood

Blood from Fas -/- mice had lower osteoclastogenic potential than B6 and FasL -/- in the first week after the tibial fracture. In week 2 and week 3, the number of osteoclasts per well was higher in Fas -/- than in B6 and FasL-/-. FasL -/- blood had a higher number of differentiated osteoclasts in cultures than B6 control in the first week, but lower than the control in week 2. (Figure 5)

When the number of osteoclasts per well was compared between the animals with fractured tibiae and those from the control and pin control group, there was a higher number of osteoclasts per well in the cultures of mice that had the tibial fracture, than in pin control and control group in B6 and Fas -/-. In FasL-/-, number of osteoclasts per well was higher in the control group than in fracture and pin control (Figure 6).

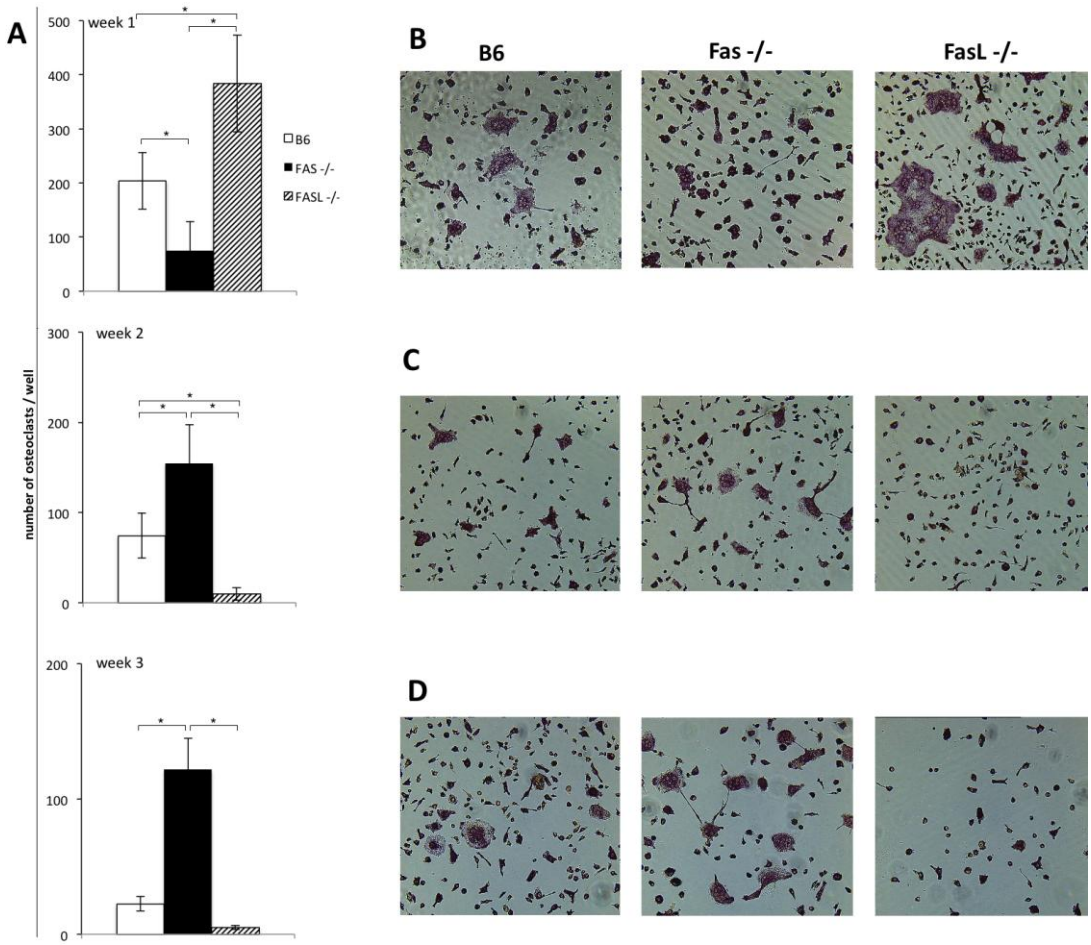


Figure 5. Osteoclastogenic potential of cells from peripheral blood 1, 2, and 3 weeks after the fracture. Cell suspensions were made by combining blood samples obtained from 2 mice per strain. Cells were cultured in 96 well plate at density $0{,}25\times10^6$ cell per well. 20ng/ml rmM-CSF and 40ng/ml rmRANKL were added to stimulate osteoclast differentiation. **A.** Number of osteoclasts per well is presented as mean +/- standard deviation. **B, C & D.** Morphology of osteoclast cell cultures in the first (B), second (C) and third (D) week after the fracture under 100× magnification. Osteoclasts are TRAP positive purple cells with 3 or more nuclei. * denotes a statistically significant difference at $p < 0{,}05$.

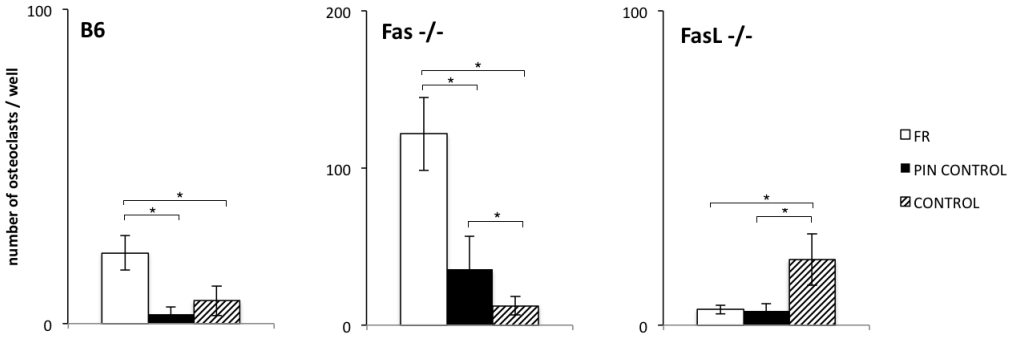


Figure 6. Osteoclastogenic potential of cells from peripheral blood three weeks after the fracture procedure ('FR'), sham procedure ('PIN CONTROL') or in control group in B6, Fas -/-, and FasL -/- mice. Cell suspensions were made by combining blood samples obtained from 2 mice per strain. Cells were cultured in 96 well plate at density $0{,}25\times10^6$ cell per well. 20ng/ml rmM-CSF and 40ng/ml rmRANKL were added to stimulate osteoclast differentiation. Number of osteoclasts per well is presented as mean +/- standard deviation. * denotes a statistically significant difference at $p < 0{,}05$.

2. Histological analysis

Histological sections of tibiae, fractured and those from the pin control group, were stained for tartrate-resistant acid phosphatase (TRAP) enzyme activity to identify and count osteoclasts on bone or cartilage surfaces. The number of osteoclasts per bone surface (N.Oc) and percent of eroded surface (ES%) was determined on trabeculae of newly formed callus, trabeculae outside of the callus area and below the cortical bone, to evaluate how the fracture influenced the number of osteoclasts on those surface and how this number was influenced by loss of Fas receptor or its ligand, FasL. Osteoclasts adjacent to cartilaginous surface were also counted on epiphyseal cartilage and on cartilage formed in fracture callus.

Changes in number of osteoclasts per bone or cartilage surface and percent of eroded surface were analyzed at three different timepoints following the fracture or sham procedure – 1, 2, and 3 weeks after.

2.1. Healing of controlled tibial fractures occurred through endochondral ossification

Histology showed that healing occurred through endochondral ossification in all three strains (B6, Fas -/-, and FasL -/-). In the first week after the fracture,

undifferentiated mesenchymal tissue was predominant in fracture calluses of all three mouse strains (Figure 7). Cartilage tissue was also abundant, and bony trabeculae were evident at some parts of the callus with few TRAP positive osteoclasts on their surface. In the second week, fracture callus was composed of hyaline cartilage and newly formed bony trabeculae (Figure 8). Numerous TRAP positive osteoclasts were observed on the trabeculae. In the third week, cartilage tissue (soft callus) was replaced by trabecular bone which formed the hard callus (Figure 9).

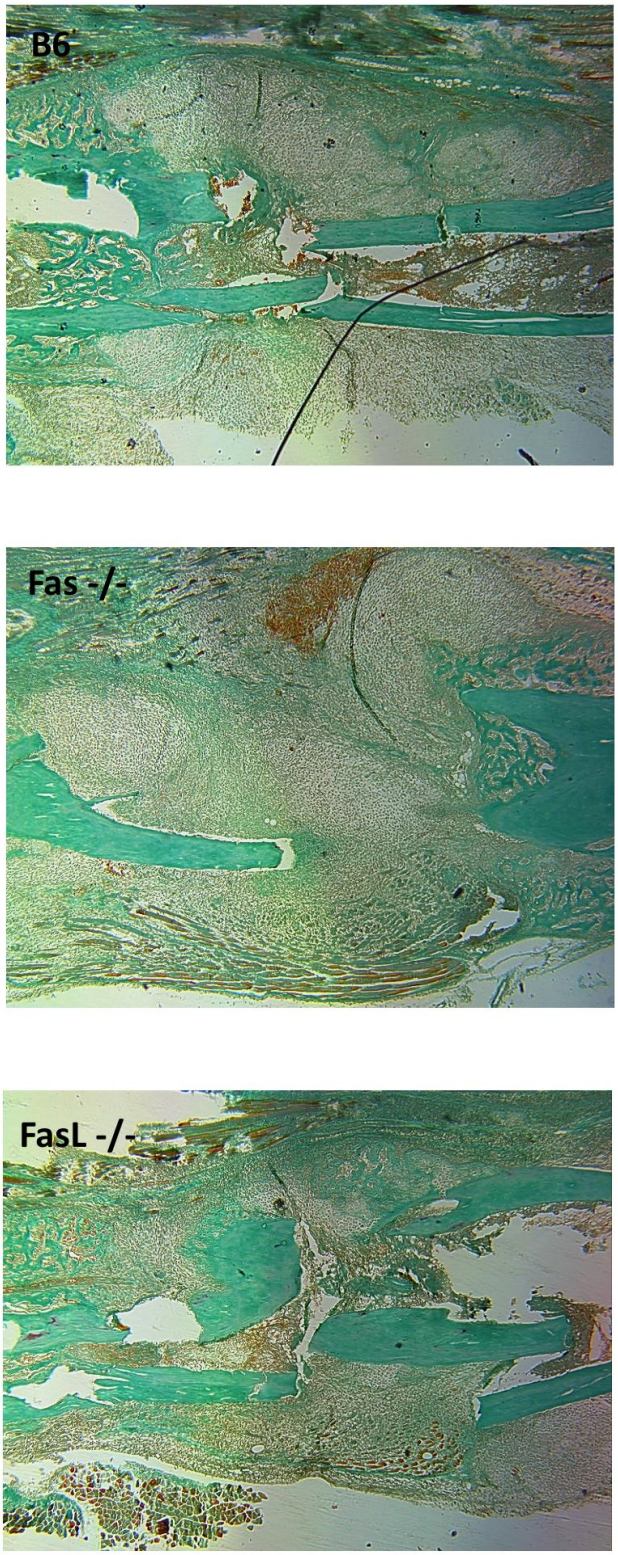


Figure 7. Histological presentation of fracture healing in the first week after the fracture in B6, Fas -/-, and FasL -/- tibiae. Representative sections of fracture calluses of tibiae under 25× magnification stained with Goldner-trichrome stain.

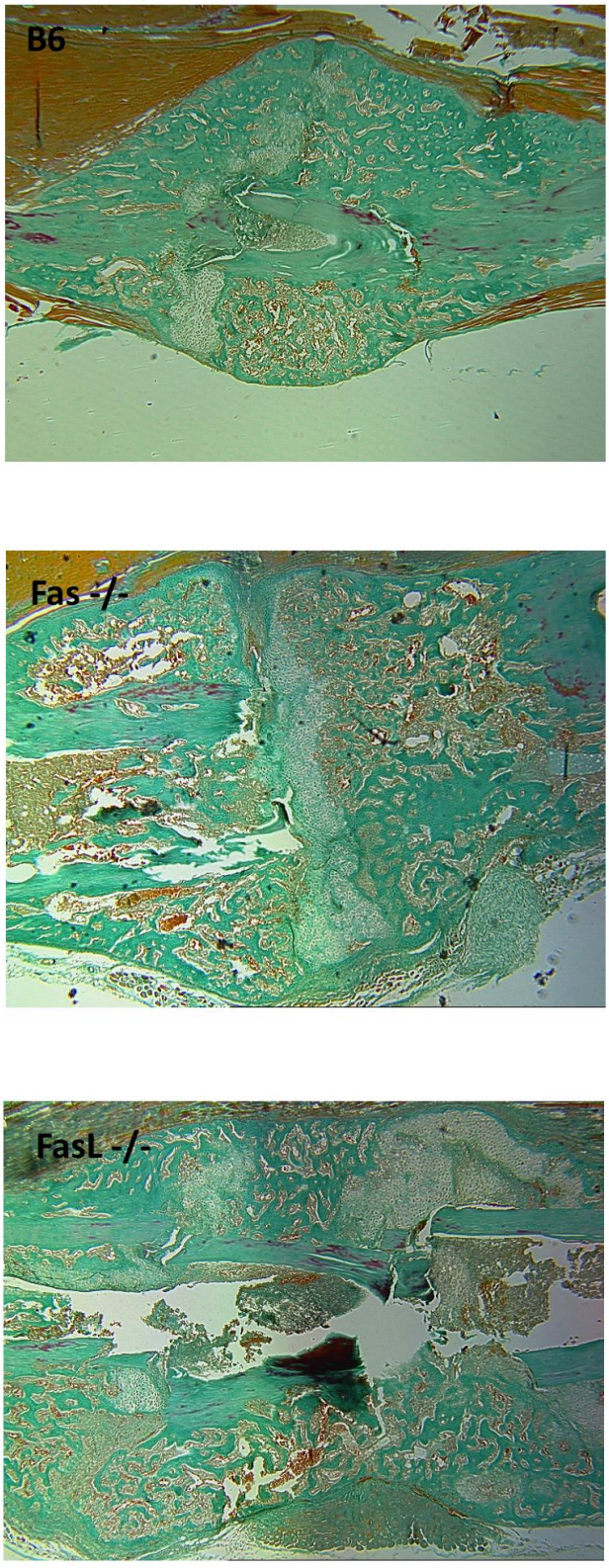


Figure 8. Histological presentation of fracture healing in the second week after the fracture in B6, Fas -/-, and FasL -/- tibiae. Representative sections of fracture calluses of tibiae under 25× magnification stained with Goldner-trichrome stain.

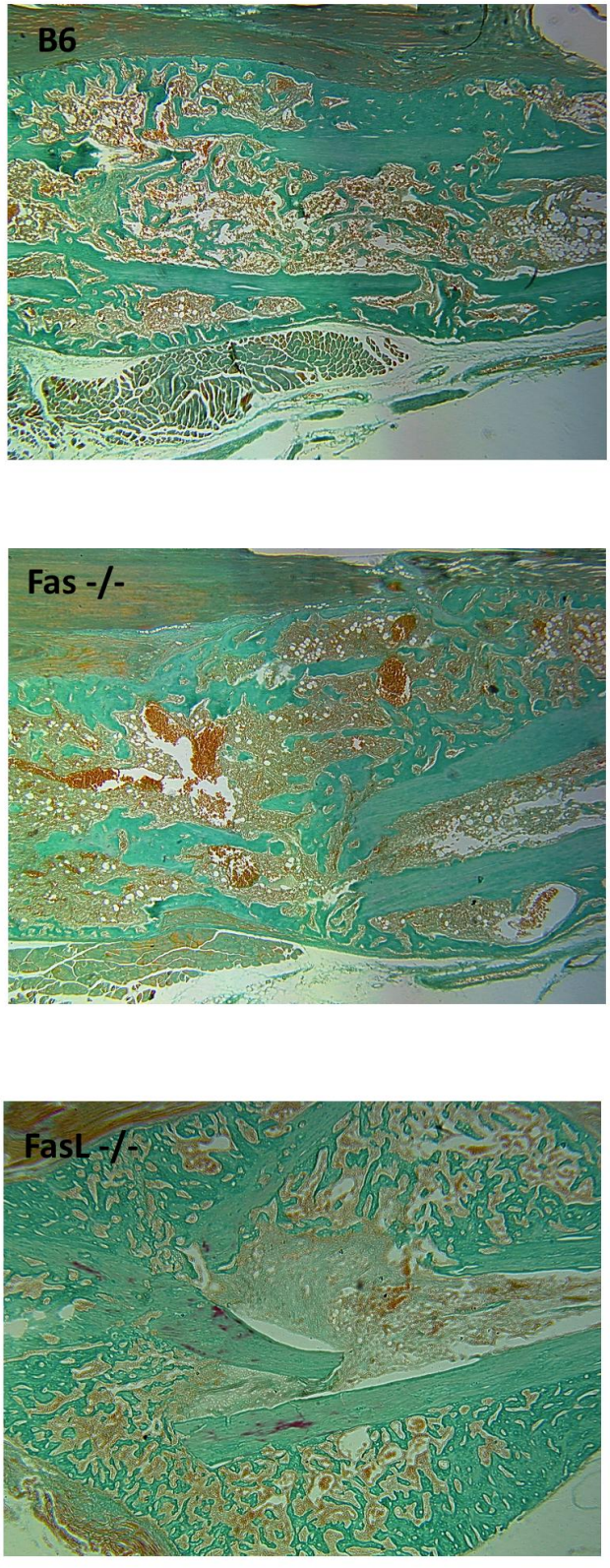


Figure 9. Histological presentation of fracture healing in the third week after the fracture in B6, Fas -/-, and FasL -/- tibiae. Representative sections of fracture calluses of tibiae under 25× magnification stained with Goldner-trichrome stain.

2.2. Effect of loss of Fas or FasL on the number of osteoclasts and percent of eroded surface on trabecular bone of tibiae

2.2.1. Trabeculae of fracture callus

The number of osteoclasts on trabeculae of fractured callus was lowest in the first week after the fracture. At that time point, tibiae of B6, Fas -/- and FasL -/- showed no significant difference (Figure 11). The number doubled in the second week after the fracture in Fas -/- and FasL -/- tibiae, but did not significantly change in the B6 control group (Figure 10). The number of osteoclasts at that time point (week 2) was significantly higher in the knockout strains than in the control group (Figure 12). The number of osteoclasts per bone surface in the knockout strains (Fas -/- and FasL -/-) did not change from second to third week (Figure 10). Mice from B6 control group showed and increase in number of osteoclasts from 2 to 3 week (Figure 10). In the third week, the number was not statistically different from knockout strains (Figure 13).

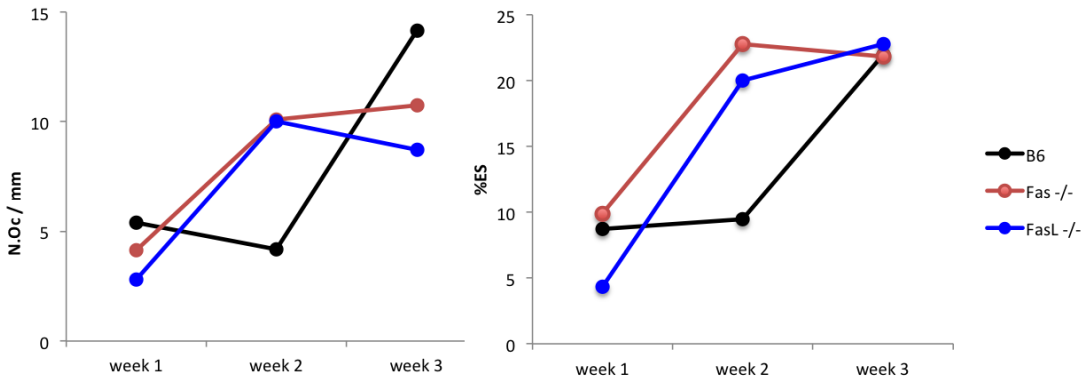


Figure 10. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on the trabeculae of fracture callus during three time points (week 1, week 2, and week 3) after the fracture of B6 control, Fas -/-, and FasL -/- tibiae. The number of TRAP positive osteoclasts is counted per mm of bone surface and presented as their mean value.

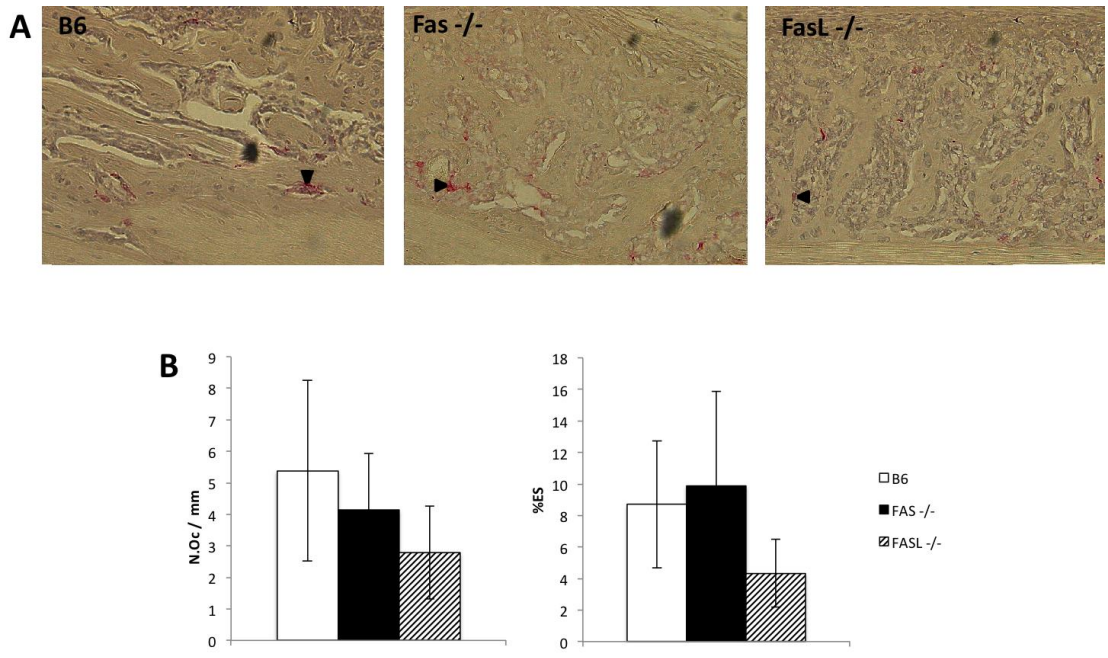


Figure 11. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on the trabeculae of fracture callus in the first week after the fracture of B6 control, Fas -/-, and FasL -/- tibiae. A. Representative sections of trabeculae in callus of fractured tibiae of B6, Fas -/- and FasL -/- mice in the first week after the fracture under 200× magnification stained with TRAP. Arrows denote TRAP positive osteoclasts. **B.** The number of TRAP positive osteoclasts is counted per mm of bone surface. Number of osteoclasts /mm and percent of eroded surface are presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

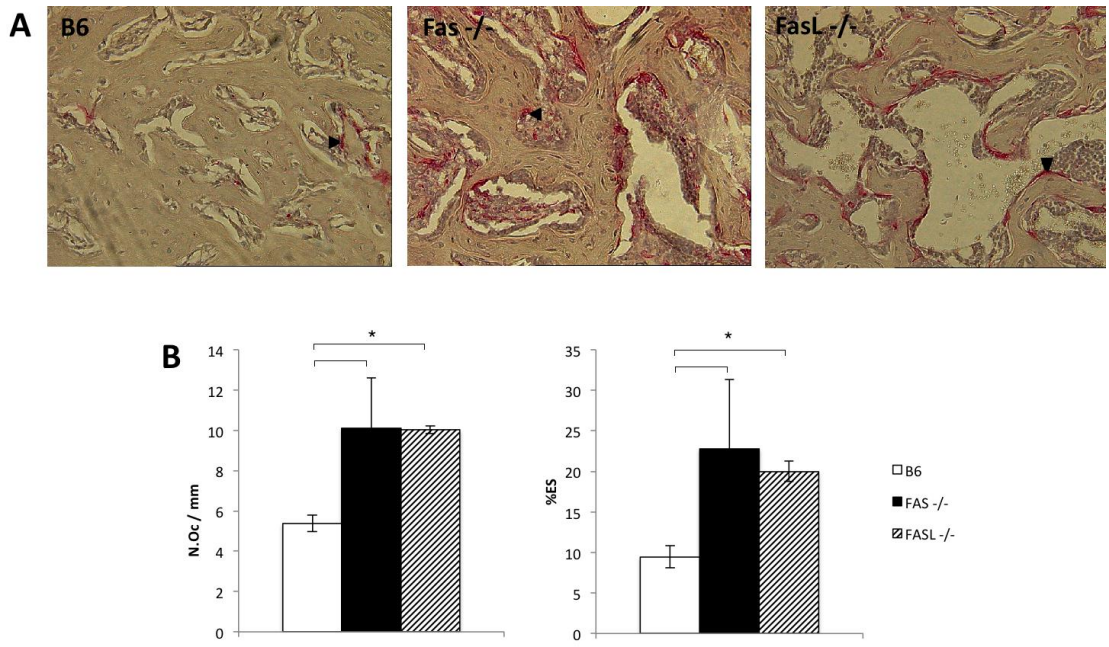


Figure 12. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on the trabeculae of fracture callus in the second week after the fracture of B6 control, Fas -/-, and FasL -/- tibiae. A. Representative sections of trabeculae in callus of fractured tibiae of B6, Fas -/-, and FasL -/- mice in the second week after the fracture under 200× magnification stained with TRAP. Arrows denote TRAP positive osteoclasts. **B.** The number of TRAP positive osteoclasts is counted per mm of bone surface. Number of osteoclasts / mm and percent of eroded surface are presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

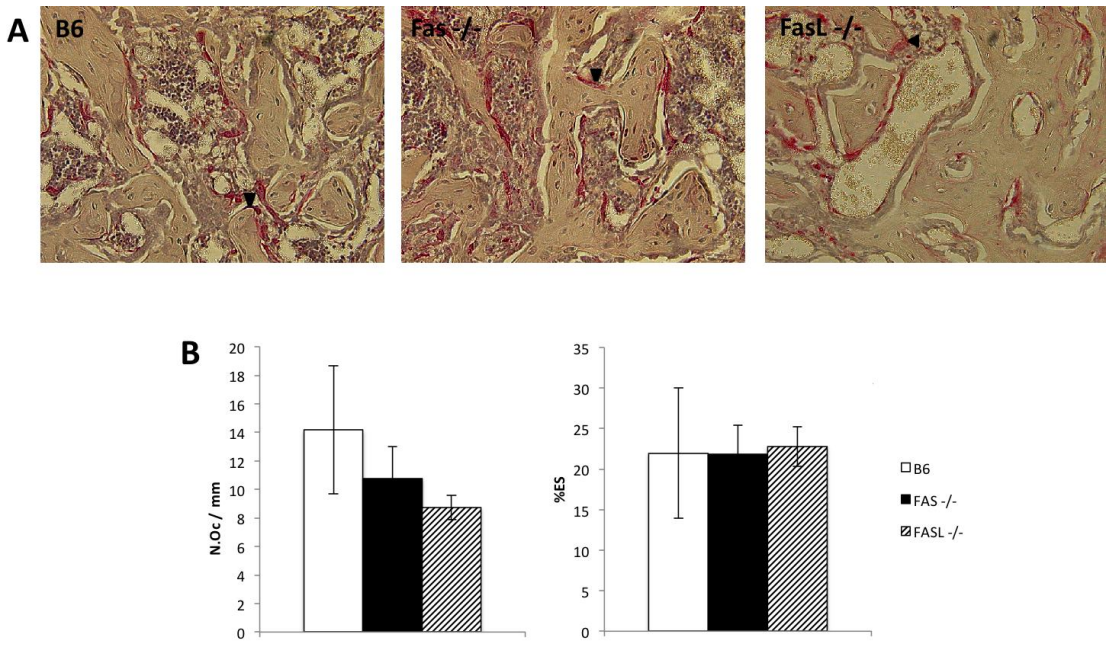


Figure 13. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on the trabeculae of fracture callus in the third week after the fracture of B6 control, Fas -/- and FasL -/- tibiae. A. Representative sections of trabeculae in callus of fractured tibiae of B6, Fas -/- and FasL -/- mice in the third week after the fracture under 200× magnification stained with TRAP. Arrows denote TRAP positive osteoclasts. **B.** The number of TRAP positive osteoclasts is counted per mm of bone surface. Number of osteoclasts / mm and percent of eroded surface are presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

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2.2.2. Trabeculae outside of callus area

In the fractured tibiae of Fas -/- and FasL -/- mice, trabeculae of tibiae outside of callus did not show significant differences in the number of osteoclasts per bone surface and in percent of eroded surface between different time points. In the fractured tibiae of B6 control mice, there was a slight difference in the number of osteoclasts per surface between the first and second week, and a slight difference between eroded surface between the third and second week. The three different strains differed only by the number of osteoclasts per bone surface in the first week after the fracture, when B6 control mice had a higher number of osteoclasts than Fas and FasL knockout mice. At other time points, there were no significant differences between strains for both N.Oc and %ES. (Figure 14)

When fractured tibiae were compared to tibiae of the pin control group, fractured tibiae of B6 control mice had and a higher number of osteoclasts on bone surface in first and second week, but lower in the third week. Similar changes were observed with percent of eroded surface. Fas -/- mice showed no difference between the fractured and pin control tibiae, except for a slightly higher number of osteoclasts per surface in the pin control tibiae in the first week. FasL -/- mice had a higher number of osteoclasts per surface and a higher percentage of eroded surface in the pin control tibiae in second week after the fracture. This difference was not observed at other time points. (Figure 15, 16, 17)

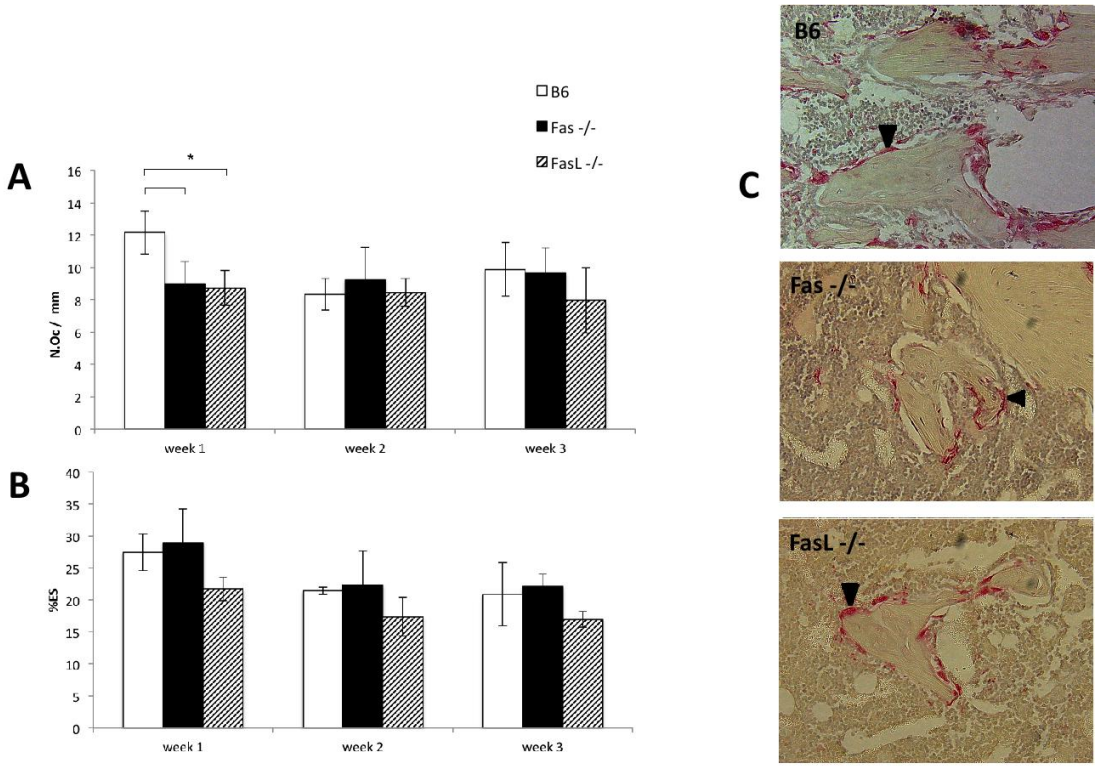


Figure 14. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on the trabeculae outside of fracture callus in the first, second and third week after the fracture of B6 control, Fas -/- ,and FasL -/- tibiae. A & B. The number of TRAP positive osteoclasts is counted per mm of bone surface. Number of osteoclasts /mm and percent of eroded surface are presented as mean +/- standard deviation. **C.** Representative sections of fractured tibiae of B6, Fas -/-, and FasL -/- mice in the first week after the fracture under 200× magnification stained with TRAP. Arrows denote TRAP positive osteoclasts. * denotes a statistically significant difference at p <0,05.

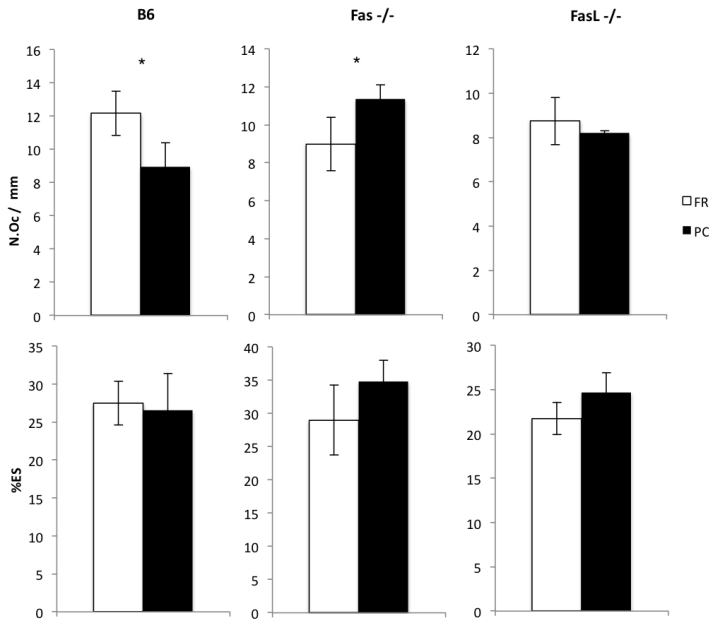


Figure 15. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on the trabeculae outside of fracture callus in the fractured (FR) and pin control (PC) tibiae in the first week after the fracture of B6 control, Fas -/-, and FasL -/- tibiae. The number of TRAP positive osteoclasts is counted per mm of bone surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

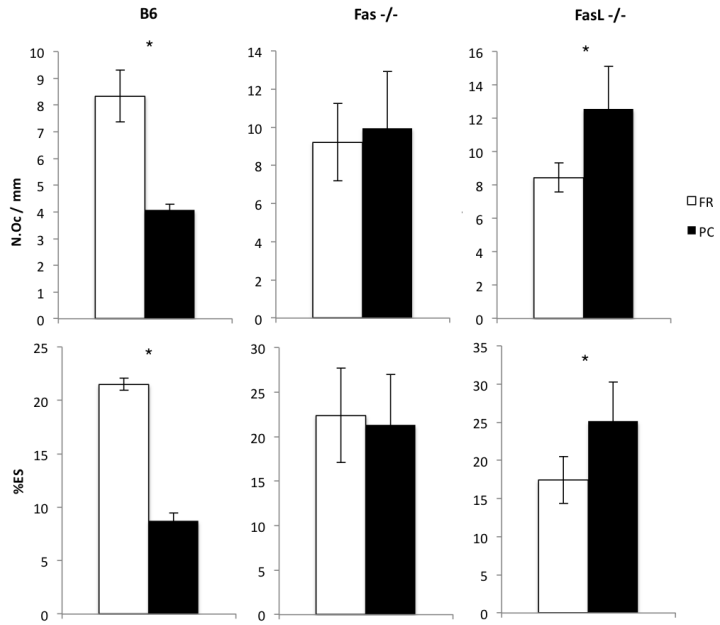


Figure 16. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on the trabeculae outside of fracture callus in the fractured (FR) and pin control (PC) tibiae in the second week after the fracture of B6 control, Fas -/- and FasL -/- tibiae. The number of TRAP positive osteoclasts is counted per mm of bone surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

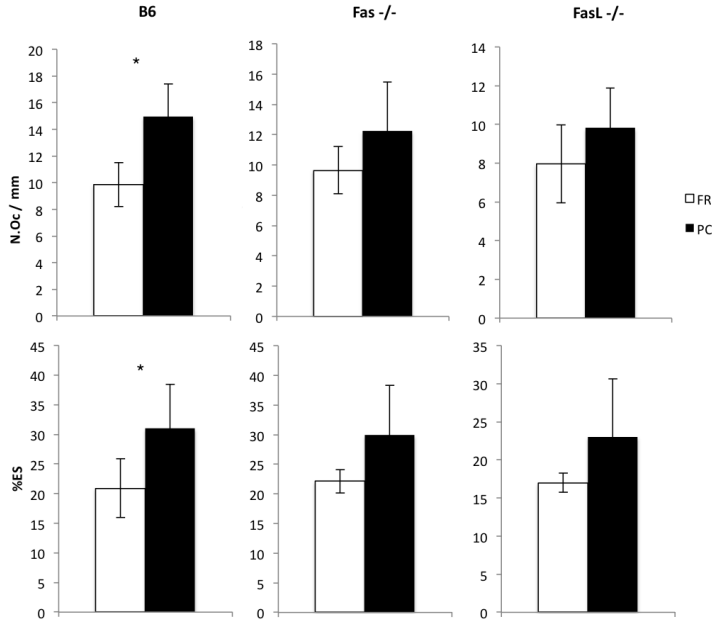


Figure 17. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on the trabeculae outside of fracture callus in the fractured (FR) and pinc control (PC) tibiae in the third week after the fracture of B6 control, Fas -/- and FasL -/- tibiae. The number of TRAP positive osteoclasts is counted per mm of bone surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

2.2.3. Comparison of trabeculae outside the callus area and newly formed trabeculae in callus

Number of osteoclasts (N.Oc) and eroded surface (%ES) were measured and compared on trabecular bone outside of callus and on trabeculae of newly formed callus. In the first week after the fracture, the number of osteoclasts per bone surface was significantly higher on the trabeculae that were not in fracture callus than on newly formed trabeculae of fracture callus in all mouse strains (Figure 18). In the second week, the number stayed higher only in the B6 control group, while it was the same and lower in Fas -/- and FasL -/- mice, respectively (Figure 19). In the third week, there were no significant differences (Figure 20).

The percent of eroded surface followed a similar fashion – the percentage was significantly lower on the trabeculae of fracture callus in all three stranis in the first week (Figure 18). In second week, this percentage stayed higher only in the B6 control group, while there was no difference between trabecullae in and out of callus in the knockout strains (Figure 19). In the third weeks, there were no differences, except of a sight increase in the eroded surface on traeculae of callus of FasL -/- tibiae (Figure 20).

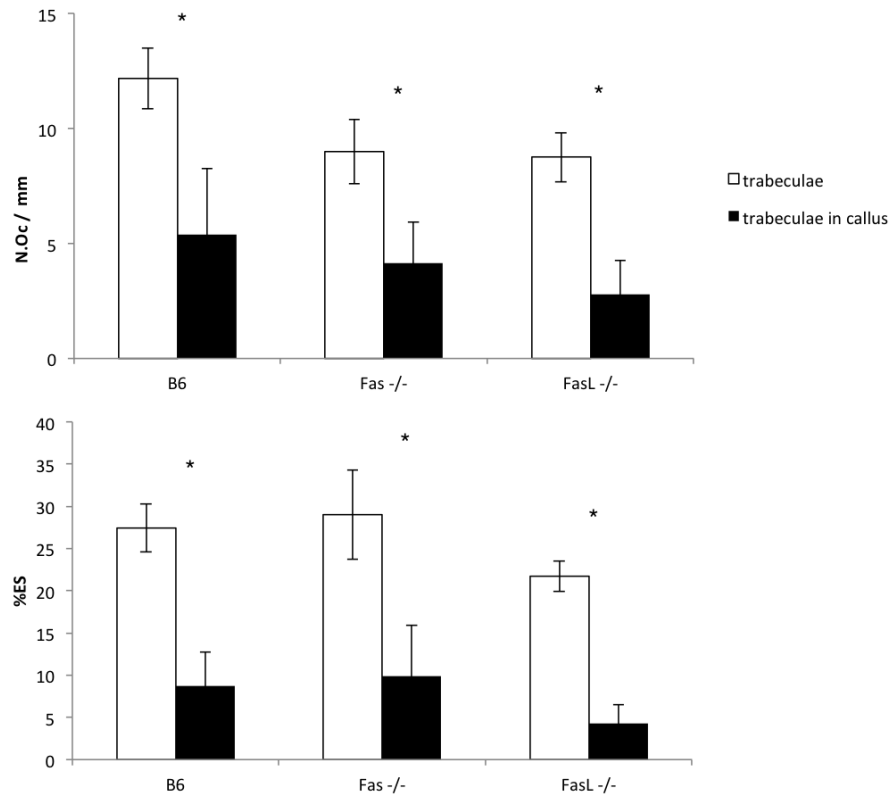


Figure 18. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on trabeculae of fracture callus and trabeculae outside of callus in the first week after the fracture of B6 control, Fas -/- ,and FasL -/- tibiae. The number of TRAP positive osteoclasts is counted per mm of bone surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

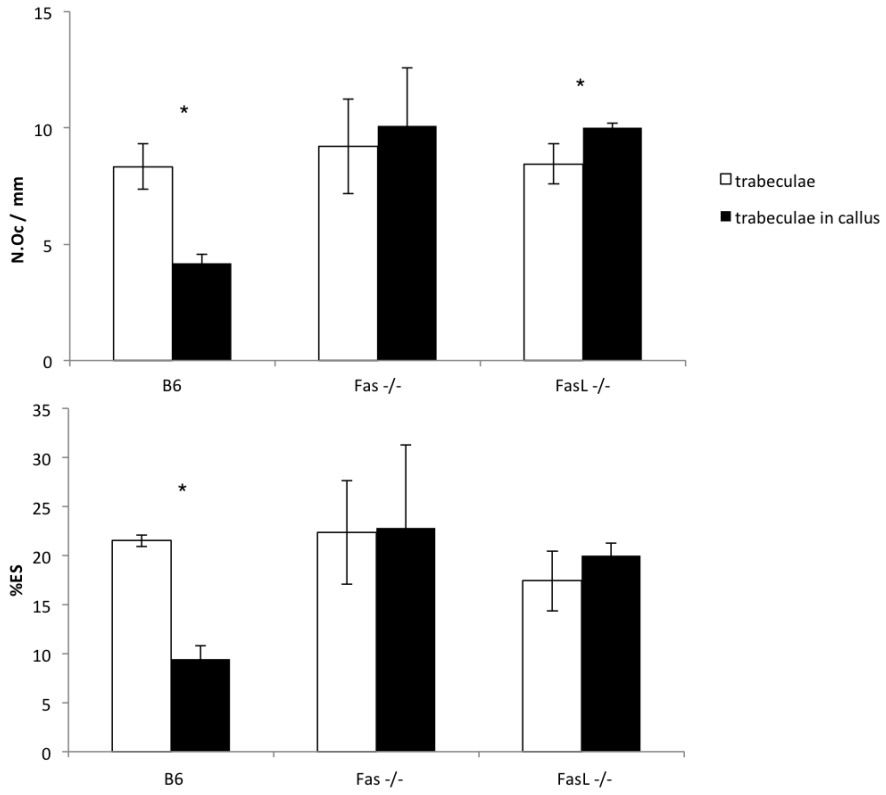


Figure 19. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on trabeculae of fracture callus and trabeculae outside of callus in the second week after the fracture of B6 control, Fas -/-, and FasL -/- tibiae. The number of TRAP positive osteoclasts is counted per mm of bone surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05**.**

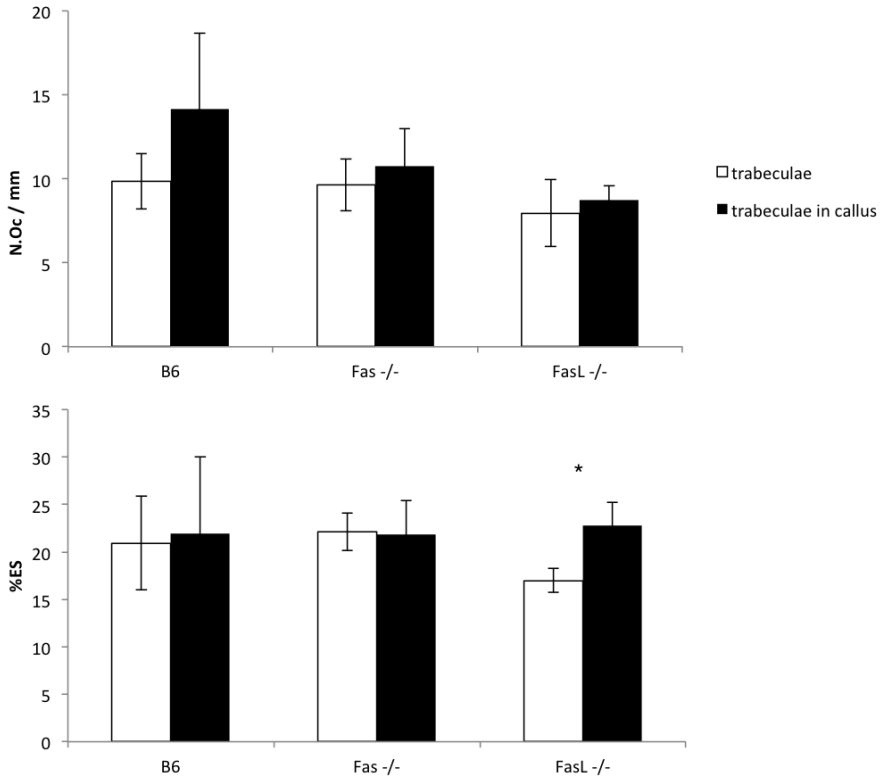


Figure 20. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on trabeculae of fracture callus and trabeculae outside of callus in the third week after the fracture of B6 control, Fas -/-, and FasL -/- tibiae. The number of TRAP positive osteoclasts is counted per mm of bone surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

2.2.4. Subcortical bone

The number of osteoclasts per surface of subcortical bone of fractured tibiae was the lowest in the first week after the fracture in all strains. The number increased in the second week, but the difference was statistically significant only in Fas -/- and FasL -/-. The number stayed high in the third week in B6 control and Fas -/- fractured tibiae, but decreased to values similar to those from first week in FasL -/- mice. Eroded surface followed a similar fashion, but the differences between weeks were only significant in fractured tibiae of Fas -/- mice (Figure 21).

There were no differences between number of osteoclasts on bone surfaces and percentage of eroded surface at first and second week after the fracture, but in the third week, B6 control mice had a significantly higher number of osteoclasts per subcortical bone than the knockout strains and a higher percentage of eroded surface than FasL -/- mice. The knockout strains also differed between each other in the third week – Fas -/- mice had both higher number of osteoclasts per bone surface and a hiher percentage of eroded surface than FasL -/- mice. (Figure 22)

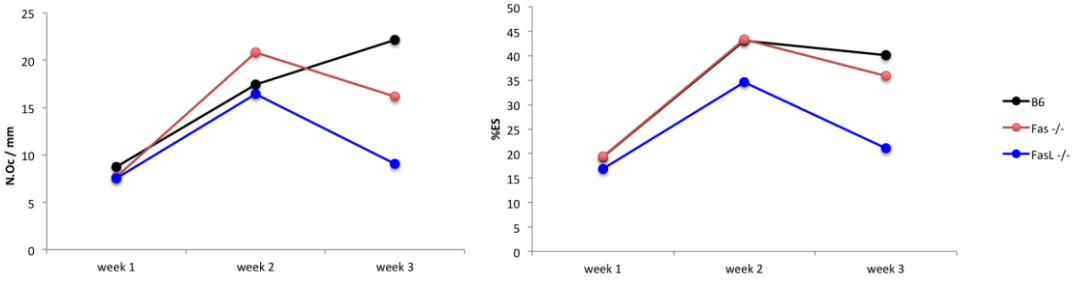


Figure 21. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on subcortical bone in fractured tibiae 1, 2 and 3 weeks after the fracture in B6, Fas -/- and FasL -/- mice. The number of TRAP positive osteoclasts is counted per mm of bone surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

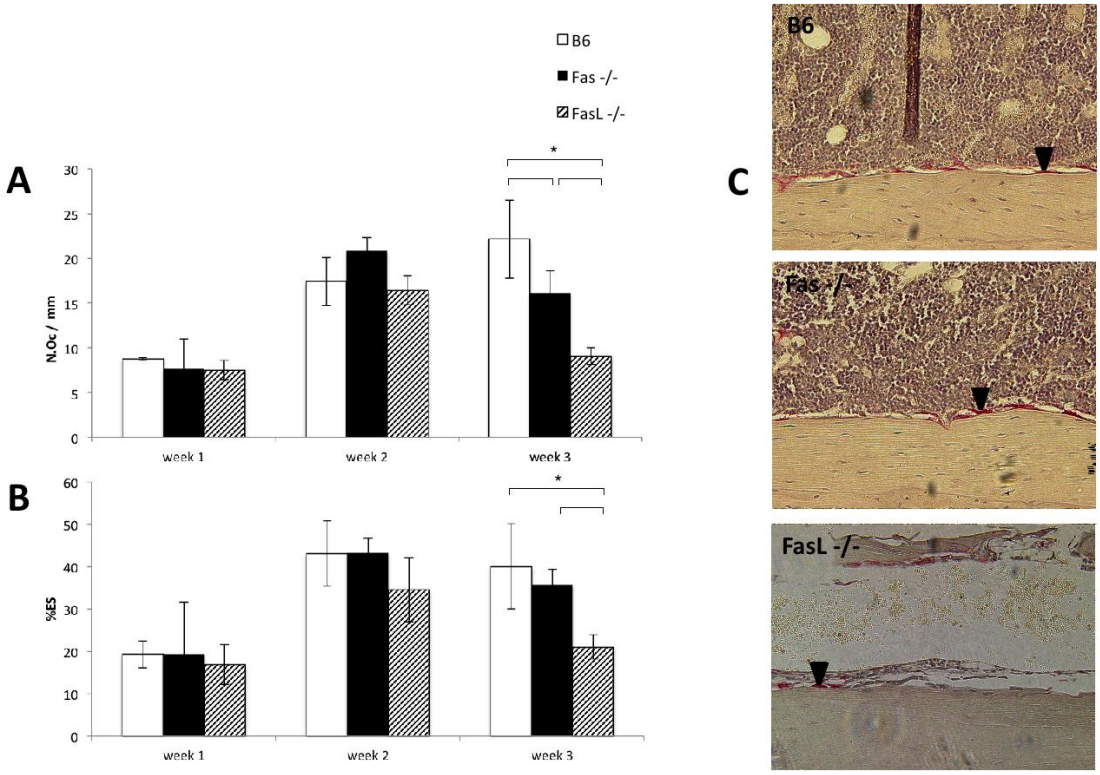


Figure 22. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) and percent of eroded surface (%ES) on subcortical bone in fractured tibiae 1, 2 and 3 weeks after the fracture in B6, Fas -/- ,and FasL -/- mice. A & B. The number of TRAP positive osteoclasts is counted per mm of bone surface. Number of osteoclasts / mm and percent of eroded surface are presented as mean +/- standard deviation. **C.** Representative sections of fractured tibiae of B6, Fas -/- and FasL -/- mice in the third week after the fracture under 200x magnification stained with TRAP. Arrows denote TRAP positive osteoclasts. * denotes a statistically significant difference at p <0,05.

2.3. Effect of loss of Fas and FasL on the number of osteoclasts on cartilage surface

The number of osteoclasts per cartilage surface were counted adjacent to the epiphyseal (growth plate) cartilage and in the fracture callus in the second week after the fracture.

The number of osteoclasts per epiphyseal cartilage surface of fractured tibiae did not differ between Fas -/-, FasL -/- ad B6 control mice at any time point after the fracture, and did not significantly change between different time points, except in FasL -/- tibiae when the number of osteoclasts was slightly higher in second week after the fracture. (Figure 23)

When mice from the pin control group were compared to mice from the fracture group, there were no significant differences between numbers of osteoclasts adjacent to growth plate cartilage, except of lower numbers of osteoclast per cartlage surface fractured tibiae of B6 control mice in the first and second week and in FasL -/- mice in third week after the fracture. (Figure 24)

Number of osteoclast per cartilage surface in the knockout strains (Fas -/- and FasL -/-) in the fracture callus second week after the fracture did not significantly differ from the B6 control group. (Figure 25)

Comparison of cartilage surface on the growth plate of fractured tibiae and cartilage in fracture callus showed there were no significant differences between the numbers of osteoclasts on cartilage surfaces. (Figure 26)

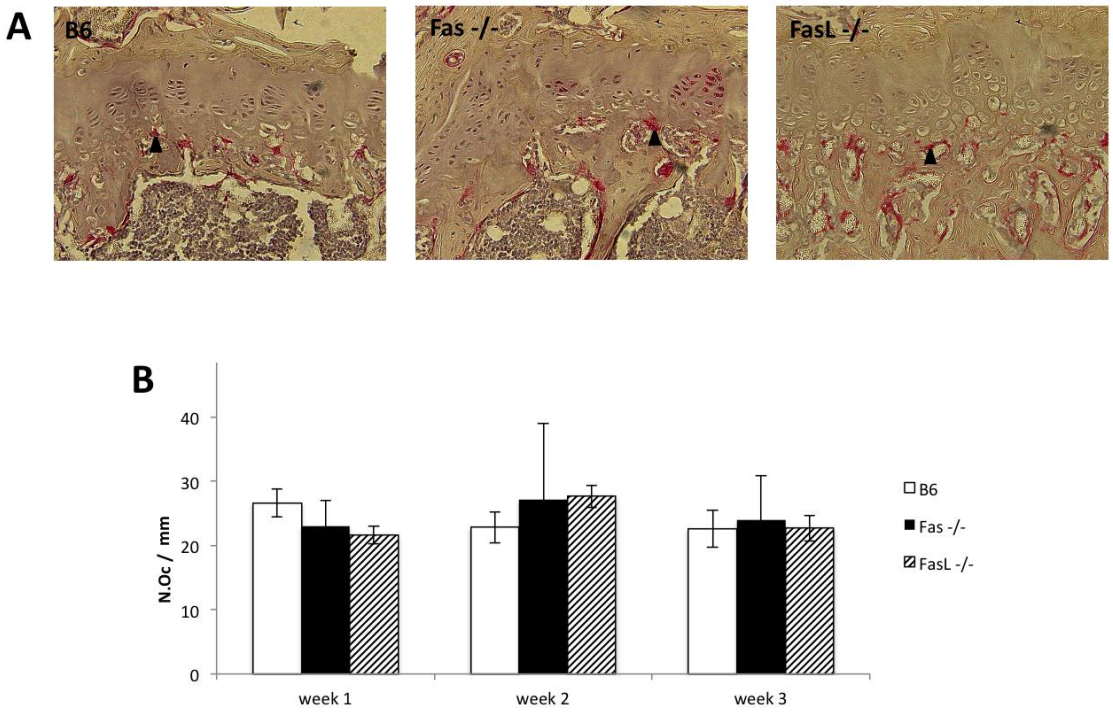


Figure 23. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) on cartilage surface of epiphyseal plates of fractured tibiae in B6, Fas -/- and FasL -/- mice at 1, 2 and 3 weeks after the fracture. A. Representative sections of epiphyses of fractured tibiae of B6, Fas -/- and FasL -/- mice in the first week after the fracture under 200x magnification stained with TRAP. Picture shows growth plate cartilage. Arrows denote TRAP positive osteoclasts. **B.** The number of TRAP positive osteoclasts is counted per mm of cartilage surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

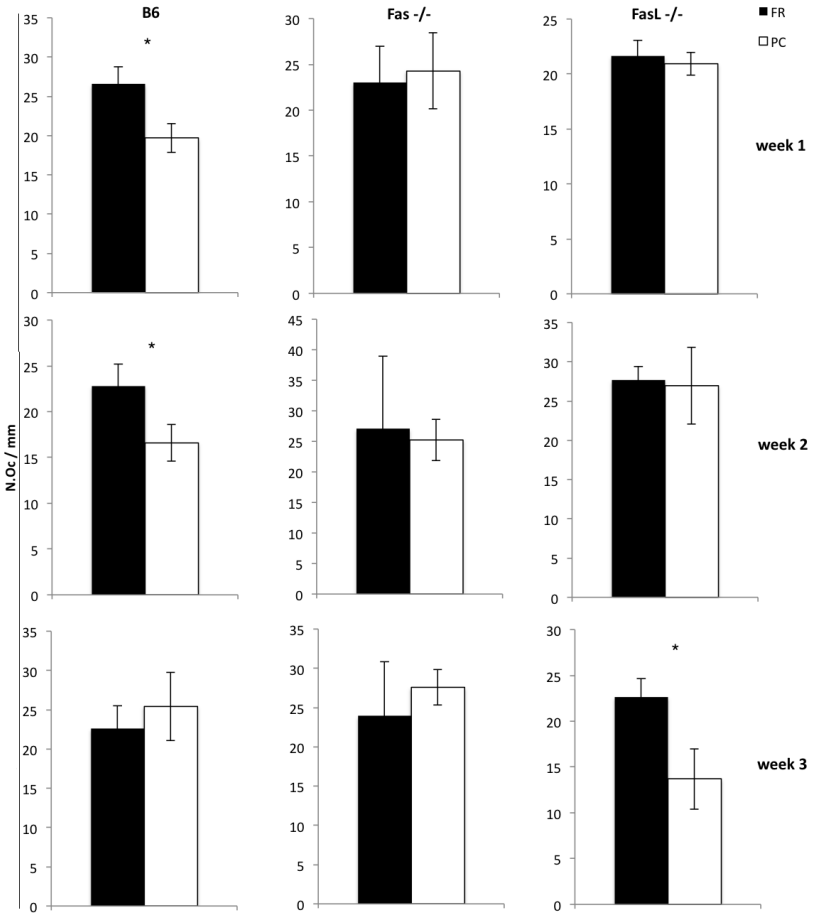


Figure 24. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) on cartilage surface of epiphyseal plates of fractured tibiae compared to tibiae of pin control group of B6, Fas -/- and FasL -/- mice at 1, 2 and 3 weeks after the fracture. The number of TRAP positive osteoclasts is counted per mm of cartilage surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

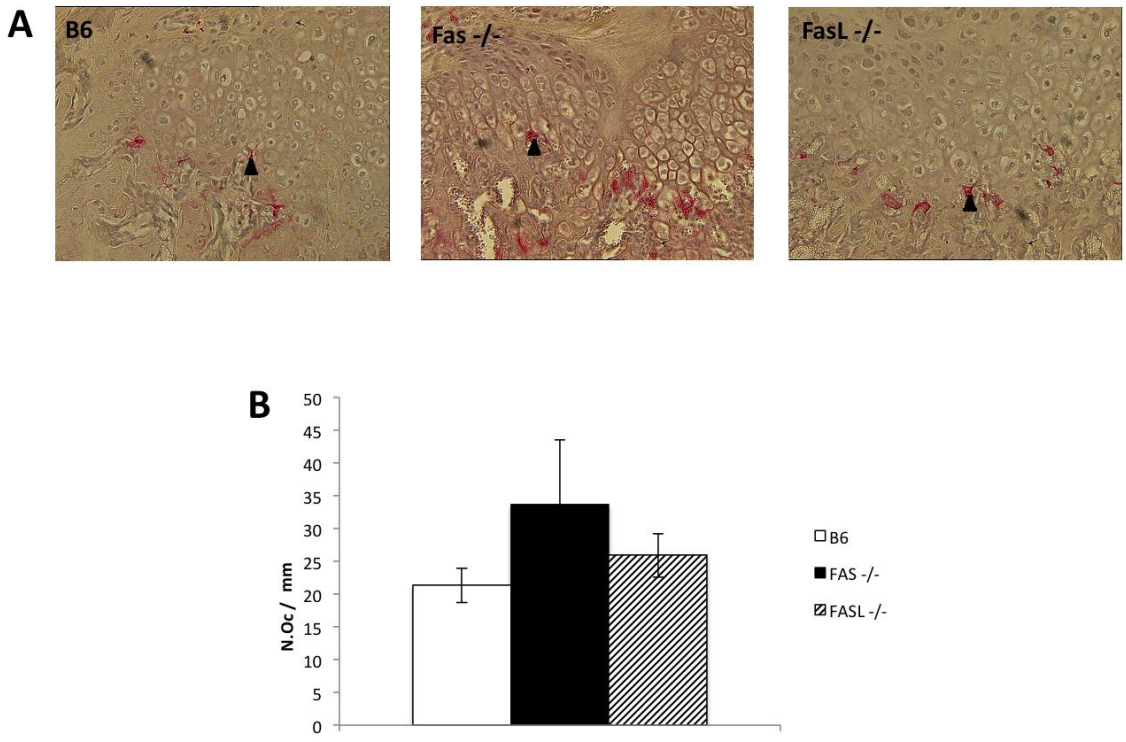


Figure 25. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) on cartilage surface in the fracture calluses of tibiae in the second week after the fracture in B6, Fas -/- and FasL -/- mice. A. Representative sections of callus of fractured tibiae of B6, Fas -/- and FasL -/- mice in the second week after the fracture under 200x magnification stained with TRAP. Picture shows hyaline cartilage present in the fracture callus. Arrows denote TRAP positive osteoclasts. **B.** The number of TRAP positive osteoclasts is counted per mm of cartilage surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at $p < 0,05$.

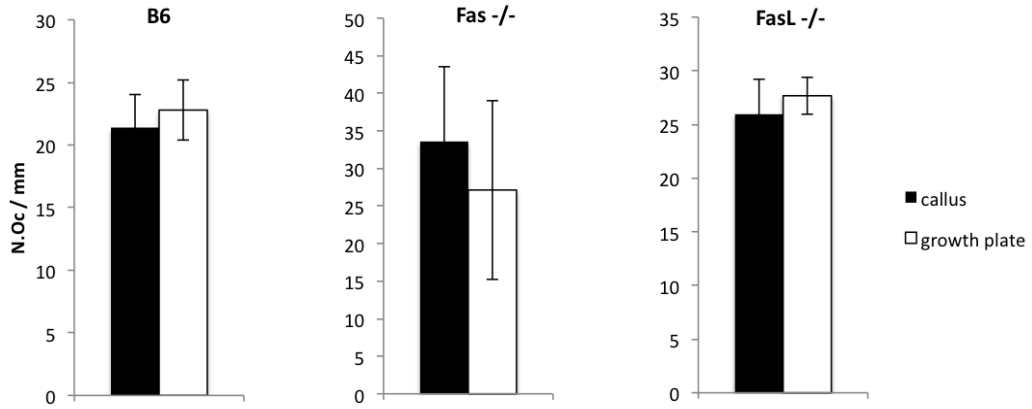


Figure 26. Histological analysis of the number of TRAP positive osteoclasts (N.Oc) on cartilage surface in the fracture calluses of tibiae in the second week after the fracture and the number of osteoclasts on surface of growth plate cartilage at that time point in B6, Fas -/-, and FasL -/- mice. The number of TRAP positive osteoclasts is counted per mm of cartilage surface and presented as mean +/- standard deviation. * denotes a statistically significant difference at p <0,05.

DISCUSSION

It has been previously described that the Fas/FasL apoptotic system has an influence on osteoclast life span and differentiation and plays a role in regulation of bone homeostasis (Katavic et al. 2003, Wu et al. 2003, Roux et al. 2005, Wang et al. 2015, Park et al. 2005b). With our experiment, we tried to define the role of Fas/FasL system on osteoclatsogenesis *in vitro* and *in vivo.*

By culturing osteoclast progenitor cells from bone marrow and blood, we tried to define the influence of loss of Fas and FasL on the *in vitro* ability of those progenitors to differentiate into mature osteoclast as well as evaluate the influence of fracture on their differentiation in B6 control and Fas and FasL deficient phenotypes. Other studies have shown that a spontaneous mutation of FasL (in *gld* mice) does not alter the potential of bone marrow progenitors in comparison to the control groups, but a complete loss of functional Fas receptor in Fas knockout (Fas -/-) mice caused a higher osteoclastogenic potential in comparison to the control (Kovacic et al. 2007).

When Fas -/- and FasL -/- mice were compared with B6 control in this experiment, osteoclast cell cultures showed differences between strains that were difficult to interpret at almost all time points in all three samples; bone marrow of tibiae, bone marrow of femurs, and blood. We also tried to evaluate the effect of the fracture procedure on the ability of progenitors to differentiate into mature osteoclasts. In bone marrow of tibiae, the fracture procedure caused lower osteoclastogenic potential of progenitor cells than the potential of those who's tibias were not fractured (the control group), but this was not apparent in bone marrow of femurs and blood. Since it was difficult to find an explanation for our results, the experiment should be repeated in order to be able to correctly interpret the differences between strains.

Histological analysis of fractured and pin control tibiae from B6, Fas -/-, and FasL -/- mice allowed us to evaluate the effect of loss of the death receptor or its ligand on osteoclastogenesis *in vivo*. Sections of tibiae were analyzed to see how loss of Fas an FasL affected the number of osteoclasts at different stages of fracture healing and to provide an insight to how the fracture itself influenced the measured parameters, by comparing the

fractured tibiae to the ones that only had the stainless steel wire inserted (pin control group). The model of controlled tibial fracture was used since fracture healing recapitulates the embryonic processes that occur during bone formation (Ferguson et al. 1999) and follows steps of endochondral ossification if there is some degree of mechanical instability present during the fracture healing process (Einhorn & Gerstenfeld 2015, Schindeler et al. 2008). In our experiment, histological observations of fractured tibiae confirmed that fracture healing occurred through a typical endochondral ossification pathway in B6, Fas -/-, and FasL -/- mice. Fracture calluses in the first and second week after the fracture showed abundant cartilage tissue. Bone formation was evident even in the first week, and newly formed trabeculae were evident at multiple sites in the callus. This early occurrence of bony trabeculae (by day 7 after the fracture) has also been described by Manigrasso et al in the C57BL/6 strain (Manigrasso & O'Connor 2008). The cartilage tissue was almost completely replaced by bone in the third week in all fracture groups.

Fracture healing proceeds through several steps – an initial inflammatory stage with hematoma formation that happens early after the fracture (happens earlier than our earliest time-points and, hence, was not observed in our experiment); a proliferative stage with soft and hard callus formation and the remodeling stage (Schindeler et al. 2008); and a resorptive phase. The phases of healing can also be defined as anabolic and catabolic. The catabolic phase, caused by osteoclast activity, starts with cartilage resorption and then continues through primary bone resorption and the remodeling of the callus (Einhorn & Gerstenfeld 2015). We analyzed the fracture calluses for numbers of osteoclasts on the surfaces of newly formed trabeculae, to see how the loss of the Fas system affected those cells during the period of new bone formation. As expected, the lowest numbers of osteoclasts on bone surfaces were found in the first week, when the bone was not that abundant yet. The numbers did not differ between B6 control and the knockout strains (Fas -/- and FasL -/-), indicating that loss of Fas receptor or its ligand did not cause a change at this time point. It has been previously proposed that loss of functional Fas signaling does not appear to alter early callus development, but causes differences in the later stages of removal of cartilage and remodeling of bone (Al-Sebaei et al. 2014). The number of

osteoclasts and the percent of eroded bone surface both almost doubled by the second week and stayed at this level until the third week in tibiae with no functional Fas signaling (Fas -/- and FasL -/- mice). In the B6 control, the number in the second week was approximately the same as in first week, but then increased in the third week, showing that the increase in osteoclast numbers occurred later when Fas signaling was normal and suggesting that the remodeling of the fracture calluses started early when there was no Fas signaling. Al-Sebaei et al (Al-Sebaei et al. 2014) also described a higher number of osteoclasts in second week calluses in *lpr* mice compared to the controls. It is also possible that the parts of the calluses that were visible on the sections were not representative in the B6 controls, because of the small number of samples used, since there should be some increase in remodeling observed by the second week after the fracture. Outside of the callus, on the same tibiae, the number of osteoclasts on the trabeculae in the second and third week did not differ between the B6 control group and Fas -/-, and FasL -/- mice, indicating that if this change that occurred in the second week with loss of functional Fas signaling was correctly observed, it only occurred during new bone formation.

We also compared osteoclast numbers in the callus to the numbers on the trabecular bone outside of the callus. In the first week, we found that the number of osteoclasts in calluses are lower, indicating there was almost no resorption going on in the callus at this time point. The same change was evident even in the second week in the B6 calluses, consistent with the result that there was no increase in the number of osteoclasts in callus at this time point, but not in the calluses where Fas signaling was dysfunctional – the numbers of osteoclasts have increased there, as was also described before. There were only rare differences between number of osteoclasts and the percent of eroded surface in callus and on trabecular bone outside of callus at the third week in B6 control and at second and third week in knockout strains, suggesting that the resorption in the callus was of the same intensity as the resorption of bone not involved in the fracture healing, or that the healing process caused an increase in osteoclasts at both locations.

When trabeculae outside of callus of fractured tibiae were compared to tibiae of the pin control group, there were slight differences between them in

all three strains at different time points that were difficult to explain. Fractured tibiae of B6 control mice had a higher number of osteoclasts on bone surface in first and second week, but lower in the third week, suggesting that the fracture is responsible for the increase in the number of osteoclasts in the early (first 2 weeks) stages of fracture healing. Similar change was not evident in the knockout strains. The differences might be due to the fact that the insertion of the pin also caused some trauma to the bones (in both fracture and pin control groups) and evoked a response with new bone formation and remodeling at those places.

We also observed the osteoclasts below the cortical bone, where there was an increase in the number of osteoclasts from the first to second week after the fracture in the control and the Fas and FasL deficient groups, indicating that the start of the remodeling in the fracture calluses also caused a certain increase in the number of osteoclasts on cortical bone, that was not dependent on Fas signaling.

When hypertrophic chondrocytes die by apoptosis during endochondral ossification, osteoclasts are responsible for the removal of the cartilaginous matrix (Einhorn & Gerstenfeld 2015, Gerstenfeld et al. 2003) and can be observed on the cartilaginous surfaces on histological sections. Cartilage surfaces at two places in the fractured tibiae were analyzed in our experiment – at the border between the epiphysis and diaphysis of the bone, where hyaline cartilage forms the epiphyseal plate and is responsible for bone growth (Mescher 2013), and in the fracture calluses, where the surface was particularly well defined in the second week after the fracture, when osteoclasts were numerous on the borders where cartilage is being replaced by bone. It is evident from our results, that the loss of Fas or FasL did not influence the number of osteoclasts on any of the cartilage surfaces. The numbers on cartilage of week 2 calluses were not different between strains. When comparing the numbers of osteoclasts on the border of cartilage surface on growth plate and in second week, the analysis also showed no significant differences, suggesting that the resorption of cartilaginous matrix by osteoclasts was the same in the epiphyses as in the fracture callus and that Fas signaling had no apparent influence on the number of osteoclasts present on cartilaginous surfaces during new bone formation by endochondral

ossification. Comparison of the fracture and pin control groups showed that the numbers on the growth plate were only mildly influenced by the fracture procedure, which caused an increase only at two time points in B6 control and one on FasL. This may be attributed to the fact that the pin also caused trauma to the growth plate and caused a change in the number of osteoclasts on the surfaces.

To further evaluate the role of Fas and FasL on osteoclastogenesis and the behavior and activity of osteoclasts, it would be necessary to analyze the parameters describing trabecular bone morphology (trabecular thickens, number and separation) by histomorphometric or micro CT analysis and explore the gene expression of osteoclast-specific genes at different time points, but this was beyond the scope of this research.

CONCLUSIONS

In the *in vitro* part of the experiment, our conclusion was that repetition of the experiment would be necessary to correctly define the role of Fas/FasL pathway on osteoclastogenic potential of bone marrow of tibiae, femur and peripheral blood in the model of controlled tibial fracture.

In the *in vivo* part, we observed that the fracture healing occurred by endochondral ossification. We concluded that an increase in the number of osteoclasts on trabeculae in fracture calluses occurred earlier, in the second week after the fracture, when Fas signaling was dysfunctional, whereas in the control groups, it was observed in the third week. We also concluded that Fas/FasL system has no apparent role in regulation of number of osteoclasts resent on cartilage surfaces of growth plate and the week 2 fracture callus.

Our conclusion was that Fas/FasL system has a role in osteoclastogenesis during new bone formation in fracture healing, but further research is necessary to correctly interpret its exact function.

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