Reduced Terminal Complement Complex Formation in Mice Manifests in Low Bone Mass and Impaired Fracture Healing

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The terminal complement complex (TCC) is formed on activation of the complement system, a crucial arm of innate immunity. TCC formation on cell membranes results in a transmembrane pore leading to cell lysis. In addition, sublytic TCC concentrations can modulate various cellular functions. TCC-induced effects may play a role in the pathomechanisms of inflammatory disorders of the bone, including rheumatoid arthritis and osteoarthritis. In this study, we investigated the effect of the TCC on bone turnover and repair. Mice deficient for complement component 6 (C6), an essential component for TCC assembly, and mice with a knockout of CD59, which is a negative regulator of TCC formation, were used in this study. The bone phenotype was analyzed in vivo, and bone cell behavior was analyzed ex vivo. In addition, the mice were subjected to a femur osteotomy. Under homeostatic conditions, C6-deficient mice displayed a reduced bone mass, mainly because of increased osteoclast activity. After femur fracture, the inflammatory response was altered and bone formation was disturbed, which negatively affected the healing outcome. By contrast, CD59-knockout mice only displayed minor skeletal alterations and uneventful bone healing, although the early inflammatory reaction to femur fracture was marginally enhanced. These results demonstrate that TCC-mediated effects regulate bone turnover and promote an adequate response to fracture, contributing to an uneventful healing outcome.

TCC comprise the induction of multiple cell signaling pathways (eg, by inducing calcium influx or the generation of other second messengers),
stimulation of proinflammatory mediators,
and modulation of cell cycle and apoptosis.
are further examples of sublytic TCC activity.

There is growing evidence that the complement system affects the skeletal system under both homeostatic and inflammatory conditions.
A recent study investigated the impact of the TCC on bone architecture and found that deletion of CD59 resulted in reduced cortical bone mineral density.
In addition, increased osteoclastogenesis was reported when CD59 was absent,
suggesting that bone cells could be directly affected by altered TCC activity. Bone cells express CD59, and Cd59a gene expression is up-regulated during osteogenic differentiation of human mesenchymal stem cells.
Therefore, the skeleton protects itself from complement attack under physiological conditions.

In addition to its effect on bone under homeostatic conditions, TCC-mediated effects may be particularly relevant during inflammatory diseases affecting bone and the surrounding tissues. Indeed, elevated TCC levels were found in conditions, TCC-mediated effects may be particularly relevant during inflammatory conditions.

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Materials and Methods

Animal Care and Mouse Models

Animal experiments were performed according to the European Union Directive 2010/63/EU and the international regulations for the care and use of laboratory animals [Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines] and were approved by the responsible local ethical committee (Regierungspräsidium Tübingen, Germany, number 1284). All animals were housed in groups of up to five mice per cage with a 14-hour light, 10-hour dark rhythm and received a standard mouse feed (ssniff R/M-H, V1535-300; Ssniff, Soest, Germany) and water ad libitum. Male mice were used in the study, which were C6-def (n = 59) or CD59-ko (n = 55). Wild-type (WT; C57BL/6j) mice were used as controls (n = 53). C6-def mice were derived from mice with natural C6 mutations and backcrossed from a C3H/He background to a C57BL/6 background, as previously described in detail.

C6-def mice were kindly provided by John D. Lambris (University of Pennsylvania, Philadelphia, PA). CD59-ko mice were generated by targeted deletion of exon 2 of the Cd59a gene, as previously described.
CD59-ko mice with a C57BL/6 background were kindly provided by Wen-Chao Song (University of Pennsylvania). WT mice were obtained from Charles River (Sulzfeld, Germany).

Cultivation of Primary Mouse Osteoblasts

Primary osteoblasts were isolated from long bones of 8- to 12-week-old mice, as previously described.
Briefly, long bones were harvested and diaphyses were cut into pieces and digested using 125 U/mL collagenase type II (Sigma-Aldrich, Taufkirchen, Germany). For osteoblast expansion, bone fragments were cultivated in culture medium, consisting of modified minimal essential medium (Biochrom, Berlin, Germany), 1% l-glutamine (PAN-Biotech, Aidenbach, Germany), 100 U/mL penicillin/streptomycin, and 0.5% amphotericin B (Fungizone), supplemented with 10% heat-inactivated fetal calf serum (all from Gibco, Darmstadt, Germany) at 37°C under 5% CO2. Passage 2 osteoblasts were used for the experiments. Osteogenic differentiation was induced in the presence of 0.2 mmol/L ascorbate-2-phosphate and 10 mmol/L β-glycerophosphate (both from Sigma-Aldrich). Alkaline phosphatase and alizarin red (both from Sigma-Aldrich) stainings were performed to confirm osteogenic differentiation after 14 or 19 days of osteogenic differentiation, respectively (n = 4 to 6). Images were obtained using a DMI 6000B microscope (Leica, Heerbrugg, Switzerland). Alizarin red was dissolved from the calcium phosphate, and the staining intensity of the solution was quantified spectrophotometrically (405 nm). In addition, mRNA samples were obtained after 14 days of osteogenic differentiation (n = 4 to 6). Experiments were performed twice in triplicate.

Cultivation of Primary Mouse Osteoclasts

Osteoclast precursor cells were extracted from bone marrow cells and cultured for 3 days in culture medium, supplemented with 10% heat-inactivated fetal calf serum and 35 ng/mL recombinant human macrophage colony-stimulating factor (Merck, Darmstadt, Germany), as...
Experiments were performed twice in triplicate. In addition, mRNA samples were obtained (and images were obtained using a DMI 6000B microscope) from osteoclasts (more than two nuclei) were counted as osteoclasts, and images were obtained using DMM 6000B microscope. In addition, mRNA samples were obtained (n = 4 to 6). Experiments were performed twice in triplicate.

Bromodeoxyuridine Cell Proliferation Assay

Cell proliferation of osteoblasts was assessed using the colorimetric BrdU Cell Proliferation Assay Kit (Cell Signaling Technology, Danvers, MA), according to manufacturer's instructions. Briefly, cells were seeded in 96-well plates and bromodeoxyuridine (1:100) was added to the culture medium for 24 hours at 37°C. Cells were incubated with a bromodeoxyuridine detection antibody and a horse-radish peroxidase–conjugated secondary antibody for 1 hour at room temperature, before adding TMB substrate for 25 minutes. The reaction was terminated and analyzed by spectrophotometry at 450 nm (n = 5 to 6).

RT-PCR

Total RNA isolation and RT-PCR of osteoclasts and osteoblasts were performed, as described previously. Gene expression analysis was performed relative to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (Gapdh), using the ΔΔCt method with PCR-efficiency correction using LinRegPCR software version 2015.3 (Academic Medical Center, Amsterdam, the Netherlands), as described previously. Primers were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA). Primer sequences are as follows: Acp5 (forward) and 5′-GGCGCTCTGACAGAT TGAT-3′ (reverse); Bglap (forward) and 5′-GGCGCTCTGTCTCTGACT-3′ (reverse); Ctsk (forward) and 5′-ACCTTTATGGGCTCCCTGCT-3′ (reverse); Csk (forward) and 5′-GGAGGCCGCTATATGACCA-3′ (reverse) and 5′-GGAGTTTATACATAACACCTTTATCC-3′ (reverse); Gapdh (forward) and 5′-GGTCAACTGCTGATGGGG-3′ (reverse) and 5′-GGTGAAGGTCCAGAGATGG-3′ (reverse) and 5′-GAAAACCGTCAGAGGACA-3′ (reverse).

Animal Experiments

Bone Phenotyping

To investigate whether a lack of C6 or CD59 influences bone metabolism, the skeleton of male 17- and 34-week-old mice was analyzed by micro–computed tomography (μCT) and histomorphometry, as described below. For dynamic bone histomorphometry, all mice received i.p. injections of calcine green (0.03 g/kg) and alizarin-3-methylindolodiacetic acid (0.045 g/kg) both from Sigma-Aldrich) after 9 and 2 days before femur harvesting, respectively (n = 5 to 8).

Femur Osteotomy

Twelve-week-old CD59-ko, C6-def, and WT mice were subjected to a femur osteotomy (0.4 mm) at the midshaft of the right femur, stabilized by an external fixator (axial stiffness, 3 N/mm; RISystems, Davos, Switzerland) mounted to the femur via four mini-Schraub screws (0.45-mm diameter), as described previously. Surgery was performed under general anesthesia using 2% isoflurane (Forene; AbbVie Deutschland GmbH & Co KG, Ludwigshafen, Germany). Preoperatively, mice received a s.c. single-dose injection of 45 mg/kg clindamycin-2-dihydrogen-phosphate (Ratiopharm, Ulm, Germany) as antibiotic. Pain medication (25 mg/L tramadol hydrochloride; Tramal; Gruententhal GmbH, Aachen, Germany) was administered via the drinking water starting 1 day preoperatively until 3 days postoperatively (n = 6 to 8).

Tissue Harvesting and Preparation

Animals were euthanized using an isoflurane overdose, and blood was withdrawn by cardiac puncture 6 hours or 1, 3, 10, or 21 days after osteotomy. Using microvettes (Sarstedt, Nuembrecht, Germany), serum and plasma samples were collected and centrifuged (serum, 800 × g for 5 minutes; plasma, 4000 × g for 10 minutes) and the supernatants were stored at −80°C until further use. The osteotomized and nonosteotomized femora were harvested and either fixed immediately in 4% saline-buffered formalin for (immuno-) histochemical staining or placed in 0.9% NaCl (Melsungen AG, Melsungen, Germany) to allow biomechanical testing under hydrated conditions (n = 6 to 8). Fracture hematomas were harvested 6 hours after fracture and placed in 150 μL lysis buffer (10 mmol/L tris, pH 7.5, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5 mmol/L Triton X-100, 0.02% NaN3, and 0.2 mmol/L phenylmethylsulfonyl fluoride; all from Sigma-Aldrich), supplemented with 1.5 μL protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Single-Use Cocktail; Thermo Fisher Scientific). Samples were homogenized using micropostles and kept on ice for 30 minutes before centrifugation at 4°C for 30 minutes at 10,000 × g. Protein concentration of the collected supernatant was determined by Pierce bicinchoninic acid Protein Assay Kit (Thermo Fisher Scientific), before sample storage at −80°C until further use (n = 6).

Analysis in Plasma, Serum, and Fracture Hematoma

To assess posttraumatic inflammation, cytokine levels in the plasma and in fracture hematoma homogenates were determined 6 hours after surgery using a customized mouse...
multiplex immunoassay (ProcartaPlex; Invitrogen, Thermo Fisher Scientific), according to the manufacturer’s instructions. Measurement was performed using a Luminex 100 Total System (Bio-Rad Laboratories, Hercules, CA), and data were analyzed using ProcartaPlex Analyst 1.0 software (Thermo Fisher Scientific). Cytokine values were determined using a standard curve of cytokine standards and normalized to the homogenate total protein concentration. Levels of soluble C5b-9 in plasma and callus homogenates were analyzed using a mouse C5b-9/TCC enzyme-linked immunosorbent assay kit (LifeSpan Biosciences, Seattle, WA). Serum complement activity and lytic TCC efficiency were analyzed by erythrocyte hemolysis assay. In total, 10 μL of antibody-sensitized sheep erythrocytes was pretreated with 15 μL mouse complement assay reagent (both from Complement Technology, Inc., Tyler, TX), for 5 minutes at room temperature, before being incubated with 30 μL mouse serum for 30 minutes at 37°C. The absorbance of released hemoglobin was determined at 415 nm. Negative control values were subtracted, and results are presented as the percentage erythrocyte lysis compared with the positive control.

μCT Analysis

After fixation in 4% phosphate-buffered formaldehyde solution, femora and lumbar vertebrae (L6) were scanned using a μCT scanning device (Skyscan 1172 version 1.5; Skyscan, Kontich, Belgium), operating at 200 μA, 50 kV, and 8-μm resolution. All analyses and calibration steps were performed according to the guidelines of the American Society for Bone and Mineral Research. Bone mineral density was determined on the basis of two defined hydroxyapatite phantoms (250 and 750 mg hydroxyapatite/cm³) within each scan. Global thresholds for cortical bone and fracture callus (641.9 mg hydroxyapatite/cm³) and for trabecular bone (394.8 mg hydroxyapatite/cm³) were applied to distinguish mineralized from nonmineralized tissue. Volumes of interest were chosen, as previously described. Briefly, in the intact femur, cortical bone was assessed over a length of 168 μm within the middiaphyseal region, and trabecular bone was determined over a length of 280 μm, starting from 200 μm proximal of the metaphyseal growth plate. Trabecular bone in the lumbar vertebral body was evaluated within a spherical area (0.8-mm diameter), excluding the cortex. To analyze fracture healing, volumes of interest were defined as the periosteal fracture callus between the two inner pinholes, excluding the fracture gap. Analyses were performed by means of SkyScan software (NRecon version 1.7.1.0, DataViewer version 1.5.1.2, and CTAn version 1.17.2.2). The number of bridged cortices per callus was counted in two perpendicular planes using Data viewer (Skyscan). Femora were considered as healed when calli displayed bridging of three or four of the four possible cortices.

Histomorphometrical Analysis

Fractured femora, explanted 10 or 21 days after surgery, were fixed in 4% phosphate-buffered formaldehyde solution, decalcified using 20% EDTA (pH 7.2 to 7.4), and embedded in paraffin. Fixed femora and lumbar vertebrae (L4 or L5) obtained from unfractured animals were embedded in methyl methacrylate. The OsteoMeasure system 4.1.0.0 (OsteoMetrics, Inc., Decatur, GA) was used to evaluate the growth plate morphology and to determine the number of osteoblasts in toluidine blue-stained sections and the number of osteoclasts in TRAP-stained sections. Growth plate thickness and numbers of chondrocytes per chondrocyte column were determined at 10 different locations along the growth plate in Safranin O-stained sections. Osteoblasts and osteoclasts were counted in a 0.6 × 0.6-mm region centered in the vertebral body and in a 1.8 × 0.9-mm region in the middle of the fracture callus. Analyses of the administered fluorescent dyes allowed the determination of the bone formation rate/bone surface, mineral apposition rate, and mineralized surface/bone surface using the OsteoMeasure system (OsteoMetrics, Inc.). The relative amount of bone, cartilage, and fibrous tissue in the newly formed tissue of the callus, within the area between the two inner pinholes, was determined in Safranin O-stained sections using image analysis software (Leica MMAF 1.4.0; MetaMorph Imaging System; Leica, Wetzlar, Germany). Images were obtained using a Leica microscope (DMI 6000B). All analyses were performed in a blinded manner (Y.M., A.V., and Z.R.) and according to American Society for Bone and Mineral Research standards (n = 6 to 8).

Immunohistochemistry

Decalcified deparaffinized longitudinal sections of intact femora of untreated animals and fractured femora, explanted 1 or 3 days after surgery, were immunohistochemically stained. After rehydration and blocking with 10% goat serum for 60 minutes at room temperature, sections were incubated with the primary antibodies against F4/80 (number MCA497GA; AbD Serotec GmbH, Puchheim, Germany; 1:500, rat), Ly6G (number 127632; BioLegend, San Diego, CA; 1:300, rat), or COL10A1 [collagen X (ColX); number PAC156Mu01; Cloud-Clone Corp., Houston, TX; 1:200, rabbit] overnight at 4°C or against CD8 (number bs-0648R; Bioss Inc., Woburn, MA; 1:500, rabbit) for 2 hours at room temperature. Species-specific IgGs were used as isotype controls. Incubation with a secondary biotinylated antibody (goat anti-rabbit number B2770 or goat anti-rat number A10517; both from Invitrogen, Thermo Fisher Scientific; 1:200) was performed for 30 minutes or 2 hours at room temperature, respectively. For antibody detection, Avidin-Biotin Complex kit (Vectastain Elite) and NovaRED (both from Vector Laboratories Inc., Burlingame, CA) were applied, according to the manufacturer’s instructions. Sections were counterstained with hematoxylin and eosin.
(Waldeck, Muenster, Germany) and analyzed using a DMI 6000B microscope in a blinded manner (Y.M., A.E.R., A.V., and Z.R.). Ly6G+ cells were counted in a 0.27-mm² area in the periosteal callus. CD8+ and F4/80+ cells were counted in a 0.405-mm² area at the fracture site between the two cortices. ColX+ cells were counted along the femoral growth plate (n = 5 to 6).

Biomechanical Testing

The biomechanical competence of intact and fractured femora explanted 21 days after osteotomy was assessed by a nondestructive three-point bending test using a material-testing machine (1454; Zwick GmbH, Ulm, Germany), as described previously. Briefly, after fixator removal, a maximum axial load of 4 N was applied to the midshaft on top of the callus. The linear section of the force-deflection curve was used to calculate the flexural rigidity (bending stiffness) of the femur, as described previously (n = 6 to 8).

Statistical Analysis

Group size for in vivo experiments was n = 5 to 8 per group and time point. Group sizes were calculated on the basis of previous experiments using the main outcome measures of flexural rigidity and cytokine concentrations in the fracture hematoma. In vitro experiments were performed twice in triplicate (n = 4 to 6). Group data were tested for normal distribution using the Shapiro-Wilk test, and data sets were normally distributed. Statistical testing was performed using one-way analysis of variance and Fisher’s least significant difference post hoc test using GraphPad Prism 6.07 (GraphPad Software, La Jolla, CA). The level of significance was set at P ≤ 0.05. Results are presented as the means ± SD or as box-and-whisker plots, showing the median, the upper and lower quartiles, and the maximum and minimum.

Results

Reduced Systemic TCC Activity and Local TCC Levels in C6-Def Mice

An erythrocyte hemolysis assay was performed to determine the systemic TCC lytic activity in sera of WT, C6-def, and CD59-ko mice. C6-def serum possessed significantly less lytic activity compared with WT and CD59-ko sera, as displayed by their percentage activity of the positive control (Figure 1A), confirming the functional effect of C6 deficiency on TCC formation and erythrocyte lysis. In addition, significantly reduced levels of soluble TCC (soluble C5b-9) were detected locally in the fracture hematoma of C6-def mice compared with WT controls (Figure 1B). In contrast, the erythrocyte lysis capability of CD59-ko serum and the C5b-9 levels in the hematoma of CD59-ko mice were similar to the controls (Figure 1, A and B).

C6 Deficiency Results in Low Bone Mass

To assess the effect of TCC formation on bone homeostasis, bone strength and structure were analyzed in femora and lumbar vertebrae of 17-week—old WT, C6-def, and CD59-ko mice by biomechanical testing and μCT analyses. Analyses of the cortical bone demonstrated a slight, but nonsignificant, reduction in cortical thickness in C6-def mice compared with WT controls (Table 1). Moreover, the moment of inertia and the bone formation rate/bone surface were significantly reduced compared with both WT and CD59-ko animals, demonstrating reduced bone strength in C6-def mice (Table 1). Cortical tissue mineral density did not differ between the genotypes (Table 1). In trabecular bone, the relative bone volume bone volume/tissue volume ratio was significantly reduced in C6-def mice compared with both WT and CD59-ko mice. Further analyzed parameters, including the decreased trabecular number and trabecular thickness and the slightly increased trabecular separation, all corroborate the low bone mass phenotype (Table 1). Representative μCT images of lumbar vertebrae show the reduction in bone mass in C6-def mice (Figure 2A). Moreover, the bone formation rate/bone surface was significantly decreased in C6-def mice, shown by the reduced distance between the fluorescent labels (Figure 2B), whereas the mineral apposition rate and mineralized surface/bone surface were slightly, but not significantly, reduced (Table 1). Cortical and trabecular bone properties did not differ between CD59-ko mice and WT controls (Table 1).

In addition, histomorphometry revealed that, although the osteoblast numbers and their surface appeared unchanged, osteoclast numbers and their active surface were significantly enhanced in C6-def mice (Table 1), demonstrated by increased numbers of TRAP-positive osteoclasts (Figure 2C). C6-def mice at the age of 34 weeks displayed a similar bone phenotype, characterized by a reduction in bone mass and high numbers of osteoclasts (data not shown). CD59-ko mice displayed unchanged cellular parameters at the age of 17 weeks (Table 1) and 34 weeks (data not shown), compared with age-matched WT mice.
C6-Def and CD59-Ko Mice Display Morphologic Changes in the Femoral Growth Plate

Femora of C6-def mice were significantly shorter compared with WT and CD59-ko bones, which was visible macroscopically and by μCT scan (Figure 3, A and B). Detailed analysis of the femoral growth plate morphology in toluidine blue–stained sections (Figure 3C) demonstrated diminished chondrocyte numbers per chondrocyte column in both C6-def and CD59-ko mice (Figure 3D) and a reduced growth plate thickness in C6-def mice (Figure 3E). Immunohistochemical staining of the growth plate for CoIX,

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Bone Phenotype in WT, C6-Def, and CD59-Ko Mice, Aged 17 Weeks</th>
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<tr>
<td>Variable</td>
<td>WT mice</td>
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<tr>
<td>----------</td>
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</tr>
<tr>
<td>Cortical bone</td>
<td></td>
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<tr>
<td>EI, Nmm²</td>
<td>3592.85 ± 148.93</td>
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<tr>
<td>TMD, mg HA/cm³</td>
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<td>1x, mm²</td>
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<td>Ct.Th, mm</td>
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<td>Marrow area, mm²</td>
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<td>Trabecular bone</td>
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<td>BV/TV, %</td>
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<td>TMD, mg HA/cm³</td>
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<td>Tb.N, 1/mm</td>
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<td>Tb.Th, mm</td>
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<td>N.Oc/B.Pm, 1/mm</td>
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<tr>
<td>BFR/BS, μm²/μm² per year</td>
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<td>MAR, μm/day</td>
<td>1.16 ± 0.12</td>
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<td>MS/BS, %</td>
<td>67.47 ± 7.11</td>
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</table>

Seventeen-week–old WT, C6-def, and CD59-ko mice were used to assess bone structure and density by micro–computed tomography of femora and lumbar vertebrae (L4). Cortical bone parameters are derived from femora. Trabecular bone parameters and cellular parameters, evaluated by histomorphometry, are derived from vertebra. Bone turnover markers were determined in serum. Data are expressed as means ± SD. One-way analysis of variance was performed. n = 5 to 7 per group. Statistically significant values are shown in bold.

*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 versus WT control.

**P < 0.05, ***P < 0.01, ****P < 0.001, and *****P < 0.0001 between C6-def and CD59-ko mice.

BFR, bone formation rate; B.Pm, bone perimeter; BS, bone surface; BV/TV, bone volume fraction (bone volume/tissue volume); Ct.Th, cortical thickness; EI, bending stiffness; HA, hydroxyapatite; 1x, moment of inertia; MAR, mineral apposition rate; marrow area, mean cross-sectional tissue area — mean cross-sectional bone area; MS, mineralized surface; N.Ob, osteoblast number; N.Oc, osteoclast number; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; TMD, tissue mineral density.

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a marker of hypertrophic chondrocytes, revealed increased numbers of ColX-positive cells in CD59-ko sections (Figure 3, F and G).

C6 Deficiency Negatively Affects Osteoblast Differentiation and Slightly Enhances Osteoclast Formation in Vitro

To evaluate whether C6 deficiency or the knockout of CD59 directly influences bone cells, primary osteoblasts and osteoclasts were cultivated in vitro. C6-def osteoblasts displayed no alterations in proliferation capacity (Figure 4A), but a slightly diminished osteogenic differentiation capacity, as shown by the reduced staining intensity for alkaline phosphatase (Figure 4B) and slightly lowered levels of osteogenic marker genes (Alpl, Ibsp, and Bgalp) compared with both WT and CD59-ko cells (Figure 4C–E). CD59-ko cells, by contrast, appeared to be more intensely stained for alkaline phosphatase compared with WT and C6-def cells (Figure 4B). Interestingly, Bglap levels in CD59-ko cells were significantly enhanced compared with both WT and C6-def cells (Figure 4E). Moreover, C6-def osteoblasts displayed impaired mineralization capacities, demonstrated by reduced alizarin red–stained mineralized nodules and by quantification of the alizarin red stain (Figure 4, F and H).

Osteoclast formation was analyzed to see whether the high numbers of osteoclasts and, thus, the reduction in bone mass observed in C6-def mice in vivo could also be recapitulated in vitro. Numbers of osteoclast-like, TRAP-positive multinucleated cells derived from C6-def mice were slightly, but not significantly, increased (Figure 4, G and I). However, expression of the osteoclast marker genes encoding for TRAP (Acp5) and cathepsin K (Ctsk) was significantly enhanced in C6-def osteoclast-like cells compared with WT cells (Figure 4J). Values in CD59-ko cells did not significantly differ from WT or C6-def cells (Figure 4J).

Altered Early Inflammatory Response to Femur Fracture in C6-Def and CD59-Ko Mice

Besides physiological effects of the TCC on bone, the role of the TCC in the early inflammatory response to bone femur fracture was investigated by analyzing cytokine and
chemokine levels 6 hours after osteotomy locally in the fracture hematoma and systemically in plasma. Enhanced levels of macrophage inflammatory protein-1α, tumor necrosis factor-α, and IL-1β were found in the hematoma of CD59-ko compared with both WT and C6-def animals (Figure 5A). In C6-def hematomas, the levels of IL-6 and interferon-γ were slightly, but not significantly, elevated (Figure 5A). Systemically in plasma, a strong up-regulation was observed for CXCL1 and IL-6 on femur fracture in all genotypes, compared with nonfracture controls (Figure 5B). Although IL-6 levels after 6 hours were similar between genotypes, CXCL1 levels were significantly higher in C6-def compared with both WT and CD59-ko animals (Figure 5B).

Immune cells were immunohistochemically stained and quantified locally in the fracture hematoma 24 and 72 hours after fracture. Although no differences were detected in neutrophil (Ly6G<sup>+</sup> cell) numbers at either time points (Figure 6, A and E), a significantly higher number of macrophages (F4/80<sup>+</sup> cells) were present in CD59-ko fracture calli compared with C6-def animals (Figure 6, B and F). Representative immunohistochemical staining of macrophages in the medullary cavity at the fracture site are shown at both 24 and 72 hours after fracture (Figure 6, D and H). Similar numbers of CD8<sup>+</sup> cytotoxic T cells were present in all groups after 24 hours (Figure 6C), whereas slightly, but not significantly, fewer T cells were detected in C6-def calli 72 hours after fracture (Figure 6G). Images of CD8-positive—stained cells are shown 72 hours post-operatively (Figure 6I).

**Bone Healing Is Impaired in C6-Def Mice**

Fracture callus morphology and the abundance of bone cells were evaluated 10 days after fracture, during the bone formation phase and the cartilage to bone transition phase. The callus of neither C6-def mice nor of CD59-ko mice displayed differences in total callus area or in the relative amount of bone, cartilage, or fibrous tissue (Table 2). C6-def calli presented with significantly decreased numbers of...
osteoblasts with a slightly decreased surface (Table 2), whereas osteoclasts did not significantly differ in number or surface between the genotypes (Table 2). To assess bone healing at a later time point, fractured femora were biomechanically tested 21 days after osteotomy and fracture callus morphology was investigated by micro-CT and histomorphometry. Fracture calli of C6-def mice were poorly bridged, as shown by representative two-dimensional micro-CT images (Figure 7A). Evaluation revealed that only 63% of fractures healed completely compared with 100% in WT and 88% in CD59-ko animals (Table 3). The flexural rigidity, and thus the biomechanical competence of the fractured femora of C6-def mice, was significantly reduced; however, no differences were observed between CD59-ko and WT animals (Figure 7H). In addition, the bone volume/tissue volume ratio and bone mineral density were significantly reduced in the callus area of C6-def compared with both WT and CD59-ko mice (Figure 7, I and J), also visible by color-coding in the three-dimensional reconstructive images of the fracture calli (Figure 7K). In agreement with this, a slightly enhanced trabecular separation (Figure 7L) and a significant decrease in trabecular thickness (Figure 7M) were observed in C6-def calli. Total numbers of trabeculae remained, however, unchanged between the genotypes (Figure 7K). Histomorphometrical analyses revealed that the relative amount of bone, cartilage, and fibrous tissue did not significantly differ between the groups (Figure 7, C–F). Although osteoblast numbers and osteoblast surface were not altered between the genotypes (Figure 7, N and O), osteoclasts displayed a significantly increased surface in C6-def calli (Figure 7Q), as shown in TRAP-stained images of the fracture calli (Figure 7G); however, differences in osteoclast numbers did not reach significance (Figure 7P).

Discussion

In this study, the TCC influenced bone turnover and repair. Altered TCC levels and activity directly affected bone cells. It was confirmed that TCC-mediated erythrocyte lysis was significantly reduced in the serum of C6-def mice; however, this occurred with low levels of TCC activity remaining. Four amino acid substitutions due to point mutations in the C6 gene were identified in the C6-deficient mouse strain used in this study.27,33 Those sequence alterations lead to a lack of C6 protein on Western blot analysis and a deficiency for complement-mediated lysis.27 The latter was confirmed in the present study.

By contrast, and as expected, serum hemolytic activity in CD59-ko serum was comparable to the positive control. The bone phenotype of these mice was analyzed to detect whether altered levels of the TCC affect bone structure under physiological conditions. The absence of the TCC led to an osteopenic phenotype and to high numbers of osteoclasts in C6-def mice. To assess whether these alterations in bone structure arise from bone cell autonomous effects, C6-def osteoblasts and osteoclasts were investigated ex vivo.

To assess bone healing at a later time point, fractured femora were biomechanically tested 21 days after osteotomy and fracture callus morphology was investigated by micro-CT and histomorphometry. Fracture calli of C6-def mice were poorly bridged, as shown by representative two-dimensional micro-CT images (Figure 7A). Evaluation revealed that only 63% of fractures healed completely compared with 100% in WT and 88% in CD59-ko animals (Table 3). The flexural rigidity, and thus the biomechanical competence of the
The TCC can induce necrotic and apoptotic cell death (eg, by the activation of caspases). Moreover, sublytic concentrations of the TCC were described to result in mitochondrial damage via inflammasome activation and can, therefore, lead to an energy crisis of the cell. TCC-mediated inflammasome activation was also found in dendritic cells, which share the same progenitor cells of the monocyte lineage with osteoclasts. In addition, constant efforts to remove forming TCC complexes from the cell membrane can deplete the cells’ energy stores. Thus, the absence of such signals might favor osteoclast viability and proliferation. However, investigations on the mechanistic role of the TCC in osteoclasts are missing so far. Further studies investigating TCC-induced lysis and intracellular signaling pathways in osteoclasts should, therefore, aid in strengthening the herein described link.
between C6 deficiency and osteoclastogenesis. In contrast to C6-def mice, CD59-ko mice did not display significant alterations in bone structure or bone turnover. However, ex vivo, the knockout of CD59 slightly enhanced osteoblast differentiation, suggesting that TCC-mediated effects might be beneficial for osteoblast function, but without being sufficiently prominent to affect bone mass in vivo. Those findings are in contrast to previously presented data, in which male CD59-ko mice, aged 8 and 20 weeks, presented with increased femur length, mineral apposition rate, and bone resorption. These differing results could be because of the different ages and skeletal locations analyzed. Notably, the effects described by Bloom and colleagues were sex dependent because only male, but not female, CD59-ko mice displayed the above-mentioned alterations. Significantly reduced serum levels of C6 and C9, and a nearly absent complement activity at the C9 level, were described in female C57/BL6 mice, which presumably accounts for the sex-specific differences. Therefore, at present, it remains unclear whether and to what extent uncontrolled TCC activity affects bone homeostasis and whether this effect is age and/or sex dependent. Additional investigations to unravel the detailed mechanistic impact of the TCC on bone cells are, therefore, required, particularly regarding intracellular signaling pathways and inflammatory reactions induced by TCC formation.

In addition to the TCC-mediated effects on bone, morphologic changes were detected in the femoral growth plate, displaying fewer chondrocytes in both C6-def and CD59-ko mice. Although growth plate alterations in C6-def mice correlated with a significantly reduced femur length, CD59-ko mice displayed no growth retardation. However, less restricted TCC action in CD59-ko mice appeared to affect chondrocyte hypertrophy, shown herein by high numbers of ColX-positive chondrocytes. TCC-mediated effects on chondrocyte hypertrophy potentially affect the replacement or the transdifferentiation of chondrocytes toward osteoblasts. However, this awaits further investigation. Indeed, it could be shown that the TCC influences human chondrocytes by inducing inflammatory cytokines, complement proteins, and matrix metalloproteinases.

After the characterization of the skeleton under physiological conditions, it was investigated whether C6 deficiency, and the associated low bone mass observed, also affected bone regeneration. The animals were, thus, subjected to a femur osteotomy and assessed regarding their inflammatory response and regeneration capacity during fracture healing. Bone fracture healing is initiated by an early inflammatory phase, which aids in initiating the later bone repair and remodeling phases. However, uncontrolled or overshooting inflammatory responses can negatively affect the healing outcome, particularly when they emerge from a locally restricted to a systemic inflammatory reaction.

In the present study, both C6-def and CD59-ko mice displayed marginally altered early inflammatory responses to femur fracture. In C6-def mice, slightly reduced numbers of macrophages and cytotoxic T cells were observed that migrated into the fracture callus and that may have negatively affected the later fracture healing outcome. In CD59-ko mice, high numbers of macrophages and elevated levels of IL-1β, tumor necrosis factor-α, and macrophage inflammatory protein-1α were present locally at the fracture site. These inflammatory cytokines (IL-1β and tumor necrosis factor-α) and the chemotactic mediator macrophage inflammatory protein-1α are secreted by macrophages, and their release induces further leukocyte infiltration, but may also contribute to the recruitment of mesenchymal stem cells and osteoprogenitor cells to the injury site. Particularly tumor necrosis factor-α, also in synergism with IL-1β, was shown to promote bone formation and regeneration in vivo. The local presence of these factors, although in low concentrations, in CD59-ko but not in C6-def mice might account, at least in part, for the differences in the later bone healing process.

Systemically, enhanced levels of CXCL1 were present in the plasma of C6-def mice. CXCL1 is an important immunoattractive chemokine, recruiting mainly neutrophils, which, in turn, attract monocytes and macrophages. Herein, the systemic increase of CXCL1 did, however, not result in a greater recruitment of neutrophils to

<p>| Table 2 | Histomorphometrical Analysis of the Fracture Callus of WT, C6-Def, and Cd59-Ko Mice, 10 Days After Fracture |</p>
<table>
<thead>
<tr>
<th>Variable</th>
<th>WT mice</th>
<th>C6-def mice</th>
<th>CD59-ko mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callus morphology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Callus area, mm²</td>
<td>4.47 ± 1.3</td>
<td>4.32 ± 0.55</td>
<td>4.79 ± 0.81</td>
</tr>
<tr>
<td>Osseous tissue, mm²</td>
<td>0.36 ± 0.13</td>
<td>0.31 ± 0.1</td>
<td>0.51 ± 0.11</td>
</tr>
<tr>
<td>Cartilaginous tissue, mm²</td>
<td>1.17 ± 0.7</td>
<td>1.42 ± 0.24</td>
<td>1.72 ± 0.36</td>
</tr>
<tr>
<td>Fibrous tissue, mm²</td>
<td>3.01 ± 0.97</td>
<td>2.59 ± 0.35</td>
<td>2.56 ± 0.54</td>
</tr>
<tr>
<td>Cellular parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N.Ob/B.Pm, 1/mm</td>
<td>21.68 ± 5.77</td>
<td>13.49 ± 3.22*</td>
<td>20.39 ± 7.6</td>
</tr>
<tr>
<td>Ob.S/BS, %</td>
<td>17.28 ± 6.07</td>
<td>12.28 ± 2.99</td>
<td>19.16 ± 8.92</td>
</tr>
<tr>
<td>N.Oc/B.Pm, 1/mm</td>
<td>7.59 ± 4.4</td>
<td>7.99 ± 1.07</td>
<td>7.64 ± 2.75</td>
</tr>
<tr>
<td>Oc.S/BS, %</td>
<td>13.2 ± 4.61</td>
<td>12.3 ± 3.29</td>
<td>11.28 ± 5.39</td>
</tr>
</tbody>
</table>

Fracture callus area and the relative amount of osseous tissue, cartilaginous tissue, and fibrous tissue were evaluated in Safranin O-stained sections. Cellular parameters were analyzed in toluidine blue-stained sections (osteoblast number and surface) and in tartrate-resistant acid phosphatase-stained sections (osteoclast number and surface). Data are expressed as means ± SD. n = 6. Statistically significant values are shown in bold.

*P ≤ 0.05 versus WT control.

B.Pm, bone perimeter; BS, bone surface; N.Ob, osteoblast number; N.Oc, osteoclast number; Ob.S, osteoblast surface; Oc.S, osteoclast surface.
the injury site of C6-def mice, 1 or 3 days after fracture. In the early fracture hematoma, neutrophils are highly abundant and crucially affect bone repair.\textsuperscript{56,57} Thus, investigating neutrophil recruitment within hours after injury might have revealed differences due to C6 deficiency.

In general, except for IL-6 and CXCL1, levels of locally and systemically measured cytokines were rather low compared with other murine fracture models, including those with a traumatic event in addition to bone fracture.\textsuperscript{30,56,58} The minor differences in the inflammatory response between the genotypes suggest that the observed disturbance in bone healing outcome in C6-def mice may be primarily attributed to a TCC-mediated altered bone cell function rather than a disturbed immune cell activity. The absence of major differences between the genotypes during the early bone regeneration phase underlines this assumption. Major differences in C6-def fracture callus composition and healing outcome only became evident during the late bone remodeling phase, during which a balanced bone cell activity is crucial. The absence of C6 hereby seems to shift this balance toward the bone-resorbing phase.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bridged cortices, n</th>
<th>Fracture-healing outcome, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0 0 0 2 6</td>
<td>8 (100) 0 (0) 8</td>
</tr>
<tr>
<td>C6-def</td>
<td>0 0 3 4 1</td>
<td>5 (63) 3 (37) 8</td>
</tr>
<tr>
<td>CD59-ko</td>
<td>0 1 0 5 2</td>
<td>7 (88) 1 (12) 8</td>
</tr>
</tbody>
</table>

Number of bridged cortices of the fracture callus evaluated by micro–computed tomographic analysis 21 days after fracture in two perpendicular planes. Femora displaying three or more bridged cortices (of four possible) were considered as successfully healed.
osteoclasts, possibly by reduced lysis or reduced sublytic TCC effects, which might negatively affect osteoclast metabolism. The initial low bone mass observed in the C6-def animals before fracture presumably further negatively affected bone repair in response to injury.

In contrast to C6-def mice, CD59-ko mice did not differ significantly from WT control mice in the healing outcome measures.

In conclusion, under inflammatory conditions during fracture healing, the presence of a certain amount of TCC concentration appears to be critical to ensure a regulated early inflammatory response and efficient bone regeneration. These data are in agreement with our previous study, in which C5-deficient mice, unable to generate C5b and thus the TCC, were assessed for bone healing and regeneration. Fracture healing was impaired in C5-deficient mice, which displayed a reduced callus size and bending stiffness of the femur. These findings contrast the current view of TCC actions during cartilage degeneration in experimental models of arthritis. Herein, it was shown that C6-def mice are protected from cartilage degeneration in an osteoarthritic mouse model, whereas the knockout of CD59 appeared to enhance disease severity. Similarly, C6-deficient mice were protected against the development of experimental arthritis, modeling human rheumatoid arthritis, whereas CD59-ko mice displayed increased disease severity and joint damage. These data imply that uncontrolled TCC formation in inflammatory joint diseases triggers joint inflammation and cartilage destruction. It remains elusive as to why the lack of CD59, and thus deregulated TCC assembly, was not detrimental in the context of fracture healing in the present study. One possibility might be that the inflammation triggered by the osteotomy was not sufficiently prominent to induce deleterious effects of the TCC. In addition, other regulator proteins of TCC assembly and TCC activity, such as clusterin or S-protein, could be active in this model, thus resulting in a certain level of remaining TCC inhibition.

TCC inhibition might, however, have an effect in the case of an additional traumatic injury, which is known to induce a strong systemic inflammation and appears to affect TCC levels and activity. High systemic levels of soluble TCC (soluble C5b-9) were found immediately after the accident in humans experiencing multiple injuries. In addition, elevated soluble C5b-9 levels were recently found in synovial fluid of patients experiencing knee injuries and osteochondral fractures. These findings show that the level of TCC formation might affect bone fractures in humans.

In conclusion, TCC-mediated lytic or sublytic effects are necessary for a balance in bone metabolism and bone regeneration. These findings might have wider implications for the adequate treatment of bone defects under sterile or infectious inflammatory conditions, involving complement activation. Further studies are required to investigate complement modulation as a therapeutic option in fracture patients.

Acknowledgments

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