Mast Cells Trigger Disturbed Bone Healing in Osteoporotic Mice

Verena Fischer,1,† Deniz Ragipoglu,1,† Johanna Diedrich,1 Lena Steppe,1 Anne Dudeck,2 Konrad Schütze,3 Miriam Kalbitz,3,4 Florian Gebhard,3 Melanie Haffner-Luntzer,1,† and Anita Ignatius1,†

1Institute of Orthopedic Research and Biomechanics, University Medical Center Ulm, Ulm, Germany
2Institute for Molecular and Clinical Immunology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany
3Department of Traumatology, Hand-, Plastic-, and Reconstructive Surgery, University Medical Center Ulm, Ulm, Germany
4Department of Trauma and Orthopedic Surgery, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nürnberg (FAU), Erlangen-Nürnberg, Germany

ABSTRACT

Mast cells are important tissue-resident sensor and effector immune cells but also play a major role in osteoporosis development. Mast cells are increased in numbers in the bone marrow of postmenopausal osteoporotic patients, and mast cell–deficient mice are protected from ovariectomy (OVX)-induced bone loss. In this study, we showed that mast cell–deficient Mcpt5-Cre R-DTA mice were protected from OVX-induced disturbed fracture healing, indicating a critical role for mast cells in the pathomechanisms of impaired bone repair under estrogen-deficient conditions. We revealed that mast cells trigger the fracture-induced inflammatory response by releasing inflammatory mediators, including interleukin-6, midkine (Mdk), and C-X-C motif chemokine ligand 10 (CXCL10), and promote neutrophil infiltration into the fracture site in OVX mice. Furthermore, mast cells were responsible for reduced osteoblast and increased osteoclast activities in OVX mice callus, as well as increased receptor activator of NF-κB ligand levels in OVX mice. Additional in vitro studies with human cells showed that mast cells stimulate osteoclastogenesis by releasing the osteoclastogenic mediators Mdk and CXCL10 in an estrogen-dependent manner, which was mediated via the estrogen receptor alpha on mast cells. In conclusion, mast cells negatively affect the healing of bone fractures under estrogen-deficient conditions. Hence, targeting mast cells might provide a therapeutic strategy to improve disturbed bone repair in postmenopausal osteoporosis.

KEY WORDS: MAST CELLS; OSTEOPOROSIS; FRACTURE HEALING; INFLAMMATION; OSTEOCLASTOGENESIS

Introduction

Mast cells are important tissue-resident immune cells and play an important role in regulating inflammatory reactions and tissue homeostasis.11 A unique characteristic is the vast number of secretory granules storing preformed mediators, including histamine, heparin, proteases (eg, tryptase, chymase), cytokines (eg, interleukin-6 [IL-6], tumor necrosis factor [TNF]), and growth factors (eg, vascular endothelial growth factor, transforming growth factor-β [TGF-β], fibroblast growth factor [FGF]).12 The rapid degradation of these granules upon mast cell activation can be followed by de novo synthesis of several factors, including IL-6, interferon-γ (IFN-γ), IL-10, and receptor activator of NF-κB ligand (RANKL), thus modulating inflammatory responses.13,14 Many mast cell mediators can exert osteoclastic (eg, histamine, IL-6, TNF, RANKL) and osteo-anabolic functions (eg, TGF-β, FGF), implying that mast cells contribute to the regulation of bone turnover in health and disease.5–7

Indeed, mast cells appear to play a critical role in osteoporosis development. Their numbers are significantly increased in the bone marrow in postmenopausal osteoporosis.15 Moreover, the treatment of postmenopausal osteoporotic women with promethazine, a histamine H1 receptor inhibitor, increased bone mineral density,10 suggesting that mast cells, the major source of histamine, could be involved in bone loss.11 These clinical indications were confirmed by experimental studies of...
ovariectomized rodents. Likewise, an accumulation of mast cells was observed in the bone marrow after the ovariectomy (OVX)-induced estrogen decline.\(^{12,13}\) This increase of mast cells directly correlated with the increase of osteoclast numbers and bone resorption.\(^{14}\) These phenomenological findings were crucially substantiated by a recent study of our group, demonstrating that mast cells were essential to induce bone resorption after OVX.\(^{15}\) Mast cell–deficient mice were protected from OVX-induced bone loss, and mast cells promoted osteoclast formation, partially by histamine.\(^{15}\) It is presumed that mast cells are more sensitive under estrogen-deficient conditions, resulting in an increased release of pro-inflammatory and osteo-catabolic mediators, including IL-6, IL-1, TNF, and granulocyte-macrophage colony-stimulating factor (GM-CSF).\(^{12,15,16,17}\)

Mast cells may also play a regulatory role in bone fracture healing, a process that compromises consecutive and overlapping stages of inflammation, repair, and remodeling.\(^{18}\) They were found in the early fracture hematoma, and their numbers progressively increased during the course of healing.\(^{19-21}\) Using Mcpt5-Cre R-DTA mice, we demonstrated that mast cells trigger the immune response after fracture by inducing inflammatory mediator release, including IL-6 and IL-1\(\beta\), and innate immune cell recruitment, including neutrophils and macrophages.\(^{15}\) Mast cells may also augment angiogenesis, bone formation, and mineralization during the repair phase.\(^{22,23}\) Finally, when the bony callus is remodeled to restore the original bone structure, mast cells promote osteoclast formation.\(^{15}\)

In osteoporotic patients, bone fractures are frequently associated with healing complications, prolonged hospitalization periods, and an increased morbidity and mortality.\(^{24-26}\) Preclinically, studies in OVX rodents reported maladaptation in angiogenesis and osteo-anabolic pathways and increased numbers of osteoclasts in the fracture callus.\(^{27-29}\) Our group reported that OVX mice displayed a disturbed inflammatory response with increased numbers of neutrophils and a higher expression of IL-6 and the pro-inflammatory and osteo-catabolic mediator midkine (Mdk) in the fracture hematoma.\(^{30,31}\) However, to date, the cellular and molecular mechanisms leading to reduced healing capacity of osteoporotic bones remain poorly understood. Specifically, the role of mast cells has not been elucidated despite their abundance in osteoporotic bone.

Based on their presumed roles in the development of osteoporosis and in bone repair, we hypothesize that mast cells play a critical role in the pathomechanisms of compromised osteoporotic fracture healing. To prove this hypothesis, we herein investigated fracture healing in mast cell–competent (MC+), and mast cell–deficient (MC–) Mcpt5-Cre R-DTA mice after OVX. Indeed, MC– mice were protected from OVX-induced impaired fracture healing. We revealed that mast cells trigger the fracture-induced inflammation and modulate osteoblast and osteoclast activities in the fracture callus of OVX mice. Further in vitro studies revealed that mast cells stimulate osteoclastogenesis via the release of the osteoclastogenic factors Mdk and C-X-C motif chemokine ligand 10 (CXCL10) in an estrogen-dependent manner and via the estrogen receptor alpha (ER\(\alpha\)) on mast cells.

### Materials and Methods

#### Animals

Female C57BL/6J control mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Mcpt5-Cre R-DTA mice were bred as described previously.\(^{15}\) Diphtheria toxin (DT) expression under the mast cell–specific promoter Mcpt5 leads to connective tissue–mast cell depletion in Cre-expressing mice (MC–). Mcpt5-Cre– R-DTA littermate female mice were used as MC–competent (MC+) control mice. Female Esr1-knockout mice (ER\(\alpha\) knockout; homozygous B6.129P2-Esr1\(^{tm1Ksk}\)/) were obtained from Charles River Laboratories. The genetic background of all the mice was C57BL/6J. All animals were housed in groups of 2 to 5 mice under standard rodent conditions. The mice received a standard mouse feed (ssniff R/M-H, V1535-300, Ssniff GmbH, Soest, Germany) until the day of sham-OVX or OVX, when food was switched to a phytoestrogen-free diet (Ssniff).

#### Study approval

All animal experiments were in compliance with international regulations for the care and use of laboratory animals (ARRIVE guidelines and EU Directive 2010/63/EU for animal experiments) with the approval of the Local Ethical Committee (nos. 1026, 1113, 1248, 1386, Regierungspräsidium Tübingen, Germany).

#### Surgical procedures

When aged 12 weeks, the mice were randomly assigned to the different treatment groups either subjected to bilateral OVX as described previously or sham operated (sham-OVX). After 4 to 8 weeks, all mice underwent standardized unilateral femur osteotomy as described previously.\(^{30,32}\) Briefly, the osteotomy was created at the femur diaphysis using a 0.4 mm Gigli wire saw (RISystem, Davos, Switzerland) and stabilized by a semi-rigid external fixator (RISystem). Mice were euthanized at different time points after surgery (6 hours, 3, 5, 10, 21 days) using an isoflurane overdose and cardiac blood withdrawal. The following analyses were performed at the different time points: serum analyses (6 hours, 3, 21 days), histological analyses (3, 5, 10, 21 days), micro-computed tomography (\(\mu\)CT), biomechanical and fracture callus gene expression analyses (21 days). Samples were allocated in a blinded manner to the different analyses, which were performed on a subset of samples because several analyses were mutually exclusive.

#### Multiplex cytokine enzyme-linked immunosorbent assay (ELISA)

Using a customized mouse Multiplex Cytokine Kit (ProcartaPlex; ebioscience, Frankfurt, Germany), blood serum IL-6, IL-1\(\beta\), TNF, CXCL1, and CXCL10 concentrations were determined 6 hours post fracture. Mdk (R&D Systems, Minneapolis, MN, USA) and RANKL (Abcam, Cambridge, UK) single ELISAs were performed according to the manufacturer’s instructions of serum samples 3 and 21 days after fracture, respectively. Serum of 4 to 8 mice was analyzed per group per time point. Furthermore, cytokine and chemokine concentrations in the cell culture supernatants from human HMC-1.2 cells were determined using a human Multiplex Kit (ProcartaPlex). Concentrations of bone morphogenic protein-2 (BMP-2), C3a, eotaxin, granulocyte-colony-stimulating factor, GM-CSF, CXCL1, IFN-\(\gamma\), IL-1\(\beta\), IL-10, IL-12p70, IL-13, IL-17A, IL-2, IL-4, IL-5, IL-6, IL-8, CXCL10, leukemia inhibitory factor (LIF), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-\(\alpha\) (MIP1-\(\alpha\)), RANKL, S100A8/A9, and TNF were measured. Mdk single ELISA (R&D Systems) was performed according to the manufacturer’s instructions.
Biomechanical testing
To analyze the flexural rigidity of the fracture callus, a functional parameter for the healing outcome, a nondestructive three-point-bending test of the fractured femurs was performed on day 21 after fracture as described previously.(33) Briefly, after fixator removal, the bone was loaded with up to a maximum of 2 N in a material-testing machine and the load and deflection were recorded (Zwick Roell, Ulm, Germany). The flexural rigidity of the fracture callus was calculated using the slope of the load-deflection curve as described previously.(33) Six to 8 mice were analyzed per group and investigators were blinded during data quantification.

μCT analysis
Fractured femurs were fixed in 4% paraformaldehyde for 48 hours. μCT scanning was performed using the Skyscan 1172 (Bruker, Kontich, Belgium) operating at 50 kV and 200 mA. isotropic voxel resolution was set at 8 μm. Three-dimensional analysis was conducted using CT analyzer and CT volume software (Bruker) according to guidelines of the American Society for Bone and Mineral Research. The volume of interest was defined as the entire periosteal callus between the two inner pinholes. Bone mineral density was assessed using two phantoms with defined hydroxyapatite (HA) contents (250 and 750 mgHA/cm³). The threshold for mineralized tissue was set at 642 mgHA/cm³. Seven to 8 mice were analyzed per group, and investigators were blinded during data quantification.

Histomorphometry of the fracture callus
After μCT scans, femurs were subjected to decalcified histology as described previously.(33) Sections of 7 μm were stained with Safranin O. The amounts of bone, cartilage, and fibrous tissue were determined by light microscopy and using image-analysis software on day 21 after fracture (Leica DMi6000B, Software MMAF 1.4.0 MetaMorph, Leica, Wetzlar, Germany). The region of interest was the entire fracture callus between the inner pins, including the periosteal callus and the fracture gap. Manually, callus, bone, and cartilage were marked, whereas fibrous tissue was calculated by subtracting the bone and cartilage from the entire callus tissue. Relative amounts of bone, cartilage, and fibrous tissue were determined in percent of the whole callus size. Osteoblasts and osteoclasts were counted in a rectangular area (650 × 450 μm) in the middle of the periosteal fracture callus using the Osteomeasure system (Osteometrics, Decatur, GA, USA) in Toluidine blue- or tartrate-resistant acid phosphatase (TRAP)-stained sections, respectively. Four to 7 mice were analyzed per group. In human fracture hemotma, mast cells were counted in Toluidine blue-stained sections, identified by their deep blue-purple staining, size, and multiple granules. Five samples were analyzed per group. Investigators were blinded during data quantification.

Immunohistochemistry and immunofluorescence staining
Longitudinal sections of 4 μm were prepared for immunohistochemical and immunofluorescence staining. Staining for Mcpt5, Ly6G, and F4/80 was performed using the following primary antibodies incubated overnight at 4°C: rabbit anti-mouse MC Protease 5 (1:100; #orb11030, Biorbyt, Cambridge, UK), rat anti-mouse Ly6G (1:200; 127632, BioLegend, San Diego, CA, USA), and rat anti-mouse F4/80 (1:500; #MCA497GA, BioRad, Hercules, CA, USA). As secondary antibodies, goat-anti rabbit IgG-biotin (1:200; #B2770, Life Technologies, Carlsbad, CA, USA) and goat anti-rat IgG-biotin (1:100 and 1:200, respectively, for Ly6G and F4/80 staining; A10517, Invitrogen, Carlsbad, CA, USA) were used and incubated at room temperature (RT) for 30 minutes or 1 hour, respectively. For signal detection, horseradish peroxidase (HRP)-conjugated streptavidin (#PK-6100, VECTASTAIN Elite ABC-HRP Kit, Peroxidase, Vector Laboratories, Burlingame, UK) was applied according to the manufacturer’s protocols. NovaRED (#SK-4800, Vector NovaRED Substrate Kit, Peroxidase (HRP), Vector Laboratories) was used as chromogen and the sections were counterstained with hematoxylin (1:2000; #2C-306, Waldeck, Münster, Germany). Three to 7 mice were analyzed per group, and time point and investigators were blinded during data quantification.

Immunofluorescence staining for Mcpt5, ERα, Mdk, CXCL10, and Avidin was performed using the following antibodies: rabbit anti-mouse MC Protease 5 (1:100; #orb11030, Biorbyt) incubated overnight at 4°C, rabbit anti-mouse ERα (1:50; #PA5-16440, Invitrogen) incubated at RT for 2 hours, rabbit anti-mouse Mdk (1:100; #MBS714488, MyBioSource, San Diego, CA, USA), goat anti-mouse CXCL10 (1:50; #AF-466-NA, R&D Systems), and Avidin Texas Red (1:150 for ERα and 1:200 for Mdk and CXCL10 staining; A820, Thermo Fisher Scientific, Waltham, MA, USA) incubated at RT for 1 hour. Donkey anti-rabbit Alexa Fluor 488 (#21206, Life Technologies) was used in a concentration of 1:2000 and 1:100, respectively, for Mcpt5 and both ERα and Mdk staining as the secondary antibody and incubated at RT for 1 hour. Rabbit anti-goat IgG (H + L) FITC (#A16143, Life Technologies) was used in a concentration of 1:50 for CXCL10 staining as the secondary antibody. Species-specific non-targeting immunoglobulins were used as isotype controls. Sections were deparaffinized, rehydrated, and blocked with 5% goat serum in tween-tris-buffered saline (TTBS) for Mcpt5 and F4/80, 5% goat serum in TTBS for Ly6G, and 5% bovine serum albumin in phosphate-buffered saline for Avidin staining at RT for 1 hour.

RNA isolation from fracture callus and cells
For fracture callus RNA isolation, RNase-free longitudinal paraffin fracture callus sections of 15 μm were prepared. After heparinase treatment, RNA was isolated using the RNeasy FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA from cells was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The amount and purity of RNA (A260/A280 ratio) was determined photometrically using the Tecan NanoPlate (Tecan, Männedorf, Switzerland).

Cell culture experiments
To analyze the effect of mast cell mediators on human osteoclasts, the HMC-1.2 human mast cell line was used. Cells were cultivated and expanded in isove medium (EMD Millipore, Burlington, MA, USA) supplemented with 1.2 mM β-thioglycerol (Sigma-Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (FBS, EMD Millipore), and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific). To induce mast cell degranulation, 2 × 10⁶ mast cells/mL were stimulated with anaphylatoxin C5a (R&D Systems) in the presence or absence of estrogen (10 nM, Sigma-Aldrich). C5a induces an immediate and massive degranulation of mast cells and the release of preformed stored
mediators.\(^{36}\) Mast cell supernatants were collected after 1 hour stimulation.

Primary human monocytes were freshly isolated, as described previously,\(^{37}\) from the peripheral blood of healthy donors after obtaining informed consent in accordance with the terms of the ethics committee of the University of Ulm, Germany. To induce osteoclast formation, 500,000 cells/cm\(^2\) were seeded on 96-well plates and cultured in minimum essential medium-\(\alpha\) (MEM-\(\alpha\)) medium (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS, Gibco, Thermo Fisher Scientific), 1% L-glutamine (Gibco, Thermo Fisher Scientific), 1% penicillin/streptomycin, 25 ng/mL recombinant human (rh) RANKL, and 10 ng/mL rhM-CSF. Cultures were stimulated with the collected mast cell supernatants (1:20 v/v in medium) to analyze the effect of mast cell mediators on osteoclastogenesis. Additionally, a Mdk-antibody (2\(\times\)10\(^{-5}\) g/ml, Santa Cruz Biotechnology) was added to the cultures to inhibit mast cell-mediated osteoclastogenesis.\(^{38}\) Furthermore, mast cell supernatants were collected after 1 hour stimulation, the formation of TRAP\(^+\) multinucleated cells was investigated by TRAP staining (Acid Phosphatase Leukocyte Kit, Sigma-Aldrich) and visualized by light microscopy.

The human bone osteosarcoma cell line SaOs-2 was cultured as described previously.\(^{39}\) To induce osteogenic differentiation, 5000 cells/cm\(^2\) were seeded on 24-well plates and cultured in MEM-\(\alpha\) (Gibco, Thermo Fisher Scientific) supplemented with 10% FCS (Superior, Biochrom Merck, Darmstadt, Germany), 1% L-glutamine (Gibco, Thermo Fisher Scientific), 1% penicillin/streptomycin, 0.2 nM Ascorbate-2-Phosphate, 10 mM \(\beta\)-glycerophosphate, and 0.1 \(\mu\)M dexamethasone. Cultures were stimulated with the collected mast cell supernatants (1:20 v/v in medium) to analyze the effect of mast cell mediators on osteogenesis. To investigate the effects of the stimulation reagents C5a and estrogen in the mast cell supernatants on osteoclast formation, media containing only these factors were used as controls. Cells were maintained at 37\(^\circ\)C with 5% CO\(_2\) under saturated humidity. At day 7 after stimulation, the formation of TRAP\(^+\) multinucleated cells was investigated by TRAP staining (Acid Phosphatase Leukocyte Kit, Sigma-Aldrich) and visualized by light microscopy.

At day 7 after stimulation, osteogenic differentiation was investigated by ALPL staining (Acid Phosphatase Leukocyte Kit, Sigma-Aldrich) and visualized by light microscopy. The human bone osteosarcoma cell line SaOs-2 was cultured as described previously.\(^{39}\) The human bone osteosarcoma cell line SaOs-2 was cultured in MEM-\(\alpha\) (Gibco, Thermo Fisher Scientific) supplemented with 10% FCS (Superior, Biochrom Merck, Darmstadt, Germany), 1% L-glutamine (Gibco, Thermo Fisher Scientific), 1% penicillin/streptomycin, 0.2 nM Ascorbate-2-Phosphate, 10 mM \(\beta\)-glycerophosphate, and 0.1 \(\mu\)M dexamethasone. Cultures were stimulated with the collected mast cell supernatants (1:20 v/v in medium) to analyze the effect of mast cell mediators on osteogenesis. To investigate the effects of the stimulation reagents C5a and estrogen in the mast cell supernatants on osteogenesis, media containing only these factors were used as controls. Cells were maintained at 37\(^\circ\)C with 5% CO\(_2\) under saturated humidity. At day 7 after stimulation, osteogenic differentiation was investigated by ALPL staining (Acid Phosphatase Leukocyte Kit, Sigma-Aldrich) and visualized by light microscopy.

RNA interference experiments

RNA interference was performed using the commercially available siRNA for Esr1 (Silencer Select siRNA, Thermo Fisher Scientific, s4824) or nontargeting control siRNA (Silencer Negative Control No.1 siRNA, Thermo Fisher Scientific). In total, 2 \(\times\) 10\(^6\) HMC-1.2 cells/mL were transfected in normal HMC-1.2 culture medium without antibiotics but with reduced FBS (1%) supplemented with Opti-MEM and lipofectamine (all Thermo Fisher Scientific). Subsequently, 2.5 pmol siRNA were added to the transfection medium for 24 hours. After a 24-hour incubation, the transfection medium was changed to normal culture medium and the cells were stimulated with 10 ng/mL CsA for 1 hour. For estrogen supplementation experiments, cells were stimulated with 10 nM estrogen alone or combined with CsA. At 1 hour after stimulation, cell culture supernatants and pellets were collected for further analysis and the osteoclast stimulation assay.

Real-time polymerase chain reaction (RT-PCR)

Total RNA isolation and RT-PCR was performed as described previously.\(^{39}\) Quantitative RT-PCR was performed using the SensiFAST SYBR Hi-ROX One-Step Kit (Bioline, Memphis, TN, USA) for the mouse samples and the Brilliant Sybr Green qPCR Master Mix Kit (Stratagene, Amsterdam, Netherlands) for the human samples according to the manufacturer’s instructions. B2m was used as the housekeeping gene for mouse samples (F: 5’-ccc ggc tca cat tga aat cc-3’, R: 5’-ttc tga act ctg cgg tct-3’) and \(\beta\)-ACTIN for human samples (F: 5’-gcc gtc tcc ccc ttc att c-3’, R: 5’-cac acg cag ctc att gta ga-3’). Relative gene expression of Ertx Esr1 (F: 5’-tcc ggc aca tga aca aa-3’, R: 5’-cca gga gca ggt cat aga gg-3’), osteoclast-specific Cpa3 (F: 5’-cca ttc cta ctc gca gat gct-3’, R: 5’-ttc aac ttt cca tag gtc ctg g-3’), and the osteogenic markers ALPL (F: 5’-cct cgg aag aca ctc tga cc-3’, R: 5’-cca cca act tgg aag acg tg-3’) and IBSP (F: 5’-cga 999 gta cga ata ca-3’, R: 5’-agg ttc ccc gtc ctt act tt-3’) was calculated using the delta–delta CT method.

Collection and analysis of human material

Two clinical studies were conducted, which were both approved by the Ethical Committee of the University Medical Center Ulm and conducted in accordance with the declaration of Helsinki (approval numbers 219/18 and 438/15). For the first study, in which fracture hematomas were collected, 10 female patients with upper ankle fractures treated surgically at the University Medical Center Ulm between August 2018 and August 2021 were included. For the second study, in which serum from fracture patients was collected, a total of 24 male and female patients with metaphyseal/diaphyseal fractures of the femur/hip treated surgically at the University Medical Center Ulm between January 2016 and January 2018 were included. All patients provided informed written consent to be enrolled in the studies. Exclusion criteria were polytrauma, pregnancy, bone diseases except primary osteoporosis, intake of bisphosphonates or parathyroid hormone, rheumatoid arthritis, open fractures of grades 3 or 4 according to Tscherne and Oestern, hepatic or renal insufficiency, cancer, intake of steroids or immunosuppressive medication, chemotherapy in the last 3 months and artificial ventilation after surgery. Fracture hematomas were collected during the surgery, which was conducted on days 3 to 5 after injury. Patients were assigned to two groups: young female fracture patients (\(n = 5\), aged \(<50\) years, mean 35.6 years) and elderly menopausal female fracture patients (\(n = 5\), aged \(>50\) years, mean 59 years). For the second study, blood samples were taken from the fracture patients within the first 24 hours after the fracture event. Patients were assigned to two groups: male fracture patients (\(n = 6\), aged 32 to 97 years, mean 69 years) and elderly female menopausal fracture patients (\(n = 13\), aged 57 to 87 years, mean 78 years). Age did not differ significantly between the two groups. Blood samples were centrifuged to obtain serum and stored at \(-80^\circ\)C until analysis. sCD25 ELISA (R&D Systems) was performed according to the manufacturer’s instructions.

Statistics

Data in figures are expressed as box-and-whisker plots (with median and interquartile range) from maximum to minimum, showing all data points. Data in tables are expressed as mean \pm standard deviation. Data were tested for normal distribution using Shapiro–Wilk normality test. Comparisons between
two groups were performed using two-tailed Student’s t test in case of normal distribution or nonparametric Mann–Whitney U test if the data were not normally distributed. Comparisons between more than two groups were performed by one-way analysis of variance (ANOVA) with post hoc Fisher’s LSD test in case of normal distribution or with Kruskal–Wallis with post hoc Dunn’s test if data were not normally distributed. Values of \( p < 0.05 \) were considered to be statistically significant. Statistical analysis was performed using Graph Pad Prism 9.0 software (GraphPad Software, La Jolla, CA, USA).

**Results**

Increased mast cell numbers in the fracture callus under estrogen-deficient conditions

Mast cell presence during osteoporotic fracture healing was analyzed by staining for Mcpt5 expression and counting in the bone marrow near the fracture and in the callus of C57BL/6J control sham-OVX and OVX mice (Fig. 1A–C; Supplemental Fig. S1A). OVX mice displayed a significantly higher number of mast cells at the fracture site on days 3, 10, and 21 after fracture, whereas there was no difference between the groups on day 5. Furthermore, gene expression of the mast cell–specific protease carboxypeptidase A3 (Cpa3) was significantly increased in OVX mice fracture callus on day 21 after fracture compared with sham-OVX mice (Supplemental Fig. S1B). At early time points after fracture (days 3 and 5), mast cells were mainly located in the bone marrow next to the fracture gap. On days 10 and 21, they were present in the bone marrow and the endosteal periosteal bony callus.

Moreover, mast cells were detected in the early fracture hematoma (days 3 to 5 after fracture) of upper ankle fracture patients (Supplemental Fig. S2A). Young female patients displayed significantly fewer mast cells in the fracture hematoma compared with elderly menopausal women (Supplemental Fig. S2B). In addition, we analyzed sCD25, a surrogate marker for mast cell activity, in the serum of age-matched male and female menopausal fracture patients after long bone fracture. Old menopausal women displayed significantly higher serum sCD25 levels within the first 24 hours after fracture compared with age-matched male patients (Supplemental Fig. S2C).

Mast-cell deficiency protects against impaired fracture healing after OVX

To investigate the relevance of mast cells for the OVX-induced disturbed fracture healing, we subjected mast cell–deficient (MC−) Mcpt5-Cre+/− R-DTA mice and their Cre+ littermates to sham-OVX and OVX surgery 4 weeks before inducing the fracture. MC− sham-OVX mice displayed decreased IL-6 serum levels compared with MC+ sham-OVX mice, whereas all other cytokines did not differ 6 hours after fracture (Table 1). In MC− mice, OVX led to an increased inflammatory response 6 hours after fracture, as indicated by significantly increased IL-6 and CXCL10 serum levels (Table 1). In addition, MC+ OVX mice displayed significantly increased Mdk serum levels on day 3 after fracture (Table 1). Notably, double-staining of both Mdk and CXCL10 with avidin in the fracture callus at day 21 after fracture showed a clear co-localization of these proteins (Supplemental Fig. S3A, B), suggesting that mast cells produce both Mdk and CXCL10. In MC+ OVX mice, the number of neutrophils was significantly increased in the fracture hematoma compared with sham-OVX mice (Fig. 2A, C). Macrophage numbers did not differ between the groups (Fig. 2B, C). Notably, the OVX-induced increase in inflammatory mediators and neutrophils was abolished in MC− mice (Table 1; Fig. 2A, C).

On day 21 after fracture, no significant differences between MC+ sham-OVX and MC− mice were found regarding the flexural rigidity of the fractured bones (Fig. 3A) or the callus composition as determined by μCT (Fig. 3B–E) or histomorphometry (Fig. 4A–E), except for a smaller callus size in the MC− sham-OVX mice (Fig. 4D). By contrast, MC− sham-OVX mice displayed significantly reduced numbers of osteoblasts and osteoclasts, as well as osteoclast surface in the fracture callus compared with MC+ sham-OVX (Fig. 4F–J). In MC− mice, OVX led to a significantly reduced flexural rigidity (Fig. 3A) and mineralization of the fracture callus (Fig. 3B), as well as a reduced bone volume and callus size (Figs. 3C–E and 4A, D, E). Furthermore, MC+ OVX mice displayed increased amounts of fibrous tissue in the callus (Fig. 4C). These results indicate delayed fracture healing because of a decreased osteoblast number and activity, as well as an increased osteoclast number and activity (Fig. 4F–J). Supporting this, RANKL serum levels were significantly increased in MC+ mice after OVX (Table 1). Strikingly, none of these differences between MC− sham and OVX mice were observed in the MC− groups, indicating that mast-cell deficiency protects against the negative effects of estrogen depletion on fracture healing.

**Estrogen attenuated osteoclastogenesis via the ERα on mast cells**

To investigate the molecular signaling pathways that are responsible for the effects of estrogen deficiency on mast cells in the fracture callus, we analyzed mast cell numbers in the fracture callus of ERα-knockout mice after OVX. In contrast to C57BL/6J control mice, mast cell numbers were not significantly different in the sham-OVX and OVX mice in the absence of the ERα (Fig. 5A, C), indicating an estrogen-dependent regulation of mast cell numbers in the fracture callus via ERα signaling. Furthermore, both sham and OVX ERα-knockout mice displayed fewer mast cells compared with wild-type control C57BL/6J sham and OVX mice in the fracture callus at day 21 after fracture (Figs. 5 and 1A). Moreover, there was no significant difference in osteoclast number or activity between sham-OVX and OVX ERα-knockout mice (Fig. 5B). Of note, the expression of ERα on mast cells located in the callus of C57BL/6J control mice was confirmed (Fig. 5D).

To analyze the role of ERα in mast cell and osteoclast interaction, we performed in vitro experiments using the human mast cell line HMC-1.2 and human primary osteoclasts. Mast cells were cultivated and stimulated with the complement anaphylatoxin C5a for 1 hour in the presence or absence of estrogen (Fig. 6A). Conditioned medium was then added to osteoclast precursor cells cultivated in normal osteoclastogenic medium containing RANKL and M-CSF. First, we could confirm the promoting effect of mast cell–derived conditioned medium on osteoclastogenesis as shown in our previous study with murine cells(15) (Fig. 6B, D). Mast cells stimulated with C5a displayed a strong osteoclastogenic effect compared with unstimulated mast cells. Incubating mast cells with estrogen attenuated the osteoclastogenic effect of mast cell–derived conditioned medium from both unstimulated and C5a-stimulated mast cells (Fig. 6B, D). To exclude the effect of stimulation reagents in the supernatants
on osteoclasts, control experiments with the same C5a and estrogen concentrations but without MCs were conducted (dashed line). To investigate the role of ERα signaling, we induced Esr1-knockdown in HMC-1.2 cells by siRNA treatment. Knockdown efficiency was approximately 50% (Supplemental Fig. S4). We could confirm that ERα mediates the estrogen effects on mast cell–induced osteoclastogenesis because the inhibiting effect of estrogen was abolished when ERα expression was reduced in mast cells (Fig. 6C). The treatment of osteoblastic cells (SaOs cells) with MC-conditioned medium did not alter AP staining and the gene expression of the osteogenic markers ALPL and IBSP (Supplemental Fig. S5A–C).

Subsequently, we aimed to determine the mast cell mediators inducing the osteoclastogenic effects. Therefore, we analyzed the mast cell supernatants by human multiplex cytokine assay. Supplemental Table S1 and Fig. 6E–G displayed all the cytokines that were detectable in the mast cell supernatants. Three cytokines correlated with the stimulating effect of the conditioned medium on osteoclastogenesis. The Mdk and CXCL10 concentrations were significantly increased in the supernatant of C5a-
Table 1. Serum Cytokine Levels After Fracture in pg/mL

|       | MC+ sham | MC+ OVX | MC- sham | MC- OVX |
|-------|----------|---------|----------|---------|
| 6 hours |          |         |          |         |
| IL-1β | 0.5 ± 1.2 | 4.0 ± 9.0 | 5.4 ± 3.2 | 5.3 ± 6.2 |
| IL-6  | 536.1 ± 162.9 | 997.8 ± 138.0a | 114.8 ± 163.4a | 276.0 ± 378.0b |
| TNFα  | 0.0 ± 0.0 | 4.9 ± 5.0 | 6.7 ± 10.0 | 3.4 ± 6.8 |
| CXCL1 | 666.4 ± 153.7 | 614.6 ± 162.1 | 561.8 ± 197.2 | 582.6 ± 174.1 |
| CXCL10| 27.3 ± 8.8 | 48.2 ± 30.8a | 8.0 ± 8.8 | 17.4 ± 21.2b |
| 3 days |          |         |          |         |
| Mdk   | 108.5 ± 24.4 | 337.3 ± 196.5a | 162.7 ± 92.8 | 158.4 ± 72.2b |
| 21 days |        |         |          |         |
| RANKL | 26.0 ± 5.3 | 72.3 ± 23.7a | 29.1 ± 10.3 | 42.4 ± 20.6b |

MC+ = mast cell competent; MC− = mast cell deficient; IL-1β = interleukin-1β; TNFα = tumor necrosis factor α; CXCL = C-X-C motif chemokine ligand; Mdk = midkine; RANKL = receptor activator of NF-κB ligand.

Data are presented as mean ± standard deviation.

aSignificantly different (p < 0.05) compared with MC+ sham.
bSignificantly different (p < 0.05) compared with MC+ OVX.

Fig 2. Presence of neutrophils and macrophages in the fracture hematoma on day 3. (A) Number of neutrophils (Ly6G+) per mm². (B) Number of macrophages (F4/80+) per mm². (C) Representative images of neutrophils and macrophages from an area near the fracture gap. Cells are marked with black arrows. Gray boxes represent sham-ovariectomized (OVX) mice; red boxes represent OVX mice. G = fracture gap; C = cortex; MC+ = mast cell-competent; MC− = mast cell-deficient. Scale bar = 100 μm. Data are shown as box-and-whisker plots (with median and interquartile range) from maximum to minimum, showing all data points. The p values were determined using one-way ANOVA with post hoc Fisher’s LSD.
stimulated mast cells, whereas adding estrogen to the medium blocked these effects (Fig. 6E, F). To prove the strong effects on Mdk observed in these experiments, we performed blocking experiments. Notably, the addition of an anti-Mdk antibody completely abolished the osteoclastogenic effect of the mast cell supernatants (Fig. 6H), indicating that Mdk is a key mediator of activated mast cells. By contrast, MCP-1 was significantly down-regulated when mast cells were stimulated with C5a. This effect was also blocked in the presence of estrogen (Fig. 6G). Because CXCL10 and Mdk are known osteoclastogenic mediators, whereas MCP-1 might rather stimulate macrophage development, this might explain the observed results of mast cell-conditioned medium on human osteoclast progenitor cells.

**Discussion**

In this study, we demonstrate that mast cells play a critical role in the pathomechanisms of impaired fracture healing under estrogen-deficient conditions. Using mast cell-deficient Mcpt5-Cre R-DTA mice, we showed for the first time that mast cells were responsible for the increased fracture-induced inflammatory reaction, as well as the reduced osteoblast and increased osteoclast activities in the fracture callus in OVX-mice. Importantly, these results were supported by in vitro studies using human cells. We demonstrated that mast cells released the pro-inflammatory and osteoclastogenic mediators Mdk and CXCL10 and stimulated osteoclastogenesis in an estrogen-dependent manner. Hence, our results identify mast cells as a promising target for therapeutic strategies to improve disturbed bone healing in postmenopausal osteoporosis.

Mast cells are regarded to have dispensable functions in physiological bone turnover but are critically involved in pathological conditions affecting the bone, including osteoporosis, and after bone fracture. Both conditions are characterized by mast cell accumulation in the bone marrow or fracture callus, respectively, where these cells stimulated osteoclastogenesis. Importantly, our study revealed that mast cell presence at the
Fig 4. Histomorphometrical analysis from fractured femurs on day 21. Sections of fractured femurs were stained with Safranin O and (A) relative bone area, (B) relative cartilage area, (C) relative fibrous tissue area, and (D) callus area were determined by histomorphometrical analysis. (E) Representative images from the periosteal fracture callus stained with Safranin O. Scale bar = 500 μm. (F) Number of osteoblasts per bone perimeter (NOb/BPm) and (G) osteoblast surface per bone surface (ObS/BS) determined by Toluidine blue staining. (H) Number of osteoclasts per bone perimeter (NOc/BPm) and (I) osteoclast surface per bone surface (OcS/BS) determined by TRAP staining. (J) Representative images from an area within the periosteal fracture callus stained with TRAP. Scale bar = 100 μm. Gray boxes represent sham-ovariectomized (OVX) mice; red boxes represent OVX mice. MC + = mast cell-competent; MC - = mast cell-deficient. Data are shown as box-and-whisker plots (with median and interquartile range) from maximum to minimum, showing all data points. The p values were determined using one-way ANOVA with post hoc Fisher’s LSD.
fracture site and their responses are significantly increased under estrogen-deficient conditions both in mice and humans. During the entire fracture-healing process, callus mast cell numbers were considerably higher in C57BL/6J OVX mice compared with sham mice. Mast cell recruitment to the fracture site could be stimulated by inflammatory mediators, including C5a, IL-6, and MCP-1, secreted from cells at the fracture site, including mesenchymal cells, neutrophils, and macrophages. However, being a limitation of our study, we cannot discriminate whether the increased mast cell numbers result from an enhanced mast cell accumulation or formation. Given the presence of mast cell progenitors in the bone marrow, we expect both mechanisms to be involved, but this needs to be investigated, for example, by lineage tracing or longitudinal live imaging in future studies.

Interestingly, ERα−knockout mice did not display increased mast cell numbers result from an enhanced mast cell accumulation or formation. However, the effects of estrogen on mast cells are mediated by the ERα. For example, uterine mast cell migration and maturation were directly affected by the estrogen status. In addition, estrogen regulated the expression of the C-C chemokine receptor type 4 (CCR4) and CCR5 in HMC-1 cells. In humans, we found more mast cells in the early fracture hematoma of postmenopausal females compared with young female patients after ankle fracture. Moreover, serum sCD25 levels, a mast cell surrogate marker, were significantly higher in postmenopausal fracture patients compared with age-matched male fracture patients, although the optimal control group of premenopausal female fracture patients is still missing. Additional mast cell-specific serum markers such as tryptase or histamine would add important information, but these factors are very short-lived and therefore difficult to measure in patient material. On the basis of these data, we hypothesize that increased mast cell presence and activity at the fracture site might contribute to the impaired fracture healing observed under estrogen-deficient conditions.

In confirming this hypothesis, we revealed that MC− Mcpt5-Cre R-DTA mice were protected from impaired bone healing after OVX. In MC− mice, OVX resulted in an increased inflammatory response toward the fracture, with increased serum levels of the pro-inflammatory cytokines IL-6, CXCL10, and Mdk, and increased neutrophil infiltration to the fracture hematoma. Of note, the local neutrophil accumulation was already shown to impair the fracture healing outcome. Moreover, we and others showed that wild-type OVX mice were characterized by changes in the systemic and local pro-inflammatory factor expression, including IL-6 and Mdk, and neutrophilia in the fracture hematoma in response to the fracture. Interestingly, Mdk serum levels were also increased in postmenopausal female fracture patients. Upregulation of the pro-inflammatory and osteocatabolic cytokine Mdk occurs under estrogen-deficient conditions and in several inflammatory settings. Moreover, Mdk can regulate neutrophil recruitment to inflammation sites. Interestingly, treatment of OVX mice with an
Fig 6. Influence of estrogen and ERα signaling on mast cell–induced osteoclastogenesis. (A) Schematic illustration of the in vitro experimental design. (B) Relative numbers of TRAP⁺ osteoclasts per well. Values from osteoclasts treated with the conditioned medium of mast cells stimulated with different compounds (complement anaphylatoxin C5a, E2 = estrogen, C5a + E2) are compared with osteoclasts treated with the same conditioned medium without the use of mast cells as controls. Medium controls were set to 100% (dashed line). Significant differences to the respective medium controls are indicated by #. (C) Number of TRAP⁺ osteoclasts per well. Osteoclasts were treated with conditioned medium from mast cells either incubated with negative control siRNA (neg co) or Esr1-siRNA (siRNA, estrogen receptor α [Erα]). After transfection, mast cells were stimulated with C5a and/or E2. Significant differences to the negative control are indicated by #. (D) Representative images of TRAP⁺ osteoclasts identified by their increased size, TRAP⁺ red staining, and multiple nuclei (exemplified by black arrows). Scale bar = 100 μm. (E–G) Cytokine analysis in the supernatant of stimulated mast cells: (E) C-X-C motif chemokine ligand 10 (CXCL10) concentration, (F) midkine (Mdk) concentration, (G) monocyte chemotactic protein 1 (MCP-1) concentration. (H) Number of TRAP⁺ osteoclasts per well. Osteoclasts were treated with conditioned medium from mast cells stimulated with C5a and/or E2 in the presence or absence of an inhibitory Mdk-antibody or a control IgG antibody. Significant differences to the IgG control are indicated by #. Data are shown as box-and-whisker plots (with median and interquartile range) from maximum to minimum, showing all data points. The p values were determined using one-way ANOVA with post hoc Fisher’s LSD or Kruskal–Wallis test with post hoc Dunn’s.
inhibitory Mdk antibody prevented the increased inflammatory response toward fracture and inhibited the neutrophil recruitment to the fracture site, improving the fracture healing outcome.\textsuperscript{53} Similarly, increased IL-6 expression was demonstrated under inflammatory conditions and in estrogen deficiency.\textsuperscript{55,56} IL-6 is crucial for inflammatory cell recruitment and directs fracture hematoma infiltration by neutrophils.\textsuperscript{49,57} Likewise, CXCL10 is known for its chemoattracting function, mainly recruiting neutrophils and macrophages.\textsuperscript{58} Importantly, our study revealed that the increased levels of pro-inflammatory cytokines and neutrophil numbers after fracture are abrogated in MC–OVX mice. Because intact, healthy male and female MC– mice did not display a pre-existing immune phenotype with any changes in systemic cytokine or chemokine levels or immune cell populations of different organs,\textsuperscript{59} the effects observed in MC–OVX mice were unlikely to be influenced by a pre-existing immune phenotype. Consequently, mast cells trigger the fracture-induced inflammation and neutrophil recruitment under estrogen-deficient conditions via the mentioned factors. Indeed, mast cells release IL-6 after bone fracture in vivo\textsuperscript{15} or after LPS stimulation in vitro.\textsuperscript{60} Moreover, Mdk can regulate cutaneous mast cell activation and degranulation.\textsuperscript{61} In asthma, mast cell presence was significantly correlated with CXCL10 gene expression,\textsuperscript{62} and in the lung, mast cells could regulate the production of CXCL10 by airway smooth muscle cells.\textsuperscript{63} In addition, respiratory virus infection stimulated human mast cell CXCL10 production in vitro.\textsuperscript{64} Further supporting, we showed that mast cells in the fracture callus on day 21 after fracture produce both Mdk and CXCL10.

During later healing stages, bone repair is considerably disturbed in MC+ OVX mice 21 days after fracture, indicated by reduced bending stiffness, bone content, and fracture callus size, thus confirming earlier studies.\textsuperscript{27,65} Mechanistically, osteoblast numbers were significantly reduced, whereas osteoclast parameters and RANKL serum levels were significantly increased compared with MC+ sham mice. Importantly, mast cell deficiency protected against the OVX-induced impaired bone repair. Osteoblast parameters were unchanged in MC– mice after OVX, hence suggesting that mast cells might suppress osteoblast activity in estrogen deficiency. However, osteogenic differentiation of human SaOs cells was not affected by treatment with supernatants from stimulated mast cells, indicating that mast cells do not directly influence osteogenic differentiation under estrogen-deficient conditions. Interestingly, in mastocytosis patients, osteoblast numbers were significantly increased\textsuperscript{66} as well as in patients with hyperparathyroidism, where mast cells and osteoblasts were frequently co-localized.\textsuperscript{67} Although very few and controversial studies describe effects of mast cells and their mediators on osteoblast functions,\textsuperscript{68,69} Mdk is known to inhibit osteoblastogenesis,\textsuperscript{70} thereby likely contributing to the effects observed in MC+ mice after OVX. Concluding, the cross-talk between mast cells and osteoblasts needs to be investigated in more detail in the future. Moreover, MC– OVX mice displayed significantly diminished osteoclast numbers and reduced RANKL serum levels compared with MC+ OVX mice. These results suggest that mast cells promote osteoclast formation and activity during fracture healing in estrogen-deficient conditions. This conclusion is supported by our recent report that MC– Mcpt5-Cre R-DTA mice were protected from OVX-induced bone loss by impeding the increased osteoclast formation and activity normally occurring after OVX-induced estrogen depletion.\textsuperscript{15} Our previous study revealed that mast cells stimulate osteoclastogenesis during callus remodeling also independently of estrogen,\textsuperscript{15} confirmed also by others.\textsuperscript{22,23} We recently revealed in a murine system that mast cell mediators rapidly released by degranulation stimulate osteoclastogenesis in vitro, where especially histamine is one of the key factors.\textsuperscript{15} The results of the present study indicate for the first time that mast cells’ osteoclastogenic effects might also be mediated partly via RANKL. Indeed, mast cells were shown to release RANKL in the inflammatory conditions of atherosclerosis or rheumatoid arthritis\textsuperscript{3,71} and after bone fracture.\textsuperscript{15}

In the present study, we aimed to investigate the cross-talk between mast cells and osteoclasts in more detail and with more translational relevance. First, using human cells, we confirmed our previous results in murine mast cells and osteoclast-like RAW 264.7 cells activated with CsA. We used CsA as mast cell activator because it is strongly activated after injury and bone fracture both locally and systemically and crucially involved in bone repair.\textsuperscript{72} Moreover, CsA is a strong activator of mast cells and triggers the rapid release of preformed granular mediators,\textsuperscript{73,74} which were mainly responsible for the osteoclastogenic effects observed in our previous study.\textsuperscript{15} We showed that CsA-activated human HMC-1.2 cell supernatant increased osteoclast formation, which was attenuated by estrogen treatment. Several studies demonstrated that estrogen regulates mast cell numbers, activity, and degranulation in different tissues and inflammatory settings;\textsuperscript{75} however, these effects were strongly dependent on the investigated tissue and experimental setup. For example, estrogen inhibits the release of IL-6 and TNF by mast cells in vitro\textsuperscript{76} and attenuated mast cell activation and degranulation after trauma in the heart.\textsuperscript{77} It also improved the outcome of inflammatory bowel disease in rats by reducing mast cell activation.\textsuperscript{78} By contrast, others showed that estrogen induced the degranulation of mast cells in the mammary gland, the bladder, and the uterus.\textsuperscript{82,83} We also investigated whether mast cell osteoclastogenic effects are dependent on their ERα signaling, since we observed that ERα knockout mice failed to increase mast cell or osteoclast numbers in the fracture callus after OVX. Notably, both sham and OVX ERα knockout mice displayed fewer mast cells in the fracture callus compared with wild-type controls, corroborating the important function of the ERα for mast cell development.\textsuperscript{84} Furthermore, we confirmed ERα expression on mast cells in the fracture callus, in line with describing ERα expression on mast cells in other tissues.\textsuperscript{85,86} We observed that ERα knockdown attenuated the inhibitory effects of estrogen on HMC-1.2 cells and their osteoclastogenic potential, indicating that estrogen effects are mediated via the ERα. Confirming these results, estrogen treatment attenuated degranulation of mast cells isolated from ERα knockout mice.\textsuperscript{75} The upregulation of osteoclastogenic cytokines Mdk and CXCL10 in the supernatants of CsA-activated HMC-1.2 cells further confirmed our in vivo data. Importantly, estrogen reduced Mdk and CXCL10 concentrations, indicating that estrogen suppressed mast cell degranulation and their mediator release. Interestingly, both the CXCL10 and Mdk promoters express estrogen response elements.\textsuperscript{87,88} Both CXCL10 and Mdk can display osteoclastogenic potential. CXCL10 can stimulate osteoclastogenesis by inducing RANKL in T cells and osteoblasts and is involved in the bone destruction pathology in rheumatoid arthritis.\textsuperscript{85,86} Mdk is a negative regulator of loading-induced bone remodeling and repair by inhibiting bone formation and inducing bone resorption.\textsuperscript{70,87} Our in vitro results indicated that activated mast cells secrete Mdk and CXCL10 under estrogen-deficient conditions, which might explain the observed effects on osteoclast formation in vivo. In fact, the blockade of Mdk completely abolished the strong osteoclastogenic effect of mast cell supernatant in the absence of estrogen, suggesting that Mdk is an estrogen-dependent key mediator in mast cell–induced osteoclastogenesis. Conversely, MCP-1 levels
significantly reduced in the supernatant of activated mast cells but increased upon estrogen treatment. Mast cell MCP-1 release was previously demonstrated, as well as the influence of estrogen on endometrial stroma cell MCP-1 expression. MCP-1 regulates macrophage migration and infiltration, as these cells originate from the same hematopoietic precursor cells as osteoclasts. Therefore, estrogen might modulate macrophage and osteoclast formation in opposite directions by influencing mast cell mediator release. Notably, we detected no changes in other mediators of the mast cell supernatants, particularly of the osteoclastic factors IL-6 and RANKL, which were changed in OVX mice serum. However, because supernatants were collected 1 hour after stimulation, RANKL and IL-6 release may be insufficient or delayed. Both factors are mainly de novo synthesized by mast cells, which is why longer stimulation might lead to increased RANKL and IL-6 release. It was previously shown that estrogen modulates RANKL expression via ERα on osteoblastic cells, which might similarly apply to mast cells, because we found mast cell ERα expressed in the fracture callus. However, specific mast cell-mediator knockout models are needed to investigate the effects of certain mediators on bone homeostasis and repair, particularly under estrogen-deficient conditions.

In conclusion, our data suggest that mast cells are involved in the pathomechanisms of disturbed fracture healing in osteoporotic bone by triggering the immune response after fracture and regulating bone formation and resorption during the later healing phases. Mechanistically, mast cells release the pro-inflammatory, anti-anabolic, and osteoclastogenic factors Mdk, CXCL10, IL-6, and RANKL and RANKL under estrogen-deficient conditions. Herein, the osteoclastogenic potential appears to be dependent on mast cell ERα signaling. Our results imply that mast cells are main drivers of osteoblast/osteoclast disbalance under osteoporotic conditions. Consequently, targeting mast cells might be a therapeutic option to prevent the maladaptation of bone metabolism and improve bone regeneration in postmenopausal osteoporosis.

Disclosures

All authors state that they have no conflicts of interests.

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Data Availability Statement

All data are available in the main text or the supplementary materials and are available upon request from the corresponding author.

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