

TNF/TNFR modulates the Traf2-Gas axis to promote aging-related bone loss and impair PTH (1-34) efficacy

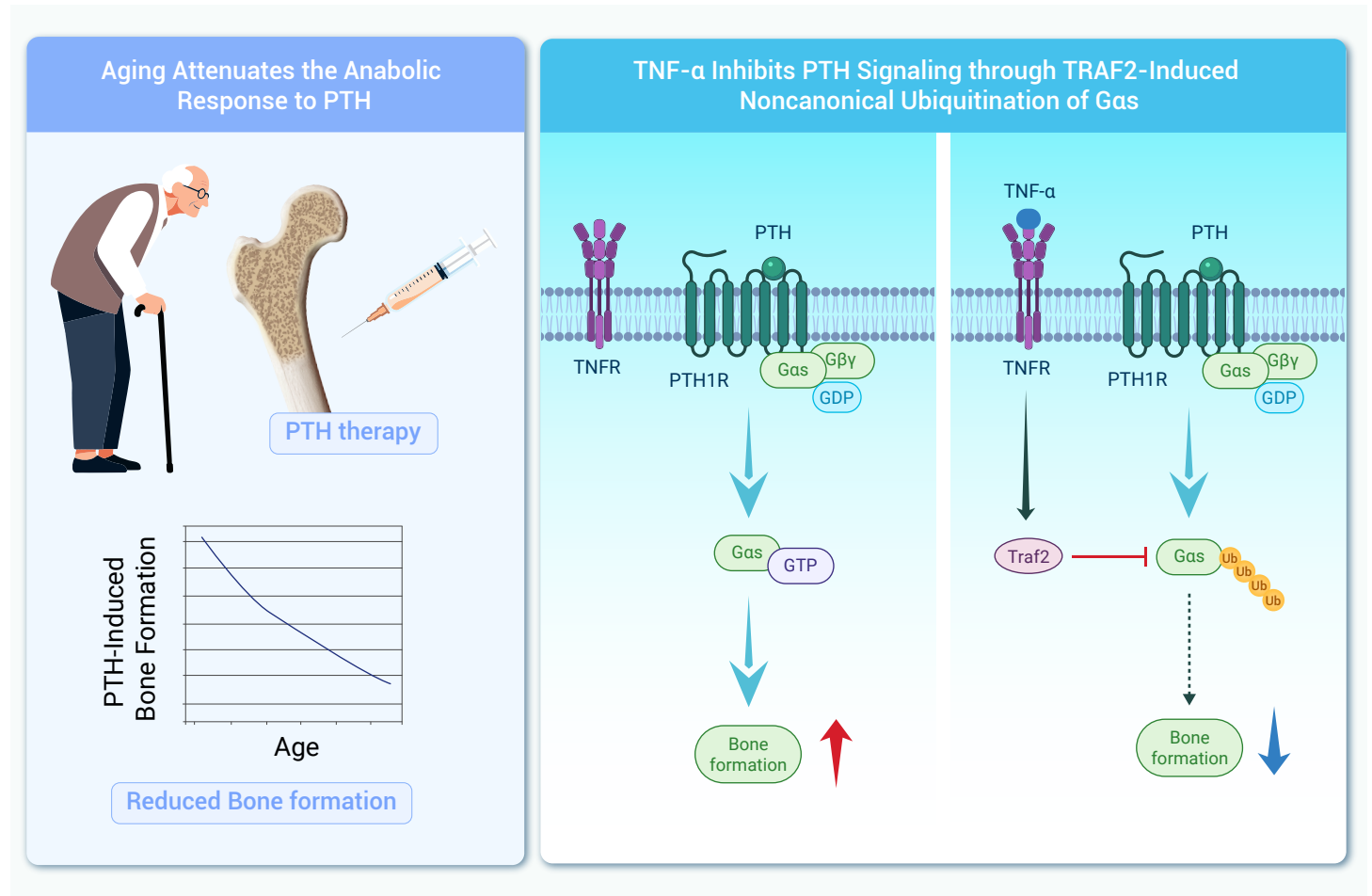
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Tumor necrosis factor (TNF) increases in aging bones and weakens bone formation.
- TNF reduces the effectiveness of parathyroid hormone (PTH) therapy in elderly bone.
- Blocking TNF signaling prevents age-related bone loss in mice.
- Combining TNF inhibition with PTH improves bone-building effects in aged animals.

TNF/TNFR modulates the Traf2-Gas axis to promote aging-related bone loss and impair PTH (1-34) efficacy

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The anti-osteoporotic effects of parathyroid hormone (PTH) analogs for patients with severe osteoporosis decrease with age; however, the underlying mechanism(s) are poorly understood. Here we provide evidence that enhanced TNF-mediated chronic inflammation during aging may play a critical role in promoting bone loss and attenuating the anabolic effects of PTH on bone in aged individuals. Specifically, we find that abolishment of TNF signaling activation by global deletion of the *Tnfr1/2* genes, which encode the TNF receptors, prevents aging-related bone mass loss and improves the anabolic effects of PTH (1-34) analog to increase bone mass and bone mineral density in aged, but not young adult, mice. These effects are achieved mainly by enhancing PTH signaling in osteoblastic cells, leading to accelerated bone formation. TNF- α inhibition of PTH signaling is mediated via TRAF2, an E3 ligase. TRAF2 promotes the non-canonical K27- and K33-linked ubiquitination of Gas, which increases Gas protein stability but blocks Gas activation and downstream signaling. Of clinical significance, infliximab, a TNF- α neutralizing antibody, reduces the bone loss in aged mice and improves the skeletal response to PTH. Collectively, we demonstrate a novel mechanism whereby TNF/TNFR/TRAF2 promotes aging-related bone loss and impairs the anabolic effects of PTH on bone in aged animals by inhibiting Gas activation, and our findings suggest that modulating the TNF/TNFR signaling pathway may improve the effectiveness of PTH treatment in senile osteoporosis patients.

INTRODUCTION

Osteoporosis (OP) is a skeletal disorder leading to reduced bone mass and bone mineral density, which causes bone fragility and fracture. OP affects more than 200 million people worldwide.¹ Above 70% of OP patients are over 65 years old, and the consequences of OP for elders are profoundly devastating.^{1,2} OP management to prevent fractures in elderly patients has always been challenging.³ Anabolic drugs, such as teriparatide and abaloparatide, can stimulate new bone formation, thus providing a possibility of replacing lost bone resulting in greater enhancement of bone mineral density and lower fracture risks.³⁻⁶ It is well established that PTH signaling promotes phosphorylation of the CREB protein to activate downstream target genes and thereby promote osteoblast and bone formation.^{7,8} Teriparatide, human PTH (1-34), activates PTH receptors and stimulates trabecular and cortical bone formation by increasing osteoblastogenesis, inhibiting osteoblast apoptosis, and activating bone-lining cells.⁹⁻¹¹ It was reported that the sensitivity and effect of PTH (1-34) administration on bones were decreased in elderly patients.¹²⁻¹⁴ Zhou and coworkers showed that the sensitivity of hMSCs to PTH decreased with age. They assessed the response of hMSCs to PTH by measuring the

expression of p-CREB, and they found that the level of PTH-induced p-CREB protein was significantly reduced with age, and that PTH-induced osteoblast differentiation of MSCs from elderly individuals was diminished. Galceran et al. showed that the ability to release cAMP in response to PTH (1-34) in the bones of young dogs was greater than that of old dogs. However, the mechanism underlying this phenomenon is not clear. It is important to understand the cellular and molecular mechanisms of PTH treatment in order to improve the anabolic effects of PTH in the treatment of elderly OP patients.

It has been known that aging is accompanied by chronic inflammation (inflammatory aging), characterized by elevated systemic levels of a variety of pro-inflammatory cytokines. Chronic, low-grade systemic inflammation is a common feature of aging, even in the absence of overt disease.¹⁵⁻¹⁷ TNF- α , a well-known biomarker of inflammation, plays an essential role in the aging process.^{18,19} There is accumulating evidence showing that inflammation can mediate the aging process and age-related diseases.^{20,21} How TNF/TNFR signaling impacts bone function, and homeostasis in aging human beings and animals is still poorly defined.

In this study, we demonstrate that TNF/TNFR signaling is activated and PTH signaling is suppressed in the bones of aged mice and humans. Genetic ablation of the genes encoding the TNF receptors reduces aging-related bone loss and enhances PTH(1-34) induced increases in bone volume (BV) and bone mineral density (BMD) in aged, but not young adult, mice. We demonstrate that Traf2 plays a critical role in mediating the TNF/TNFR inhibition of PTH signaling in osteoblastic cells. Traf2 interacts with Gas and promotes Gas ubiquitination, leading to Gas inactivation and blocking PTH downstream signaling. Finally, blocking TNF- α signaling with neutralizing antibodies improves the therapeutic effect of PTH on bone loss in aged mice.

MATERIALS AND METHODS

Data acquisition and preprocessing

Human bone tissues RNA-seq count data were obtained from the Gene Expression Omnibus (GSE141614),²² retaining only young and aged samples from the control group. For mouse osteocytes, raw RNA-seq data were downloaded from PRJNA695408 and processed using a standardized workflow. Read quality was assessed using FastQC, followed by adapter trimming and quality filtering. Cleaned reads were aligned to the Ensembl GRCm38 mouse genome using HISAT2.²³ Post-alignment processing—including read sorting, indexing, and quality control—was performed using SAMtools.²⁴ Gene-level quantification was conducted with featureCounts to generate raw read counts for downstream analyses.²⁵

Differential expression and functional enrichment analysis

Differentially expressed genes (DEGs) between young and aged groups were identified using DESeq2,²⁶ which applies a negative binomial generalized linear model and assesses significance via the Wald test. P-values were adjusted for multiple testing using the Benjamini-Hochberg method, with an FDR threshold of <0.05 considered statistically significant.

To elucidate enriched biological processes and pathways, we performed functional enrichment analysis on DEGs using the clusterProfiler R package.²⁷ Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was conducted via the enrichKEGG function, with terms at an adjusted *p*-value <0.05 deemed significant. Additionally, GSEA was performed using clusterProfiler to further characterize gene expression patterns.

Patient trabecular bone samples

In this study, samples of vertebral body were collected from 16 male patients who underwent lumbar spine surgery. Variables, such as gender, age, obesity or trauma, and presence of other diseases, were considered, and samples from patients with other diseases or significant differences in variables were excluded. All experiments involving patients were conducted in accordance with the ethical rules and procedures approved by the First Affiliated Hospital of Sun Yat-sen University (Approval no. 2013176). Informed consent was obtained from the subjects for all experiments. Trabecular bone samples were fixed with 4% paraformaldehyde, decalcified with 15% ethylenediaminetetraacetic acid, and embedded in paraffin for further histological and IF analysis.

Animal studies

Tnfrsf1a^{-/-} and *Tnfrsf1b*^{-/-} mice were obtained from the Shanghai Model Organism. *Tnfrsf1a*^{-/-} and *Tnfrsf1b*^{-/-} mice were generated in C57BL/6 genetic background mice using CRISPR/Cas9 technology. The C57BL/6 wild-type mice in the same genetic background were used as controls in this study. To conserve consistency and reduce the use of mice, male mice were used for all experiments except Supplementary Figure 4, which used female mice. Mice were injected subcutaneously with PTH (1-34) (Bachem) (80 µg/kg body weight) daily for 28 d. Infliximab (MCE) was administered via intraperitoneal injection at 10 mg/kg body weight once weekly for 4 weeks. Mice were kept on a 12-hour light/dark cycle with free access to food and water. All animal experiments were permitted and undertaken in the specific pathogen-free (SPF) Experimental Animal Center of the Southern University of Science and Technology.

Micro-computerized tomography (µCT) analysis

Fixed non-demineralized femurs were used for micro-computerized tomography (µCT) analysis in the Southern University of Science and Technology using a Bruker µCT (SkyScan 1172 Micro-CT, Bruker MicroCT, Kontich, Belgium) as previously described.²⁸ The analysis of the specimens involved the following bone measurements: the bone volume/tissue volume fraction (BV/TV) and bone mineral density (BMD). VOIs of trabecular bone selected for analysis was a 1.5 mm length of the metaphyseal secondary spongiosa, originating 0.5 mm below the epiphyseal growth plate and extending caudally.

H/E staining

Serial 5-µm paraffin sections were used for H/E staining as previously described.²⁹

TRAP staining

The TRAP staining was performed as previously described.³⁰ The osteoclast surface and osteoclast number were determined as previously described.³¹

Calcein double labeling

Calcein double labeling, MAR, and BFR were measured as we previously described.³² Briefly, mice were injected i.p. with calcein (20 mg/kg) at 6 and 2 days before sacrifice. Non-demineralized tibiae were embedded using an Osteo-Bed Bone Embedding kit (EM0200; MilliporeSigma) and sectioned at

30 µm. Images were captured using a fluorescence microscope (Olympus-BX53).

Von Kossa staining

Von Kossa staining was performed as previously described.³² Briefly, the undecalcified sections were stained with 1% silver nitrate solution under 100 W light bulb for 1 h. After being rinsed in three changes of distilled water, sections were incubated with Eosin for 5 min.

Enzyme-linked immunosorbent assay (ELISA)

ELISA assay was performed as previously described.⁹ MLO-Y4 cells were cultured in serum-free medium for 12 h, and then treated with and without PTH (1-34) (10⁻⁷ M) in the presence and absence of 2.5 ng/mL TNF-α for 1 h. Quantitative determination of cyclic AMP (cAMP) in cell lysates was performed using the cAMP Assay Kit (R&D Systems) according to the manufacturer's instructions.

Western blot analysis

Protein isolation and Western blot analyses were performed as we previously described.³³ The antibodies used in this study are listed in Table S1.

Cell culture and transfection

MLO-Y4 cells were generated as previously described.³² Cells were cultivated in α-MEM (10% FBS; 1% penicillin/streptomycin) and were grown in 37 °C, 5% CO₂ cell culture incubator. The MLO-Y4 cells were seeded the day before transfection, and transfection was performed at a density of 70–80%. For siRNA transfection, siRNAs were transfected by the Lipofectamine® RNAiMAX Reagent (ThermoFisher Scientific; cat# 13778150) according to the manufacturer's instructions. For plasmid transfection, plasmids were transfected by Lipofectamine TM 3000 Transfection Reagent (Invitrogen; cat# L3000015). Cells were collected and analyzed after 48h transfection.

Protein expression and purification

The pSUMO-His-Gas plasmid was generated by linking the Gas CDS to the EcoRI/ HindIII linearized pSUMO vector with a 6x His tag at the c-terminus. The CDS fragment of Traf2 was ligated into the BamHI/ SalI linearized pGEX-5x-1 vector with a c-terminal tag fused to GST to generate the pGEX-GST-Traf2-plasmid. Recombinant proteins were expressed by incubating in standard LB at 20°C for 8 to 14 h. Cells were centrifuged at 5,000 g for 10 min at 4°C, resuspended in TBS buffer (20 mM Tris-HCl, pH 7.5, 1 M NaCl and 1x complete protease inhibitor cocktail [Roche]), and disrupted by ultrasound at 4°C. For His-tagged proteins, cell lysates were incubated with Ni-NTA agarose (Qiagen) for 30 min at 4°C. After washing 4 to 6 times with TBS buffer containing 20 mM imidazole, bound proteins were eluted with TBS buffer containing 500 mM imidazole. For GST-tagged proteins, cell lysates were incubated with glutathione Sepharose 4B (Cytiva) for 4 to 12 h at 4°C. After washing four times with TBS buffer, the bound proteins were eluted with TBS buffer (pH 7.5) containing 50 mM glutathione. The resulting proteins are immediately assayed in vitro or stored at -80°C for a short period of time.³⁴

GST pull-down assay

For GST pull-down, GST-Traf2 fusion proteins were expressed and purified as described above and incubated with glutathione Sepharose 4B (Cytiva) in pull-down buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20 and 1x complete protease inhibitor cocktail [Roche]) for 2 h at 4°C, followed by 5 washes in the same buffer. Save 2.5% of the sample protein as input. Then incubate with purified Gas-His, or His control, respectively, for 2 h at 4°C. After 5 washes in the same buffer, boil in 2x SDS sample buffer for 10 min, followed by PAGE and immunoblotting.

Immunohistochemistry (IHC) and immunofluorescence (IF) analyses

IHC and IF staining was performed as previously described.^{32,35} Briefly, for IHC staining, 5-µm paraffin sections were incubated with the indicated antibodies or control IgG using the EnVision+System-HRP (DAB) kit (Dako North America Inc, Carpinteria, CA, USA). Images were obtained using a light microscope equipped with a digital camera. For IF staining, 5-µm paraffin sections were incubated with the indicated antibodies. Cells were grown on glass

coverslips (Corning, cat# 354236). Cultured cells were fixed with 4% PFA for 10 min. After permeabilization cells were penetrated with 0.25% Triton X-100, blocked with QuickBlock (cat# P0260, Beyotime, Shanghai, China) and then incubated with antibodies. Sections or cells were visualized by SP8 lighting confocal microscopy (Leica) with 40x and 63x objectives. The antibodies used in this study are listed in Table S1.

IP assay

IP assay was performed as previously described.³⁶ Briefly, cells were transfected with corresponding expression plasmids. After 48 h, cells were collected in 500 μ L RIPA buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 1 mM PMSF, 1 mM NaF, 1 mM β -glycerolphosphate, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate) containing proteinase inhibitor cocktail. Cell lysates were incubated with the corresponding primary antibody overnight and then with protein A or G beads at room temperature for 1 h. The beads were washed three times with RIPA buffer and resuspended with 100 μ L 1 $\times$ SDS sample buffer, and boiled at 100 $^{\circ}$ C for 5 min, followed by SDS-PAGE and western blotting.

Quantitative real-time RT-PCR (qPCR) analysis

qRT-PCR analysis was performed as previously described.³⁷ Briefly, total mRNA was isolated from tissue samples using Trizol (Takara), following the manufacturer's instructions. mRNA was reverse-transcribed into DNA by the PrimeScript RT Reagent Kit with gDNA Eraser (Takara) following the manufacturer's protocol. SYBR Green (Takara) was used to quantify PCR amplification. Primer sequences are described in Table S2.

Active Gas pull-down assay

The activation of Gas proteins were measured using the Gas Activation Assay Kit (NewEastbio, 80801) according to the manufacturer's instructions. Briefly, cell lysates were added to centrifuge tubes with 1 μ L of anti-active Gas monoclonal antibody and protein A/G agarose beads slurry. Incubate the tubes overnight at 4 $^{\circ}$ C with gentle stirring. Centrifuge at 12,000 $\times$ g to precipitate the beads. Aspirate and discard the supernatant. Wash the beads 3 times with Lysis Buffer, centrifuge and aspirate the supernatant each time, and resuspend the microbead precipitate in 20 μ L of 2X reducing SDS-PAGE sample buffer. Boil each sample for 5 minutes. Centrifuge each sample at 12,000 $\times$ g and collect the supernatant.

Statistical analysis

The sample size for each experiment of this study was determined as previously described.^{29,36} Animals were randomly grouped in experiments in this study. IF, IHC and histology were performed and analyzed in a double-blinded way. Data analysis was performed using Prism GraphPad software. Normality was tested using the Kolmogorov-Smirnov test, and variance homogeneity was verified by Levene's test. Group differences were analyzed by one-way ANOVA with Tukey's post hoc comparisons. The results are presented as means $\pm$ SD. $P < 0.05$ was considered statistically significant.

RESULTS

TNF/TNFR signaling is upregulated and PTH signaling is downregulated in aged skeletal tissues

To identify key signaling pathways involved in skeletal aging, we re-analyzed the RNA-seq dataset (GSE141614).²² Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed the TNF and NF- κ B signaling pathways were prominently upregulated in aged human bone, whereas the cAMP signaling pathway was markedly downregulated (Figure 1A). Further Gene Set Enrichment Analysis (GSEA) confirmed that genes involved in the p53, TNF, and NF- κ B pathways were enriched at the top of the ranked gene list in aged osteocytes, indicating activation of these pathways. In contrast, related genes of the cAMP pathway were enriched at the bottom of the list, suggesting suppression of these signaling genes (Figure 1B). Similarly, analysis of a murine RNA-seq dataset (BioProject PRJNA695408) revealed a similar pattern of pathway alterations in aged mouse osteocytes (Figures S1A-B).³⁸ Consistent with these findings, analysis of single-cell RNA-Seq data (GSE253355) from human bone mesenchymal stromal cell (MSCs) revealed upregulated TNF/TNFR signaling and downregulated PTH-respon-

sive gene clusters in aged human BMSCs, paralleling the alterations observed in murine and human osteocyte cells (Figures S1C-D).³⁹ Notably, since PTH is known to activate the cAMP pathway, this age-associated downregulation of cAMP signaling may imply a mechanistic link to impaired PTH signaling during skeletal aging. To validate these findings, we initially investigated the activation of TNF- α and PTH signaling pathways in adult and elderly human bones. We collected 16 vertebral trabecular bone samples from patients undergoing lumbar spine surgery. We divided the 16 samples into two groups (i.e., adult group, 27-40 -year-olds; elderly group, 66-80-year-olds). Trabecular bones of these patient samples were uniformly intercepted, and paraffin sections were prepared after fixing and decalcification. The expression levels of PTH signaling pathway proteins, including phosphorylated-Creb (p-Creb) and total Creb, were examined by immunofluorescence (IF) staining, and results showed that the numbers of p-Creb positive osteoblasts on the trabecular bone surfaces was significantly decreased in the elderly group compared to those in the adult group (Figures 1C-D). Note: the expression of total Creb was slightly reduced in osteoblasts in aged bones (Figures 1C & E). In the meantime, the expression levels of TNF- α were examined by IF staining on the same samples, and results showed that TNF- α expression was both markedly increased in trabecular bone in the elderly group versus the adult group (Figures 1C & F). We next determined the expression patterns of PTH signaling and TNF- α signaling-associated proteins in young (3 m) and aged (18 m) C57BL/6 mice. In agreement with the data obtained in human bones, there was a dramatic activation of the TNF- α signaling pathway, as revealed by markedly increased area of trabecular bone with TNF- α positive immunofluorescence staining, there was significant inhibition of PTH signaling detected in the trabecular bones of aged C57BL/6 mice (Figures S1E-H).

TNFR deletion reduces aging-related bone loss in mice

To elucidate the role of TNF- α activation in bone homeostasis, we purchased a global *Tnfrsf1a* and *Tnfrsf1b* double knockout mouse line generated using CRISPR/Cas9 technology from the Shanghai Model Organisms. We examined the bone mass and bone mineral density (BMD) of the double knock-out mice (*Tnfr^{-/-}*), in which the tumor necrosis factor receptor genes (*Tnfrsf1a*, which encodes p55, and *Tnfrsf1b*, which encodes p75) were both deleted, leading to a global inactivation of TNF/TNFR signaling. As expected, expression of both *Tnfrsf1a* and *Tnfrsf1b* mRNAs and proteins was abolished in the KO mice (Figures S2A-D). We then measured bone parameters of 3-, 14-, and 20-month-old control and KO mice. X-ray micro-computed tomography (μ CT) analyses of distal femurs showed no significant difference in bone mass between control and KO mice in 3 months (Figures 1G-I). However, at 14 and 20 months of ages, the KO mice displayed significant increases in BMD and bone volume fraction (BV/TV) (Figures 1G-I). Results from in vitro studies showed that TNF- α dramatically reduced the alkaline phosphatase (ALP) activity in primary calvarial osteoblast cultures from control mice, which was largely reversed in KO group (Figure 1J).

TNFR deletion enhances PTH-induced bone mass in aged mice, but not in young adult mice

We next determined whether deleting TNFR expression had any effects on PTH-induced bone volume and BMD in mice. At 3 months of age, the control and KO mice were subjected to daily PTH (1-34) (80 μ g/kg) injection for 28d. Femur samples were collected after 2 days of the final injection and BMD, BV/TV were determined by μ CT analyses. As expected, although PTH increased BV/TV and BMD in both control and KO mice, it did not exhibit any significant differences in PTH responses between the two genotypes (Figures S3A-C). Twenty-month-old control and KO mice were administered with daily PTH (1-34) (80 μ g/kg) for 28 days, and femurs were collected 2 days after the final injection. Interestingly, different from the young ones, the 20-month-old KO mice showed a significantly greater enhancement of bone volume and BMD compared to controls after PTH administration (Figures 2A-C). Similar results were observed in the skull and spines (Figures 2D-G). In line with observations in male mice, PTH induced higher increases in BMD and BV/TV in 20-month-old KO female mice than in age-matched control female mice (Figures S4A-C). While PTH increased the number of Osx(+) osteoblasts in both control and KO mice, the magnitude of the increase was much greater in KO bones than in control bones (Figures 2H-I). The calcein double-labeling

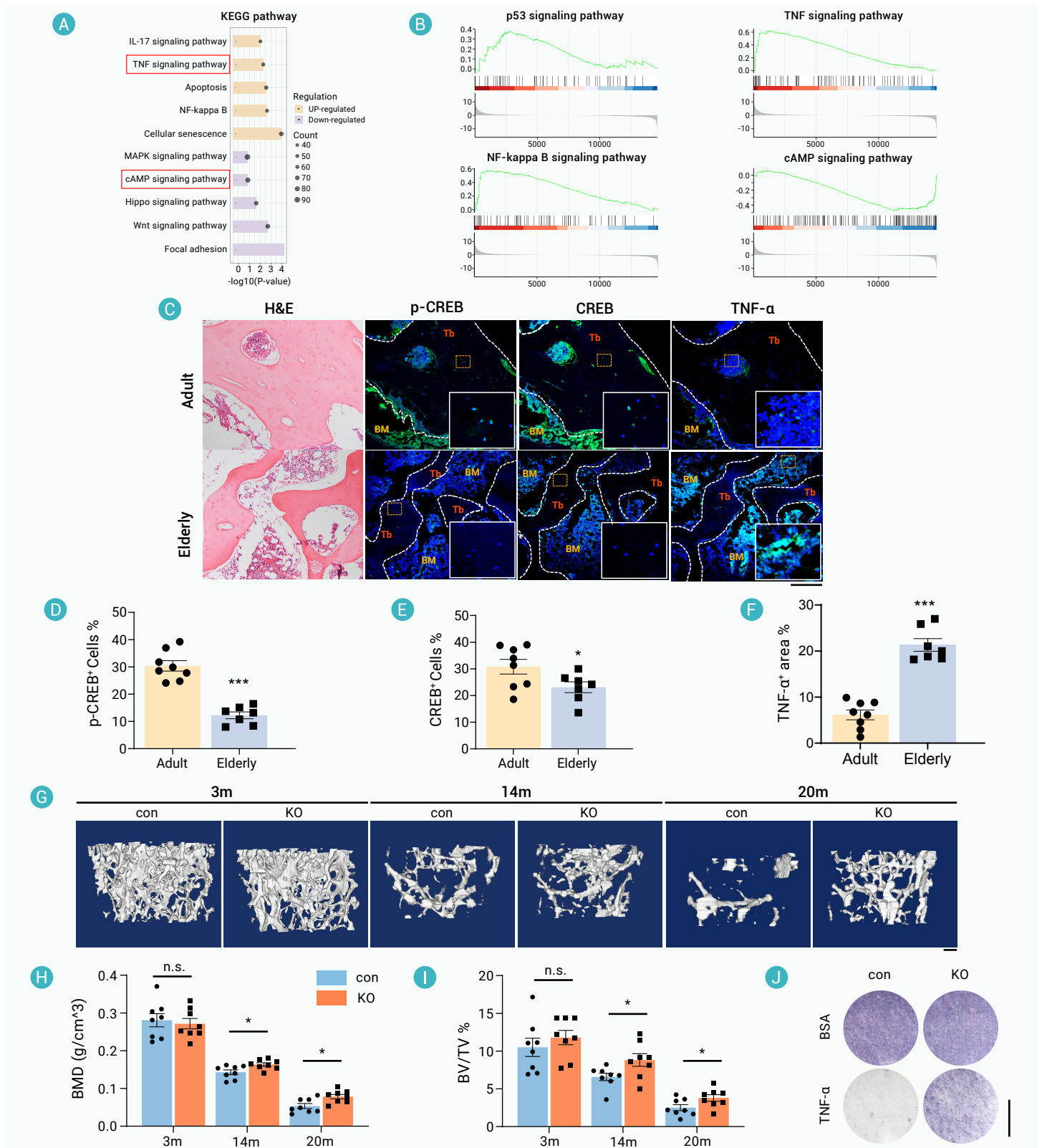


Figure 1. Elevated TNF and reduced PTH signaling in aged human bone tissues and deleting TNFR expression reduces aging-related bone loss in mice (A) The numbers of up- and down-regulated genes within enriched KEGG pathways in aged human osteocytes. (B) GSEA showing enrichment of p53, TNF, and NF-κB signaling pathways and suppression of the cAMP signaling pathway in aged human bone/osteocytes. (C-F) Hematoxylin and eosin (H&E) staining and immunofluorescence (IF) staining. Sections of adults (27-40 years old) and elderly (66-80 years old) human vertebral trabecular bone were subjected to H&E staining and IF staining with anti-p-CREB, CREB and TNF-α antibodies. White dashed lines indicate the bone surfaces, Tb indicates the trabecular bone area, BM indicates the bone marrow area, yellow solid boxes indicate the higher magnification images in the lower right panels (C). Scale bar, 100 μm. Quantitative measurements of the expression levels of p-CREB (D), CREB (E), and TNF-α (F), $n = 8$ samples per group. (G) Three-dimensional (3D) images of μCT of distal femurs from control and KO male mice at 3, 14 and 20 months of ages. Scale bar, 250 μm. (H,I) Quantitative analyses of BMD (H) and BV/TV (I). $n = 8$ mice per group. (J) Alkaline phosphatase (ALP) staining. Primary calvarial osteoblasts were isolated from 3-day-old control and KO mice and cultured with osteoblast differentiation medium with BSA or TNF-α (20 ng/mL) for 7 days, followed by ALP staining. All data are expressed as mean $\pm$ standard deviation (s.d.) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

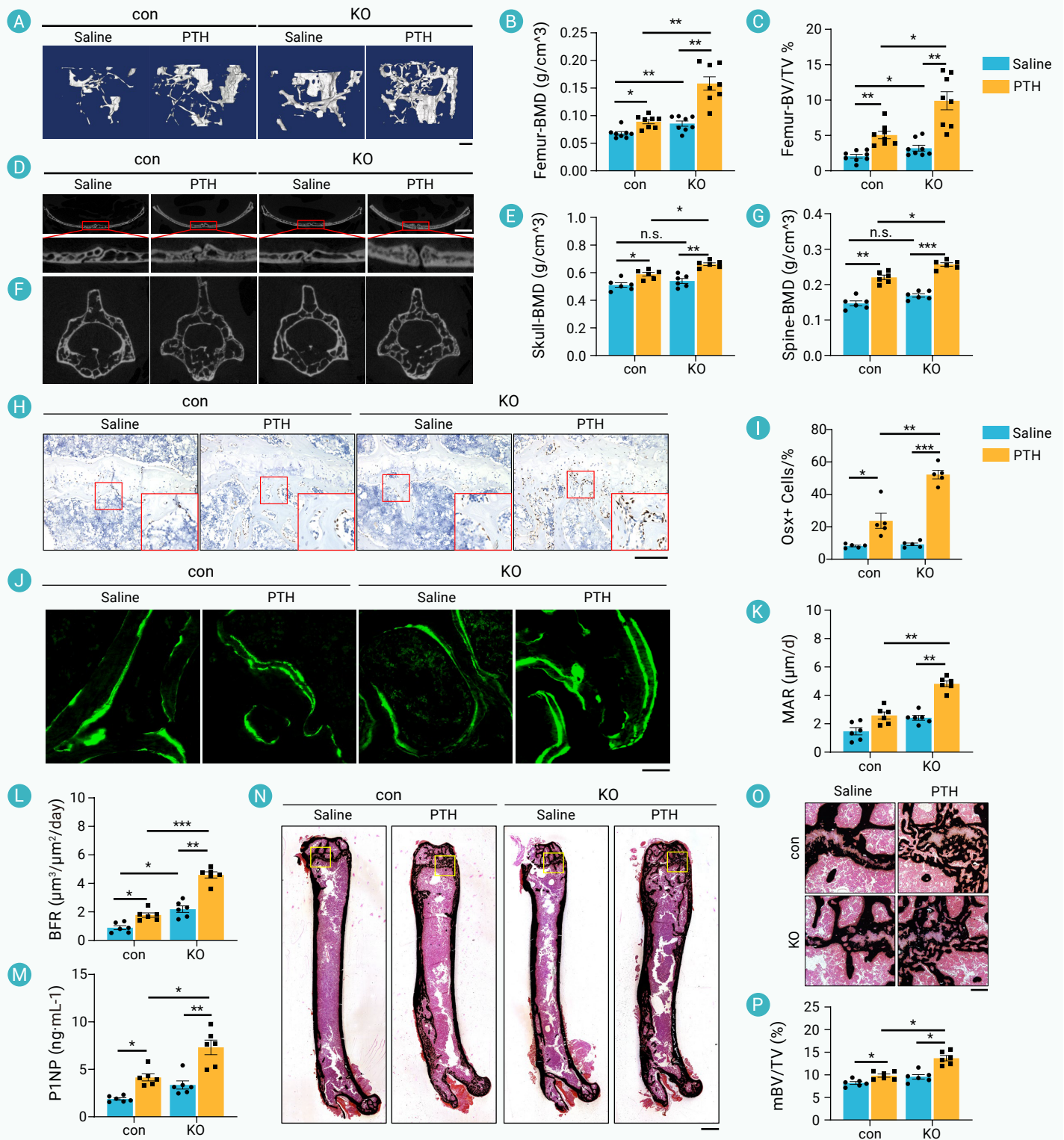


Figure 2. *Tnfr* loss enhances PTH-induced bone mass and BMD in aged mice (A–P) Control and KO male mice with and without PTH treatment for 28 d starting at the age of 20 months. (A) 3D images of μ CT of distal femurs. Scale bar, 250 μ m. (B, C) Quantitative analyses of BMD (B) and BV/TV (C). $n = 8$ mice per group. (D) μ CT cross-section view of the suture of parietal and interparietal bone. Scale bar, 250 μ m. (E) Quantification of BMD from D of calvaria ROI. $n = 6$ mice per group. (F) 2D images of cross-section images of the L5 lumbar spines. Scale bar, 250 μ m. (G) Quantitative analyses of BMD from F. $n = 6$ mice per group. (H, I) Immunohistochemistry (IHC) staining. Sections of tibial were subjected to IHC staining with anti-Osx antibody (H). Scale bar, 150 μ m. Quantification of Osx-positive cell number (I), $n = 5$ mice per group. (J–L) Calcein double-labeling. Images of the mineralized surface of the non-deminerized distal femoral sections (J). Scale bar, 50 μ m. Quantitative analyses of the mineral apposition rate (MAR) (K) and bone formation rate (BFR) (L). $n = 6$ mice per group. (M) Serum levels of procollagen type 1 N-terminal propeptide (P1NP). $n = 6$ mice per group. (N–P) von Kossa staining. von Kossa staining of undecalcified sections of femora (N). Scale bar, 1 mm. Higher magnification of N, highlighted in yellow box is showed in (O). Scale bar, 200 μ m. Quantitative mineralized bone volume/tissue volume (mBV/TV) data (P). $n = 6$ mice per group. Results are expressed as mean $\pm$ standard deviation (s.d.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

experiment confirmed that the mineral apposition rate (MAR) and bone formation rate (BFR) were significantly increased in PTH-induced KO bone compared to those in control bone (Figures 2J–L). In addition, the level of

serum N-terminal propeptide of type I procollagen (P1NP), a bone formation marker, was significantly elevated in PTH-induced KO versus control mice (Figure 2M). The calcium deposition level was promoted in PTH-induced KO

bones versus control bones, as revealed by von Kossa staining (Figures 2N-P). Note: PTH exhibited similar stimulatory effects on both osteoclast and osteoblast formation in 3-month-old KO versus control bones (Figure S5). We then analyzed the osteoclast formation of PTH-treated KO mice by tartaric acid-resistant acid phosphatase (TRAP) staining. Results showed that PTH treatment promoted osteoclast formation in both control and KO mice, with slightly higher increases in osteoclast surface/bone surface (Oc. S/BS) and osteoclast number/bone circumference (Oc. Nb/BPm) in KO bone than in control bone (Figures S6A-D).

TNF- α suppresses PTH signaling in cultured cells

Based on above in vivo results, our investigation aimed to elucidate whether TNF- α exerts an inhibitory influence on PTH signaling in vitro. To explore this, MLO-Y4 cells, an osteocyte-like cell line, were cultured in serum-free α -MEM medium containing TNF- α or BSA for 12 h, and then treated with and without PTH (10^{-7} M) for varying time intervals (10, 30, 60 and 180 min). The level of cyclic adenosine monophosphate (cAMP), which plays a pivotal role in PTH signaling, was determined. Results from ELISA showed that PTH induced increase in cAMP production was significantly decreased by pre-incubation with TNF- α at different time points in MLO-Y4 cells (Figure 3A). Additionally, we examined the impact of TNF- α on PTH-stimulated p-Creb production in vitro. As expected, exposure of MLO-Y4 cells to PTH upregulated the level of p-Creb protein (Figures 3B-C). However, pre-treatment with TNF- α decreased the PTH-induced increase in the p-Creb protein level without affecting its total protein level at various time points in MLO-Y4 cells (Figures 3B-C). Notably, TNF- α increased the level of p-p65 protein, while PTH did not alter the level of p-p65 or total p65 protein in MLO-Y4 cells (Figures 3B & S7). In parallel, we determined variation in the degree of inhibition due to changes in TNF- α dose. Results from western blotting analysis showed that pretreatment of TNF- α dose-dependently decreased the PTH-induced increase in the p-Creb protein level in MLO-Y4 cells (Figures 3D-E). Note: Various doses of TNF- α treatment also decreased p-Creb protein level in MLO-Y4 cells without PTH administer treatment (Figures S8A-B). PTH alone robustly induced Creb phosphorylation and increased its level in the nucleus in MLO-Y4 cells (Figure 3F). TNF- α significantly inhibited PTH-induced p-Creb production and increased its level in the nucleus of MLO-Y4 cells (Figures 3F-G). To further identify the inhibitory effect of TNF- α on PTH signaling in bone, an ex vivo bone tissue culture model was used. Freshly marrow-removed central femoral epiphyses from 3-month-old control and KO male mice were prepared and cut into small pieces. These bone tissue pieces were cultured in serum-free α -MEM medium containing TNF- α or BSA for 12 h, then treated with and without PTH (10^{-7} M) for 1h. Results showed that PTH treatment strongly induced the phosphorylation of Creb without affecting its total protein level in both control and KO groups (Figures 3H-I). However, pre-incubation with TNF- α significantly inhibited PTH-stimulated Creb phosphorylation in control bone but had no effect in the KO bone (Figures 3H-I). Furthermore, Infliximab, a TNF- α neutralizing monoclonal antibody, blocked the inhibition of TNF- α on PTH-induced upregulation of p-Creb in MLO-Y4 cells (Figure 3J).

Traf2 directly interacts with Gas and suppresses PTH signaling

The TNF ligands and cognate receptors are involved in diverse biological processes ranging from the induction of cell death to the stimulation of immunity and inflammation.^{40,41} Key factors in conducting TNFR signaling are TNF receptor-associated factor proteins (Trafs) that can be recruited to activated TNF receptors either by direct interactions with the receptors or indirectly via the adaptor protein TRADD.⁴¹ Trafs contribute to signaling events by TNF-receptor superfamily members, such as Tnfrsf1a, Tnfrsf1b, or CD40, and inhibit TLR-induced TRIF signaling.^{42,43} To date, seven different Trafs (Trafs1-7) have been discovered and characterized.⁴³ All Trafs except for Traf7 share a common domain of 180 amino acids at the C-terminal end, the "Traf domain," which is required for the oligomerization of Traf proteins and for the interaction with upstream receptors and downstream effector proteins.^{44,45} Therefore, to investigate whether Trafs proteins play a functional role in the crosstalk of the TNF- α and PTH signaling pathways, we targeted Trafs1-6. We overexpressed Traf1-6 plasmids, respectively, in MLO-Y4 cells under the condition of PTH (10^{-7} M) treatment and examined the expression level of p-

Creb protein induced by PTH. Results showed that overexpression of Traf2 but not other Trafs markedly inhibited PTH-induced increase in p-Creb protein level (Figures 4A-B). In addition, we knocked down Traf2 expression in MLO-Y4 cells by si-RNA under the condition of TNF- α pretreatment and PTH and found that knocking down Traf2 significantly alleviated TNF- α inhibition of PTH signaling (Figures 4C-D). Taken together, our in vitro experiments suggest that p-Creb activation by PTH signaling can be blocked by Traf2. However, co-IP assays showed that Pth1r did not interact with Tnfr1 or p65 proteins (Figures S9A-B). It is known that PTH triggers cAMP/Creb signaling through coupling with the G protein, which activates the adenylyl cyclases (ACs) to produce cAMP. To dissect the mechanism of how PTH/cAMP /CREB signaling is regulated by TNF- α , we chose the forskolin (FSK) and 3-isobutyl 1-methylxanthine (IBMX). FSK directly activates ACs and triggers the activation of the downstream p-Creb protein, IBMX is a broad-spectrum phosphodiesterase (PDE) inhibitor.⁴⁶⁻⁴⁸ MLO-Y4 cells were treated with PTH ($1-34$) (10^{-7} M), FSK (30 μ M) and IBMX (100 μ M) for 1 h, respectively, in the presence and absence 12h preincubation of 2.5 ng/ml TNF- α . As expected, all of PTH, FSK and IBMX potently induced Creb phosphorylation (Figures 4E-F). Interestingly, TNF- α dramatically suppresses the PTH- and IBMX-induced increase in the protein level of p-Creb, but FSK-induced activation of Creb phosphorylation was not affected by TNF- α (Figures 4E-F). Results from ELISA are consistent with western blotting results (Figure 4G). The above result suggests that TNF- α inhibits PTH signaling by impacting ACs and their upstream events. ACs activation is controlled by the G proteins,⁴⁹ which are composed of three subunits: α , β and γ . The α subunit is widely known for its ability to be activated and bind to GTP.^{49,50} G proteins are classified into four subfamilies, in which it is known that Gas mediates the cAMP/PKA/CREB signaling.^{49,50} To explore mechanisms of how Traf2 mediates the PTH pathway through G protein, we next determined whether Traf2 interacts with Gas. Co-immunoprecipitation (co-IP) assays using whole-cell extracts isolated from MLO-Y4 cells showed that Gas and Traf2 interacted with each other in these cells (Figures 4H-I). In addition, Flag-Traf2 and GFP-Gas were co-expressed in HEK293T cells, and co-IP assays showed that Flag-Traf2 was co-immunoprecipitated with an antibody against GFP-Gas (Figures 4J-K). To test whether this is a direct interaction, full-length His-Gas and GST-Traf2 proteins were expressed and purified from E.coli, and the GST pull-down assays were performed. Results showed that purified Traf2 directly interacted with purified Gas in the absence of other proteins (Figure 4L). To map the binding region between Gas and Traf2, four truncated constructs of Traf2 were generated (Figure 4M). Traf2 contains a RING domain at the N terminus, a Zn Fingers domain in the middle, and a TRAF domain. The four truncated Traf2 constructs were co-transfected with GFP-Gas expression plasmids into 293T cells, and co-IP assays were performed (Figure 4N). Results showed that both the aa1-100 region and aa101-501 region of Traf2 protein could bind to Gas (Figure 4N). These results may suggest the presence of more than one binding site between the two molecules.

Traf2 mediates K27- and K33-linked ubiquitination of Gas without inducing protein degradation

Traf2 acts as a powerful E3 ligase downstream of TNF- α to mediate the TNF/TNFR downstream pathway.⁴¹ To investigate how Traf2 regulates Gas, GFP-Gas and Flag-Traf2 constructs were co-transduced into MLO-Y4 cells to overexpress Gas and Traf2 protein, and ubiquitination assay showed that the ubiquitination of exogenous Gas was significantly increased after overexpression of Traf2 (Figure 5A). Results of ubiquitination assays showed that the ubiquitination of endogenous Gas was also increased after over-expression of Traf2 (Figure S10A). We further determined the ubiquitination levels of Gas in MLO-Y4 cells treated with PTH ($1-34$) in the presence of TNF- α pretreatment, and results showed that TNF- α dramatically elevated the level of Gas ubiquitination in MLO-Y4 cells, which was significantly inhibited by siRNA knockdown of Traf2 (Figure 5B). To determine which type of ubiquitination was connected to Gas, a series of ubiquitin mutant plasmids were transduced into MLO-Y4 cells, including Ub-K6R, Ub-K11R, Ub-K27R, Ub-K29R, Ub-K33R, Ub-K48R and Ub-K63R. Results revealed that Ub-K27R and Ub-K33R partially inhibited Traf2-mediated Gas ubiquitination, not by any other chain types (Figure 5C). Traf2 specifically promoted ubiquitination of

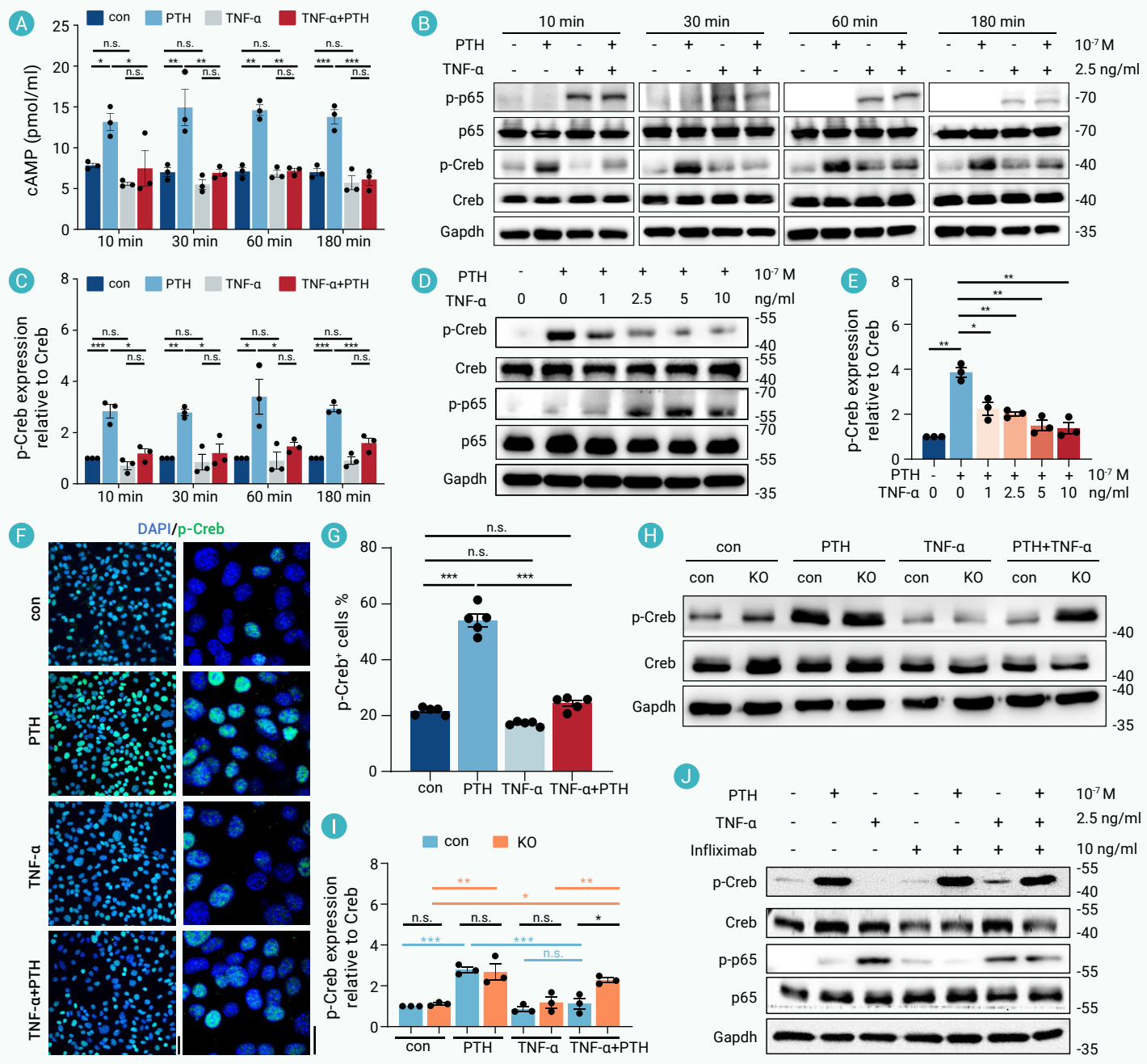


Figure 3. TNF- α suppresses PTH-induced cAMP production and Creb phosphorylation in MLO-Y4 cells in vitro (A) cAMP ELISA. MLO-Y4 cells were cultured with serum-free medium containing TNF- α (2.5 ng/mL) for 12 h, then treated with PTH (10^{-7} M) for the indicated times, followed by cAMP ELISA. $n = 3$ independent biological replicates. (B,C) Western blot analysis. MLO-Y4 cells were cultured in serum-free medium with TNF- α (2.5 ng/mL) for 12 h, then treated with PTH (10^{-7} M) for the indicated times, followed by Western blotting (B). Quantitative data (C). $n = 3$ independent biological replicates. (D,E) Western blot analysis. MLO-Y4 cells were cultured in serum-free medium containing increasing concentrations of TNF- α for 12 h, then treated with PTH (10^{-7} M) for 1 h, followed by Western blotting (D). Quantitative data (E). $n = 3$ independent biological replicates. (F,G) IF staining. MLO-Y4 cells were cultured in serum-free medium with TNF- α (2.5 ng/mL) or BSA for 12 h, then treated with and without PTH (10^{-7} M) for 1 h. Fixed cells were subjected to IF staining with p-Creb antibody (F). Scale bar, 50 μ m. Quantification of p-Creb-positive cells (G). $n = 5$ independent biological replicates. (H,I) Western blot analysis. Freshly marrow-removed central femoral epiphyses of 3-month-old control and KO male mice were cut into small pieces and cultured in serum-free α -MEM medium with TNF- α (2.5 ng/mL) or BSA for 12 h, then following with PTH (10^{-7} M) or saline for 1 h, p-Creb expression was determined by Western blotting (H). Quantitative data (I). $n = 3$ independent biological replicates. (J) MLO-Y4 cells were cultured in serum-free medium with TNF- α (2.5 ng/mL) or infliximab (10 ng/mL) for 12 h, then treated with PTH (10^{-7} M) for 1 h, followed by Western blotting for expression of the indicated proteins. Results are expressed as mean $\pm$ standard deviation (s.d.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Gas by K27- and K33-ubiquitin in the same pattern as wild-type HA-Ub (Figure 5D). Importantly, the K27/33R double mutant completely inhibited Traf2-induced Gas ubiquitination (Figure 5E).

Furthermore, to investigate whether Traf2 affects the stability of Gas protein, we measured the turnover rate of Gas in the presence or absence of Traf2 by the cycloheximide (CHX) assay. Transfection of Traf2 in MLO-Y4 cells resulted in an obvious stabilization of endogenous Gas compared to the empty vector group (Figures 5F-G). Likewise, overexpression of Traf2 extended the half-life time of exogenously expressed GFP-Gas proteins

(Figures 5H-I).

Traf2 exhibits an enhanced affinity for Gas in the inactive conformation compared to the active conformation

Given that the location of Gas at the plasma membrane is essential in its activation, we next tested whether Traf2 regulates Gas localization. Gas protein was detected by IF staining in MLO-Y4 cells under the condition of TNF- α and PTH. Results showed that pretreatment TNF- α largely altered the localization pattern of Gas protein in cells with or without PTH treatment

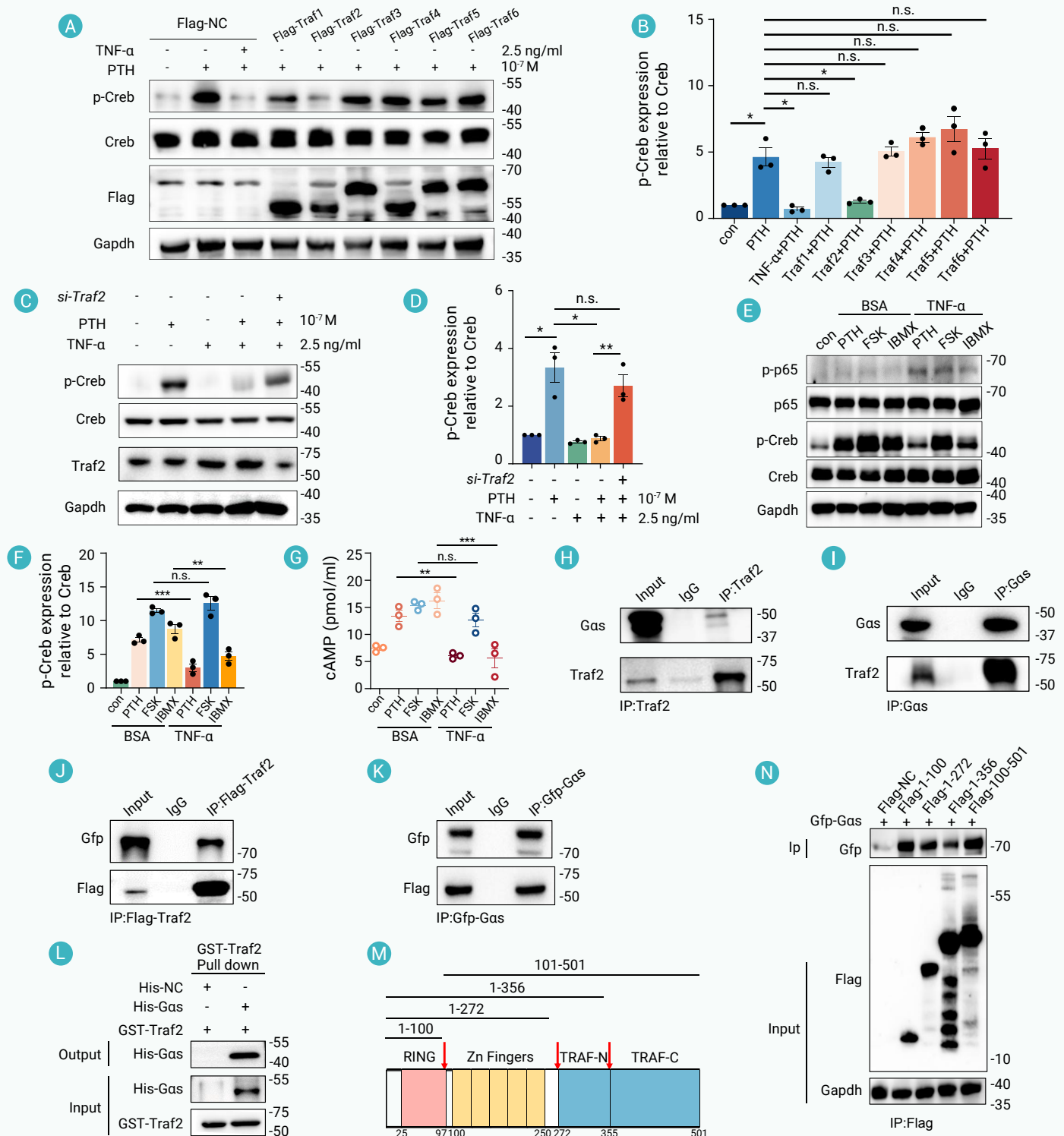


Figure 4. Tra2 directly interacts with Gas and mediates TNF- α inhibition of PTH signaling activation (A,B) Western blot analysis. MLO-Y4 cells transfected with the indicated plasmids for 36 h, and then cultured in serum-free medium with TNF- α (2.5 ng/mL) or BSA for 12 h, treated with and without PTH (1-34) (10^{-7} M) for 1 h. p-CreB expression was determined by Western blotting(A). Quantitative data (B). $n = 3$ independent biological replicates. (C,D) Western blot analysis. MLO-Y4 cells were transfected with indicated siRNAs for 36 h, after then cultured in serum-free medium with TNF- α (2.5 ng/mL) or BSA for 12 h, treated with and without PTH (1-34) (10^{-7} M) for 1 h. Protein extracts isolated from the cells were subjected to the indicated antibodies (C). Quantitative data (D). $n = 3$ independent biological replicates. (E-G) Western blot analysis and cAMP ELISA. MLO-Y4 cells were cultured in serum-free medium with TNF- α (2.5 ng/mL) or BSA for 12 h, and treated with PTH (1-34) (10^{-7} M), FSK (30 μ M) or IBMX (100 μ M) for 1 h. Protein extracts isolated from the cells were subjected to western blot analysis (E) and cAMP ELISA(G). Quantitative data for (E,F). $n = 3$ independent biological replicates. (H, I) Co-immunoprecipitation (co-IP) assay. Protein extracts from MLO-Y4 cells were incubated with Traf2, Gas antibody or IgG, and the immunocomplexes were separated by SDS-PAGE, followed by western blotting with antibodies against Traf2 and Gas.(J, K) co-IP assay. Cell lysates from HEK293T cells transfected with Flag-Traf2 and GFP-Gas, after then, cell lysates were incubated with Flag, GFP antibody or IgG, and the immunocomplexes were separated by SDS-PAGE, followed by western blotting with antibodies against Flag and GFP. (L) GST pull-down assay. Purified GST-Traf2, His and His-Gas protein were analyzed by GST pull-down assay. (M) A diagram of the Tra2 protein structure topology. (N) co-IP assays. HEK293T cells were co-transfected with plasmid constructs expressing Gas and truncated Traf2. 48h later, whole-cell extracts were prepared and subjected to co-IP assays. Results are expressed as mean $\pm$ standard deviation (s.d.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

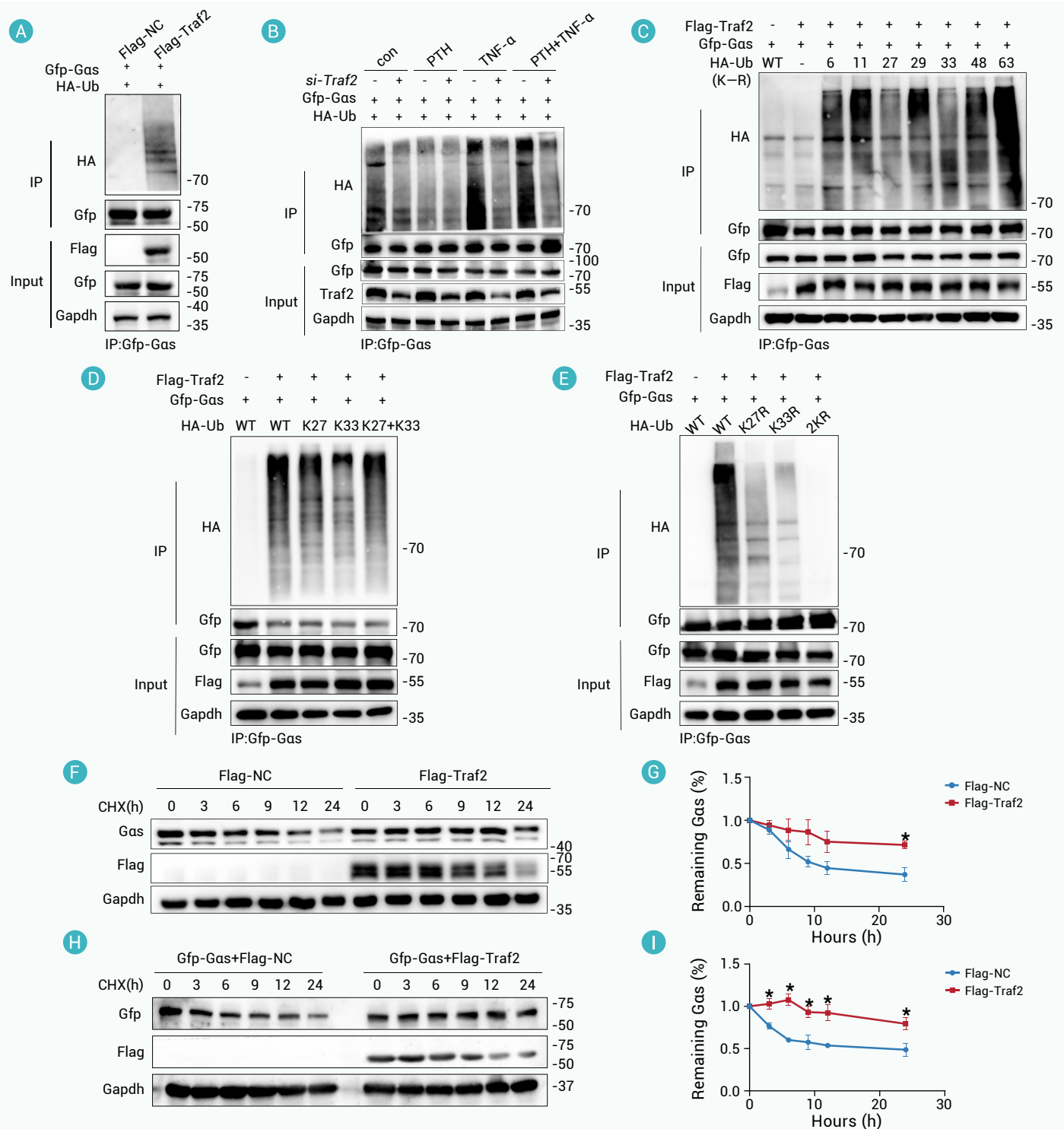


Figure 5. Traf2 induces polyubiquitination of Gas (A) Ubiquitination analysis. GFP-Gas and HA-Ub were transfected into MLO-Y4 cells with Flag-NC or Flag-Traf2 plasmids for 48 h and pretreated with proteasome inhibitor MG132 (10 μ M) for 6 h. Ubiquitination of GFP-Gas was detected by anti-HA antibody. (B) Ubiquitination analysis. MLO-Y4 cells transfected with indicated siRNAs for 36 h, after then cultured in serum-free medium with TNF- α (2.5 ng/mL) or BSA for 12 h, treated with and without PTH (1-34) (10^{-7} M) for 1 h, protein extracts followed by IP and IB assay with the indicated antibodies. (C) Ubiquitination analysis. MLO-Y4 cells were transfected with GFP-Gas, Flag-Traf2, and various Ub mutant plasmids. 48 h after transfection, ubiquitination assay was performed. (D) Ubiquitination analysis. HA-Ub WT or K27 and K33 linkage of Ub were transfected into MLO-Y4 cells together with GFP-Gas and Flag-Traf2 or control vector. GFP-Gas ubiquitination was detected. (E) Ubiquitination analysis. HA-Ub WT or mutants of K27 and K33 linkage of Ub were transfected into MLO-Y4 cells together with GFP-Gas and Flag-Traf2 or control vector. GFP-Gas ubiquitination was detected. (F,G) Cycloheximide (CHX) experiments. MLO-Y4 cells were transfected with Flag-NC or Flag-Traf2 plasmid. At 36 h after transfection, cells were treated with CHX at 100 μ g/mL for the indicated times. The half-life of Gas protein was measured by Western blot and analyzed (F). Quantitative data (G). $n = 3$ independent biological replicates. (H,I) CHX experiments. Gfp-Gas plasmid was transfected into MLO-Y4 cells together with Flag-NC or Flag-Traf2 plasmid. At 36 h after transfection, cells were treated with CHX at 100 μ g/mL for the indicated times. The half-life of exogenous GFP-Gas protein was measured by western blotting and analyzed (H). Quantitative data (I). $n = 3$ independent biological replicates. Results are expressed as mean $\pm$ standard deviation (s.d.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Figure 6A). Authentication of the Gas antibody was conducted using Gas siRNA (Figures S10B-C). Additionally, we found that PTH activated Gas,

which was abolished by TNF- α in MLO-Y4 cells (Figures 6B-C). Next, MLO-Y4 cells were pretreated with TNF- α (2.5 ng/mL) and PTH (10^{-7} M), followed

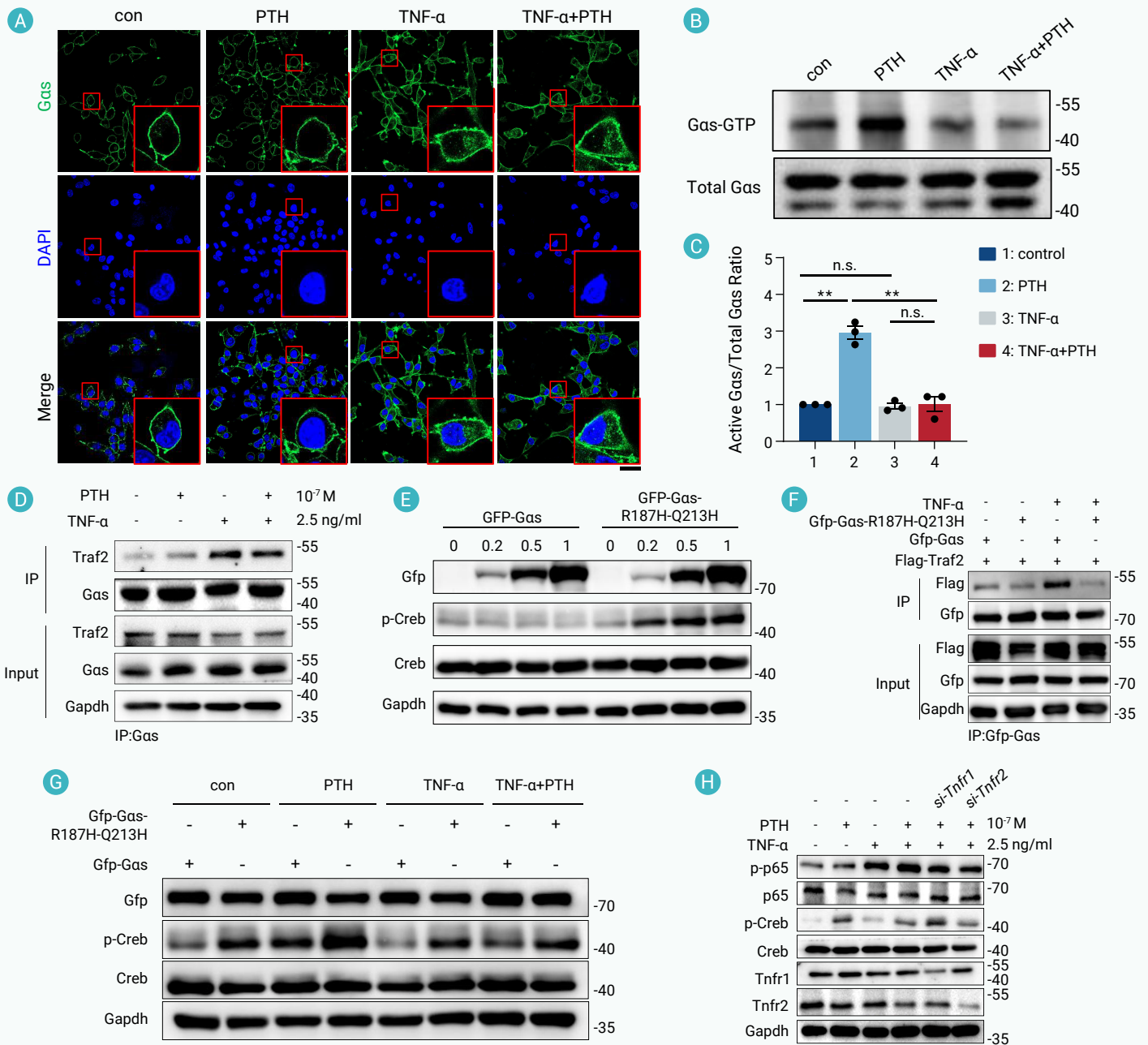


Figure 6. Traf2-dependent Gas inactivation mediates TNF/TNFR inhibition of PTH signaling (A) IF staining. MLO-Y4 cells were cultured in serum-free medium with TNF- α (2.5 ng/mL) or BSA for 12 h, then treated with and without PTH (1-34) (10^{-7} M) for 1 h. Fixed cells were subjected to IF staining with Gas antibody. Scale bar, 20 μ m. (B,C) MLO-Y4 cells were cultured in serum-free medium with TNF- α (2.5 ng/mL) or BSA for 12 h, then treated with and without PTH (1-34) (10^{-7} M) for 1 h, protein extracts followed by IP and IB assay used to determine the level of activated GTP-bound Gas (B). Quantitative data (C). $n = 3$ independent biological replicates. (D) co-IP assay. MLO-Y4 cells treated in the same way as B, protein extracts were incubated with Gas antibody and the immunocomplexes were separated by SDS-PAGE, followed by western blotting. (E) Western blot analysis. MLO-Y4 cells were transfected with increasing amounts of GFP-Gas or GFP-Gas-R187H-Q213H plasmids for 36 h, and then cultured in serum-free medium for 12 h, p-Creb expression was determined by western blotting. (F) co-IP assay. MLO-Y4 cells were transfected with Flag-Traf2 and GFP-Gas or GFP-Gas-R187H-Q213H plasmids for 36 h, and then cultured in serum-free medium with TNF- α or BSA for 12 h, protein extracts were incubated with GFP antibody and the immunocomplexes were separated by SDS-PAGE, followed by western blotting. (G) Western blot analysis. MLO-Y4 cells were transfected with GFP-Gas or GFP-Gas-R187H-Q213H plasmids for 36 h, and then cultured in serum-free medium with TNF- α or BSA for 12 h, then treated with and without PTH (1-34) (10^{-7} M) for 1 h, p-Creb expression was determined by western blotting. (H) Western blot analysis. *Tnfr1* or *Tnfr2* was knocked down by siRNA interference for 36 h, after the cultured with serum-free medium with TNF- α or BSA for 12 h, then treated with and without PTH (1-34) (10^{-7} M) for 1 h, protein extracts were subjected to western blotting for expression of the indicated proteins. Results are expressed as mean $\pm$ standard deviation (s.d.). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

by IP assays with antibodies against Gas. The results showed that TNF- α significantly increased the interaction between Traf2 and Gas in the presence and absence of PTH treatment (Figure 6D). To determine whether Traf2 binds Gas in its inactive (GDP bound state) or active (GTP-bound state) conformation, we constructed the activating mutants GFP-Gas-R187H-Q213H plasmid by sequence alignment.⁵¹ Note: mutations at these sites block GTPase activity, which keeps Gas in a GTP-bound active state, leading

to its constant activation.⁵¹ MLO-Y4 cells were transfected increasing concentrations of GFP-Gas plasmid or GFP-Gas-R187H-Q213H plasmid for 36h, then changed the medium to serum-free medium for 12 h and found that overexpression of mutant plasmid, but not WT plasmid, increased the level of p-Creb in a dose-dependent manner (Figure 6E). To further identify the state of Gas binding to Traf2, MLO-Y4 cells were co-transfected with WT and mutant plasmids, along with the Traf2 expression plasmid, followed by

co-IP assays. The results showed that TNF- α significantly increased the interaction of WT Gas. Interestingly, mutations at R187 and Q213 effectively reduced the interaction with Traf2 and not affected by TNF- α (Figure 6F). Moreover, overexpression of mutant plasmid in MLO-Y4 cells significantly diminished TNF- α inhibition of PTH signaling (Figure 6G). Finally, TNF- α inhibition of PTH signaling activation was reversed by siRNA knockdown of Tnfr1, but not Tnfr2 (Figure 6H).

TNF- α -neutralizing monoclonal antibody infliximab enhances the promoting effect of PTH to increase bone mass in aged mice

Infliximab is a chimeric human-mouse anti-TNF- α monoclonal antibody, and is commonly used in the treatment of various autoimmune diseases, including Crohn's disease and rheumatoid arthritis.^{52,53} To assess its potential effect on bone mass in aging, we administered daily subcutaneous injections of PTH (1-34) (80 μ g/kg) to 18-month-old C57BL/6 mice for 28 days, with infliximab given once weekly during the same period. Femurs were harvested two days after the final injection and analyzed by micro-CT to assess BMD (Figures 7A-D). The results revealed that infliximab treatment alone reduced age-related bone loss compared to vehicle controls (Figures 7A-B). Combined infliximab and PTH treatment led to a more pronounced attenuation of bone loss than PTH alone (Figures 7A-B). Similar effects were observed in the calvarial and spinal bones (Figures 7A & C-D). Calcein double labeling showed that the MAR was significantly elevated in the combination treatment group compared to that in the PTH-only group (Figures 7E-F), with a corresponding significant increase in Osx(+) osteoblasts (Figures 7G-H). Notably, results from TRAP staining of bone sections showed that the increases in Oc.S/BS and Oc.N/BPm were lower in the combination group than those in the PTH-alone group (Figures 7I-K).

DISCUSSION

The function of activated TNF/TNFR signaling in the regulation of various important cellular processes are linked to many human diseases and disorders, including rheumatoid arthritis (RA), osteoarthritis (OA), OP, cancers, and other diseases.⁵⁴⁻⁵⁷ In this study, we demonstrate that TNF/TNFR signaling pathway is activated in the bone of aged mice and elderly humans, which is coupled with suppression of the PTH pathways. Importantly, we provide convincing genetic evidence that blocking TNF- α signaling prevents aging-induced bone loss in mice. We find that old (14m and 20m) *Tnfrsf1a/Tnfrsf1b* global double knockout (KO) mice show significant increases in bone mass and BMD compared with their control littermates; this does not occur in young adult mice. Interestingly, another *Tnfrsf1a/Tnfrsf1b* knockout mouse line, also referred to as p55/p75 KO mouse strain, was reported to obtain abnormal bone healing ability and abnormal trabecular bone morphology after bone marrow cannulation, and also the KO mice gained increased bone resorption after bacterial inoculation of the dental pulp.^{58,59} However, the authors did not investigate the effect of Tnfr deletion on aging-related bone loss. Our study demonstrates for the first time that genetic blockade of TNF signaling by global deletion of *Tnfr1/2* significantly enhances both basal bone mass and BMD in aged, but not in young adult mice.

To our knowledge, Tnfr deletion significantly enhances PTH-induced bone formation in aged mice, leading to an obvious increase in the enhancement of bone mass and BMD induced by intermittent PTH injection. These experiments clearly indicate that TNF/TNFR serves as a potent inhibitor in intermittent PTH-induced bone formation in aged bone. TNF- α plays an important role in the regulation of bone homeostasis.⁶⁰ It is also recognized as a pro-inflammatory cytokine that promotes the development of various autoimmune disorders (gout, osteomyelitis, and RA), most of which are accompanied with osteoporosis.⁶¹⁻⁶³ Previous studies have shown that TNF- α may lead to bone loss of patients with RA.^{40,64,65} Bone loss in patients with gout and osteomyelitis has also been associated with increased TNF- α levels.^{66,67} Thus, TNF- α may be an important factor regulating bone homeostasis and associated with osteoporosis. Previous views suggested that TNF- α acts as a powerful bone-resorbing cytokine, mainly on osteoclasts.⁴⁰ In addition, previous studies showed that the production of TNF- α increased with aging.⁶⁸ Whether or not TNF- α induces bone loss via inhibition of the PTH signaling was not known. In this study, we demonstrate that TNF- α inhibits osteoblast function and bone formation via suppressing PTH signal-

ing in aged mice. Thus, TNF- α may have a dual regulatory function in bone homeostasis, providing a novel mechanism for the prognosis and prevention of senile osteoporosis. In addition to TNF- α , other pro-inflammatory cytokines such as IL-1 β and IL-6 are also involved in skeletal aging.⁶⁹ IL-1 β activates NF- κ B and MAPK pathways, inhibits osteogenic differentiation, and has been associated with hormonal responsiveness in other tissues.^{70,71} It is potential that these additional cytokines may exert synergistic or inhibitory effects on the PTH signaling. Further investigations are necessary to explore the broader inflammatory signaling network that modulates PTH signaling during aging.

Interestingly, regular physical exercise has been identified as another anti-aging intervention that decreases chronic inflammation. Zhang et al. summarized that endurance training activates mitochondrial metabolism and anti-inflammatory pathways involving SIRT1, PGC-1 α , AMPK, and AKT/mTOR signaling across cardiovascular and musculoskeletal tissues, thereby improving systemic metabolism and reducing inflammatory stress.⁷² While Zhang et al. focus on a non-pharmacological anti-aging strategy driven by exercise-mediated strengthening of mitochondrial metabolism, our study identifies TNF/TNFR inhibition as a pharmacological approach that improves PTH responsiveness and bone anabolism in aged mice. Both approaches highlight the importance of suppression of chronic inflammation, whether through exercise or targeted therapy, is pivotal to maintaining musculoskeletal integrity during aging.

It should be noted that in this study we have deleted the expression of genes encoding both Tnfr1 and Tnfr2; this may complicate the interpretation of data because we do not know which one of the receptors is responsible for mediating the inhibitory effect of TNF- α on PTH signaling. However, our in vitro studies reveal that siRNA knockdown of Tnfr1, but not Tnfr2, attenuates TNF- α inhibition of PTH-induced Creb phosphorylation in osteoblastic cells. This result suggests that Tnfr1 may be the major player in the inhibition of PTH signaling to promote aging-related bone loss and decrease the anabolic actions of PTH in aged bone. Cumulative evidence from the literature suggests that TNFR1 plays an important role in promoting inflammation, while TNFR2 is anti-inflammatory in various conditions and diseases.^{73,74}

Our findings provide insights into the new function of TNF/TNFR signaling in bone formation and also uncover a novel mechanism of the interplay between PTH and TNF/TNFR signaling pathways. One highlight of this study is that we demonstrate that TNF- α suppresses PTH induction of the cAMP/CREB pathway through Traf2 interaction with Gas protein in osteoblastic cells. Results of our study support the notion that TNF- α inhibits the activation of Gas by promoting the ubiquitination of Gas without inducing its degradation. Although ubiquitination is the major way of mediating protein degradation, certain ubiquitinated proteins do not go to the degradation pathway.^{75,76} In fact, K33-ubiquitination of Crn7 mediated by KLHL20 is essential in protein trafficking.⁷⁷ Moreover, another study elucidated the distinctive role of K27-linked polyubiquitination in NS3 in function of protein recruitment.⁷⁸ The functions regulated by these atypical ubiquitin chains remain elusive. Our findings from the present study indicate that the binding of Traf2 to Gas is enhanced after TNF- α treatment. Additionally, Traf2 exhibits an enhanced affinity for Gas in the inactive conformation. Moreover, a portion of Gas protein was observed to translocate from the plasma membrane to cytoplasmic lysates after exposure to TNF- α , and the activation of Gas partially decreased the inhibitory effect of TNF on PTH. However, the mechanisms through which Traf2 regulates the subcellular localization of Gas has not been identified.

Furthermore, PTH also activates calcium/calmodulin-dependent protein kinase II (CaMK2) and Glycogen synthase kinase-3 (GSK-3 β).^{79,80} We demonstrate that TNF- α also inhibited PTH-induced p-CaMK2 production, while GSK-3 β production was unaffected (Figure S11). In addition, 12h pretreatment of TNF- α did not markedly alter RANKL levels under these conditions (Figure S11). No increase in Rankl level may be due to insufficient TNF- α treatment time as reported that osteocytes exhibit a significant increase in RANKL mRNA expression after 3 days of TNF- α incubation.⁸¹ These observations contribute to an enhanced understanding of the intricate effects of TNF- α and PTH stimulation on cells.

We also present observations on sex-specific differences. While most in vivo experiments used male mice to minimize variability, Figure S4 shows

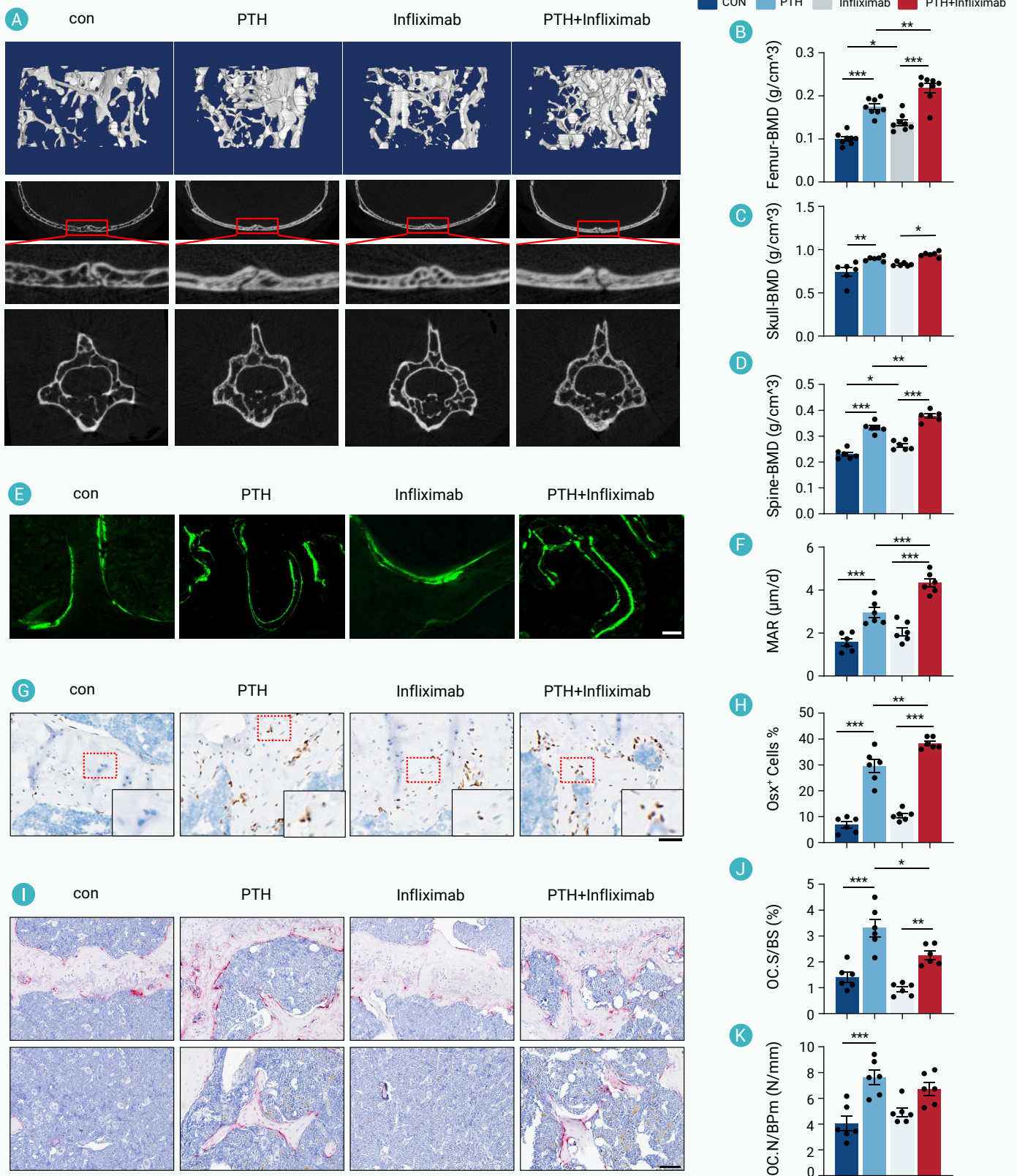


Figure 7. TNF- α -neutralizing antibody infliximab enhances the anabolic effects of PTH on bone in aged mice (A-K) C57BL/6 male mice with and without PTH (80 μ g/kg) treatment and with Infliximab (10 mg/kg) or BSA for once per week for 28 d starting at the age of 18 months. (A) 3D images of μ CT of distal femurs, cross-section view of the suture of parietal and interparietal bone and cross-section images of the L5 lumbar spines. Scale bar, 250 μ m. (B) Quantitative analyses of Femur BMD from A. $n = 8$ mice per group. (C) Quantification of BMD from A of calvaria ROI. $n = 6$ mice per group. (D) Quantitative analyses of Spine BMD from A. $n = 6$ mice per group. (E,F) Calcein double-labeling. Images of the mineralized surface of the non-demineralized distal femoral sections (E). Scale bar, 20 μ m. Quantitative analyses of MAR (F). $n = 6$ mice per group. (G,H) Immunohistochemistry (IHC) staining. Sections of tibia were subjected to IHC staining with anti-Osx antibody (G). Scale bar, 20 μ m. Quantification of Osx-positive cell number (H), $n = 6$ mice per group. (I) TRAP staining, TRAP staining of the tibia. Scale bar, 50 μ m. (J, K) Oc.S/BS (J) and Oc.N/BPm (K) of spongiosa bones from I were measured using Image-Pro Plus 7.0. $n = 6$ mice per group. Results are expressed as mean $\pm$ standard deviation (s.d.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

that aged female mice exhibited PTH responses similar to those of males after Tnfr deletion. No significant sex- treatment interaction was detected, suggesting that the TNF/TNFR regulatory mechanism is largely sex-independent under the present conditions. However, hormonal differences, particularly estrogen withdrawal, might affect Gas and PTH signaling in aged females, requiring further investigation in future studies.

To strengthen clinical relevance, we analyzed single-cell RNA-Seq data (GSE253355) from human BMSCs. Aged human BMSCs displayed increased expression of TNF/TNFR pathway genes and reduced PTH-responsive gene clusters, similar to our findings in mice. This cross-species consistency highlights the translational significance of inflammatory regulation of PTH signalling in skeletal ageing and supports the potential application of anti-TNF- α therapies in clinical settings.

From a translational perspective, blocking TNF- α signalling with clinically established biologics such as infliximab or TNFR-Fc offers a promising strategy to restore skeletal responsiveness to PTH in elderly patients. Both genetic ablation of TNF receptors and pharmacologic inhibition with infliximab enhanced bone mass and osteogenesis in aged mice receiving PTH treatment, indicating that TNF- α -driven inflammation impairs PTH-mediated bone anabolism. Given the extensive clinical use of TNF- α inhibitors in inflammatory diseases, their short-term or sequential combination with intermittent PTH therapy could potentially be adapted for age-related osteoporosis. Combined treatment may improve the aged bone microenvironment by alleviating inflammation-induced PTH resistance and improving osteoblast responsiveness, particularly in individuals with chronic low-grade inflammation. However, long-term TNF- α inhibition carries infection and immunosuppression risks; therefore, future preclinical and clinical studies should define optimal dosing schedules and evaluate safety and synergistic efficacy.

CONCLUSION

In summary, we demonstrate a novel and important molecular mechanism whereby TNF/TNFR promotes aging-related bone loss and decreases the anabolic effects of intermittent PTH on aged bones. We demonstrate that Traf2 plays a critical role in this regulation. Traf2 directly binds to, ubiquitinates, and inactivates Gas protein, thus restraining the PTH/cAMP/CREB signaling pathway from promoting bone formation. Therefore, inhibiting the TNF/TNFR signaling pathway may prevent aging-related bone loss and help improve the anabolic effects of intermittent PTH on bone in senile osteoporosis patients.

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AUTHOR CONTRIBUTIONS

G.X., C.T., and Q.Y. conceived and designed the study. C.T., Q.Y., G.X. wrote the manuscript. S.L., Y.S., Q.Y., W.G., Q.Y., X.F., D.G., P.Z., X.Z., C.L., X.B., F.W., and D.C. performed the experiments and analyzed the data. C.T. and Q.Y. take responsibility for the integrity of the data analysis. All authors contributed to and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

All research protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Southern University of Science and Technology. All relevant guidelines for the work with animals were adhered in this study. The human studies were approved by the First Affiliated Hospital of Sun Yat-sen University (No. 2013176).

DATA AND CODE AVAILABILITY

All data are available in the main text or the supplementary materials.

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-med.2026.100237>.