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ARTHRITIS

Analysis of combined deficiency of interleukin-1 and -6 versus single deficiencies in TNF-mediated arthritis and systemic bone loss

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Aims

Insufficient treatment response in rheumatoid arthritis (RA) patients requires novel treatment strategies to halt disease progression. The potential benefit of combination of cytokine-inhibitors in RA is still unclear and needs further investigation. To explore the impact of combined deficiency of two major cytokines, namely interleukin (IL)-1 and IL-6, in this study double deficient mice for IL-1αβ and IL-6 were investigated in different tumour necrosis factor (TNF)-driven inflammatory bone disorders, namely peripheral arthritis and sacroiliitis, as well as systemic bone loss.

Methods

Disease course, histopathological features of arthritis, and micro-CT (μCT) bone analysis of local and systemic bone loss were assessed in 15-week-old *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg in comparison to *IL1*$^{-/-}$hTNFtg, *IL6*$^{-/-}$hTNFtg, and hTNFtg mice. μCT bone analysis of single deficient and wild-type mice was also performed.

Results

Combined deficiency of IL-1/IL-6 markedly ameliorated TNF-mediated arthritis and bilateral sacroiliitis, but without additive benefits compared to single IL-1 deficiency. This finding confirms the important role of IL-1 and the marginal role of IL-6 in TNF-driven pathways of local joint damage, but questions the efficacy of potential combinatorial therapies of IL-1 and IL-6 in treatment of RA. In contrast, combined deficiency of IL-1/IL-6 led to an additive protective effect on TNF-driven systemic bone loss compared to single IL-1 and IL-6 deficiency. This finding clearly indicates a common contribution of both IL-1 and IL-6 in TNF-driven systemic bone loss, and points to a discrepancy of cytokine dependency in local and systemic TNF-driven mechanisms of inflammatory arthritis.

Conclusion

Combinatorial treatments in RA might provide different benefits to inflammatory local arthritis and systemic comorbidities.

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Keywords: Experimental arthritis, Cytokine, Inflammatory joint damage, Inflammatory systemic bone loss

Article focus

- This study aims to evaluate individual and combined contribution of interleukin (IL)-6 and IL-1αβ in tumour necrosis factor (TNF)-driven arthritis, sacroiliitis, and inflammatory bone loss.
- Are local and systemic bone loss governed by the same mechanisms?

Key messages

- TNF-driven peripheral arthritis and sacroiliitis are dependent on IL-1αβ, but not IL-6, with no detectable additive effect of a combined deficiency of both cytokines.
- Combined deficiency of IL-1αβ/IL-6 leads to an additive protective effect on

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TNF-driven systemic bone loss compared to single IL-1 and IL-6 deficiency.
- Combined deficiency of IL-1αβ/IL-6 points to differences in the mechanisms driving local and systemic TNF-dependent inflammatory arthritis and bone loss.

Strengths and limitations

- This was a comprehensive analysis of the effect of IL-1αβ and/or IL-6 on TNF-mediated axial and peripheral arthritis, as well as systemic bone loss.
- The study was limited by the restriction to innate immunity-driven pathways and by the use of only male mice.

Introduction

Proinflammatory cytokines such as tumour necrosis factor alpha (TNFα), interleukin (IL)-1β, and IL-6 play a pivotal role in the pathogenesis of inflammatory arthritis.[1] They have been described as central players in the initiation and perpetuation of arthritis, and therapies targeting these cytokines have been developed and successfully used in patients with various inflammatory joint diseases.[2] Although they are highly efficient drugs, there is still a substantial proportion of patients not responding sufficiently to these drugs. While the contribution of the individual cytokines has been analyzed in detail, both in translational and preclinical as well as in therapeutic settings, it is unclear whether there is a synergistic effect of combined inhibition of these cytokines. Studies in humans with combination therapies of TNF and IL-1 inhibitors have yielded disappointing results, as there was no synergistic effect on disease activity, yet there was a substantial increase in adverse events such as serious infections.[3] As one other study investigating a combination of monoclonal antibodies yielded similar results,[4] caution and good preclinical evidence of potential benefits of a combinatorial approach are necessary before more trials in humans can be planned.

Besides the clinical symptoms of arthritis, patients with inflammatory joint diseases also have a high risk of developing not only bony erosions but also systemic osteoporosis, a process that is believed to be driven by the increased inflammatory load in these patients.[5,6] However, it is currently insufficiently understood whether similar or different pathways are responsible for the development of local damage, systemic bone loss, or both. Therefore, our study focuses on details of the effect of single and combined deficiencies of IL-1αβ and IL-6 on the hTNFtg mouse model of arthritis, which is a well-studied model of TNF-driven inflammatory arthritis, as well as providing an in-depth analysis of the role of these two cytokines on systemic bone mass.

Methods

Animals. IL-6 knockout mice (B6.129S2-IL6^{tm1Kopf}/J) and IL-1α/ß knockout mice were maintained on C57BL/6 background.[7] hTNFtg mice (Tg197 strain) were originally generated by Prof G. Kollias (Fleming Institute, Greece) and also maintained on the C57BL/6 genetic background.[8] In brief, this transgenic mouse line carries a modified 3' human TNF (hTNF) gene construct leading to constitutive overexpression of hTNF and the spontaneous development of symmetrical polyarthritis starting four to five weeks after birth.[8,9] By crossing these three mouse strains we generated the following genotypes: *IL1-/-IL6-/-*hTNFtg, *IL1-/-*hTNFtg, *IL6-/-*hTNFtg mice, and hTNFtg mice, as well as non-hTNFtg mice including *IL1-/-IL6-/-*, *IL1-/-*, *IL6-/-*, and wild-type (wt) animals. Genotypes were determined by polymerase chain reaction (PCR) from DNA isolated from tail biopsies (see Supplementary Material). Investigated groups consisted of seven to eight age-matched male mice. Sample size was calculated a priori according to previous data on bone erosions testing for an effect size of 1.64, with 0.05 significance level and 90% power to detect effects corresponding to a variance in means of 0.66, and assuming a common standard deviation of 0.404. Mice were kept in the same room under conventional housing conditions (22°C ± 1°C; 50% humidity; 12-hour light/dark cycle) with free access to standard laboratory animal diet and tap water. Mice were twice weekly checked for their wellbeing and potential humane endpoints (loss of 20% of body weight, inappetence, rough hair coat, infections, etc.). Potential confounders and exclusion criteria were not detected. Mice were euthanized at the age of 15 weeks by cervical dislocation. Blood was withdrawn from orbital sinuses in anaesthetized mice. Right hind paws were used for histopathological evaluation of arthritis. Left hind paws, knee joints, and lumbar vertebrae were isolated for micro-CT (µCT) bone analysis. Front paws were isolated and shock-frozen for RNA isolation. Animal experiments were conducted according to the 3Rs and adhered to the ARRIVE guidelines; an ARRIVE checklist is included in the Supplementary Material. SH was aware of the group allocation at the different stages of the experiment. The local ethical committee of the Austrian Federal Ministry of Education, Science and Research approved all experiments.

Assessment of clinical signs of arthritis. Mice were weekly and blindly assessed by an independent, well-experienced investigator (KW) from week 4 until week 15 after birth for clinical signs of arthritis, including paw swelling and grip strength, using an established semi-quantitative scoring system (see Supplementary Methods).[9] In addition, body weight (in grams) was also monitored weekly from all animal groups.

Histology. Preparation of histological sections from hind paws and quantitative assessment of histopathological features of inflammatory arthritis were performed according to the SMASH recommendations,[10] and are described in futher detail in the Supplementary Material. Likewise, sacroiliitis was assessed in decalcified paraffin-embedded pelves sections as previously described.[11,12] Briefly, isolated pelves were fixed in 7% formalin overnight and then decalcified in 14% ethylenediaminetetraacetic acid (EDTA) (pH 7.2) at 4°C until the bones

were pliable. Serial frontal sections (2 µm) of pelves were prepared to allow evaluation of both sacroiliac joints. Haematoxylin and eosin (H&E), toluidine blue (TB), and tartrate-resistant acid phosphatase (TRAP) stainings were performed to assess the area of inflammation, bone erosion, and cartilage degradation as well as the number of TRAP+ multinucleated synovial osteoclasts as described in the Supplementary Material. Quantitative data are given as the mean of the sum of both sacroiliac joints and of two sections per mouse.

µCT bone analysis. Left hind paws, knees, and lumbar vertebrae from 15-week-old animals were fixed in 7% formaldehyde overnight and subsequently stored in 70% ethanol. µCT scans were performed using a µCT35 (Scanco Medical Solution, Switzerland) with the following settings: 55kVp; 145µA, 8 W; 300 ms, high resolution: trabecular bone microarchitecture, cortical bone density; or medium resolution: talus, ankle, knee joints; reconstruction threshold: 280 (talus, patella, ankle, knee, trabecular bone) or 310 (cortical bone from tibiae) as previously described.[13] For quantitative assessment of local bone damage, bone volume (mm^3) was evaluated in patella and talus bones. Trabecular bone variables were assessed in 350 slices of the proximal tibiae (starting 50 slices below the growth plate) and in the lumbar vertebrae body (L4, in 300 slices in between both caudal and cranial growth plates). Cortical bone variables such as thickness and bone mineral density (BMD) were evaluated in the cortex of the tibial mid-shaft (200 slices) and of the lumbar vertebrae body (L4, approximately 300 slices).

Quantitative real-time PCR. Total RNA was isolated from frozen front paws (removed from toes and skin) of 15-week-old mice by mechanical homogenization with steel beads (TissueLyser II, Qiagen, Germany) in presence of Trizol reagent. Extracted RNA was further purified using RNeasy kit (Qiagen). Complementary DNA (cDNA) sythesis was performed from 1 µg total RNA using Omniscript cDNA synthesis kit (Qiagen). Quantitative real-time PCR was performed with SYBR-Green and monitored with a LightCycler 480 (Roche Molecular Systems, USA). Primers are listed in the Supplementary Material. Crossing point (Cp) values from genes of interest were normalized to Cp values from glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. Relative expression was calculated using the 2–ΔΔCt method.

Serum variables. Blood samples were taken to determine expression levels of humanTNF by enzyme-linked immunosorbent assay (ELISA) (1:25 serum dilution, R & D Systems, USA) according to the manufacturer's protocol.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, USA). Data are expressed as mean (standard error of the mean (SEM)). Data obtained from histopathological and µCT analysis have been tested for their normal distribution using the Kolmogorov-Smirnov (K-S) test. Individual variables between two animal groups were compared using the independent-samples two-tailed *t*-test. Statistical significance between more than two groups was evaluated with one-way analysis of variance (ANOVA) and Tukey's post hoc test or two-way ANOVA with repeated measures for longitudinal analysis. Statistical significance was set at $p < 0.05$ ($p < 0.05$, $p < 0.005$, $p < 0.001$).

Results

Combined deficiency of IL-1 and IL-6 markedly ameliorates TNF-mediated arthritis, but without additive benefits compared to single IL-1 deficiency. To investigate potential synergistic effects of a combined deficiency of IL-1 and IL-6 on inflammatory, erosive arthritis, we crossed mice deficient for IL-1αβ, IL-6, or both cytokine genes with hTNFtg mice. We then compared the disease course and clinical severity of *IL1-/-*hTNFtg mice, *IL6-/-*hTNFtg mice, and *IL1-/-IL6-/-*hTNFtg mice with those of hTNFtg littermates. *IL1-/-IL6-/-*hTNFtg animals exhibited significantly reduced paw swelling over the observation period from week 4 to week 15 after birth compared to their hTNFtg littermates (Figure 1a). In line with the decreased paw swelling, *IL1-/-IL6-/-*hTNFtg mice were significantly protected from grip strength loss compared to hTNFtg animals (Figure 1a). However, when compared to *IL1-/-*hTNFtg mice, there was no significant difference in the development of clinical signs in the double deficient animals, while *IL6-/-*hTNFtg mice developed severe arthritis indistinguishable from hTNFtg littermates. Furthermore, the *IL1-/-IL6-/-*hTNFtg and *IL1-/-*hTNFtg animals' bodyweight was about 15% higher compared to sex-matched hTNFtg and *IL6-/-*hTNFtg mice (data not shown).

Next, we explored the effects of the IL-1/IL-6 double deficiency on distinct histopathological features of inflammatory arthritis. $IL1^{-/-}IL6^{-/-}$hTNFtg mice were markedly protected from synovial inflammation (mean 0.363 mm^2 (SEM 0.08)), demonstrating a 75% reduction of synovitis compared to hTNFtg mice (1.323 mm^2 (SEM 0.104), $p < 0.001$) and $IL6^{-/-}$hTNFtg mice (1.307 mm^2 (SEM 0.196), $p < 0.001$, both one-way ANOVA with Tukey's post hoc test; Figures 1b and 1c). The residual synovial inflammation was characterized by minor synovial thickness and low number of infiltrating cells, comparable to joint inflammation of $IL1^{-/-}$hTNFtg mice (mean 0.488 mm^2 (SEM 0.106)). In line with these findings, the generation of TRAP+ synovial osteoclasts was significantly reduced in $IL1^{-/-}IL6^{-/-}$hTNFtg mice, causing markedly fewer subchondral bone erosions compared to hTNFtg mice (Figures 1b and 1c; $IL1^{-/-}IL6^{-/-}$hTNFtg: mean 0.086 mm^2 (SEM 0.032), hTNFtg: 0.484 mm^2 (SEM 0.054), $p < 0.001$; $IL6^{-/-}$hTNFtg: 0.408 mm^2 (SEM 0.131), $p = 0.026$, both one-way ANOVA with Tukey's post hoc test). Likewise, inflammation-mediated proteoglycan loss and cartilage degradation were significantly protected in $IL1^{-/-}IL6^{-/-}$hTNFtg mice compared to hTNFtg mice and $IL6^{-/-}$hTNFtg mice (Figures 1b and 1c). Moreover, we did not detect any significant differences in histopathological features between $IL1^{-/-}IL6^{-/-}$hTNFtg and $IL1^{-/-}$hTNFtg mice. These data indicate that a combined deficiency of the two cytokines IL-1 and IL-6 does not

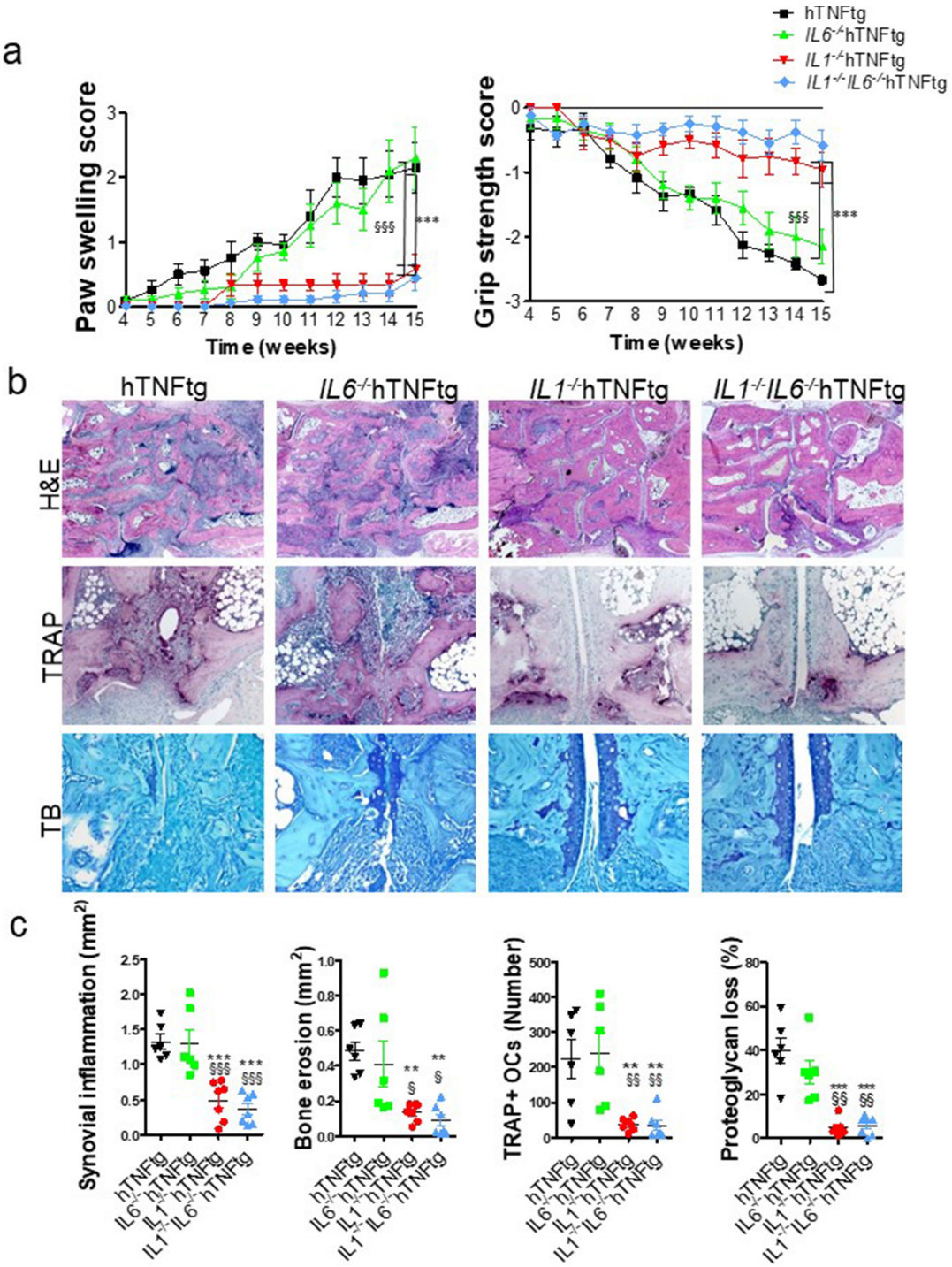

Fig. 1

Clinical course and histopathological features of inflammatory arthritis in *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg, *IL1*$^{-/-}$hTNFtg, *IL6*$^{-/-}$hTNFtg, and hTNFtg mice. a) Mean scores of paw swelling and grip strength from all four paws were weekly assessed from week 4 until week 15 after birth. Data are expressed as mean (standard error of the mean (SEM)). N = 7 male animals per group. All p-values were calculated using two-way analysis of variance (ANOVA) with repeated measures. b) Representative images from histopathological features of inflammatory arthritis in transversal sections from hind paws at the age of 15 weeks after birth. Synovial inflammation and pannus formation in the tarsal area are indicated by haematoxylin and eosin (H&E) stained sections. Tartrate-resistant acid phosphatase (TRAP) positive synovial osteoclasts (purple-coloured) and subchondral bone erosions are illustrated by TRAP-stained sections. Inflammatory cartilage damage is characterized by cartilage degradation and proteoglycan loss indicated by destaining of toluidine blue (TB)-stained sections. Original magnification is 25× (H&E), 200× (TRAP), and 400× (TB). c) Quantitative assessment of the area of synovial inflammation (mm^2), number of TRAP+ multinucleated synovial osteoclasts (OCs), area of subchondral bone erosion (mm^2), and proteoglycan loss in articular cartilage (%). Data are expressed as mean (SEM). N = 7 animals per group. All p-values were calculated using one-way ANOVA with Tukey's post hoc test. * indicates statistical significance compared to hTNFtg mice (*p < 0.05, **p < 0.005, ***p < 0.001); § indicates statistical significance compared to *IL6*$^{-/-}$hTNFtg mice (§p < 0.05, §§p < 0.005, §§§p < 0.001). hTNFtg, human tumour necrosis factor transgenic.

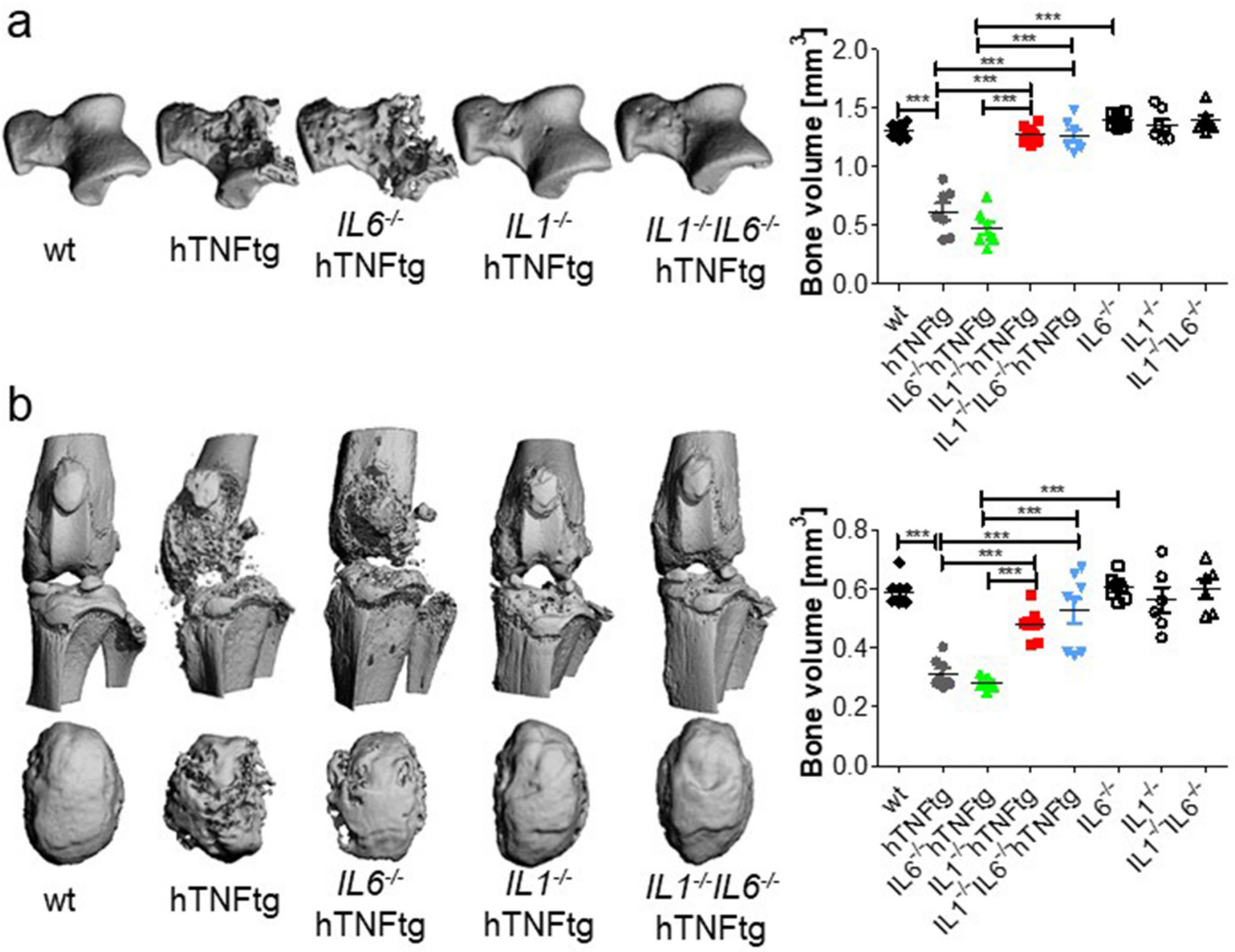

Fig. 2

Micro-CT (µCT)-based assessment of local inflammatory bone damage in ankle and knee joints from *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg, *IL1*$^{-/-}$hTNFtg, *IL6*$^{-/-}$hTNFtg, and hTNFtg mice at the age of 15 weeks after birth. a) Representative 3D image from talus bone and quantitative analysis of bone volume (mm^3) indicating local bone damage in arthritic mice. b) Representative 3D images from knee joints and patella, as well as quantitative analysis of bone volume (mm^3) from patella indicating local bone damage or preservation of bone architecture. Data are expressed as mean (standard error of the mean (SEM)). N = 7 males per group. ***p < 0.001, one-way analysis of variance (ANOVA) and Tukey's post hoc test. hTNFtg, human tumour necrosis factor transgenic; IL, interleukin; wt, wild-type.

provide any additive beneficial effects on the prevention of TNF-driven inflammatory, erosive arthritis, and moreover confirms the important role of IL-1 and the marginal role of IL-6 in this model. As histological analyses of tissue damage are limited to a small area, we used µCT analyses to explore the effect of the IL-1/IL-6 double deficiency on structural bone damage in more detail. Whereas hTNFtg and *IL6*$^{-/-}$hTNFtg mice showed massive cortical bone erosions and structural damage of ankle joints, joints from *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg mice and *IL1*$^{-/-}$hTNFtg were less affected and showed intact architecture similar to wt, *IL1*$^{-/-}$, *IL-6*$^{-/-}$, or *IL1*$^{-/-}$*IL6*$^{-/-}$ control mice (Supplementary Figure a). Quantification of the bone volume from talus bone revealed significant protection from inflammatory bone damage in *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg mice and *IL1*$^{-/-}$hTNFtg mice compared to hTNFtg and *IL6*$^{-/-}$hTNFtg animals (Figure 2a, Supplementary Table i).

To further address local bone damage in larger joints, µCT bone images were also performed from knee joints of *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg, *IL1*$^{-/-}$hTNFtg, *IL6*$^{-/-}$hTNFtg, and hTNFtg mice and their four non-tg genotypes (Figure 2b). We observed markedly fewer bone erosions in knee joints from *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg mice and *IL1*$^{-/-}$hTNFtg mice compared to hTNFtg and *IL6*$^{-/-}$hTNFtg animals, indicated by smoother and less porotic bone surfaces of the femoral and tibial bones (Figure 2b). Quantitative analyses demonstrated significantly higher bone volume of patellae in *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg mice as well as in *IL1*$^{-/-}$hTNFtg mice compared to hTNFtg and *IL6*$^{-/-}$hTNFtg, reaching similar values to their non-tg littermates (Figure 2b, Supplementary Table i). This finding indicates that TNF-mediated structural bone damage at various sites is strongly dependent on IL-1 alone and

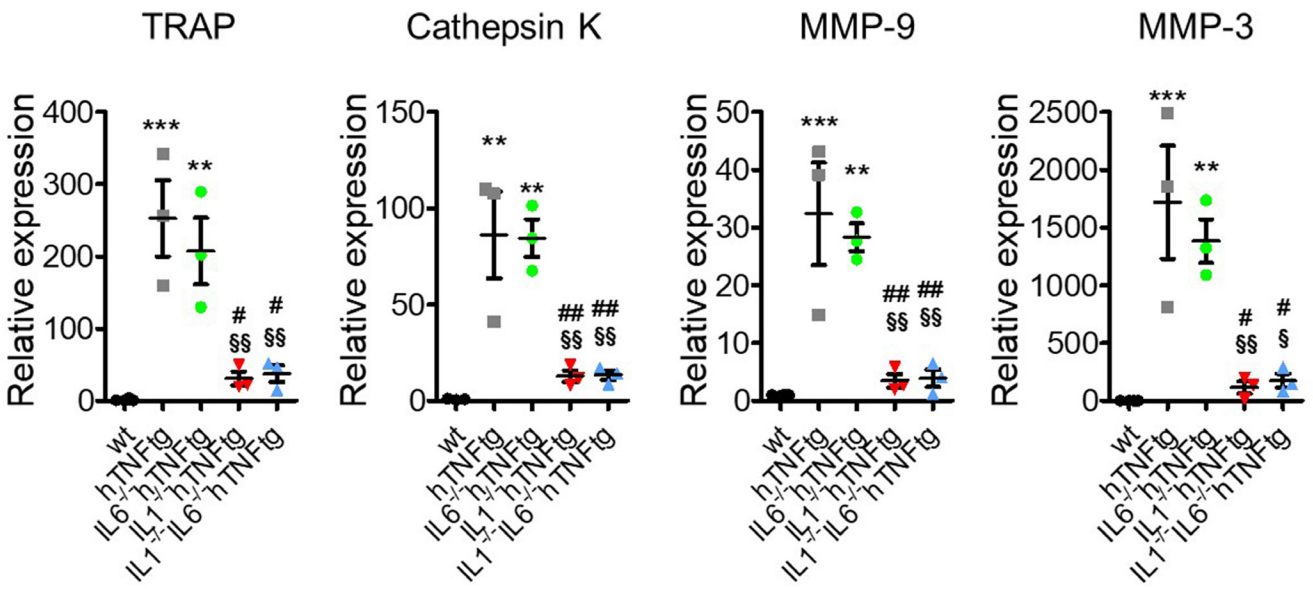

Fig. 3

Local gene expression of markers for osteoclasts and tissue-degrading enzymes. RNA extracts from front paws were analyzed for messenger RNA (mRNA) expression levels of tartrate-resistant acid phosphatase (TRAP), cathepsin K, matrix metalloproteinase 3 (MMP-3), and MMP-9 by quantitative real-time polymerase chain reaction (qRT-PCR). Data are expressed as mean (standard error of the mean (SEM)). N = 3 to 4 animals per group. Symbols indicate statistical significance compared to * wt, § hTNFtg, and # *IL6*-/-hTNFtg (**p < 0.005, ***p < 0.001; §p < 0.05, §§p < 0.005; # p < 0.05, ## p < 0.005). All p-values calculated using one-way analysis of variance (ANOVA) and Tukey's post hoc test. hTNFtg, human tumour necrosis factor transgenic; IL, interleukin; wt, wild-type.

cannot be completely halted in hTNFtg mice lacking both IL-1 and IL-6.

Combined deficiency of IL-1 and IL-6 demonstrates suppression of osteoclast-specific and tissue-degrading gene expression in joints comparable to single IL-1 deficiency. To examine the contribution of IL-1 and IL-6 on the local regulation of marker gene expression specific to TNF-driven inflammation-triggered osteoclast activity, as well as bone and cartilage damage, we investigated messenger RNA (mRNA) expression levels of matrix metalloproteinase 3 (MMP-3), MMP-9, cathepsin K, and TRAP from RNA extracts of front paws. We observed a massive downregulation of inflammation-specific MMP-3 in *IL1-/-IL6-/-*hTNFtg compared to hTNFtg littermates and *IL6-/-*hTNFtg mice, and similar levels compared to *IL1-/-*hTNFtg mice. Similarly, osteoclast-specific genes such as TRAP, cathepsin K, and MMP-9 were significantly decreased in *IL1-/-IL6-/-*hTNFtg mice and *IL1-/-*hTNFtg mice compared to *IL6-/-*hTNFtg and hTNFtg mice (Figure 3). These findings indicate that IL-1 but not IL-6 is an essential downstream regulator for TNF-mediated gene expression of tissue-degrading enzymes.

Combined deficiency of IL-1 and IL-6 ameliorates bilateral sacroiliitis comparable to single IL-1 deficiency. In addition to the development of peripheral inflammatory, erosive arthritis, hTNFtg mice also suffer from bilateral, erosive sacroiliitis.[11] To address the effects of IL-1 and/or IL-6 deficiency on axial inflammation, we compared histopathological features of sacroiliac joints from *IL1-/-IL6-/-*hTNFtg with *IL1-/-*hTNFtg, *IL6-/-*hTNFtg, and hTNFtg mice. In line with peripheral joint inflammation, *IL1-/-IL6-/-*hTNFtg demonstrated less inflamed sacroiliac joints comparable to single *IL1-/-*hTNFtg mice, whereas both hTNFtg and *IL6-/-*hTNFtg mice showed comparable inflammation (Figure 4a). Similar results were obtained in the analysis of bone-resorbing osteoclasts and the formation of bone erosions, as well as cartilage degradation (Figures 4a and 4b). These findings indicate that, in line with peripheral arthritis, TNF-driven bilateral sacroiliitis seems to be strongly dependent on IL-1 alone, but not on IL-6.

Additive benefit of combined deficiency of IL-1 and IL-6 on TNF-mediated systemic osteopenia. Several reports have demonstrated that local and systemic bone loss can be differentially regulated in hTNFtg mice.[14,15] We therefore analyzed cytokine-dependent effects on TNF-driven systemic bone loss by assessing tibial bones and lumbar vertebrae in hTNFtg mice by quantitative µCT bone analysis. Consistent with previous analyses, we detected significantly lower bone mass in tibial bones of hTNFtg mice compared to wt mice (Figure 5a).[16] In addition, hTNFtg mice also showed reduced bone mass in their vertebrae and display a reduced cortical thickness (Figure 5b, Supplementary Figure b). In contrast to erosive bone loss in arthritis, *IL1-/-*hTNFtg mice were not fully protected from TNF-induced systemic bone loss, as their bone mass was not significantly higher than that of hTNFtg mice. However, double deficient *IL1-/-IL6-/-*hTNFtg mice showed a significant protection from systemic osteopenia as indicated by markedly increased BV/TV compared to hTNFtg and *IL6-/-*hTNFtg mice (Figure 5a). Combined

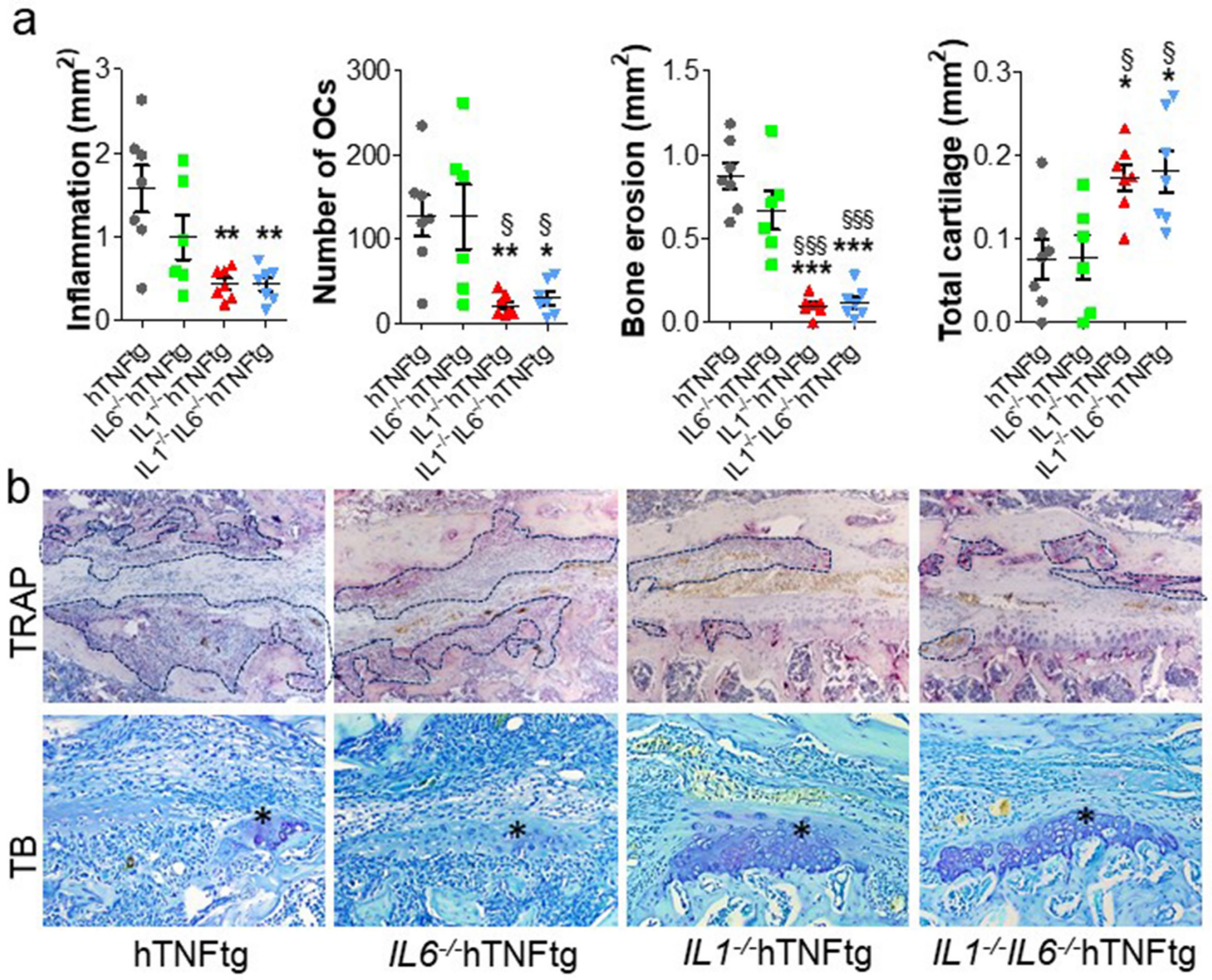

Fig. 4

Histopathological analysis of bilateral erosive sacroiliitis in 15-week-old *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg, *IL1*$^{-/-}$hTNFtg, *IL6*$^{-/-}$hTNFtg, and hTNFtg mice. a) Quantitative assessment of the area of inflammation (in mm²), the number of osteoclasts (OCs), area of bone erosions (in mm²), and cartilage tissue (in mm²) assessed in both left and right sacroiliac joints. b) Representative images from tartrate-resistant acid phosphatase (TRAP) (upper row) and toluidine blue (TB) (lower row) stained sections of sacroiliac joints indicating inflammation, pannus formation (black dotted lines), erosions, and osteoclast and cartilage degradation (asterisk). Data are expressed as mean (standard error of the mean (SEM)). N = 7 to 8 animals per group. Symbols indicate statistical significance compared to * hTNFtg and § *IL6*$^{-/-}$hTNFtg (*p < 0.05, **p < 0.005, ***p < 0.001; §p < 0.05, §§p < 0.005, §§§p < 0.001). All p-values calculated using one-way analysis of variance (ANOVA) and Tukey's post hoc test. hTNFtg, human tumour necrosis factor transgenic; IL, interleukin.

deficiency of IL-1 and IL-6 also revealed significantly higher trabecular thickness and connectivity density compared to hTNFtg mice. In control experiments, single and double deficiencies of IL-1 and/or IL-6 in non-hTNFtg mice did not show any alteration of tibial trabecular or cortical bone parameters compared to wt mice (Figure 5a, Supplementary Figure b, Supplementary Table i).

Next, to address other regions usually evaluated when assessing systemic bone mass, we analyzed trabecular bone mass and morphology in lumbar vertebrae of these animals. Here, we observed a significantly reduced trabecular bone mass in hTNFtg, *IL-6*$^{-/-}$hTNFtg, and *IL1*$^{-/-}$hTNFtg mice, which demonstrated approximately 31%, 39%, and 31% bone loss compared to wt mice (Figure 5b, Supplementary Table i). In contrast, in the presence of double IL-1/IL-6 deficiency there was no significant bone loss in lumbar vertebra body compared to wt mice.

Investigating cortical bone variables in the tibial midshaft, we observed a significant reduction in cortical thickness in *IL6*$^{-/-}$hTNFtg and hTNFtg mice, however not in *IL1*$^{-/-}$hTNFtg and *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg (Supplementary Figure ca), compared to wt mice. In contrast, double deficiency in IL-1 and IL-6 led to a significantly higher cortical BMD compared to hTNFtg mice, whereas single deficiencies revealed no difference. Moreover, cortical thickness in lumbar vertebrae was also found to be significantly higher in *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg, although bone density was not altered compared to hTNFtg mice (Supplementary Figure cb). In line with the results on tibial bones, single and double deficiencies in IL-1 and/or IL-6 alone in

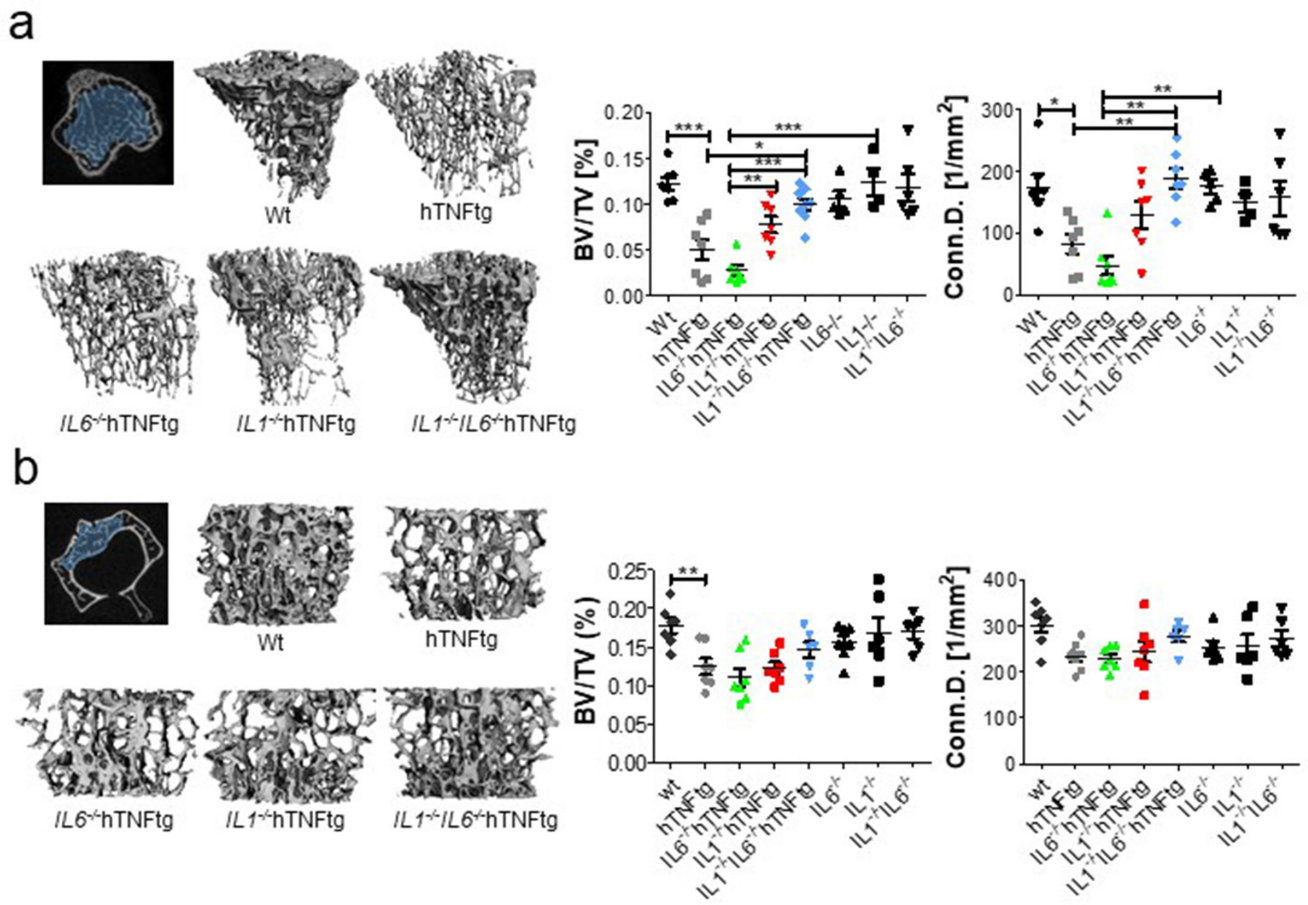

Fig. 5

Micro-CT (μCT)-based quantitative assessment of systemic bone parameters in tibial bones and lumbar vertebrae from 15-week-old *IL1*$^{-/-}$*IL6*$^{-/-}$hTNFtg, *IL1*$^{-/-}$hTNFtg, *IL6*$^{-/-}$hTNFtg, and hTNFtg mice in comparison to wt, *IL1*$^{-/-}$, *IL6*$^{-/-}$, and *IL1*$^{-/-}$*IL6*$^{-/-}$ mice. a) 3D images of trabecular network and quantitative analysis of trabecular bone parameters in proximal tibial bone, including bone volume/tissue volume (BV/TV) and connectivity density (Conn.D.). b) 3D images of trabecular bone and quantitative analysis of bone parameters in lumbar vertebrae body (L4). Data are expressed as mean (standard error of the mean (SEM)). N = 7 to 8 animals per group. *p < 0.05, **p < 0.005, ***p < 0.001. All p-values calculated using one-way analysis of variance (ANOVA) and Tukey's post hoc test. hTNFtg, human tumour necrosis factor transgenic; IL, interleukin; wt, wild-type.

non-hTNFtg mice did not show any alteration on trabecular and cortical bone parameters in lumbar vertebrae compared to wt mice (Figure 5b, Supplementary Figures bb and cb). These findings demonstrate that, in contrast to local joint inflammation, both IL-1 and IL-6 contribute to TNF-driven systemic bone loss.

Discussion

Chronic inflammatory, destructive RA depends on a complex network of multiple proinflammatory cytokines and activated signalling pathways.[1] Targeted therapies against single cytokines such as TNF, IL-6, or IL-1 are beneficial in many RA patients, but are often non-responsive or insufficient in some patients to halt progression of inflammatory, destructive processes of the disease.[1] Thus, there are ongoing discussions whether insufficiently responsive patients should receive a combination of targeted synthetic and/or biological disease-modifying anti-rheumatic drugs (DMARDs).[2]

Animal models of arthritis allow us to address these basic questions. Using the well-established hTNFtg model of arthritis, we generated a combinatorial knockout of two of the most important cytokines in arthritis, IL-1 and IL-6, as well as IL-1αβ/IL-6 double deficient hTNFtg mice. Our study aimed to explore in detail whether a combined deficiency of two cytokines conveys additive benefits on TNF-driven joint inflammation, local damage, or systemic bone loss. Our data reveal that peripheral and axial arthritis are dependent on IL-1, but not IL-6, with no detectable additive effect of a combined deficiency of both cytokines. However, we could demonstrate that a combined deficiency of IL-1αβ/IL-6 leads to an additive protective effect on TNF-driven systemic bone loss compared to single IL-1 and IL-6 deficiency.

Proinflammatory cytokines such as TNF, IL-1, and IL-6 are key players in the pathogenesis of RA.[1,17,18] Their increased presence in synovial tissue and peripheral blood cells perpetuates and amplifies chronic

inflammatory processes through various autocrine and paracrine pathways, which finally cause destructive subchondral and periarticular bone damage as well as systemic bone loss. Preclinical studies using cytokine knockout animals or blocking reagents for TNF or its receptors, IL-1 or IL-6, demonstrated individual cytokine dependencies in various models.[17,19,20] Whereas T cell- and B cell-dependent models such as collagen-induced arthritis have been found to be more dependent on IL-6, IL-1, and IL-17, innate immune-driven models such as K/BxN serum transfer model or hTNFtg mice are strongly regulated by TNF and IL-1.[21-25] Even with the analysis of various sites (paws, knees, ankles, patella) and using both histological and µCT analysis, we were not able to detect synergistic effects of IL-1 and IL-6 in the development of erosive joint damage or cartilage degradation, which is in line with previous studies.[21,22] In addition to peripheral arthritis, hTNFtg mice are also characterized by the development of bilateral sacroiliitis, a typical feature of spondyloarthropathies.[11] Consistent with the observations found in peripheral joints, we show a similar amelioration of sacroiliitis in hTNFtg mice deficient for IL-1/IL-6 as well as for IL-1 alone. Absence of IL-6 did not alter TNF-mediated sacroiliitis as previously described,[12] indicating that TNF-driven peripheral and axial inflammatory processes are both dependent on similar pathways in our model, which involve downstream action of IL-1.

It was interesting to note that in contrast to local bone loss, systemic bone loss in this model seems to be dependent on both IL-1 and IL-6. This is remarkable, as blocking the IL-6 receptor in this model had the opposite effect (amelioration of arthritis, no effect on systemic bone loss),[26] possibly indicating differential functions of the cytokine and the complex receptor system in arthritis. Given the abundance of circulating soluble IL-6 receptor, it is conceivable that this may not have been fully blocked in these previous experiments, whereas deleting the IL-6 gene eliminates all of the circulating IL-6 and thus the ligand for the cognate soluble and cell membrane receptor. Bone mass as well as µCT and histomorphometric analyses showed no alterations in only IL-6 deficient animals compared to wt mice, in line with previously published reports.[27,28] IL-6 has, however, been noted to play an important role in ovariectomy-induced bone loss, a model of postmenopausal osteoporosis,[29] and human SNP analyses have linked IL-6 to osteopenia,[28] suggesting a role for IL-6 in bone biology under challenged conditions, in our case systemic inflammation due to TNF overexpression. One limitation of our study is that we used only male mice. However, in our experience with the hTNFtg model, there are no sex differences regarding phenotypes in these mice.[12,21,30] Previous observations suggested a more fundamental role of IL-1 in the regulation of TNF-induced systemic bone loss. However, this might be explained by the timepoint of the analysis, as we investigated a later timepoint than before, when the contribution of IL-6 may have become more apparent.[30]

Taken together, combined deficiency of IL-1 and IL-6 achieved additive benefits in TNF-driven systemic bone loss, pointing to differences in the mechanisms driving local and systemic TNF-dependent inflammatory arthritis. Based on the lack of additive clinical and histopathological effects on local inflammatory, erosive arthritis and bilateral sacroiliitis, and the fact that IL-1 and IL-6 receptor antagonists are already in clinical use for the treatment of RA, our study suggests that a potential combination of those two drugs may be particularly efficacious in treating all aspects of RA, including systemic inflammatory bone loss.

Supplementary material

Supplementary material provides detailed information on genotyping, assessment of clinical signs, and quantitative real-time polymerase chain reaction; representative 3D images of ankle joints; microCT based trabecular and cortical bone assessments from all eight genotypes, as well as a summary table of results from one-way analysis of variance (Tukey's post hoc test) of bone parameter bone volume per tissue volume in talus, patella, tibiae, and lumbar vertebrae.

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