SUPPLEMENTARY FILE

Materials and Methods:

Antibodies and reagents. Human monocytic leukemia THP-1 cells were from ATCC (Manassas, VA). Phorbol 12-myristate 13-acetate (PMA) (Catalog no: tlrl-pma), monosodium urate (Catalog no. tlrl-msu) crystals were obtained from Invivogen (San Diego, CA). For cell culture RPMI-1640 were sourced from Corning. Duoset ELISA kits for human IL-1β, TNF-α, MCP-1 and IL-8 were purchased from R&D Systems (Minneapolis, MN). Inhibitors of the signaling protein IRAK-1/4 (*N*-[2-morpholinylethyl]-2-[3-nitrobenzoylamido]-benzimidazole) and TAK1 ([5Z]-7-oxozeaenol) were purchased from EMD Millipore (Burlington, MA) and TRAF6 control and inhibitor peptides were from Imgenex/Novus (San Diego, CA). Rabbit monoclonal or polyclonal antibodies specific for MyD88 (Catalog no. 4283), IRAK-4 (Catalog no. 4363), pIRAK-4 Thr^{345}/Ser^{346} (Catalog no. 11927), pTAK1 $Thr^{184/187}$ (Catalog no. 4531), anti-IL-1β (Catalog no. 12703 & 83186), anti-IL-1β mouse specific (Catalog no. 12507), anti-K^{63}-linked polyubiquitin (Catalog no. 5621), anti-K^{48}-linked polyubiquitin (Catalog no. 8081), goat anti-rabbit and goat anti-mouse horseradish peroxidase–linked secondary antibodies were purchased from Cell Signaling Technologies (Beverly, MA). pIRAK-1 Thr^{209} (Catalog no. ab61799), pTAK1 Ser^{439} (Catalog no. ab109404), total TAK1(Catalog no. ab109526) pIRAK-1 Thr^{387} (Catalog no. ab139739), anti-TRAF6 (Catalog no. ab33915) were purchased from Abcam (Cambridge, MA). Mouse monoclonal IRAK-1 (Catalog no. sc-55530), mouse monoclonal TRAF6 (Catalog no. sc-8409), mouse monoclonal TAK1 (Catalog no. sc-166562) and mouse monoclonal β-actin (Catalog no. sc-47778) antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–FK-2 antibody (Catalog no. BML-PW8810) was purchased from Enzo Life Sciences.

Western immunoblotting. To study the effects of MSU, whole cell extracts were prepared using RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X100, 1mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor and phospho-Stop tablets (Roche, Indianapolis, IN). Protein

was measured using a Bio-Rad DC method (Bio-Rad, CA). Equal amounts of protein (35 µg) were loaded and separated by SDS-polyacrylamide gel electrophoresis in 4-15% TGX gradient gel (Bio-Rad) and transferred onto PVDF (EMD Millipore). For phosphorylated TAK1 proteins, 8.5% gel were used. Blots were probed using rabbit mono- or polyclonal antibodies specific for MyD88, IRAK-4, pIRAK-4 (Thr^{345}/Ser^{346}), IRAK-1, pTAK1 (Thr$^{184/187}$), pTAK1 (Ser^{439}), total TAK1, TRAF6, K^{63}, K^{48}, FK-2, β-actin, and other signaling proteins. The protein bands were visualized by the Bio Rad XRS system or developed using films. Blots were stripped and re-probed with β-actin or other normalization protein to ensure equal loading. Western blots from mouse were prepared using de-skinned right paw treated with PBS, MSU, or MSU plus inhibitor. Whole cell extract (30 µg) were used to analyze the expression of pTAK1 Thr$^{184/187}$, pTAK1 Ser^{439}, TAK1, TRAF6, or β-actin. Densitometric analysis of the relative expression of each protein was done as described in our earlier studies [12].

Differentiation of Primary human PBMCs to macrophage and treatment: Human normal peripheral blood mononuclear cells (PBMCs) were procured from a commercial source (Zen-Bio Inc., Research Triangle, NC). Briefly, human PBMC were seeded in 6-well format of 35 mm dishes at 3 x 10^6 cells per well in RPMI 1640 with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured for 7 d in M-CSF (50 ng/ml) to differentiate them into monocyte-derived macrophages as described previously by Lacey et al [20]. On day 8, PBMC-derived human macrophages were starved overnight, followed by the treatment with MSU as described in the results.

Expression of TAK1-TAB1 fusion protein.TAK1/TAB1 fusion protein clone (DU Number 3339) acquired from Medical Research Council, UK. This clone contains amino acid sequences 1-303 from TAK1 followed by fusion of amino acid sequences 371-504 of TAB1 protein. cDNA from clone 3339 were prepared using forward primer **5'CGGGATCCATGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGATGTC TACAGCCTCTGCCGCCTCC3'** and reverse primer **'5GGGGTACCCTACGGTGCTGTCACCACGCTCTGCTCG3'**. PCR amplified fragments were

digested with BamH1 and Kpn1 followed by cloning in a BamH1/Kpn1 cut pFastBac1 (Invitrogen) plasmid. Since V5 tag was part of forward primer V5 TAK1-TAB1 pFastBac plasmids; we further verified the plasmid sequence before transposing it on DH10Bac bacmid using BluoGal and IPTG selection as per manufacturer's instructions (Invitrogen). The bacmid was purified using miniprep and transfected into *sf9* cells using Cellfectin reagent (Invitrogen). Supernatant from lysed cells 5 days post transfection was collected and used for a fresh round of transduction in normal *sf9* cells and an additional two times to obtain high titer baculovirus. Expression of TAK1/TAB1 protein was confirmed using p-TAK1 ($Thr^{184/187}$) and V5 tag antibodies.

***In Vitro* Kinase assay of TAK1/TAB1 fusion protein.** *Sf9* cells were lysed in PBS+ 1% Triton X100 buffer containing protease and phosphatase inhibitors. V5 beads (50 µl) were added to 4 mg whole cell extract of *sf9* cells overnight at 4^0 C. Next day V5 beads were washed 3 times with 50mM Tris pH7.6 + 150mM NaCl + 0.5% Triton X100 buffer (TNT buffer). V5 beads saturated with TAK1/TAB1 fusion protein were diluted 10-fold and dephosphorylated using 100 U of calf intestine alkaline phosphatase (Cat no.: M0290S, New England Biolabs). Dephosphorylated TAK1/TAB1 fusion protein (10 µl each tube) was used in a kinase reaction by incubating a different amount of MSU crystals (1-50 µg per reaction) with 1mM ATP. Final volume of kinase reaction was brought to 50 µl using 1X kinase reaction buffer A (Cat no: K03-09; SignalChem, BC, Canada). The phosphorylation of TAK1/TAB1 fusion protein by MSU was determined at room temperature for 20 min. *In vitro* kinase assay was terminated by adding 10 µl of 6X SDS-sample buffer. V5-TAK1/TAB1-bound beads were then eluted by heating the reaction mixture at 75^0 C for 20 min. Beads were separated by high-speed centrifugation and collected supernatants were subjected to SDS-PAGE gel for probing p-TAK1 $Thr^{184/187}$. Coomassie staining confirmed the elution of total recombinant TAK1/TAB1 fusion protein.

Molecular modeling studies and ligand preparation. All computational studies have been performed using different module of Schrodinger suite 15.3 as described earlier [12, 18, 19]. The ligand as a uric acid was prepared using LigPrep. For docking study, the default protocol employed in Glide 4.5 has been used for the docking of uric acid in the binding site of TAK1. Molecular dynamics (MD) simulation were carried

out using Desmond 4.2.

The X-ray crystal structure of TAK1 protein (PDB ID: 2EVA) was obtained from protein data bank. The protein preparation wizard was used to prepare protein in order to add hydrogen, assign correct bond order and generate protonation state of titratable residues at physiological pH 7.4. Missing side chains and loops were built using prime and termini were capped with neutral groups (N-terminal acetyl and C-terminal amide). The ligand as a uric acid was prepared using LigPrep. The default protocol employed in Glide 4.5 was used for the docking of uric acid in the binding site of TAK1. A 20Å cubical grid was generated by defining the co-crystallized ligand as grid center. The default standard precision (SP) docking settings were initially used. The 10 lowest energy ligand-receptor complexes obtained from all the different SP results were used as initial structures for a more refined extra precision (XP) search. Again, ten different binding poses of the ligands were obtained. The lowest energy conformations of docked ligand were kept as is and 5 more uric acids were arbitrarily kept around the binding site near phosphorylation loop.

MD simulation were carried out by using Desmond 4.2. The final complex structure of TAK1 with docked uric acid was prepared using system builder panel of Desmond. The orthorhombic TIP3P water box with 10 Å buffer region between the protein-ligand complex structure and the simulation box boundary on all sides. The Cl^- ions were added to neutralize the total system charge. The prepared system was first equilibrated using the default relax protocol implemented in Desmond. In which, a series of restraint minimization and short molecular dynamics simulation were performed in order to slowly relax the system without any substantial deviation from the initial protein coordinates. The 400ns production run were then submitted by applying isobaric-isothermic ensemble (NPT) ensemble using Nose-Hoover chain thermostat and Martyna-Tobias-Klein with isotropic coupling at constant temperature 300K and pressure of 1atm. A cutoff distance of 9 Å was used for the Columbic and short-range interactions and the long-range electrostatic interactions were computed with the Smooth particle-mesh Ewald summation method with Ewald tolerance 1e-09. The energy and trajectory were recorded for every 10ps and 20ps,

respectively. The trajectory was analyzed using simulation interaction diagram panel of Desmond.

Animal experiment. All animal experiments were approved by the WSU IACUC committee (protocol approval number: 04775). We chose C57BL/6 male mice since they are susceptible to the development of paw inflammation when injected with MSU crystal (0.5 mg, Invivogen) suspended in 20 µl endotoxin free PBS using 27 gauge microliter syringes (Cat: 705SN, Hamilton, NY) as described recently [21]. PBS, as a vehicle control, was injected into the metatarsal region of ankles. Mice were assessed for inflammation up to 4 days. Paw diameter was scored using Vernier caliper for circumference calculation. Paw circumference was calculated using formula and statistical analysis was performed [12, 17]. To obtained Δ paw circumference, we subtracted the measurement from different time points from day 0 reading to calculate the net change in paw diameter. Values obtained were plotted using GraphPad Prism. Based on the time when paw inflammation peaks with MSU injection, we studied the potential therapeutic effect of 5Z-7-oxozeaenol and We divided 22 mice in 4 groups as follows: Group 1: Naïve (PBS injected; 4 mice), Group 2: MSU (0.5 mg/mice/right paw; 6 mice), Group 3: 5Z-7-oxozeaenol (7-ZO, 5 mg/kg; a single p.o. dose 1 h prior to MSU injection; 6 mice), and Group 4: 7-ZO (15 mg/kg; a single p.o. 1 h prior to MSU injection; 6 mice). The clinical measurements were taken at 0, 3, 6, 12, 24, and 48 h post MSU injections and mean ± SEM values were plotted for paw circumference. Mice were sacrificed, and right and left hind paws were collected. Right paws were subjected for whole cell extract preparation as described previously for Western blot analysis [12] or for H&E staining to examine infiltrates [17, 22].

Fig. S1

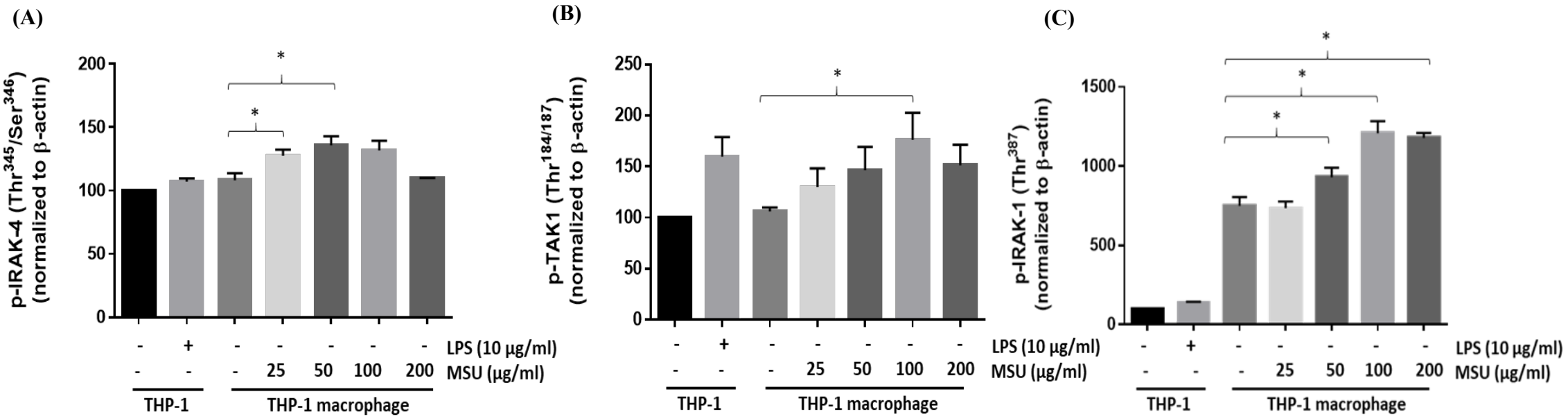


Fig. S1: (**A-C**) Densitometric analysis of the Western blots results obtained for pIRAK4 (Thr^{345}/Ser^{346}), pTAK1 ($Thr^{184/187}$), and pIRAK1 (Thr^{387}) in Fig. 1A from THP-1 macrophages treated with MSU (25-200 µg/ml) or LPS (10 µg/ml) for 30 min. *$p<0.05$.

Fig. S2

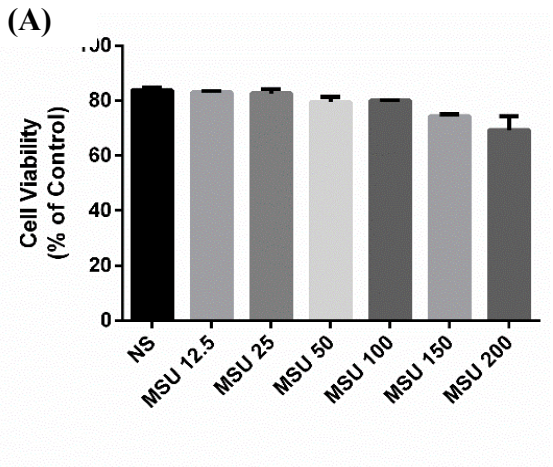


Fig. S2A: THP-1 macrophages were treated with MSU (12.5-200 µg/ml) for 24 h in 96-well plates. The effect of MSU on THP-1 macrophage viability was determined using a MTT viability assay on as described previously [12]. Our MTT results showed that MSU has no significant effect on the viability of THP-1 macrophages. The results shown are obtained from the experiment performed in triplicate and presented as mean ± SEM.

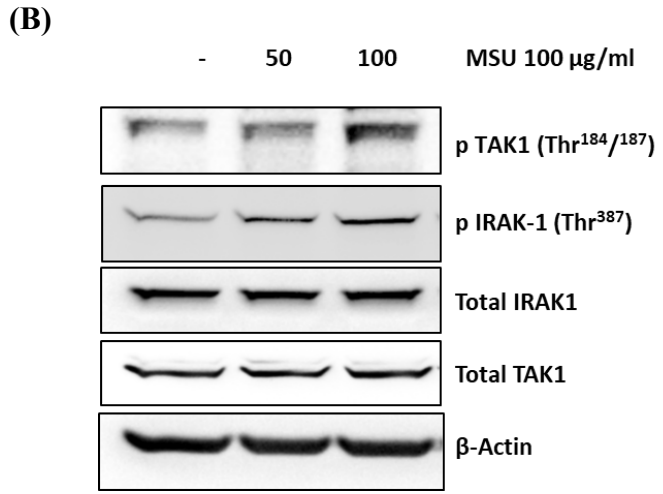


Fig. S2B: Undifferentiated THP1 monocytes were treated for 30 min with MSU (50 and 100 µg/ml) in a 6-well 35 mm plates. Whole cell extract were evaluated for the phosphorylation of IRAK1 and TAK1. The results shown are representation of the experiment performed in triplicate.

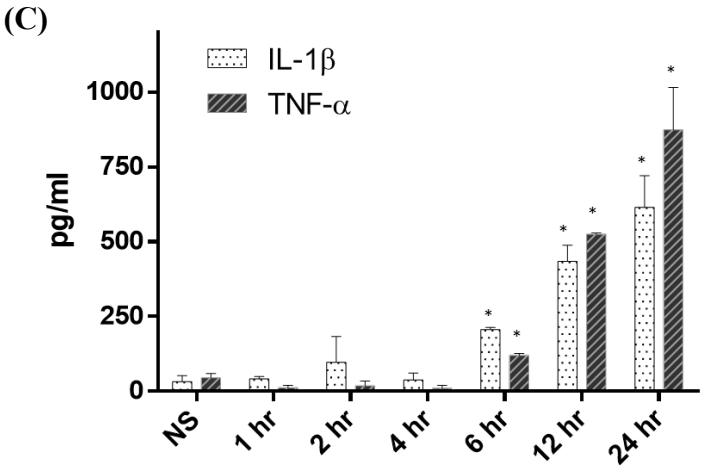


Fig. S2C: THP-1 macrophages were treated with MSU (100 µg/ml) for 1, 2, 4, 6, 12, and 24 h in 12-well plates. Conditioned media was collected and utilized for the quantification of IL-1β and TNF-α using ELISA methods. Our results showed that MSU induces IL-1β and TNF-α production only after 6 h of stimulation, which peaks around 24 h. The results shown are obtained from three independent experiments performed in duplicate and presented as mean ± SEM.

Fig. S3

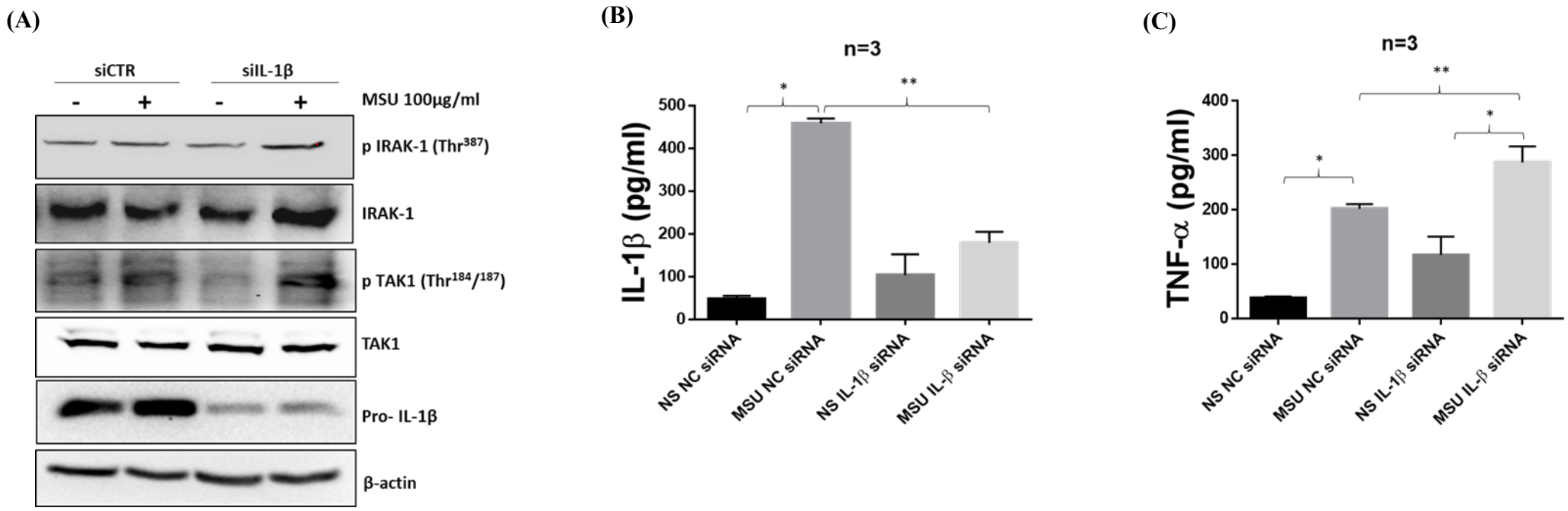


Fig. S3: (**A-C**) THP-1 macrophages were transfected with 120 pmoles of either scrambled (siCTR) or IL-1β siRNA (siIL-1β) for 48 h in 6-well plates. After 48 h of transfection, cells were serum starved overnight prior to MSU simulation for 24 h. Cell were lysed with cell lysis buffer and lysates were analyzed for pIRAK1 (Thr^{387}), total IRAK1, pTAK1 ($Thr^{184/187}$), total TAK1, pro-IL-1β, and β-actin. Conditioned media was collected and used to quantitate the production of IL-1β and TNF-α. The results shown are obtained from three independent experiments performed in duplicate and presented as mean ± SEM. *$p<0.05$, **$p<0.01$.

Fig. S4

(A)

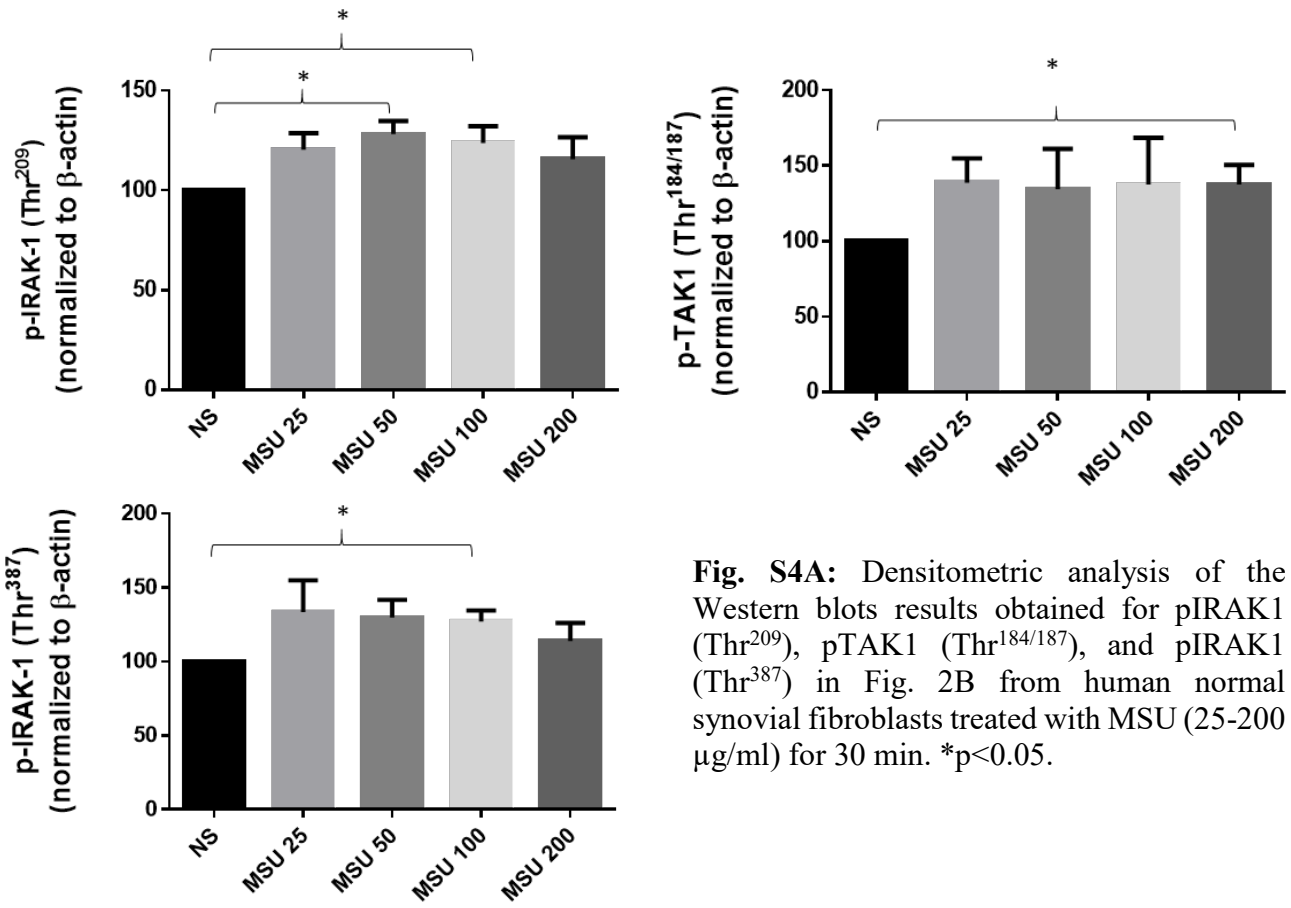


Fig. S4A: Densitometric analysis of the Western blots results obtained for pIRAK1 (Thr^{209}), pTAK1 ($Thr^{184/187}$), and pIRAK1 (Thr^{387}) in Fig. 2B from human normal synovial fibroblasts treated with MSU (25-200 µg/ml) for 30 min. *p<0.05.

(B)

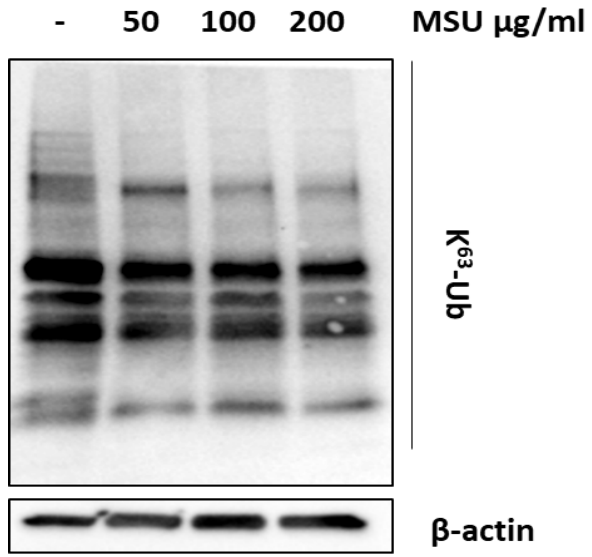
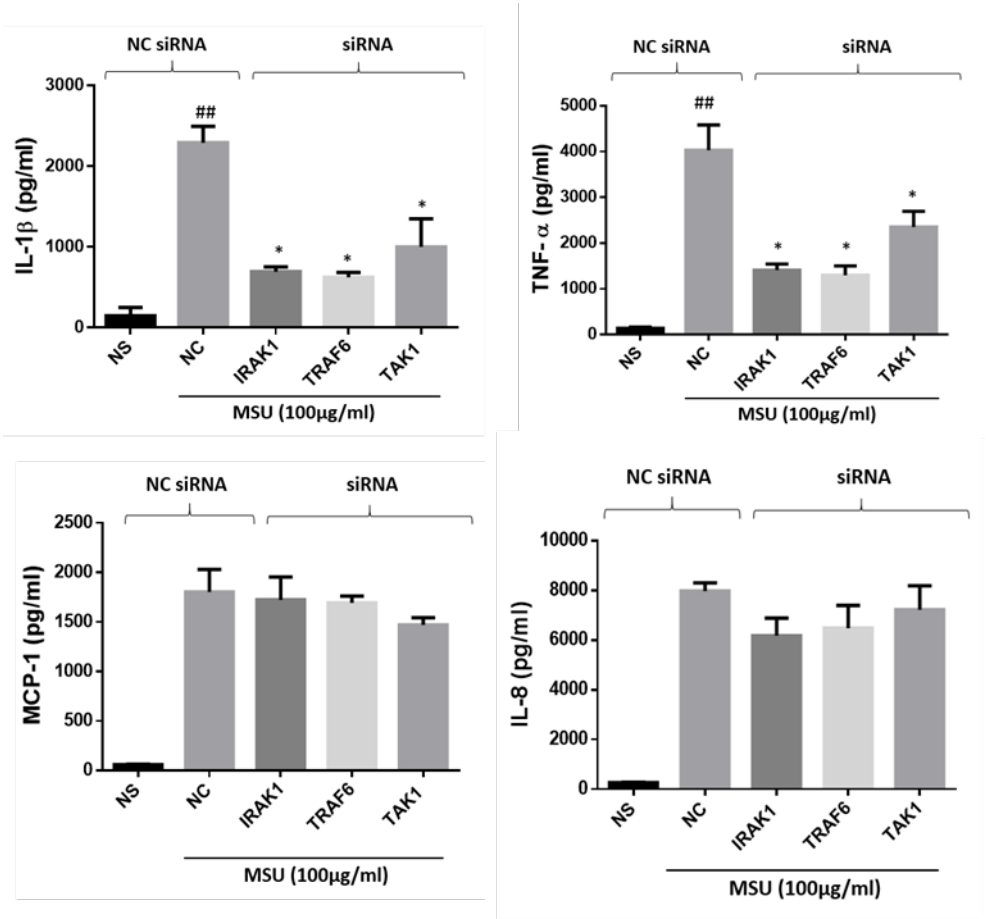


Fig. S4B: Human PBMC-derived macrophages were treated with MSU (50-200 µg/ml) for 24 h and cell lysates prepared were used in the Western blots analysis of K^{63}-ubiquitination in these cells.

Fig. S5

(A)



(B)

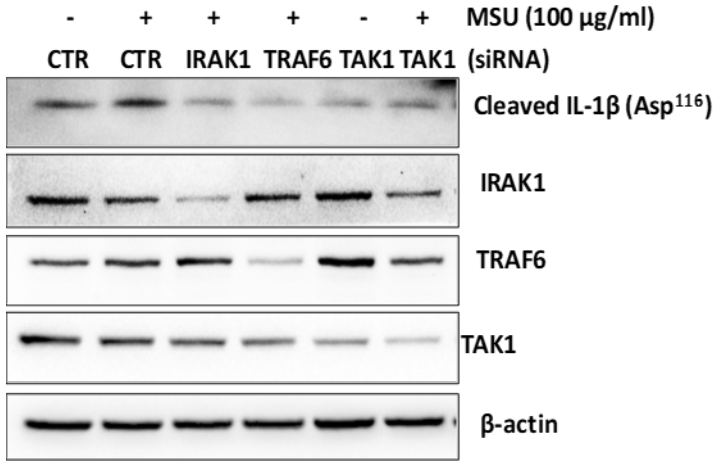


Fig. S5: (**A**)**,** THP-1 macrophages were transfected with scrambled (NC), IRAK1, TRAF6, or TAK1 siRNA (120 pmoles) for 48 h in 6-well plates. After 48 h of transfection, cells were serum starved overnight prior to MSU simulation for 24 h. Conditioned media was collected and used to quantitate the production of IL-1β, TNF-α, MCP-1 and IL-8. The values presented in the graph are mean ± SEM of three independent experiments. ##$p<0.05$ for NS (non-stimulated) versus NC (MSU); *$p<0.05$ for NC versus siRNA treatment. (**B**)**,** Western blot analysis to show knockdown of specific proteins by siRNA used.

Fig. S6

(A)

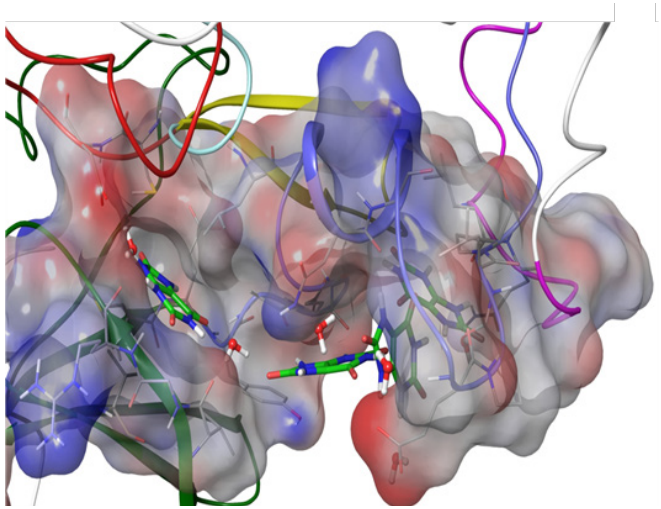


Fig. S6 (a). A docking pose at 400 ns showing TAK1-TAB1 fusion protein interacting with uric acid molecules. [Blue region: hydrophobic sites; Red region: hydrophilic sites].

(B)

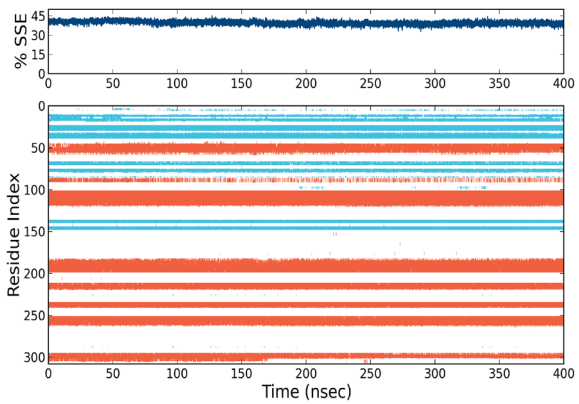


Fig. S6 (b). The plot above summarizes the Protein secondary structure elements (SSE) composition for each trajectory frame over the course of the simulation, and the plot at the below monitors each residue and its SSE assignment over time.

(C)

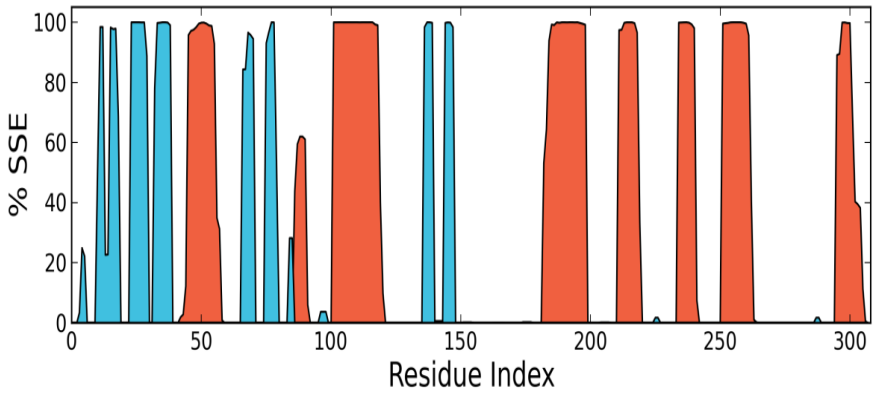


Fig. S6 (c). The plot above reports Protein secondary structure elements (SSE) like α-helices distribution by residue index throughout the protein structure.

(D)

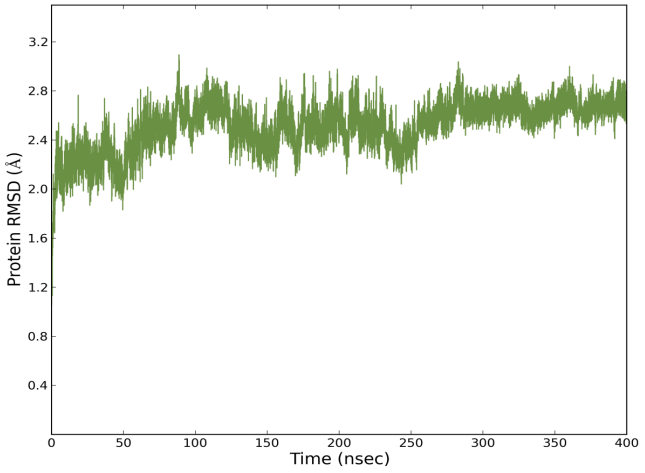


Fig. S6 (d). The plot shows the root mean square deviation (RMSD) evolution of a protein (Y-axis) with time (X-axis). All protein frames are first aligned on the reference frame backbone, and then the RMSD is calculated based on the backbone atom selection. RMSD plots indicate that the simulation has equilibrated well.

Fig. S7

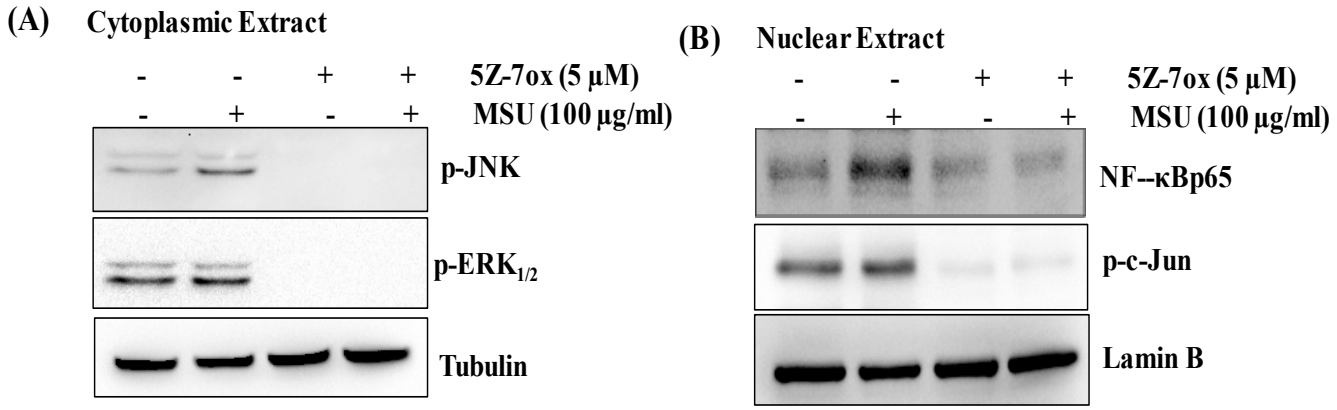


Fig. S7: (a & b), THP-1 macrophages were pretreated with 5Z-7-oxozeaenol (5 µM) followed by MSU (100 µg/ml) stimulation for 30 min. Cell were lysed to prepare cytoplasmic and nuclear fractions as described previously [13]. Cytoplasmic extracts were treated analyzed for p-JNK and p-ERK1/2 expression, whereas nuclear fractions were analyzed for the nuclear translocation of transcription factors NF-κBp65 and p-c-Jun. Our results showed that 5Z-7-oxozeaenol pretreatment inhibits MSU-induced activation of JNK and $ERK_{1/2}$ MAPKs and nuclear translocation of NF-κBp65 and p-c-Jun in THP-1 macrophages. The representative blots of three independent experiments are shown in this figure.

Fig. S8

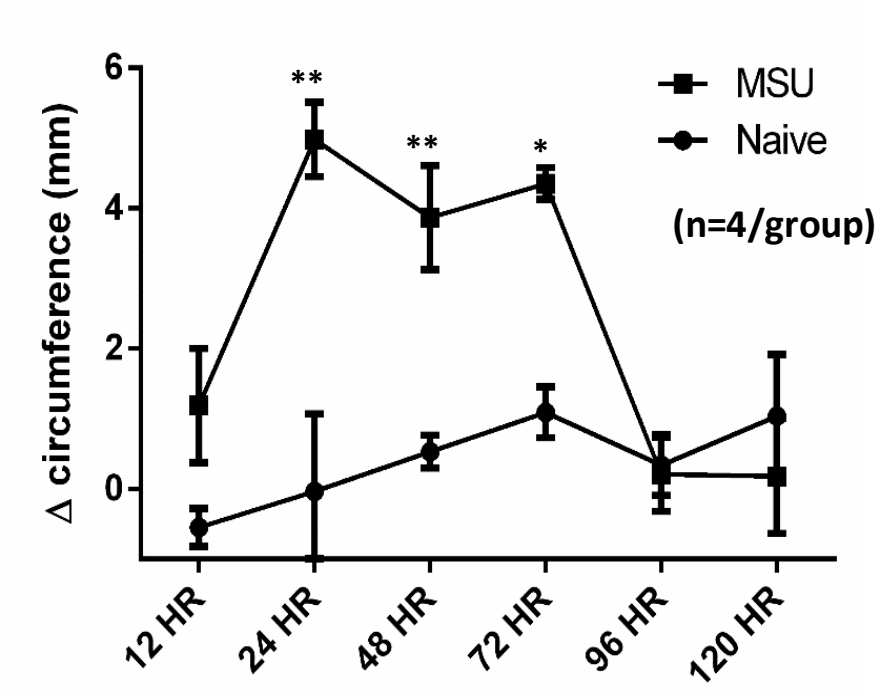


Fig. S8. The line plot above references to the ankle circumference scores measured between 12 to 120 h of mice injected MSU (0.5 mg) in the ankles. *p<0.05 or **p<0.01 versus naïve measurements.

	Inflammation of synovium and adjacent tissue											
Treatment group	**Naïve**			**MSU**			**MSU+5Z-7-ox**			**MSU+Febuxostat**		
Joint	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
-Neutrophils	0	0	0	4	4	4	1	1	1	1	1	1
- Lymphocytes	0	0	0	1	1	1	1	0	0	1	0	0
-Plasma cells	0	0	0	0	0	0	0	0	0	0	0	0
-Macrophages	0	0	0	4	4	4	1	1	1	1	1	0
-Giant cells	0	0	0	0	0	0	0	0	0	0	0	0
-Necrosis	0	0	0	0	0	0	0	0	0	0	0	0
Neovascularization	0	0	0	2	2	2	0	1	0	1	1	0
Fibrosis	0	0	0	2	2	2	1	1	1	1	1	0
Total	**0**	**0**	**0**	**13**	**13**	**13**	**4**	**4**	**3**	**5**	**4**	**1**
Group Total	**0**			**39**			**11.0**			**10.0**		
Average	**0.0**			**13.0**			**3.7**			**3.3**		
Soft tissue edema	0	0	0	3	3	3	1	0	1	1	0	1
Global inflammation severity score	0	0	0	2	3	3	1	1	1	1	1	1
Synovial lining cell layer grade	0	0	0	3	3	3	1	1	1	1	1	1
Articular cartilage surface erosion	0	0	0	3	3	3	2	1	1	2	1	1
Subarticular bone erosion	0	0	0	3	3	4	1	1	2	1	1	0
Bone loss peripheral to joint	0	0	0	3	4	3	1	1	1	1	1	0
New bone formation/ bone remodeling	0	0	0	2	2	2	1	0	1	1	1	0

Supplementary Table 1: Microscopic Evaluation for inflammation score and detailed analysis of pathology of MSU-induced paw swelling and destruction in slides stained with H&E. The pathologist blinded to the treatment scored three representative slides from each group.