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Small molecule inhibition of non-canonical (TAK1-mediated) BMP signaling results in reduced chondrogenic ossification and heterotopic ossification in a rat model of blast-associated combat-related lower limb trauma

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bone.2020.115517>.

Abstract

Heterotopic ossification (HO) is defined as ectopic bone formation around joints and in soft tissues following trauma, particularly blast-related extremity injuries, thermal injuries, central nerve injuries, or orthopaedic surgeries, leading to increased pain and diminished quality of life. Current treatment options include pharmacotherapy with non-steroidal anti-inflammatory drugs, radiotherapy, and surgical excision, but these treatments have limited efficacy and have associated complication profiles. In contrast, small molecule inhibitors have been shown to have higher specificity and less systemic cytotoxicity. Previous studies have shown that bone morphogenetic protein (BMP) signaling and downstream non-canonical (SMAD-independent) BMP signaling mediated induction of TGF- β activated kinase-1 (TAK1) contributes to HO. In the current study, small molecule inhibition of TAK1, NG-25, was evaluated for its efficacy in limiting ectopic bone formation following a rat blast-associated lower limb trauma and a murine burn tenotomy injury model. A significant decrease in total HO volume in the rat blast injury model was observed by microCT imaging with no systemic complications following NG-25 therapy. Furthermore, tissue-resident mesenchymal progenitor cells (MPCs) harvested from rats treated with NG-25 demonstrated decreased proliferation, limited osteogenic differentiation capacity, and reduced gene expression of *Tac1*, *Col10a1*, *Ibsp*, *Smad3*, and *Sox2* ($P < 0.05$). Single cell RNA-sequencing of murine cells harvested from the injury site in a burn tenotomy injury model showed increased expression of these genes in MPCs during stages of chondrogenic differentiation. Additional *in vitro* cell cultures of murine tissue-resident MPCs and osteochondrogenic progenitors (OCPs) treated with NG-25 demonstrated reduced chondrogenic differentiation by 10.2-fold ($P < 0.001$) and 133.3-fold ($P < 0.001$), respectively, as well as associated reduction in chondrogenic gene expression. Induction of HO in *Tak1* knockout mice demonstrated a 7.1-fold ($P < 0.001$) and 2.7-fold reduction ($P < 0.001$) in chondrogenic differentiation of murine MPCs and OCPs, respectively, with reduced chondrogenic gene expression. Together, our *in vivo* models and *in vitro* cell culture studies demonstrate the importance of TAK1 signaling in chondrogenic differentiation and HO formation and suggest that small molecule inhibition of TAK1 is a promising therapy to limit the formation and progression of HO.

Keywords

TAK1; TGF- β activated kinase 1; Non-canonical TGF- β signaling; Heterotopic ossification; Blast injury model; Chondrogenic differentiation

1. Introduction

Recent conflicts in Iraq and Afghanistan have increased the number of individuals affected by blast injuries [1]. Despite the rise in the severity of injuries sustained following blast-related trauma, survival has improved due to better pre-hospital care, tourniquet use, and access to healthcare personnel [2]. However, secondary effects of increased survival include trauma-related injuries, such as heterotopic ossification (HO), results in life-long debilitating consequences [2]. Traumatic HO (tHO) is characterized by painful ectopic bone formation around complex fractures and amputation sites following severe blast and burn injuries [3]. Consequences of tHO formation include pain, limited range of motion around trauma or amputation sites, and reduced capacity to perform daily activities. Non-steroid anti-

inflammatory drugs (NSAIDs) and radiation therapy have been used to target the inflammatory response associated with tHO with limited efficacy [4]. Furthermore, these treatments have off-target effects, including increased bleeding and renal impairment with NSAIDs and risk of sterility and secondary malignancies in the case of radiation [5]. Surgical excision has also been utilized in severe cases but has a high risk of recurrence [6,7]. The identification of more specific therapeutic treatments that limit the development of tHO, a disease that will have increasing prevalence in the next few decades, is therefore necessary.

Transforming growth factor beta (TGF- β) and bone morphogenic protein (BMP) canonical and non-canonical signaling pathways are critical in the formation of HO as levels these molecules have been shown to increase following traumatic injury and act on tissue-resident mesenchymal progenitor cells (MPCs) causing them to proliferate and differentiate towards chondrogenic and osteogenic lineages [8,9]. Canonical TGF- β signaling activates SMAD2/3, resulting in its nuclear localization and transcription of the *Runx2* gene, which drives osteoblast differentiation in MPCs. Non-canonical TGF- β signaling, however, acts through the mitogen-activated protein kinase (MAPK) pathway specifically phosphorylating p38, which supports osteoblast differentiation and proliferation through phosphorylation of RUNX2. RUNX2 further acts as a transcription factor in osteoblast genesis [10,11]. During endochondral ossification, BMP binds to TGF- β receptor I (TGF- β RI) and activates TGF- β -activated kinase 1 (TAK1) leading to phosphorylation of SMAD1/5/8, which binds to the co-activator SMAD4 and translocates to the nucleus to serve as transcription factor for BMP responsive genes critical in osteogenesis such as *Runx2* and others [12]. Altogether, this highlights the importance of TAK1 signaling in both TGF- β and BMP activated pathways during bone formation. Further, *Tak1* genetic knockouts display similar phenotypes to both BMP receptor 1B (*Bmpr1b*) and the growth differentiation factor 5 (*Gdf5*) knockouts, including severe chondrodysplasia with runting, impaired secondary centers of ossification, and joint abnormalities [12]. *Tak1* deficient chondrocytes display reduced phosphorylation of Smad1/5/8 and p38/JNK/ERK MAP kinases [12]. These findings highlight that TAK1 is important in chondrogenesis and maintenance of cartilage [12–15]. Thus, following traumatic injury, increased levels of TGF- β could signal through TAK1 and result in aberrant osteochondrogenic signals driving ectopic bone formation in tissue-resident MPCs and osteochondrogenic progenitors (OCPs) after traumatic injury.

Previous studies with small molecule inhibitors of TAK1 demonstrate significant promise in specificity to the target molecule with limited off target side effects [16,17]. The TAK1 inhibitor NG-25 has been shown to be effective in preventing downstream signaling of non-canonical BMP signaling in various pathologies [16,17]. Studies have shown that TAK1 inhibition may alter RUNX2-driven chondrogenic differentiation initiated during the pre-hypertrophic phase and maintained throughout the hypertrophic stage, thereby preventing further endochondral osteogenesis [16,17]. In this study, we investigated the efficacy of pharmacological TAK1 inhibition on osteo-chondral differentiation and ectopic bone formation. Our results suggest that TAK1 signaling is critical in endochondral ossification, and NG-25 may be an effective and specific small-molecule pharmacological therapeutic agent to limit HO following traumatic injury.

2. Material and methods

2.1. Animals

Adult male Sprague Dawley rats (*Rattus norvegicus*) between 11 and 12-week-old (350 to 450 g) were obtained from Taconic Biosciences (Germantown, NY). Animals were housed for a minimum of 7 days for acclimatization and quarantine purposes in the vivarium located at the Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD. All animals were housed in clean clear plastic cages and exposed to a 12-hour light/dark cycle, with free access to food (standard rodent chow) and water *ad libitum*, under veterinary care and supervision. Animal weights were assessed prior to surgery and at various intervals during the study. All experiments and animal care procedures for this research were approved by the Naval Medical Research Center (NMRC) and Walter Reed Army Institute of Research (WRAIR) Institutional Animal and Care and Use Committee (IACUC). All activities were conducted in accordance with all applicable regulations and best practices pertaining to the use of animals in research.

Adult wild-type C57BL/6 mice between 6 and 8-week old were purchased from Charles River Laboratory (Boston, MA), and TAK1 knockout (TAK1^{fl/fl}) mice were bred on a C57BL/6 background and gender and age matched to C57BL/6 controls. Animals were housed for a minimum of 7 days for acclimatization and quarantine purposes in the vivarium located at the University of Michigan, Ann Arbor, MI. All animals were housed in clean clear plastic cages and exposed to a 12 h light/dark cycle, with free access to food (standard rodent chow) and water *ad libitum*, under veterinary care and supervision. Weights were assessed prior to surgery and at various intervals during the study. All experiments and animal care procedures for this research were approved by the University of Michigan Institutional Animal and Care and Use Committee (IACUC). All activities were conducted in accordance with all applicable regulations and best practices pertaining to the use of animals in research.

2.2. Trauma-induced blast and extremity injury and TAK1 inhibitor administration

Rats were subjected to whole-body exposure to blast overpressure, femoral fracture, soft tissue quadriceps crush injury, and limb amputation through the zone of injury as previously described [18]. Starting on postoperative day (POD) 1, rats were administered 1 mg/kg of freshly prepared NG-25 through intraperitoneal injections (SML1332; Sigma-Aldrich, St. Louis, MO) dissolved in phosphate buffered saline (PBS) daily for 14 days. Animal health status and body weights were recorded daily.

2.3. Quantitative micro-computed tomography analysis

Total new bone formed at the site of amputation was quantified using a high-resolution micro-computed tomography (microCT) system (SkyScan 1176; Bruker microCT, Kontich, Belgium) and a standard phantom was used for normalization. Imaging was conducted on postoperative week eight. Scans were conducted with the same settings as previous studies; 89-kV polychromatic x-ray beam, 256 μ A current and an exposure time of 81 milliseconds per 180° rotation [19,20]. Images were processed using NRecon Reconstruction software (Bruker) to align scan images and generate reconstructed 3D and cross-sectional images.

Total new bone (differential new bone from native bone) and soft tissue ectopic bone (not associated with residual femur) formation were calculated using Bruker microCT volumetric software version 1.14.10.0 + as previously described [18].

2.4. Blood analysis for complete blood count (CBC) and comprehensive metabolic profile (CMP)

Tail-vein blood collections were performed on POD3, POD7, and POD10. CBC analysis was performed using a Sysmex XT-2000i Automated Hematology Analyzer (Sysmex America Inc., Mundelein, IL). CMP was performed using a Vitros 350 Chemistry System (Ortho-Clinical Diagnostics, Raritan, NJ). CMP analysis encompassed a renal panel, electrolyte panel (sodium, potassium, carbon dioxide, chloride, urea nitrogen, creatinine), a liver panel (AST, ALT, LDH, ALKP, bilirubin), metabolic panel (albumin, total protein, glucose, cholesterol, triglycerides) and creatine kinase (CK) levels. Samples were run on the same day and the remaining serum was stored at -80°C prior to use.

2.5. Cell proliferation, analysis of alkaline phosphatase (ALP) activity, and colony forming unit assays of tissue-resident MPCs

Biopsies of skeletal muscle were aseptically harvested from the zone of injury of vehicle- and NG-25-treated rats on POD3, POD7, and POD10 as previously described [18]. Briefly, the samples (50–100 mg) were devoid of fascia and fat, were minced, and incubated in a solution of 300 U/mL of collagenase type II (Worthington, Lakewood, NJ) for 2 h. A single-cell suspension was generated by consecutive straining through 70- μm followed by 40- μm nylon mesh cell strainers (BD BioSciences, San Jose, CA). Red blood cells were lysed by incubating filtered cells in ACK lysis buffer (Lonza, Walkersville, MD) for 5 min followed by rinsing cells with ice-cold PBS. Cells were evaluated for their osteogenic differentiation potential. Cells were serially diluted and seeded in triplicates at a concentration of 1000 cells per well of a 6-well plate in either regular mesenchymal stromal cell growth medium (Dulbecco's modified Eagle's medium-F12, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg of streptomycin; Lonza) or osteogenic differentiation medium (ODM; Lonza) and evaluated after 6 days. To evaluate cell proliferation, a BrdU cell proliferation assay was performed according to manufacturer's instructions (Cell Signaling Technology). For quantification of cellular ALP activity (ALP assay; Sigma-Aldrich), cells were lysed in 150 μL of 1% Triton X-100 for 1 to 2 min, followed by the addition of an equal volume of *p*-nitrophenyl phosphate (pNpp) substrate for 1 to 2 min, and absorbance was read immediately at 405 nm. For colony-forming cell assay, plates were rinsed with PBS, followed by fixation with 100% methanol for 3 to 5 min, air dried, stained with crystal violet for 2 to 3 min, and rinsed five times with tap water. Colony forming progenitor cells (CFP; > 50 cells) were manually counted at $15\times$ magnification using an inverted phase microscope.

2.6. RNA isolation and gene expression analysis of injured rat soft tissue

Biopsies of skeletal muscle were aseptically harvested from the zone of injury surrounding the amputation site in vehicle control ($N = 4$) and NG-25-treated rats on POD10 ($N = 4$) to evaluate changes in gene expression. RNA was stored in RNALater (Ambion Inc., Austin, TX) at 4°C for four days. Approximately 50 mg of muscle tissue was homogenized in 1 mL

of QIAzol lysis reagent (Qiagen, Germantown, MD). RNA was extracted using RNeasy Lipid Tissue Mini kits (Qiagen, Germantown, MD) according to manufacturer's instructions. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to convert 1 µg of RNA to cDNA. Selected mRNA transcripts for 83 genes consisting of chondrogenic, osteogenic, angiogenic, and cell matrix targets were examined by quantitative real-time PCR (qPCR) using a custom low-density microarray (Bio-Rad Laboratories). Cycle Threshold (CT) values per gene transcript were normalized to the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) within each sample and relative gene expression was calculated using the 2^{-Ct} method. A gene was reported as differentially regulated if there was greater than a three-fold difference in expression compared to naïve controls.

2.7. Burn/tenotomy injury model

Wildtype C57BL/6 mice were subjected to the well-established burn/tenotomy (B/T) injury model to induce HO, as previously described [15]. Briefly, mice received a 30% total body surface area (TBSA) partial-thickness burn on the shaved dorsum followed by left hind limb Achilles' tendon transection as previously described [15].

2.8. Single cell RNA-sequencing and bioinformatics

Single cell RNA sequencing data obtained from tissue harvested from the calcaneus to the proximal extent of the Achilles tendon of uninjured mice and mice subjected to the B/T injury was reanalyzed [9]. Bioinformatics analysis was performed similarly to our previously described study [9]. Briefly, the sequencing data were first pre-processed with 10× Genomics software Cell Ranger (10× Genomics Inc., Pleasanton, CA), and aligned to mm10 genome. Downstream analysis was performed with the Seurat framework. Counts were normalized (default Seurat parameters) and scaled (regressing against number of genes expressed per cell and fraction of mitochondrial expression). A pooled set was generated by aligning the filtered single time point sets *via* canonical correlation analysis (13,362 cells). Variable genes for the pooled set were defined as the intersection of the top 5000 variable genes for each time point set (1497 genes). Unsupervised clustering was used to extract provisional clusters (Louvain algorithm, resolution 0.4, using the aligned canonical correlation components). One small provisional cluster (95 cells) remained unidentified and was excluded from the rest of the analysis. Provisional clusters were then aggregated into (consolidated, final) clusters by similarity of expression profile. Cluster was labeled according to highly expressed distinguishing markers (Supplemental Figs. 1, 2). In our analysis, we considered cells from injured animals of primary importance. POD3 demonstrated low expression of all genes analyzed, with a disproportionate fraction of cells identified as inflammatory cells and a relatively low fraction of MPCs (86% of the cells were macrophages or dendritic cells; 5% were MPCs; Supplemental Fig. 3). POD7 and POD21 were evaluated to determine late response and cells responsible for osteochondrogenic differentiation that leads to robust HO formation.

2.9. Isolation and treatment of tissue-resident MPCs and bone marrow-derived OCPs from wild-type C57BL/6 and TAK1^{fl/fl} knockout mice

Murine tissue-resident MPCs were harvested from the calcaneus to the proximal extent of the Achilles tendon adjacent to the injury site and bone marrow-derived OCPs were harvested from the diaphysis of femur, tibia, and fibula of adult 6–8-week-old wildtype C57BL/6 or *Tak1^{fl/fl}* as previously described [15]. Briefly, cells were separated *via* 70 µm cell strainer and digestive enzymes were quenched in standard growth medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum [FBS] and 1% penicillin/streptomycin). Cells were spun down at 1000 rpm for 5 min. The supernatant was discarded, and the cell pellet was resuspended in standard growth media and subsequently plated. Cells used were passage 2 through 6. For the TAK1 inhibitor treated group, murine tissue-resident MPCs and OCPs were isolated from wild-type C57BL/6 mice and cultured with NG-25 (2 µM). For TAK1 knockout experiments, murine *Tak1^{fl/fl}* cells were treated with either Ad.LacZ (MOI 500) or Ad. Cre (MOI 500) in DMEM free of FBS (serum deprived) for 24 h. Cells were then cultured in standard growth media (serum-replete) for 48 h.

2.10. Chondrogenic differentiation and staining

Murine tissue-resident MPCs and bone marrow-derived OCPs were isolated as described above and was plated at a concentration of 200,000 cells per 15 mL tube in chondrogenic medium (DMEM with 1% FBS, 1% penicillin/streptomycin, 37.5 µg/mL ascorbate-2-phosphate [Sigma-Aldrich], insulin, transferrin, selenium premix [BD Biosciences, Franklin Lakes, NJ], 5 ng/mL TGF-β1). Medium was replaced every 2–3 days. After 12 days, cells were fixed in 0.1% glutaraldehyde in PBS for 20 min at room temperature. Cells were stained with ALP to evaluate early osteogenic differentiation per manufacturer's instructions (Sigma-Aldrich). After 12 days, micromasses were fixed in 4% paraformaldehyde followed by 4% sucrose for 15 min, embedded in optimal cutting temperature compound, and cryo-sectioned at 10 µm onto glass slides. For Alcian blue staining, cells were rinsed in 0.1 N HCl pH 1.0 (Sigma-Aldrich) for 5 min and stained with 1% Alcian blue 8-GX (Sigma-Aldrich) diluted in 0.1 N HCl pH 1.0 for 30 min. Cells were then rinsed in HCl solution and counterstained with nuclear fast red (Biomed, Foster City, CA).

2.11. RNA isolation and gene expression analysis of murine tissue-resident MPCs and OCPs

Murine tissue-resident MPCs and bone marrow-derived OCPs were cultured in chondrogenic differentiation media and harvested after 12 days and placed in TRIzol reagent (Ambion/Life Technologies). RNA was purified using RNeasy Mini Kit (Qiagen) following the manufacturer's procedure with added DNase digestion. The cDNA reaction was performed using the iScript Advanced cDNA Synthesis Kit for qRT-qPCR (Bio-Rad, Hercules, CA) following the manufacturer's instructions. qRT-PCR was performed with a SYBR Green supermix (SsoAdvanced™ Universal SYBR Green Supermix, Bio-Rad, Hercules, CA). Plates contained housekeeping gene beta-actin for the normalization of target gene expression (Supplemental Table 1). The thermal cycling protocol for the reaction commenced with the first stage of 90 s at 50 °C, followed by 10 min at 95 °C. The second

stage consisted of 40 amplification cycles of 15 s denaturation at 95 °C, followed by 30 s at 60 °C for annealing and extension. The final stage consisted of a melt curve analysis was then performed at the end of the protocol between 60 and 95 °C using 0.5 °C increments at 2–5 s/step. Cycle Threshold (CT) values per gene transcript were normalized to the endogenous housekeeping gene beta-actin within each sample and relative gene expression was calculated using the 2^{-Ct} method.

2.12. Statistical analysis

Intraclass correlation coefficient was used to investigate inter-rater agreement between the investigator scores in calculating the volume of ectopic bone from micro-computed tomography images. The Welch's two-sample *t*-test and Fisher test were used to determine the statistical differences in ectopic bone volume (mm³) between vehicle and NG-25-treated animals. Gene expression analysis was performed using Qbase + data analysis software, version 3.2 (Biogazelle, Zwijnaarde, Belgium), with results reported as mean values with standard error of the mean (SEM) based on an unpaired *t*-test. Statistical analysis was performed with the exception of gene expression data using Prism 8.1.2 for Mac (GraphPad Software, La Jolla, CA) and SPSS 25 for Mac (IBM Corp, Armonk, NY) software. All data are presented as mean values with SEM, and *P* values < 0.05 were considered to be statistically significant.

3. Results

3.1. TAK1 inhibitor limits ectopic bone formation

To evaluate ectopic bone formation, a well-established rat blast injury model consisting of exposure to blast overpressure followed by a femur fracture, quadriceps crush injury, and limb amputation through the zone of injury was utilized to evaluate the effect of TAK1 inhibitor NG-25 [18]. NG-25 treated rats demonstrated reduced ectopic bone formation from 48.5 mm³ in vehicle control to 26.9 mm³ in NG-25 treated animals at 8 weeks (*P* < 0.05; Fig. 1A). No statistically significant differences in weekly weights were observed between vehicle-treated and NG-25-treated animals (Fig. 1B). These results demonstrate that TAK1 inhibitor NG-25 effectively inhibited HO formation without systemic effects assessed by weight loss.

3.2. Pharmacological inhibition of TAK1 induces early but not late systemic inflammation with no negative systemic side effects

To determine the impact of NG-25 on systemic inflammation, blood was obtained from rats exposed to blast-related extremity injury. CBC revealed no significant differences in white blood cell count on POD3, POD7, or POD10 (Fig. 2A). NG-25 treatment yielded a slight increase in neutrophil (0.4 K/μL in the vehicle- to 1.9 K/μL in NG-25-treated animals; *P* < 0.05) and eosinophil count (0.06 K/μL in the vehicle- to 0.3 K/μL in NG-25-treated animals; *P* < 0.05) on POD3 (Fig. 2A). However, by POD7 and POD10, there were no significant differences in either neutrophil or eosinophil count. No significant differences in monocyte or lymphocyte count were observed. CMP analysis revealed an increase in glucose levels on POD3 (184.2 mg/dL in vehicle- to 228.4 mg/dL in NG-25-treated animals; *P* < 0.05; Fig. 2B) and a mild decrease in sodium and chloride levels on POD3 following NG-25 treatment.

However, by POD10, there was no statistically significant difference in glucose, sodium, or chloride levels, indicating no long-term side effects. Overall, no correlation was observed on any day to indicate systemic renal, hepatic, cardiovascular, or musculoskeletal injury (Fig. 2B). These results indicate that pharmacological inhibition of TAK1 with NG-25 in a blast injury model leads to no long-term systemic inflammatory response or negative side effects. Importantly, these findings indicate that the effect of NG-25 as an inhibitor of HO is independent of its effect on the inflammatory response.

3.3. TAK1 inhibitor reduced proliferation, osteogenic differentiation, and self-renewal capacity of tissue-resident MPCs with associated alterations in gene expression

To explore the impact of NG-25 on the tissue-resident MPCs, cells isolated from tissue harvested from the zone of injury surrounding the amputation site of NG-25 treated rats were evaluated for proliferation and osteogenic differentiation capacity in both stromal media and osteogenic media. The tissue-resident MPCs isolated from NG-25 treated rats were assessed by BrdU staining as a surrogate for proliferation and demonstrated reduced proliferation in NG-25-treated animals on POD7 and POD10 from 1.7 absorbance units (AU) in vehicle-treated animals to 0.3 AU (5.6-fold reduction; $P < 0.01$) and 0.5 AU (3.4-fold reduction; $P < 0.05$), respectively (Fig. 3A). There was a significant reduction in early osteogenic differentiation assessed by alkaline phosphatase in both stromal media (0.24 AU in vehicle-treated to 0.0 AU in NG-25-treated $P < 0.01$) and osteogenic media (0.19 AU in vehicle-treated to 0.0 AU in NG-25-treated $P < 0.05$; Fig. 3B). Quantification of tissue-resident MPCs isolated from vehicle-treated animals on POD3 demonstrated 7.5 colony forming progenitors (CFPs) in stromal media and 23.5 CFPs in osteogenic media. In contrast, fewer number of tissue-resident MPCs were assayed from NG-25-treated animals (4.0 CFPs (1.9-fold reduction, $P < 0.001$) in stromal media and 2.3 CFPs (10.2fold reduction, $P < 0.001$) in osteogenic media; Fig. 3C). These results establish that NG-25 treatment suppressed MPC activity.

To evaluate changes in the gene expression following NG-25 treatment, rat tissue harvested from the zone of injury surrounding the amputation site was analyzed. A total of 35 osteogenesis or chondrogenesis genes had altered gene expression with the greatest decrease in expression in *Tac1* (2.0×10^{-4} ; $P < 0.01$), *Col10a1* (1.1×10^{-3} ; $P < 0.05$), *Ibsp* (3.6×10^{-3} ; $P < 0.01$), *Smad3* (4.3×10^{-3} ; $P < 0.05$), *Sox2* (4.9×10^{-3} ; $P < 0.05$), *Bglap* (7.1×10^{-3} ; $P < 0.01$), and *IL-6* (8.5×10^{-3} ; $P < 0.001$) following treatment with NG-25 (Table 1). These results suggest that treatment with NG-25 alters TAK1 signaling with associated reduction in chondrogenic genes and TGF- β related gene expression.

3.4. Single cell RNA sequencing demonstrates MPCs are an important source of TAK1 and other response genes

Having determined that NG-25 results in changes in the gene expression profile of rat tissue, we reanalyzed our previously published dynamic single-cell RNA sequencing analysis of traumatic HO using a proven murine B/T model [9,18,21]. This analysis allowed us to determine that indeed, alterations in *Tak1* expression occurs primarily in the MPC sub-populations. To do this, we reanalyzed all cell populations harvested in our B/T model from the calcaneus to the proximal extent of the Achilles tendon to evaluate genes found to be

downregulated following NG-25 treatment in the rat blast injury model. Population identification was performed using previously described marker enrichment [9] and showed nine distinct cell clusters. The resulting nine clusters were identified by characteristic gene expression: MPC (*Pdgfra*, *Prrx1*), macrophages/dendritic cells (*Fcgr1*, *Adgre1*), endothelial cells (*Cdh5*, *Pecam1*), smooth muscle cell (*Mcam*, *Pdgfrb*), lymphocytes (*Cd2*), muscle satellite cells (*Pax7*), nerve (*Sox10*, *Plp1*), skeletal muscle (*Myoz1*, *Myh4*), and granulocyte (*Cxcr2*, *Mmp9*, Supplemental Figs. 1, 2). Global assessment of *Tak1* expression in uninjured and injured MPCs demonstrated an increase in *Tak1* expression on POD7 and POD21 (Fig. 4A). Next, we evaluated the expression of the top downregulated genes identified in the rat blast-injury following NG-25. Most notably, *Smad3* expression was increased in MPCs on POD7 and POD21 (Fig. 4B) compared to uninjured tissue. The remainder of the genes did not show an increase in expression in MPCs or other cell types on POD7 and POD21. We further explored other non-canonical TGF- β genes *Erk1/2*, *p38*, *Jnk*, *Ap-1*, *Mkk3*, and *Traf4* and found increased expression on POD7 and POD21 in MPCs (Fig. 4C).

Together, these results suggests that non-canonical TGF β signaling through TAK1 is important in MPCs to induce HO in multiple *in vivo* models at later stage in the disease progression.

3.5. Pharmacological inhibition of TAK1 on resident-tissue MPCs and OCPs reduces endochondrogenic ossification and inhibits chondrogenic gene expression

Given the identification of MPCs as the likely altered cell type, we evaluated the chondrogenic and osteogenic differentiation capacity of MPCs to form ectopic bone. We further utilized an *in vitro* murine model in order to evaluate the direct effects of TAK1 inhibition of MPCs. Wildtype harvested, murine tissue-resident MPCs were treated with NG-25 under chondrogenic differentiation conditions *in vitro*. Tissue-resident MPCs demonstrated a reduction in Alcian blue staining (26% to 2.5%) following NG-25 treatment ($P < 0.001$; Fig. 5A–B). This corresponded to an undetectable level of the chondrogenic marker *Col2a1* ($P < 0.001$; Fig. 5C) and a reduction in chondrogenic transcription factor *Sox9* (1.05-fold to 0.71-fold; $P < 0.001$; Fig. 5C) and *Aggrecan* expression (0.97-fold to 0.43-fold $P < 0.001$; Fig. 5C). To determine the efficacy of pharmacological inhibition of TAK1 on inhibiting chondrogenic differentiation of OCPs, wild-type OCPs were treated with NG-25 and evaluated with Alcian blue and Safranin O (Fig. 5D). NG-25 treatment of OCPs completely abolished cartilage deposition as demonstrated with no detectable Safranin O staining, while vehicle-treated OCPs demonstrated 98% Safranin O staining ($P < 0.001$, Fig. 5E). This corresponded to a reduction in *Sox6*, *Col2a1*, *Sox9*, and *Aggrecan* from 1.0-fold in vehicle-treated OCPs to 0.40-fold ($P < 0.01$), 0.06-fold ($P < 0.001$), 0.20-fold ($P < 0.001$), and 0.08-fold ($P < 0.001$), in NG-25-treated OCPs, respectively (Fig. 5F). Together, these results suggest that NG-25 effectively reduces osteogenic and chondrogenic signaling and differentiation of MPCs.

3.6. Genetic knockout of Tak1 in MPCs and OCPs demonstrated limited endochondral ossification and chondrogenic gene expression

Given that NG-25 could have off-target effects, we utilized a TAK1 conditional deletion model to determine the role of TAK1 in endochondral ossification as it relates to HO.

Murine *Tak1^{fl/fl}* MPCs isolated after injury were transduced with adenovirus expressing Cre recombinase (Ad-Cre) to delete *Tak1* or Adenovirus-LacZ (Ad-LacZ) to be used as a control. These cells were exposed to chondrogenic media and evaluated for ALP activity. *Tak1^{fl/fl}* Ad-Cre MPCs demonstrated a reduction in ALP staining (3.43% to 0.48%; $P < 0.001$, Fig. 6A–B). These results corresponded to a significant reduction in *Col2a1* (0.85 to 0.0004-fold; $P < 0.001$) and *Sox9* (1.05 to 0.15-fold; $P < 0.001$) and no significant difference in *Aggrecan* expression (Fig. 6C). Similarly, Ad-Cre transduced *TAK1^{fl/fl}* OCPs in chondrogenic medium demonstrated decreased Alcian blue and Safranin O staining (Fig. 6D). Safranin O staining was reduced from 90% in controls to 38% in *Tak1* knockout OCPs ($P < 0.001$, Fig. 6E), suggesting reduced cartilage deposition with the loss of TAK1 in OCPs. These results corresponded to a reduced chondrogenic gene expression, including *Sox6*, *Col2a1*, and *Aggrecan*, (1.0 to 0.67-fold; $P < 0.01$, 0.55-fold; $P < 0.001$, and 0.42-fold; $P < 0.001$; respectively, Fig. 6F). No significant reduction in *Sox9* expression was seen following *Tak1* deletion (Fig. 6F). Together, these results suggest that genetic deletion of *Tak1* in tissue-resident MPCs and OCPs inhibits osteogenic and chondrogenic gene expression and differentiation.

4. Discussion

HO remains a significant clinical challenge following severe trauma and is characterized by the development of extra-skeletal bone formation around the injury site and amputation site. Patients with tHO face debilitating pain, resulting in limited function, and non-healing wounds, which reduce their quality of life. Unfortunately, current treatment options have limited efficacy with systemic toxicity. In contrast, small molecule inhibitors have greater specificity and reduced systemic cytotoxicity to surrounding tissue. These small molecule inhibitors hold great promise for a multitude of diseases, including HO. One such small molecule inhibitor is NG-25, which was previously modified for dual activity against TAK1 and MAP4K2 [22]. This TAK1 inhibitor has been shown to have good pharmacokinetic properties that enable their use in pharmacological studies *in vivo* [22] and was utilized in this study. NG-25 binds to the ATP binding pocket of the target kinase in addition to an adjacent hydrophobic pocket that is created when the action loop containing the conserved DGF motif is in an “out” conformation [22]. In the current study, small molecule inhibition of TAK1 with NG-25 was shown to be efficacious in limiting ectopic bone formation following blast-associated lower limb trauma. Equally important in the efficacy of NG-25 is the limited systemic side effects with no identifiable renal, hepatic, cardiovascular, or musculoskeletal complications when used as short-term prophylaxis to prevent tHO following acute extremity injury. Consistent with previous studies, our analysis of single cell RNA-sequencing data revealed that MPCs are the cells that express high levels of *Tak1* and other molecules involved in downstream pathways during the osteo-chondral differentiation stages. In the current study, we further confirmed that NG-25 simultaneously inhibits chondrogenic differentiation of tissue-resident MPCs in B/T model. Therefore, NG-25 could potentially be considered an effective small molecule inhibitor to inhibit chondrogenic differentiation.

With respect to the mechanism by which tHO forms, studies have previously shown that bone morphogenetic protein (BMP) signaling and downstream non-canonical (SMAD-

independent) BMP activation of TAK1 are critical. Previous work indicates that TAK1 and TAK1 binding protein play a pivotal role as upstream signal transducers by activating the MKK3-p38 MAPK signaling cascade that leads to the induction of type I collagen expression by TGF- β [23]. These signaling pathways converge at the RUNX2 gene to control mesenchymal precursor cell differentiation into chondrogenic and osteogenic lineages [24]. These pathways also appear to differentially mediate TGF- β and BMP-2 function in osteoblasts [25]. Thus, we utilized an *in vitro* model with MPCs and OCPs to investigate the direct effect of TAK1 on chondrogenesis. Indeed, we found TAK1 inhibition in OCPs reduced their chondrogenic gene expression, suggesting that TAK1 inhibitor prevents downstream targeting of all TGF- β pathways. Additional genetic ablation of TAK1 has also been shown to mitigate HO formation in the B/T model, another tHO model [26]. While the current study focuses on tHO, it is possible that TAK1 may also be a critical factor in the formation of HO secondary to genetic mutations, such as fibrodysplasia ossificans progressive (FOP) [27,28]. FOP is caused by increased BMP signaling aberrantly induced by Activin A and downstream signaling has been shown to be mediated through signal transduction factors specific to BMPs (Smad1/5/8) and through the MAPK signaling pathways [29–32]. Activation of non-canonical MAPK signaling pathway through TAK1 may contribute to endochondral ossification in FOP, beyond its role in tHO [33]. Together, these findings suggest that TAK1 plays an integral role in osteochondrogenic cell differentiation to increase HO following trauma and potentially in FOP, and inhibition of TAK1 through NG-25 successfully limits endochondral differentiation.

Our results further support that TAK1 is a critical molecule that regulates the activation of cell cycle and that TAK1 inhibition leads to quiescence in bone marrow mesenchymal stem cells both *in vitro* and *in vivo* [34]. This inhibition ultimately leads to reduced osteogenic and chondrogenic differentiation of mesenchymal progenitor cells. Additional work on bone marrow mesenchymal stem cells highlights that TAK1 upregulates ALP, which is involved in mineralization of the bone matrix, which in turn likely directly relates to bone formation [35]. As shown, MPCs harvested from the Achilles' tendon and adjacent tissue have osteogenic potential, yet far lower than OCPs in culture. Tissue-resident MPCs and OCPs likely have different epigenetic and transcriptional differences that modulate their responsiveness to osteogenic and chondrogenic differentiation media. Additionally, this could also have an effect on the responsiveness of TAK1 inhibition, whether genetically or pharmacologically, on chondrogenic and osteogenic differentiation. Analysis of tissue-resident MPCs harvested from the zone of injury surrounding the amputation site revealed significant reduction in TAK1 expression following NG-25 treatment compared to control, indicating the specificity of the small molecule inhibitor to sites prone to tHO formation. Current treatment options include NSAIDs and radiation therapy, which lack specificity and results in damage to surrounding tissue [36]. With the advent of small molecule inhibitors, there is the potential to target specific factors. There appears to be a coordinated effort between multiple cell types that potentially leads to the final development of tHO, and it is likely that the cross talk between the two cell types culminates in ectopic bone formation. Future studies to investigate the interaction between these various cell types, including tissue-resident MPCs and bone-marrow OCPs, will be necessary to determine the coordinated effort of each cell type and the role each cell type plays in HO formation

secondary to trauma or genetics. The current study demonstrates that TAK1 was successfully targeted to the site of HO development post blast injury, and that systemic delivery of small molecule inhibitor of TAK1, NG-25, was able to reduce HO formation. Ultimately, this study demonstrates the efficacy of small molecule inhibition of TAK1 for possible prevention of HO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

TAK1 inhibitor limits heterotopic ossification. Rats were subjected to a blast injury model with whole-body exposure to blast overpressure by a pneumatically driven shock tube, followed by controlled femoral fracture, a 1-minute soft tissue quadriceps crush injury, and limb amputation through the zone of injury. Animals were then treated with vehicle (PBS) or NG-25 (1 mg/kg) for 14 days. (A) Micro-computed tomography scans were performed with 89-kV polychromatic x-ray beam, 256 μ A current and an exposure time of 81 milliseconds per 180° rotation on vehicle-treated and NG-25-treated rats following blast injury at week 8. Total new bone was determined by defining the difference between new bone and naïve bone. (B) Animals were weighed every other week. Mean $\pm$ SEM. *, $P < 0.05$, comparing vehicle and NG-25 treatment.

Fig. 2.

No systemic side effects following TAK1 inhibitor treatment. Following blast related extremity injury, rats were treated with either vehicle or NG-25 (1 mg/kg) for 3, 7, or 10 days and whole blood was analyzed on POD3, POD7, and POD10, respectively. Blood was collected from the tail vein and immediately subjected to analysis. (A) Complete blood count and (B) comprehensive metabolic profile. Mean $\pm$ SEM. *, $P < 0.05$, ***, $P < 0.001$, comparing vehicle and NG-25 treatment. WBC, white blood cells; AST, aspartate

aminotransferase; ALT, alanine aminotransferase; LDH, lactic acid dehydrogenase; CK, creatine kinase; ALKP, alkaline phosphatase.

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Fig. 3.

TAK1 inhibitor reduces the prevalence of tissue-resident MPCs capable of osteogenic differentiation. Following blast-related extremity injury, rats were treated with either vehicle or NG-25 (1 mg/kg) for 3, 7, or 10 days, and tissue-resident MPCs were quantified on POD3, POD7, and POD10, respectively. Isolated nucleated cells from muscle biopsies at the zone of injury were plated in stromal media or osteogenic media. (A) Following 6 days in stromal media or osteogenic media, cells were assessed for cell proliferation by BrdU. (B) Cells were allowed to undergo osteogenic differentiation for 6 days and assessed for early osteogenic differentiation by alkaline phosphatase activity through conversion of the substrate p-ntrophenyl phosphate (pNpp). (C) MPC CFPs were assessed after 6 days. Cultures containing CFPs (> 50 cells) were stained with crystal violet. CFPs were manually counted at 15× magnification using an inverted phase microscope. Quantification of colony forming units following exposure to stromal media or osteogenic media. Mean ± SEM. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, comparing vehicle and NG-25 treatment in stromal media. #, $P < 0.05$, ###, $P < 0.001$, comparing vehicle and NG-25 treatment in osteogenic media. ΨΨΨ, $P < 0.001$, comparing stromal media and osteogenic media in vehicle control.

Fig. 4. MPCs express high levels of *Tak1* and associated genes. Analysis of 10× single cell RNA sequencing data from uninjured and injured tissue from the calcaneus to the proximal extent of the Achilles tendon of mice subjected to burn tenotomy injury on POD7 and POD21. (A) tSNE plot of cell clusters. (B) tSNE plot of MPCs displaying *Tak1* expression with high (red) and low (blue) levels. (C) Violin plot for *Tak1* expression of MPCs. (D) tSNE plot of MPCs displaying *Smad3* expression with high (red) and low (blue) levels. (E) Violin plot for *Smad3* expression of MPCs. (F) tSNE plot of MPCs displaying gene expression of non-

canonical TGF-beta genes. (G) Violin plots for non-canonical TGF- β genes. All expression scatterplots are internally scaled for each treatment group across each individual gene. DC, dendritic cells; MPCs, mesenchymal progenitor cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Pharmacological inhibition of TAK1 demonstrates robust inhibition of endochondral ossification in MPCs and OCPs with associated gene expression. (A–C) Tissue-resident MPCs were harvested from wild-type C57BL/6 mice and exposed to chondrogenic differentiation media treated with vehicle control or NG-25 (2 µM) for a total of 12 days. (A) Representative images of cells following Alcian blue staining. (B) Quantification of Alcian blue staining relative to area stained. (C) Quantification of chondrogenic gene expression of vehicle and NG-25 treated murine MPCs. (D–F) OCPs were isolated from wild-type C57BL/6 mice after burn tenotomy injury model and cultured in chondrogenic

differentiation media treated with vehicle control or NG-25 (2 μ M) for 12 days. (D) Cartilage deposition was evaluated and representative images of cells stained with Alcian blue and Safranin O. (E) Quantification of Safranin O staining relative to area stained. (F) Quantification of chondrogenic gene expression of vehicle and NG-25 treated murine OCPs. Mean $\pm$ SEM. **, $P < 0.01$, ***, $P < 0.001$, comparing vehicle and NG-25 treatment.

Fig. 6.

Genetic inhibition of TAK1 in MPCs and OCPs demonstrates limited endochondral ossification through reduced cartilage deposition, calcium deposition, and chondrogenic gene expression. (A–C) Murine TAK1^{fl/fl} MPCs isolated 21 days after burn tenotomy injury model was evaluated to determine the role of TAK1 in chondrogenic differentiation. Isolated cells were cultured in chondrogenic differentiation media treated with Ad.LacZ or Ad.Cre for control or TAK1 knockout for 12 days, respectively. (A) Representative images of cells following ALP staining. (B) Quantification of ALP staining relative to area stained. (C)

Quantification of chondrogenic gene expression of Ad.LacZ and Ad.Cre treated murine MPCs. (D-F) TAK1^{fl/fl} OCPs were harvested from long bone after exposure to burn tenotomy injury model for 21 days. Cells were then treated with Ad.LacZ or Ad.Cre for control or TAK1 knockout for 12 days, respectively. (D) Cartilage deposition was evaluated by Alcian blue and Safranin O staining. (E) Quantification of Safranin O staining. (F) Quantification of chondrogenic gene expression of Ad.LacZ and Ad.Cre treated murine MPCs. Mean $\pm$ SEM. **, $P < 0.01$, ***, $P < 0.001$, comparing vehicle and TAK1 knockout.

Table 1

Relative gene expression profile of animals following treated with NG-25 compared to vehicle treated animals after blast injury on POD10. Following blast related extremity injury, rats were treated with either vehicle or NG-25 (1 mg/kg) for 10 days. Muscle biopsies were collected on POD10. Data is normalized to vehicle-treated rats following blast related extremity injury on POD10.

Genes	Fold Change	95% Confidence Interval		P value	Statistically significant
		Lower	Upper		
Tac1	2.00E-04	3.049E-06	0.0179	0.007	**
Col10a1	1.10E-03	1.121E-05	0.1172	0.017	*
Ibsp	3.60E-03	2.780E-04	0.0459	0.002	**
Smad3	4.30E-03	5.570E-05	0.3365	0.027	*
Sox2	4.90E-03	1.512E-04	0.1606	0.012	*
Bglap	7.10E-03	4.860E-04	0.1038	0.006	**
Il6	8.50E-03	1.968E-03	0.0367	3.92E-04	***
Lep	0.017	1.616E-03	0.1686	0.005	**
Tert	0.026	3.298E-03	0.2096	0.005	**
Col2a1	0.039	4.185E-03	0.3604	0.012	*
Il10	0.046	0.0121	0.1746	0.002	**
Cebpa	0.052	4.958E-03	0.5349	0.021	*
Bmp2	0.060	6.858E-03	0.5190	0.021	*
Ptch1	9.271	1.028	83.538	0.048	*
Itga2	9.625	1.167	79.353	0.040	*
Adipor1	11.143	1.051	118.057	0.047	*
Omd	15.614	1.579	154.396	0.029	*
Angpt2	22.373	5.464	91.602	0.002	**
Cd44	24.417	2.587	230.474	0.014	*
Ptk2	25.410	6.901	93.560	0.001	***
Tgfb1	33.100	5.708	191.951	0.004	**
Il1b	40.801	3.698	450.118	0.011	*
Pdgfa	43.857	1.371	1402.990	0.039	*
Lrp5	53.850	8.460	342.786	0.002	**
Itgam	98.151	4.374	2202.033	0.019	*
Flt1	119.646	8.076	1772.458	0.006	**
Smurf2	124.290	14.969	1031.995	0.002	**
Bmp4	143.292	3.665	5601.882	0.018	*
Sparc	144.235	5.001	4159.885	0.017	*
Jag1	168.375	51.948	545.746	5.14E-05	***
Fabp4	170.433	16.946	1714.125	0.002	**
Hdac1	214.473	18.967	2425.165	0.002	**
Vegfa	252.630	24.457	2609.525	0.001	***
Gusb	283.349	12.527	6408.627	0.011	*
Col1a1	311.417	21.962	4415.729	0.003	**

^{*}, $P < 0.05$

^{**}, $P < 0.01$

^{***}, $P < 0.001$ between vehicle-treated rats and NG-25 treated MPCs.

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