Bone & Joint Research

Supplementary Material

10.1302/2046-3758.117.BJR-2021-0481.R1

Supplementary Methods

Genotyping

Genotypes were determined by polymerase chain reaction (PCR) from DNA isolated from tail biopsies using the following primers: human tumour necrosis factor (hTNF) gene, forward 5´- TAC CCC CTC CTT CAG ACA CC -3´ and reverse 5´- GCC CTT CAT AAT ATC CCC CA –3´; interleukin (IL)-1α wild-type gene forward 5'-CTTGGCCATACTGCAAAGGTCATG-3' and reverse 5'-CAGGTTCATTTAACCAAGTGGTGCTG-3'; IL1-β wildtype gene forward 5'-GAGGTGCTGTTTCTGGTCTTCACC-3' and reverse 5'-CCATGCCACAGTCCCTCCAC-3' ; IL-1αβ knockout reverse 5'-GAGGTGCTGTTTCTGGTCTTCACC-3'. IL-6 wild-type gene forward 5´- TTC CAT CCA GTT GCC TTC TTG G–3´ and reverse 5´ TTC TCA TTT CCA CGA TTT CCC AG -3´; IL-6 knockout gene construct forward 5´- TTC CAT CCA GTT GCC TTC TTG G -3´and reverse 5´- CCG GAG AAC CTG CGT GCA ATC C -3´. The IL-1 PCR was amplified at 94°C for two minutes, 40 cycles of 94°C for 30 seconds, 66°C for two minutes and 72°C for one minute, and finally 72°C for three minutes. The IL-6 PCR was run with the following programme: 94°C for three minutes; 35 cycles of 94°C for 30 seconds, 63°C for one minute and 72°C for one minute; and finally 72°C for two minutes. The tumour necrosis factor (TNF) PCR was amplified at 94°C for two minutes; 35 cycles: 94°C for 45 seconds, 55°C for 30 seconds and 72°C for one minute 30 seconds; and finally 72°C for ten minutes. The PCR products were separated on a 1.5% agarose gel.

Assessment of clinical signs of arthritis

Mice were weekly and blindly assessed by an independent 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.[1,2] Briefly, paw swelling scores ranged from 0 to 3: 0, no swelling; 1, mild swelling of toes and ankle; 2, moderate swelling of toes and ankle; 3, severe swelling of toes and ankle. Grip strength was semi-quantitatively assessed on a metal mesh (3 mm wire in diameter) based on the ability to fully grab and hold onto the wire. Grip strength scores ranged from 0 to -3 (0, normal grip strength; -1, mildly reduced grip strength; -2, severely reduced grip strength; -3, no grip at all). In addition, body weight (in grams) was monitored weekly in all animal groups.

Quantitative real-time PCR

The following primers were used: matrix metalloproteinase 3 (MMP-3) forward 5'-CGA TGA TGA ACG ATG GAC AG-3' and reverse 5'-AGC CTT GGC TGA GTG GTA GA-3' ; MMP-9 forward 5'-CCTGTGTGTTCCCGTTCATCT-3' and reverse 5'-CGCTGGAATGATCTAAGCCCA-3' ; MMP-13 forward 5'-AGTTGACAGGCTCCGAGAAA-3' and reverse 5'-GGCACTCCACATCTTGGTTT-3' ; cathepsin K forward 5'-TGAGAGTTGTGGACTCTGTGCT-3' and reverse 5'-TTGTGCATCTCAGTGGAAGACT-3'; IL-6 forward 5'-CTGATGCTGGTGACAACCAC-3' and reverse 5'-CAGAATTGCCATTGCACAAC-3', tartrate-resistant acid phosphatase (TRAP) forward 5'-TCCTGGCTCAAAAAGCAGTT-3' and reverse 5'-ACATAGCCCACACCGTTCTC-3'; GAPDH forward 5'- TGG CAT TGT GGA AGG GCT CAT GAC-3'and reverse 5'-ATG CCA GTG AGC TTG CCG TTC AGC-3'and for human TNF forward 5´- TCCTTCAGACACCCTCAACC-3´ and reverse 5´- AGGCCCCAGTTTGAATTCTT –3´. 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 $2^{-\Delta\Delta Ct}$ method.

References

1. **Hayer S, Redlich K, Korb A, Hermann S, Smolen J, Schett G.** Tenosynovitis and osteoclast formation as the initial preclinical changes in a murine model of inflammatory arthritis. *Arthritis Rheum*. 2007;56(1):79–88.
2. **Hayer S, Zeilinger M, Weiss V, et al.** Multimodal [18 F]FDG PET/CT is a direct readout for inflammatory bone repair: a longitudinal study in TNFα transgenic mice. *J Bone Miner Res*. 2019;34(9):1632–1645.

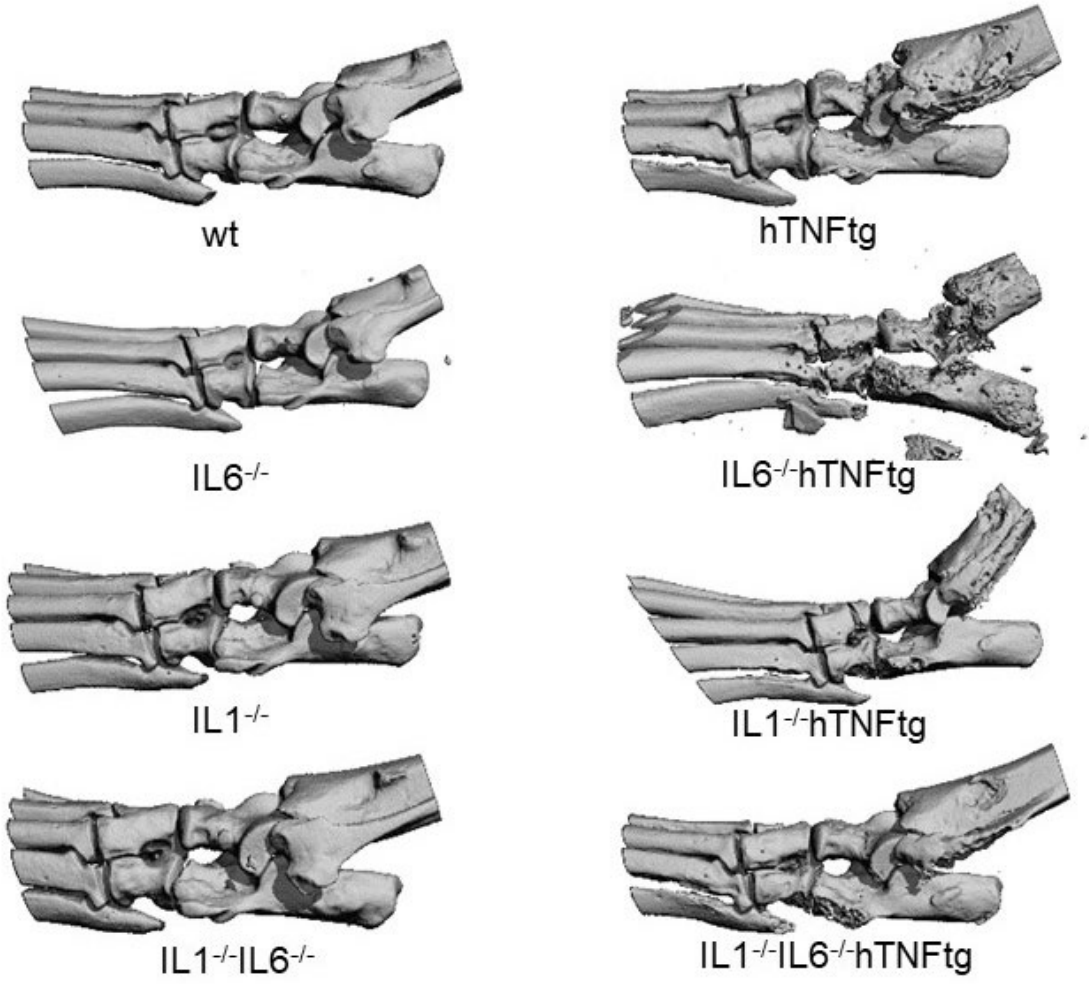

Fig a. Representative micro-CT (µCT)-based 3D images of ankle joints from 15-week-old IL1-/-IL6-/-hTNFtg, IL1-/-hTNFtg, IL6-/-hTNFtg and hTNFtg mice in comparison to wild-type (wt), IL1-/-, IL6-/- and IL1-/-IL6-/- littermates. hTNFtg, human tumour necrosis factor transgenic; IL, interleukin.

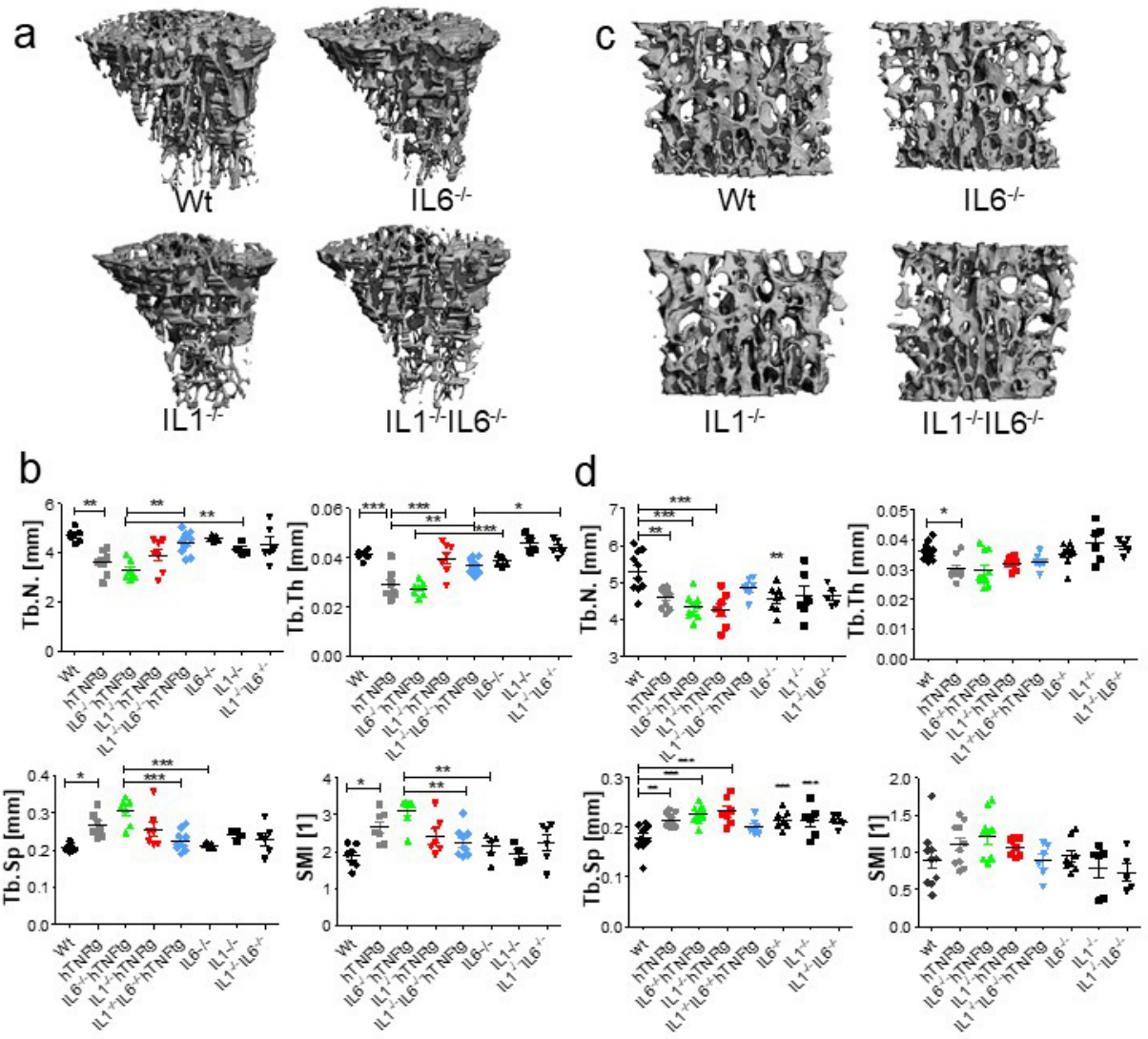

Fig b. Micro-CT (µCT)-based bone images and trabecular assessments from wild-type (wt), single deficient, and double deficient for interleukin (IL)-1 and IL-6 control animals. a) Representative 3D images of tibial trabecular network of control mice. b) Quantitative analysis of additional trabecular bone parameters in proximal tibial bone including trabecular number (Tb.N.), trabecular thickness (Tb.Th.), trabecular separation (Tb.Sp.), and structural model index (SMI) from 15-week-old IL1-/-IL6-/-hTNFtg, IL1-/-hTNFtg, IL6-/-hTNFtg, and hTNFtg mice in comparison to wt, IL1-/-, IL6-/-, and IL1-/-IL6-/- littermates. c) Representative 3D images of lumbar vertebral trabecular network. d)

Quantitatively assessed bone parameters from all eight genotypes. Data are expressed as mean (standard error of the mean (SEM)). *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. All p-values were calculated using one-way analysis of variance (ANOVA) and Tukey's post hoc test. hTNFtg, human tumour necrosis factor transgenic; IL, interleukin.

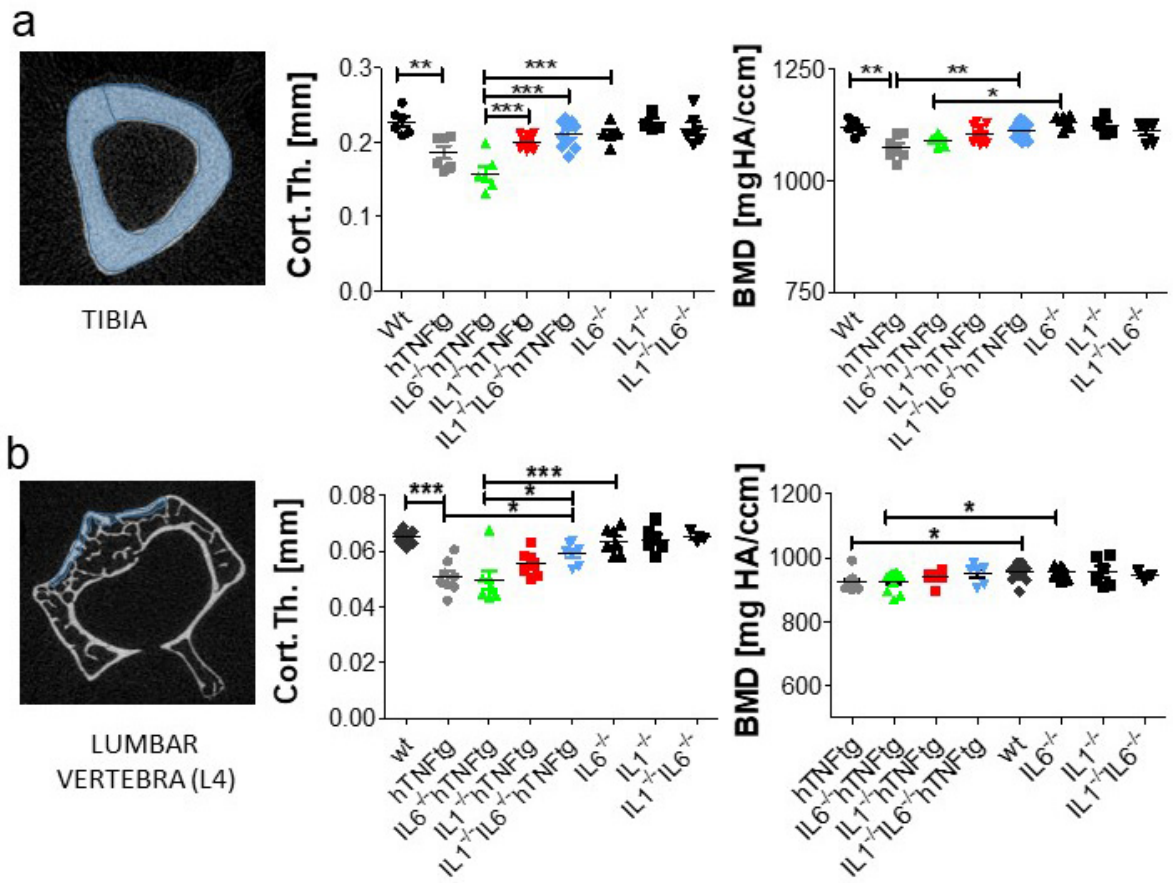

Fig c. Micro-CT (µCT)-based assessment of cortical bone thickness (Cort. Th.) and bone mineral density (BMD) in a) tibial midshaft and b) fourth lumbar vertebrae body from 15-week-old IL1-/-IL6-/-hTNFtg, IL1-/-hTNFtg, IL6-/-hTNFtg, and hTNFtg mice in comparison to wild-type (wt), IL1-/-, IL6-/-, and IL1-/-IL6-/- littermates. Data are expressed as mean (standard error of the mean (SEM)). *p < 0.05, **p < 0.005, ***p < 0.001. All p-values were calculated using one-way analysis of variance (ANOVA) and Tukey's post hoc test. hTNFtg, human tumour necrosis factor transgenic; IL, interleukin.

Table i. Results from one-way analysis of variance and Tukey's post hoc test of bone parameter bone volume/tissue volume assessed in trabecular bone of tibiae and lumbar vertebrae as well as in total bone of talus and patella from wt, $IL1^{-/-}$, $IL6^{-/-}$, $IL1^{-/-}IL6^{-/-}$, hTNFtg, $IL1^{-/-}$hTNFtg, $IL6^{-/-}$hTNFtg, and $IL1^{-/-}IL6^{-/-}$hTNFtg mice. Statistical significance was set at $p < 0.05$ (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$).

Tukey's post hoc test	**Significant changes ($p < 0.05$) in:**			
	BV/TV of talus	**BV/TV of patella**	**BV/TV of trabecular tibiae**	**BV/TV of trabecular lumbar vertebrae**
Wt vs hTNFtg	***	***	***	**
Wt vs $IL6^{-/-}$hTNFtg	***	***	***	**
Wt vs $IL1^{-/-}$hTNFtg	ns	ns	*	*
Wt vs $IL1^{-/-}IL6^{-/-}$hTNFtg	ns	ns	ns	ns
Wt vs $IL1^{-/-}$	ns	ns	ns	ns
Wt vs $IL6^{-/-}$	ns	ns	ns	ns
Wt vs $IL1^{-/-}IL6^{-/-}$	ns	ns	ns	ns
hTNFtg vs $IL6^{-/-}$hTNFtg	ns	ns	ns	ns
hTNFtg vs $IL1^{-/-}$hTNFtg	***	**	ns	ns
hTNFtg vs $IL1^{-/-}IL6^{-/-}$hTNFtg	***	***	*	ns
hTNFtg vs $IL1^{-/-}$	***	***	***	ns
hTNFtg vs $IL6^{-/-}$	***	***	**	ns
hTNFtg vs $IL1^{-/-}IL6^{-/-}$	***	***	***	ns
$IL6^{-/-}$hTNFtg vs $IL1^{-/-}$hTNFtg	***	***	**	ns
$IL6^{-/-}$hTNFtg vs $IL1^{-/-}IL6^{-/-}$hTNFtg	***	***	***	ns
$IL6^{-/-}$hTNFtg vs $IL1^{-/-}$	***	***	***	ns
$IL6^{-/-}$hTNFtg vs $IL6^{-/-}$	***	***	***	*
$IL6^{-/-}$hTNFtg vs $IL1^{-/-}IL6^{-/-}$	***	***	***	*
$IL1^{-/-}$hTNFtg vs $IL1^{-/-}IL6^{-/-}$hTNFtg	ns	ns	ns	ns
$IL1^{-/-}$hTNFtg vs $IL1^{-/-}$	ns	ns	ns	ns
$IL1^{-/-}$hTNFtg vs $IL6^{-/-}$	ns	ns	ns	ns
$IL1^{-/-}$hTNFtg vs $IL1^{-/-}IL6^{-/-}$	ns	ns	ns	ns
$IL1^{-/-}IL6^{-/-}$hTNFtg vs $IL1^{-/-}$	ns	ns	ns	ns
$IL1^{-/-}IL6^{-/-}$hTNFtg vs $IL6^{-/-}$	ns	ns	***	ns
$IL1^{-/-}IL6^{-/-}$hTNFtg vs $IL1^{-/-}IL6^{-/-}$	ns	ns	***	ns
$IL1^{-/-}$ vs $IL6^{-/-}$	ns	ns	ns	ns
$IL1^{-/-}$ vs $IL1^{-/-}IL6^{-/-}$	ns	ns	ns	ns
$IL6^{-/-}$ vs $IL1^{-/-}IL6^{-/-}$	ns	ns	ns	ns

BV/TV, bone volume/tissue volume; hTNFtg, human tumour necrosis factor transgenic; IL, interleukin; ns, not significant; wt, wild-type.