

Cortistatin protects against intervertebral disc degeneration through targeting mitochondrial ROS-dependent NLRP3 inflammasome activation

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Running title: Role of cortistatin in IVD degeneration

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Figure S1

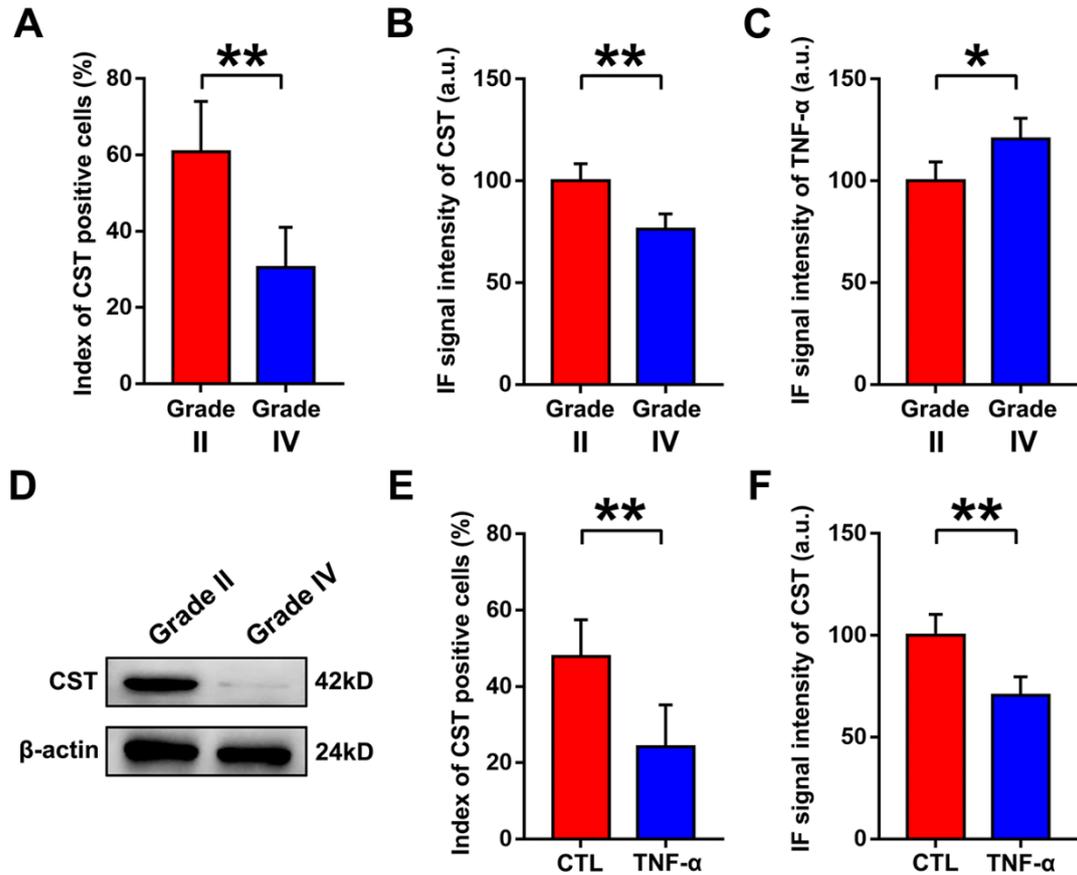


Figure S1. (A) The index of CST positive cells percentage based on immunohistochemistry of Figure 1C. (B-C) IF signal intensity of CST and TNF- α based on immunofluorescence of Figure 1D. (D) Expression of CST in NP tissues of Grade II (n=10) and Grade IV (n=7) group, as assayed through Western blot. (E) The index of CST positive cells percentage based on immunohistochemistry of Figure 1H (n=5). (F) IF signal intensity of CST and TNF- α based on immunofluorescence of Figure 1K (n=5). * p <0.05 and ** p <0.01 vs. Control group. Data are presented as mean \pm SD.

Figure S2

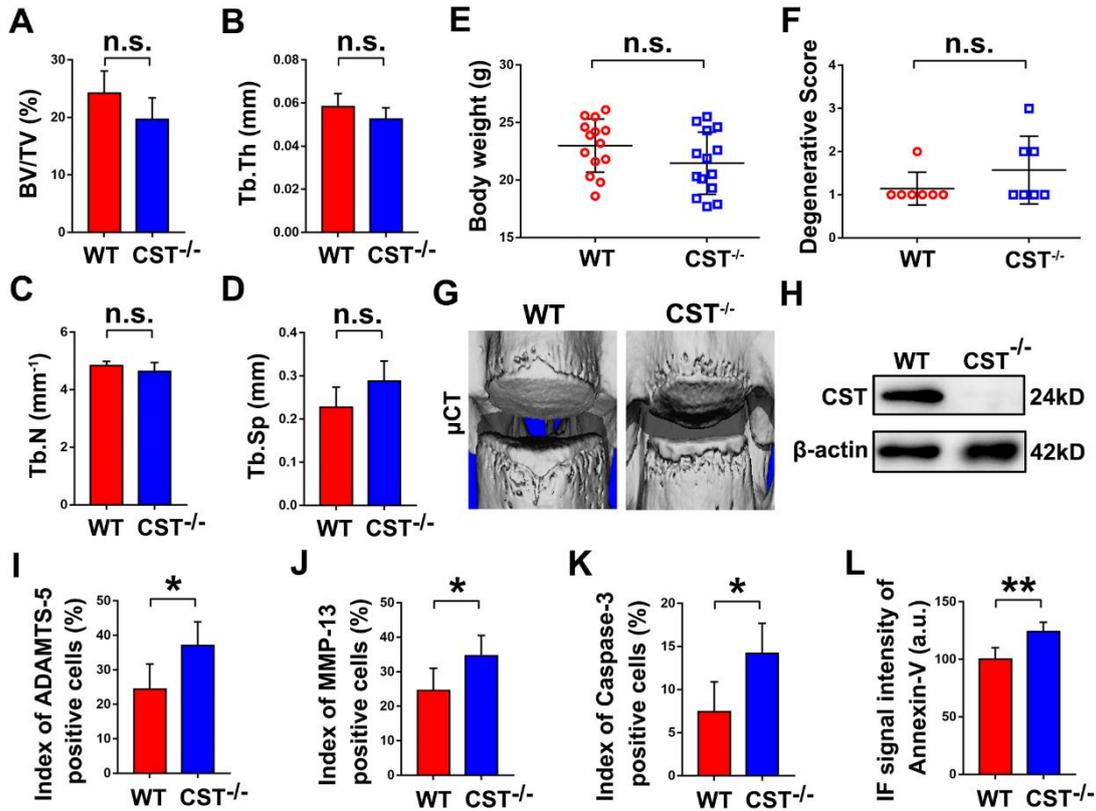


Figure S2. (A-D) The analysis of BV/TV, Tb.Th, Tb.N and Tb.Sp in vertebral body of 2-month old WT and CST^{-/-} mice based on micro-CT (n=5). (E) Data of body weight from 2-month WT and CST^{-/-} mice (n=14). (F) Degenerative score in IVD tissue from 2-month WT and CST^{-/-} mice based on Safranin O staining (n=7). (G) Representative μ CT images of intervertebral space region of 6-month old WT and CST^{-/-} mice. Abnormal formation of osteophyte and reduction of intervertebral space could be observed in CST^{-/-} mice (n=5). (H) No expression of CST was detected in NP tissue of CST^{-/-} mice compared with WT littermates (n=3), as assayed by Western blot. (I-J) The percentage of ADAMTS-5 and MMP-13 positive cells based on immunohistochemistry of Figure 2F (n=5). (K) The percentage of caspase-3 positive cells based on immunohistochemistry of Figure 2K (n=5). (L) IF signal intensity of Annexin-V based on immunofluorescence of Figure 2N (n=5). n.s., not significant. *p<0.05 and **p<0.01 vs. Control group. Data are presented as mean \pm SD.

Figure S3

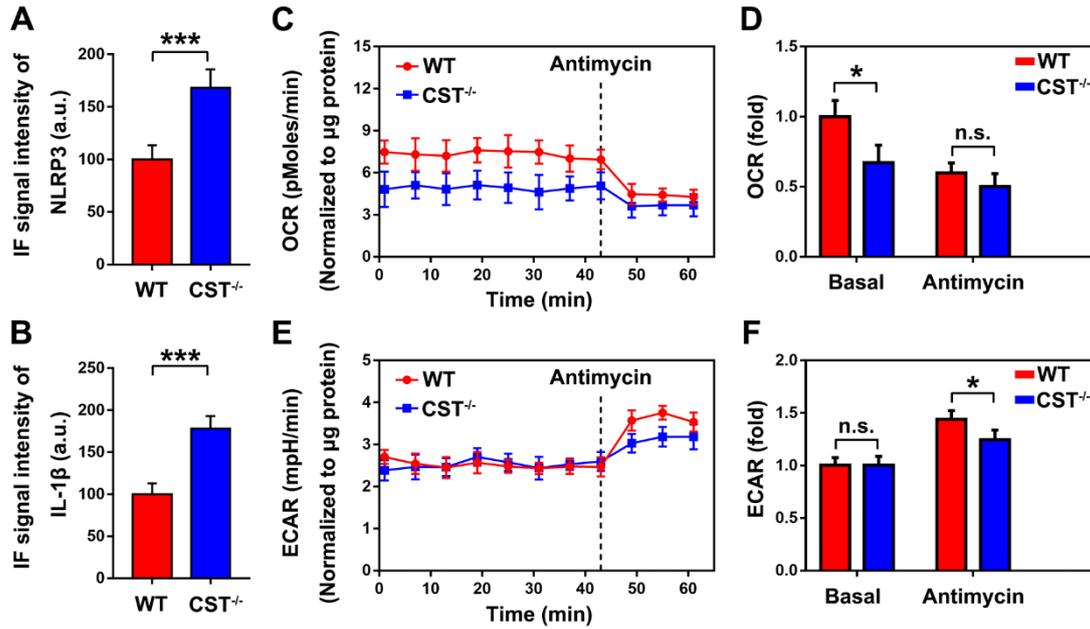


Figure S3. (A-B) IF signal intensity of NLRP3 and IL-1 β based on immunofluorescence of Figure 3K (n=5). (C-D) Oxygen consumption rate (OCR) of NP cells reveals that CST deficiency results in the reduction of basal respiration (n=3). (E-F) Extracellular acidification rate (ECAR) of NP cells indicates CST deficiency results in the decreased glycolysis capacity (n=3). n.s., not significant. *p<0.05 and ***p<0.001 vs. Control group. Data are presented as mean \pm SD.

Figure S4

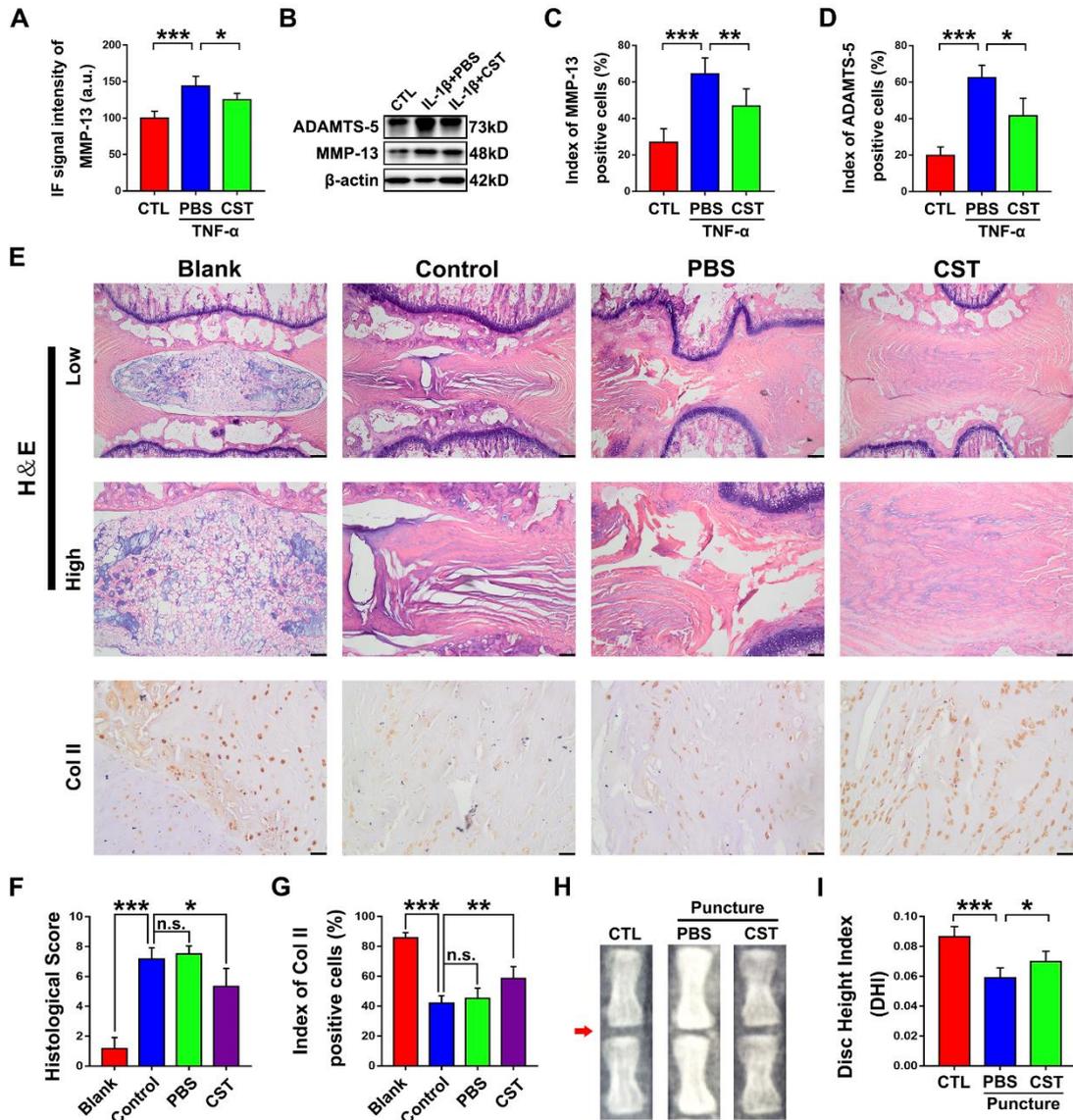


Figure S4. (A) IF signal intensity of MMP-13 based on immunofluorescence of Figure 4C (n=5). (B) Human NP cells stimulated by 10 ng/mL IL-1 β for 24h, with or without treatment of CST recombinant peptide (50 μ g/mL) and then total protein of each indicated group were extracted. CST was identified to inhibit the production of ADAMTS-5 and MMP-13 induced by IL-1 β (n=5), as assayed by Western blot. (C-D) The index of MMP-13 and ADAMTS-5 positive cells percentage based on immunohistochemistry of Figure 4K (n=5). (E) Representative hematoxylin and eosin (H&E) staining and immunohistochemistry of rat IVDs (n=6). Scale bar, 150 μ m (up panel), 100 μ m (third and bottom panel). (F) Histological score of rat IVDs in each

indicated group (n=6). **(G)** Index of Col II positive cells based on immunohistochemistry of Figure S4E (n=6). **(H-I)** Representative X-ray image of each group and associated disc height index (n=6). n.s., not significant. *p<0.05, **p<0.01 and ***p<0.001 vs. Control group. Data are presented as mean \pm SD.

Figure S5

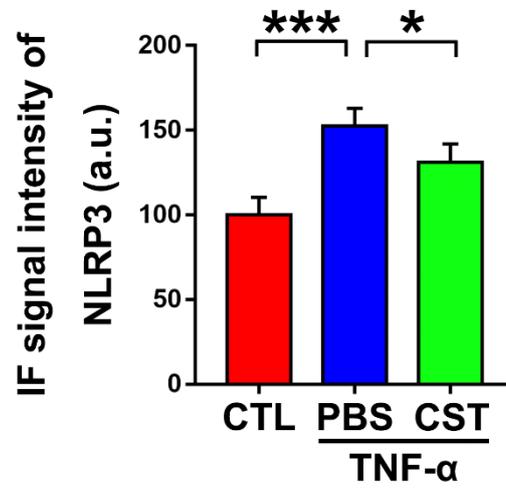


Figure S5. IF signal intensity of NLRP3 based on immunofluorescence of Figure 5G (n=5). * $p < 0.05$ and *** $p < 0.001$ vs. Control group. Data are presented as mean \pm SD.

Figure S6

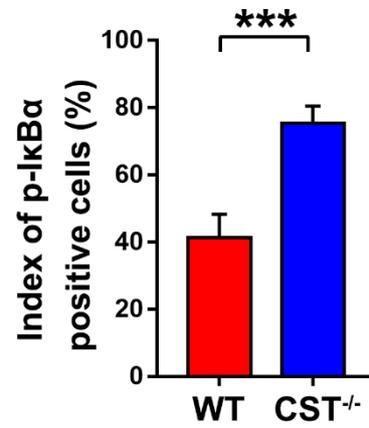


Figure S6. The index of p-IkBa positive cells percentage based on immunohistochemistry of Figure 6B (n=5). ***p<0.001 vs. Control group. Data are presented as mean \pm SD.

Figure S7

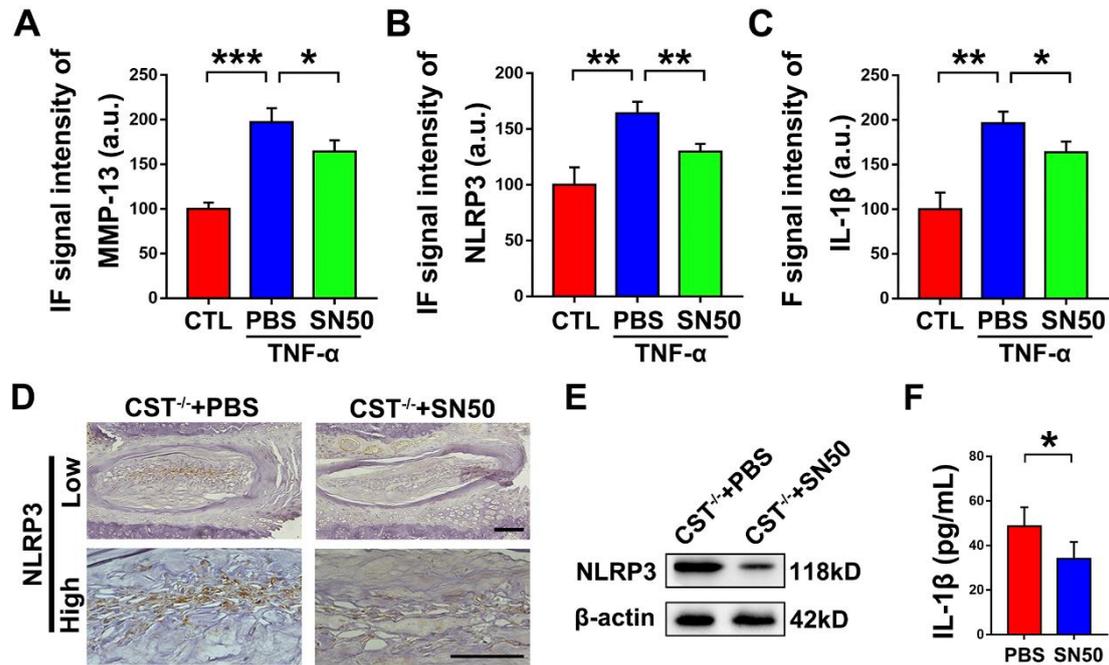


Figure S7. (A) IF signal intensity of MMP-13 based on immunofluorescence of Figure 7C (n=5). (B-C) IF signal intensity of NLRP3 and IL-1β based on immunofluorescence of Figure 7I (n=5). (D) Representative immunohistochemistry image in PBS or SN50 treated 6-month old CST^{-/-} IVD tissues (n=5). Scale bar, 150 μm. (E) Western blot analysis of PBS and SN50 treated groups (n=5). (F) The expression of IL-1β in culture media of PBS and SN50 treated groups (n=5). *p<0.05, **p<0.01 and ***p<0.001 vs. Control group. Data are presented as mean ±SD.

Figure S8

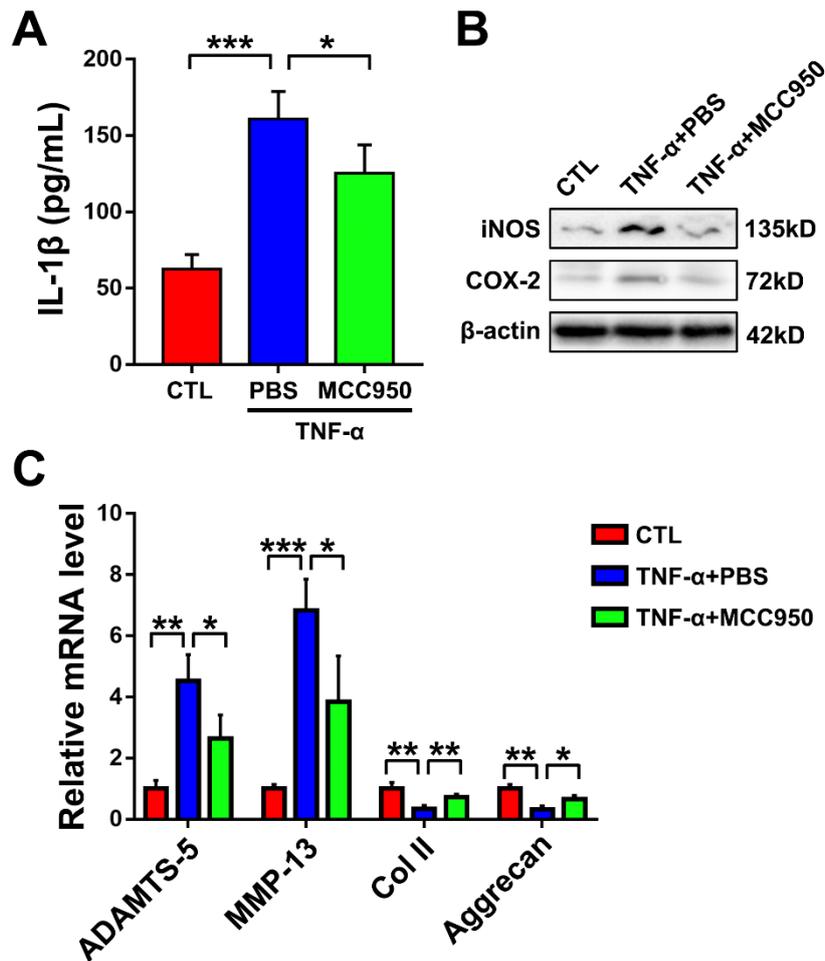


Figure S8. MCC950 attenuates impaired homeostasis of NP cells in deficiency of CST. (A) MCC950 diminished the expression of IL-1 β in culture media of 2-month old murine CST^{-/-} NP cells, as detected by ELISA (n=5). (B) Murine CST^{-/-} NP cells stimulated by 10 ng/mL TNF- α for 24h, with or without treatment of 1 μ M MCC950 and then total protein of each indicated group were extracted. MCC950 inhibits TNF- α -mediated expression of inflammatory mediators (iNOS and COX-2), as assayed by Western blot (n=5). (C) Relative mRNA expression of MMP-13, ADAMTS-5, Col 2 and Aggrecan in murine NP cells from CST^{-/-} mice, as measured by Real time PCR (n=5). *p<0.05, **p<0.01 and ***p<0.001 vs. Control group. Data are presented as mean \pm SD.

Figure S9

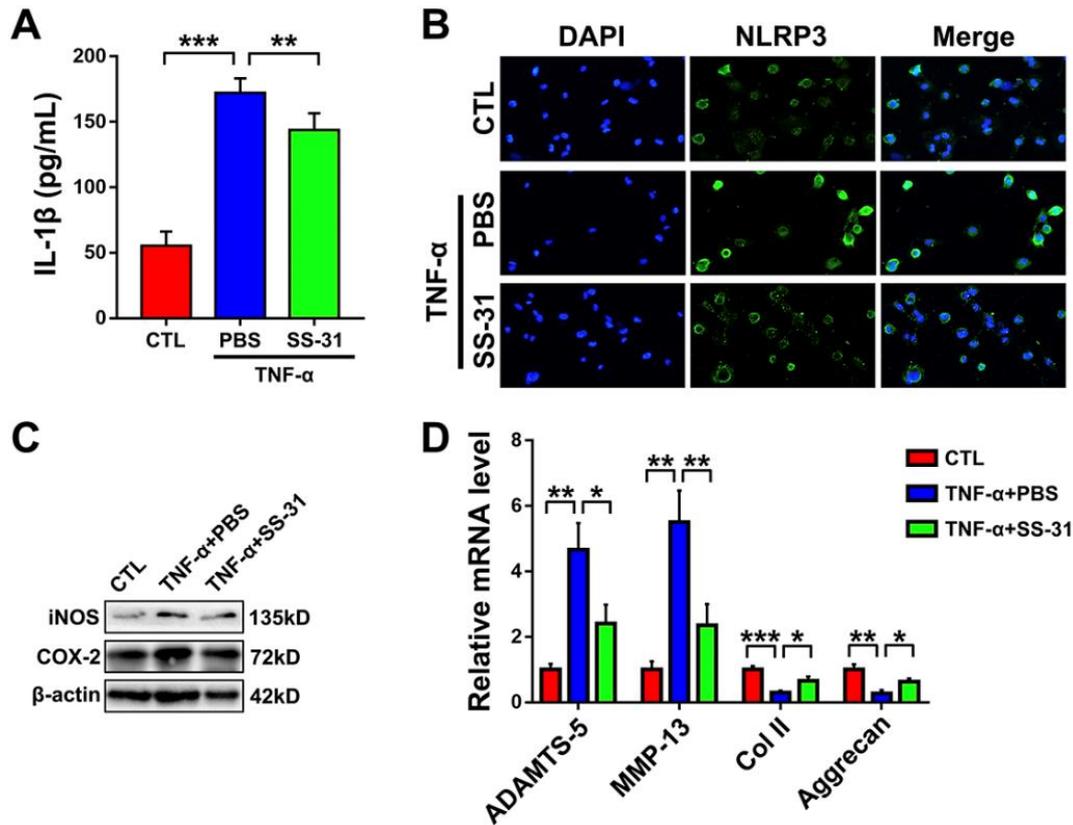


Figure S9. SS-31 alleviated NLRP3 inflammasome activation in $CST^{-/-}$ NP cells.

(A) SS-31 markedly downregulated expression of IL-1 β in culture media of 2-month old murine $CST^{-/-}$ NP cells, as detected by ELISA (n=5). (B) Murine $CST^{-/-}$ NP cells stimulated by 10 ng/mL TNF- α for 24h, with or without treatment of 5 μ M SS-31 and then total protein of each indicated group were extracted. SS-31 attenuates the TNF- α -mediated activation of NLRP3 (n=5). (C) SS-31 inhibits TNF- α -mediated the expression of inflammatory mediators (iNOS and COX-2), as assayed by Western blot (n=5). (D) Relative mRNA expression of MMP-13, ADAMTS-5, Col 2 and Aggrecan in murine NP cells from $CST^{-/-}$ mice, as measured by Real time PCR (n=5). *p<0.05, **p<0.01 and ***p<0.001 vs. Control group. Data are presented as mean \pm SD.

Table S1: Summary of clinical and demographic features of patients.

Subject Number	Gender	Age	Level	Pfarrmann grading
Grade II group				
1	F	30	L3/L4	II
2	M	29	L3/L4	II
3	F	28	L4/L5	II
4	M	29	L3/L4	II
5	M	24	L4/L5	II
6	F	32	L4/L5	II
7	M	24	L4/L5	II
8	F	26	L2/L3	II
9	F	41	L5/S1	II
10	M	27	L5/S1	II
Grade IV group				
11	M	42	L4/L5	IV
12	F	60	L5/S1	IV
13	F	51	L4/L5	IV
14	M	52	L4/L5	IV
15	F	53	L4/L5	IV
16	F	74	L5/S1	IV
17	M	65	L5/S1	IV

Table S2: Sequences of mice primers used for quantitative real-time PCR.

	Gene	Primer sequence (5'-3')
Mouse	<i>Cort</i>	Forward: GGAGCGGCCTTCTGACTTTCC Reverse: GCCTTTCCTGGCTCTTGGACA
	<i>Mmp13</i>	Forward: ACTTTGTTGCCAATTCCAGG Reverse: TTTGAGAACACGGGGAAGAC
	<i>Adams5</i>	Forward: GCATTGACGCATCCAAACCC Reverse: CGTGGTAGGTCCAGCAAACAGTTAC
	<i>Nos2</i>	Forward: ACAGGAGGGGTAAAGCTGC Reverse: TTGTCTCCAAGGGACCAGG
	<i>Cox2</i>	Forward: AATGCTGACTATGGCTACAAAA Reverse: AAAACTGATGCGTGAAGTGCTG
	<i>Col2a1</i>	Forward: ACTAGTCATCCAGCAAACAGCCAGG Reverse: TTGGCTTTGGGAAGAGAC
	<i>Acan</i>	Forward: AATGCTGGTACTCCAAACCC Reverse: CTGGATCGTTATCCAGCAAACAGC
	<i>Casp3</i>	Forward: AGGAGGGACGAACACGTCT Reverse: CAAAGAAGGTTGCCCAATCT
	<i>Bax</i>	Forward: CTGAGCTGACCTTGGAGC Reverse: GACTCCAGCCACAAAGATG
	<i>Bcl2</i>	Forward: TGTGGTCCATCTGACCCTCC Reverse: ACATCTCCCTGTTGACGCTCT
	<i>Nlrp3</i>	Forward: GAGTTCTTCGCTGCTATGT Reverse: ACCTTACGTCTCGGTTCC
	<i>Nfkb2</i>	Forward: TACAAGCTGGCTGGTGGGGA Reverse: GTCGCGGTCTCAGGACCTT
	<i>Gapdh</i>	Forward: CTTACCACCATGGAGAAGGC Reverse: GACGGACACATTGGGGGTAG

Table S3: Sequences of human primers used for quantitative real-time PCR.

Gene	Primer sequence (5'-3')
Human	<i>CORT</i> Forward: CGTGTCTTGAGTAATTTGGA Reverse: ATGAACATCAGAAGAAAAGC
	<i>ADAMTS5</i> Forward: GCAGTATGACAAGTGCGGAGT Reverse: CAGGGCTAAATAGGCAGTGAA
	<i>MMP13</i> Forward: ACTTTGTTGCCAATTCCAGG Reverse: TTTGAGAACACGGGGAAGAC
	<i>COL2A1</i> Forward: TGAGGGCGCGGTAGAGACCC Reverse: TGCACACAGCTGCCAGCCTC
	<i>ACAN</i> Forward: AATGCTGGTACTCCAAACCC Reverse: CTGGATCGTTATCCAGCAAACAGC
	<i>CASP3</i> Forward: GAGCACTGGAATGTCATCTCGCTCTG Reverse: AGACCGAGATGTCATTCCAGTGCTT
	<i>BAX</i> Forward: CCCGAGAGGTCTTTTTCCGAG Reverse: CCAGCCCATGATGGTTCTGAT
	<i>BCL2</i> Forward: ACTTCGCCGAGATGTCC Reverse: ATGACCCACCGAACTC
	<i>GAPDH</i> Forward: AGAAGGCTGGGGCTCATTG Reverse: AGGGGCCATCCACAGTCTTC

Table S4: Antibodies used for Western Blot.

Name	Source	Catalog number	Dilution
Anti-cortistatin	Santa Cruz Biotechnology	sc-393108	1:1000
Anti-MMP-13	Santa Cruz Biotechnology	sc-515284	1:1000
Anti-ADAMTS-5	Abcam	ab41037	1:500
Anti-iNOS	Proteintech	18985-1-AP	1:500
Anti-COX-2	Boster	BA0738	1:1000
Anti-Col 2	Santa Cruz Biotechnology	sc-52658	1:1000
Anti-aggrecan	Proteintech	13880-1-AP	1:1000
Anti-caspase-3	Proteintech	19677-1-AP	1:2000
Anti-Bax	Boster	BM3964	1:400
Anti-Bcl-2	Abcam	ab196495	1:1000
Anti-OPA1	Boster	PB0773	1:2000
Anti-Drp1	Proteintech	12957-1-AP	1:3000
Anti-Mfn1	Proteintech	13798-1-AP	1:1000
Anti-Mfn2	Proteintech	12186-1-AP	1:3000
Anti-pAMPK	Affinity Biosciences	AF3422	1:2000
Anti-AMPK	Affinity Biosciences	AF6423	1:2000
Anti-PGC1 α	Proteintech	66369-1-Ig	1:3000
Anti-NLRP3	Abcam	ab214185	1:1000
Anti-NF- κ B p65	Proteintech	10745-1-AP	1:3000
Anti-Tubulin	Proteintech	66240-1-Ig	1:15000
Anti-Lamin A	Santa Cruz Biotechnology	sc-71481	1:1000
Anti-I κ B α	Abcam	ab32518	1:3000
Anti-pI κ B α	Santa Cruz Biotechnology	sc-8404	1:1000
Anti- β -actin	Abcam	60008-1-Ig	1:10000

Table S5: Antibodies used for Immunohistochemistry.

Name	Source	Catalog number	Dilution
Anti-cortistatin	Santa Cruz Biotechnology	sc-393108	1:200
Anti-MMP-13	Santa Cruz Biotechnology	SC-515284	1:200
Anti-ADAMTS-5	Abcam	ab41037	1:200
Anti-caspase-3	Proteintech	19677-1-AP	1:200
Anti-NLRP3	Abcam	ab214185	1:100
Anti-p-I κ B α	Santa Cruz Biotechnology	sc-8084	1:100