Supplemental Material

Materials and Methods

Reagents

All chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Apigenin was from MedChemExpress (Monmouth Junction, NJ). Recombinant human IL-1β and human total MMP-3 and MMP-13 DuoSet enzyme-linked immunosorbent assay (ELISA) kits (DY-513 and DY-511) were from R&D Systems Inc. (Minneapolis, MN). The reagents for immunohistochemistry (IHC) were from Vector Laboratories (Burlingame, CA). Antibodies for Western blotting include CD38 (#51000), SIRT1 (#2496), phospho-NF-κB p65 Ser 536 (#3033), acetyl-NF-κB p65 Lys310 (#12629), NF-κB p65 (#8242), phospho-Stat1 Tyr701 (#9167), phospho-Stat1 Ser727 (#8826) and Stat1 (#14944) were from Cell Signaling Technology, Inc (Danvers, MA).

Studies of human articular chondrocytes

Studies were performed in compliance with institutional IRB and approved protocol of human subjects #H170130 at the VA Medical Center of San Diego. Human chondrocytes were isolated from knee cartilage of surgical waste from total knee arthroplasty using the methods described previously (1) and were cultured in Dulbecco's modified Eagle's (DMEM) high glucose medium with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37^0C. No later than first passage chondrocytes were used. Chondrocytes were plated at 2.5 x 10^5/ well in 12-well or 5×10^5 /well in 6-well plates for *in vitro* experiments. To overexpress CD38, chondrocytes were transfected with pCMV3-CD38-Myc (#HG-10818-CM, SinoBiological, Wayne, PA) plasmid and the empty plasmid vector for 48 hours using X-tremeGENE HP DNA Transfection Reagent (#XTGHP-RO, MilliporeSigma, Burlington, MA).

Quantitative RT-PCR

Total RNA was extracted from chondrocytes using the RNeasy Mini kit (Qiagen, Germantown, MD) and then used for reverse transcription to generate cDNA using the cDNA synthesis kit (MilliporeSigma, Burlington, MA). The cDNAs were subjected to quantitative PCR (SYBR Green) analysis for gene expression of *aggrecan (ACAN), collagen2a1 (Col2a1), MMP3 and MMP13* using the efficiency-adjusted ΔΔCT method. The PCR primer sequences were shown in the table below. Total RNA samples isolated directly from human knee cartilage of normal and OA donors kindly provided by Dr. Martin Lotz (The Scripps Research Institute) were used for analysis of *CD38* gene expression using TaqMan Gene Expression Assay probe sets (ThermoFisher, Waltham, MA) for CD38 (Hs01120071_m1) and endogenous control GAPDH (Hs02786624_g1). The data was analyzed using the ΔΔCT method. For each sample, ΔCT (CT_{sample}-CT_{GAPDH}) value was used to subtract the mean ΔCT of normal donors to calculate its ΔΔCT value and then relative fold of gene expression. The data was presented as fold changes relative to the mean of normal donors.

Gene	Primer sequences
Aggrecan	Forward: ACAGATGCTTCCATCCCAGC
	Reverse: GATGCTGCTCAGGTGTGACT
Col2a1	Forward: GTGAGCCATGATTCGCCTCG
	Reverse: TCACAGACACAGATCCGGCA

MMP3	Forward: TGG GCCAGGGATTAATGGAG
	Reverse: CCGAGTCAGGTCTGTGAGTG
MMP13	Forward: AGGAGCATGGCGACTTCTAC
	Reverse: AGACCTAAGGAGTGGCCGAA

Western Blotting

Cells were lysed in RIPA buffer with 2 mM sodium vanadate and protease inhibitor cocktails (Roche, Mannheim, Germany). Cell lysates (10-15 μg) were separated by gradient 4-20% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, California), probed with primary and secondary antibodies, exposed to SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Scientific, Waltham, MA), and visualized by autoradiography. Alternatively, after primary antibody incubation, the membranes were washed and then incubated with near-infrared secondary antibodies. After washing, the membranes were scanned on the Li-COR Odyssey imaging system.

Measurement of NAD glycohydrolase (NADase) activity, NAD^+ and NADH

Etheno-NAD (N2630, Sigma), a modified NAD^+ molecule which, when hydrolyzed, produces a fluorescent etheno-ADP-ribose product, was used to evaluate NADase activity. As described previously (2), cellular NADase activity was determined by following the change in fluorescence at 300 nm excitation and 410 nm emission. Intracellular NAD^+ and NADH were quantified using a NAD/NADH quantification colorimetric kit (#K337-100, BioVision, Milpitas, CA).

Measurement of production of MMP-3, MMP-13, and glycosaminoglycan (GAG) from conditioned media

The conditioned media from chondrocyte or cartilage explants experiments were used to measure production of MMP-3 and MMP-13 by ELISA analysis and GAG using the colorimetric assay with cationic 1,9 Dimethylmethlyene Blue (DMB) Dye method (3).

Experimental OA model in mice

All animal experiments were performed in compliance with the approved protocol #A14-010 from the Institutional Animal Care and Use Committee (IACUC) of VA Medical Center of San Diego. WT and CD38 KO mice in C57BL/6 background were housed in static, polysulfone, microisolation cages and maintained on a 12:12-h light: dark cycle with caging, food and water bottles changed weekly. Mice at 16 weeks of age were subjected to destabilization of the medial meniscus (DMM) surgery under anesthesia condition as previously described (4). Sham surgeries were performed on separate groups of mice. To study the effect of apigenin, a competitive inhibitor of CD38 (5), on OA development, C57BL/6 mice were randomly assigned to treatment and control (non-treated) groups after DMM surgery. The treatment group received apigenin at 20 mg/kg/day the day after DMM surgery via gavage daily. The non-treated control group received only the vehicle control. All mice were euthanized at 2 or 10 weeks after surgery by carbon dioxide inhalation, and OA phenotype of knee joints were analyzed. Only male mice were used because they develop more robust and consistent OA and associated pain behaviors than female mice following DMM (6,7). For mice euthanized at 10 weeks post-surgery, 96 mice were used including 20 WT and 20 CD38KO mice (10/group for DMM and sham) and 18 WT mice with apigenin treatment and 18 WT mice with vehicle control (9/group for sham and DMM). The sample size of 9-10/group was determined with an assumption of a pooled standard

deviation of 1.5 units to achieve a power of 80% and a level of significance of 5% for detecting a true difference in means between WT and CD38KO or non-treated and apigenin-treated groups of 2 units. Mice euthanized at 2 weeks post-DMM surgery were used for H&E staining and fluorescence IHC analysis of CD38 expression in the synovium. 6 WT and 6 CD38KO mice (2/group for sham and 4/group for DMM) and 6 WT mice treated with apigenin (2 for sham, 4 for DMM) were used.

Micro-CT assessment of subchondral bone plate

Mouse knee joints were subjected to micro-computed tomography scan (Skyscan-1076, Bruker, Kontich, Belgium) with a voxel resolution of 9 μm. Each knee joint sample was scanned in one field encompassing the distal mid-femur and proximal mid-tibia using imaging parameters of 50 kVp, 200 μA, 0.8° step rotations, and 3-frame averaging. The scanned images were evaluated using CT Analyzer software (Skyscan) for subchondral bone changes at the same thresholds to allow 2-dimensional (2-D) and 3-D structural rendering of each sample. Regions of interest (ROIs) from femur and tibia were manually contoured by drawing 2D regions of interest as described in (8). Because the bone changes occurring underneath of the articular cartilage layer (subchondral bone plate) was our focus, care was taken not to include any outgrowing osteophytes. Bone mineral density (BMD) was determined based on hydroxyapatite (HA) reference and presented as $mgHA/cm^3$.

Histology assessment of joint structural changes

Following micro-CT scanning, mouse knees were fixed, decalcified, embedded in paraffin, and coronally sectioned (5 micron). For each knee, 6-8 slides at ~40-micron intervals were stained with safranin-O/Fast green. Cartilage damage was evaluated using the OARSI score system (9) by two blinded observers. Synovitis scores were determined based on changes in synovial lining thickness and cellular density in the synovial stroma as previously described in (10). Osteophyte formation was evaluated semi-quantitatively based on both size and maturity of osteophytes (11).

Immunohistochemistry (IHC)

Sections of human knee cartilage or mouse knees were subjected to antigen retrieval using trypsin digestion for 15 min, and sequentially treated with 3% H_2O_2 for 10 min and blocked with 10% normal goat serum for 1 h. Sections were then incubated with primary antibodies CD38 (1:100, #A1680, Abclonal) and SIRT1 (1:100, #MABE426, MilliporeSigma) and the negative control rabbit IgG (1:50) overnight at 4^0C. After washing, sections were incubated with secondary biotinylated goat anti-rabbit antibody for 30 min, followed by incubation with the ImmPRESS Universal Antibody Polymer Reagent (#MP-7500, Vector Labs). IHC signals were revealed by ImmPACT DAB Peroxidase Substrate. Fluorescence method was used for IHC analysis of expression of CGRP, CD38 and F4/80. Briefly, mouse knee sections were incubated with Alexa Fluor 594 conjugated CGRP (1:100, #BS-0791R-A594, Bioss Antibodies), Alexa Fluor 594 conjugated CD38 (#102725, BioLegend) and Alexa Fluor 488 conjugated F4/80 (#123120, BioLegend) overnight at 4^0C. After washing, sections were incubated with diluted DAPI solution for 2-5 minutes and slides were mounted with the Prolong Gold Antifade reagent (#P36930, ThermoFisher).

Pain behavioral assessment

To evaluate stimulus evoked nociception, mechanical allodynia was measured using the Electric Von Frey system (Bioseb) operated under similar principles as manual von Frey (12). Prior to von Frey hind paw test, the mice were allowed to acclimate to the evaluation environment for 15-30 minutes on a wire mesh grid. The tests were performed at 1 week before (baseline) and every 2 weeks after DMM surgery in a blind manner in the same order of cages of mice each time. To assess non-stimulus evoked nociception, the static weight bearing touch was evaluated using the Incapacitance Test system (Bioseb), which allowed to measure weight distribution on both rear paws to estimate discomfort levels, indicated by ipsilateral (DMM injured) to contralateral (non-injured) weight ratio.

Statistical analyses

GraphPad PRISM 8 (San Diego, CA) was used for statistical analyses. All data were subjected to the normality test. For normally distributed data, unpaired student t-test (comparing 2 groups) or two-way *ANOVA* with Tukey multiple comparisons test (comparing 2 $\geq$ groups with 2 independent variables) were performed. For data that were not normally distributed, unpaired non-parametric Mann-Whitney test (comparing 2 groups) or Kruskal-Wallis test with multiple comparisons using Dunn's corrections (comparing > 2 groups) were used. The data were expressed as mean$\pm$SD or mean$\pm$SEM or mean$\pm$95% confidence interval (CI). $P < 0.05$ was considered statistically significant.

References

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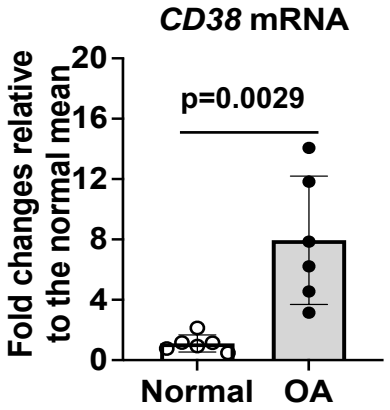
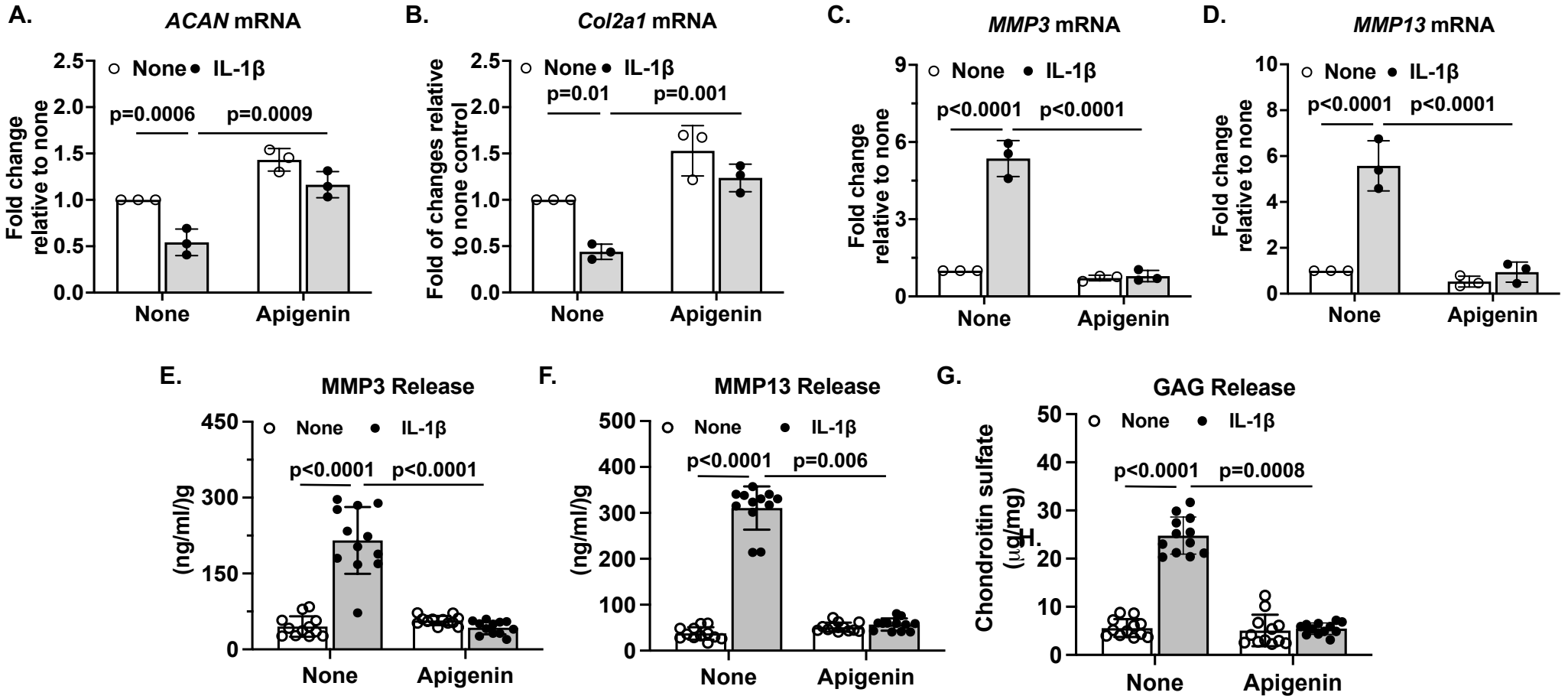
Suppl Figure 1



Normal			OA		
Subjects	Age	Sex	Subjects	Age	Sex
1	52	F	1	59	F
2	49	F	2	59	F
3	56	F	3	52	F
4	46	F	4	57	F
5	61	M	5	67	M
6	62	M	6	60	M

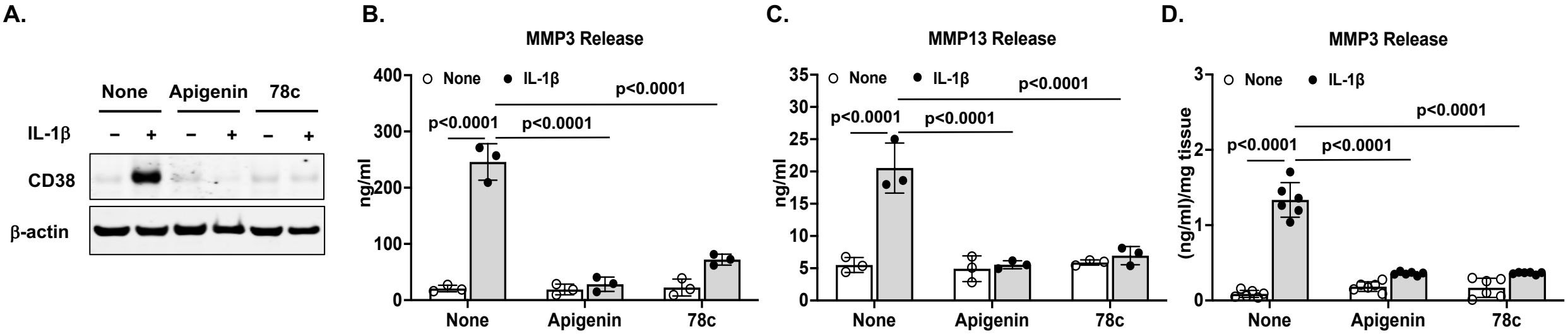
Suppl Figure 1. Upregulation of CD38 mRNA expression in human knee OA cartilage. Total RNA directly isolated from age and gender matched human knee cartilage of normal and OA donors (n=6 pair) were subjected to qRT-PCR analysis of CD38 mRNA expression (A). Information about the donors' age and gender was shown in B. Statistical analysis was performed using Student t-test. Data in A were expressed as mean±SD. p value with significance was indicated in the figure.

Suppl Figure 2



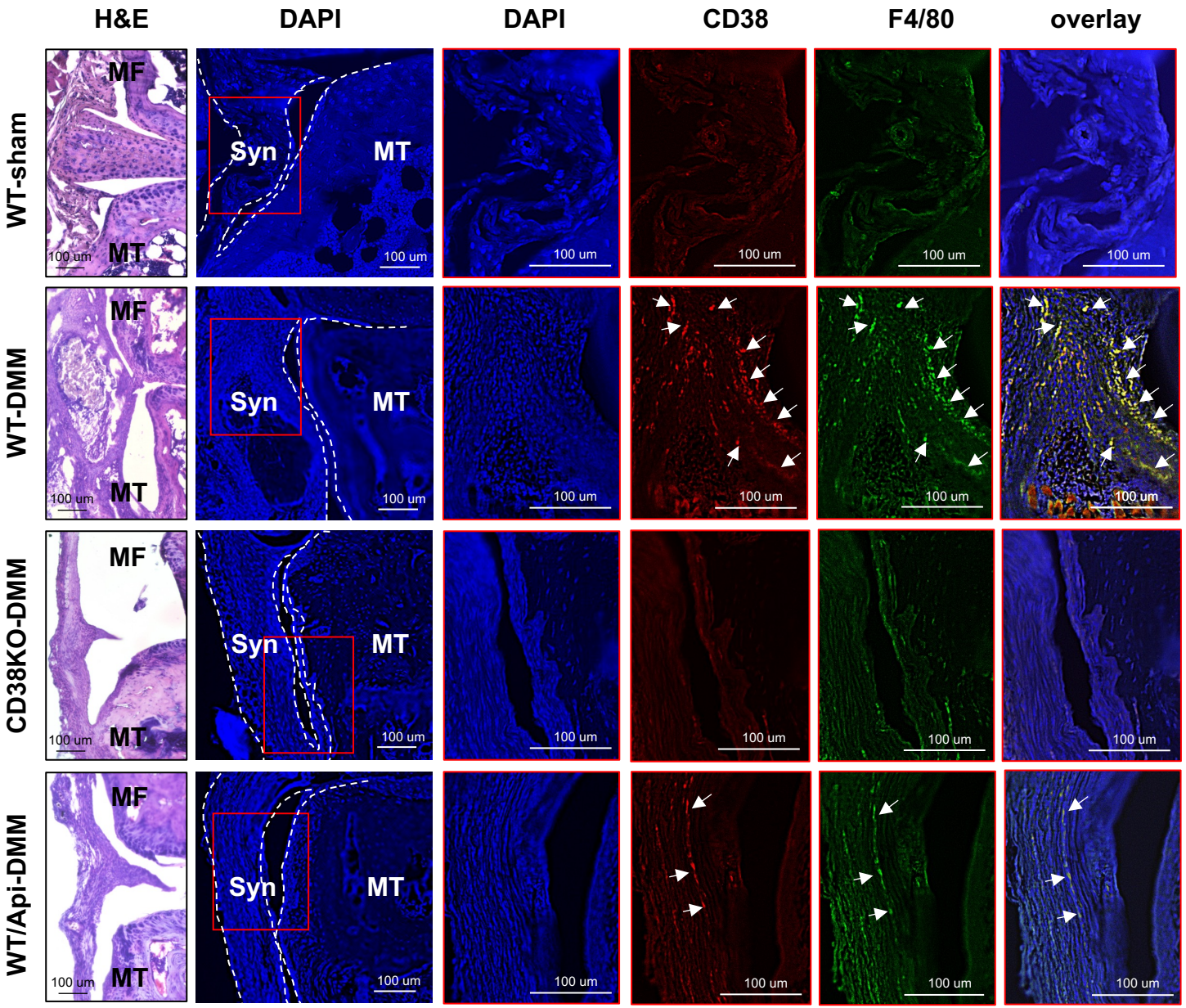
Suppl Figure 2. Attenuation of chondrocyte catabolic activities induced by IL-1β by apigenin. Human chondrocytes and human knee cartilage explants were treated with IL-1β (2 ng/ml) in the presence or absence of apigenin (25 μM) for 6 and 24 hours, respectively. The chondrocyte samples were used for evaluating mRNA expression of *ACAN*, *Col2a1*, *MMP3* and *MMP13* by qRT-PCR analysis (A-D), and the cartilage explant samples were used for measuring release of MMP3, MMP13 and glycosaminoglycan (GAG) from the conditioned media by ELISA and the Dimethylmethlyene Blue (DMB) dye methods (E-G). For data in A-D, n=3 biological replicates with each representing the mean of 3 technical replicates. For data in E-G, n=12 cartilage explants collected from 4 different donors (3 explants from each donor). Statistical analysis was performed using Two-way *ANOVA* with Tukey multiple comparison test. All data were expressed as mean±SD. p values with significance were indicated in the figures.

Suppl Figure 3.



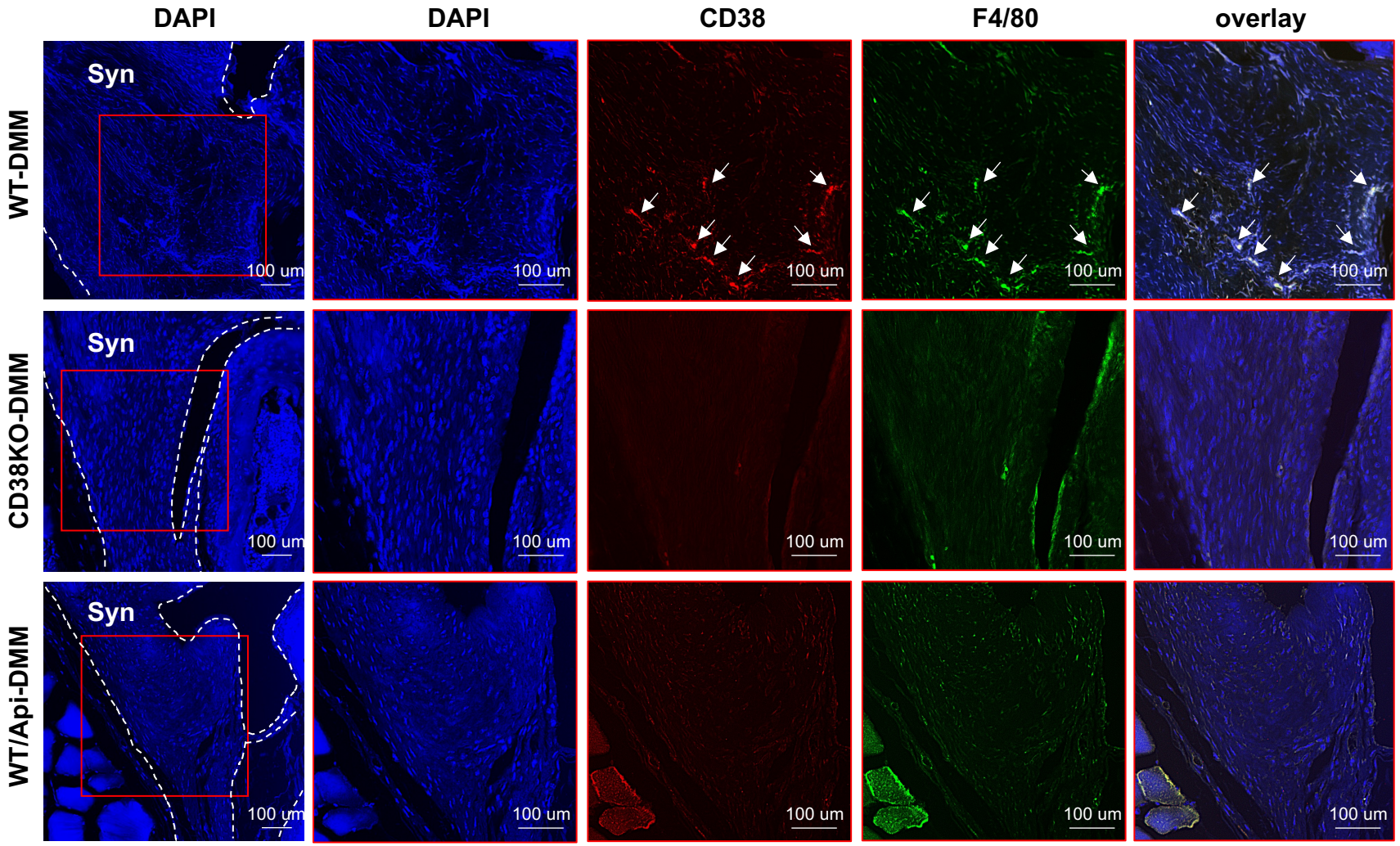
Suppl Figure 3. The CD38-specific inhibitor 78c had the same effects as apigenin on attenuation of IL-1β-induced CD38 expression and catabolic activity in chondrocytes. Human chondrocytes were stimulated with IL-1β (2 ng/ml) in the presence or absence of apigenin (25 μM) or 78c (25 μM) for 18 hours. CD38 protein expression was examined by Western blot analysis (A). Release of MMP3 and MMP13 was measured from the conditioned media by ELISA (B and C). Data in A were representative of 3 independent experiments in chondrocytes from 3 different donors. For data in B and C, n=3 biological replicates with each representing the mean of 3 technical replicates. Statistical analysis was performed using Two-way *ANOVA* with Tukey multiple comparison test. Data were expressed as mean±SD. p values with significance were indicated in the figures.

Suppl Figure 4.



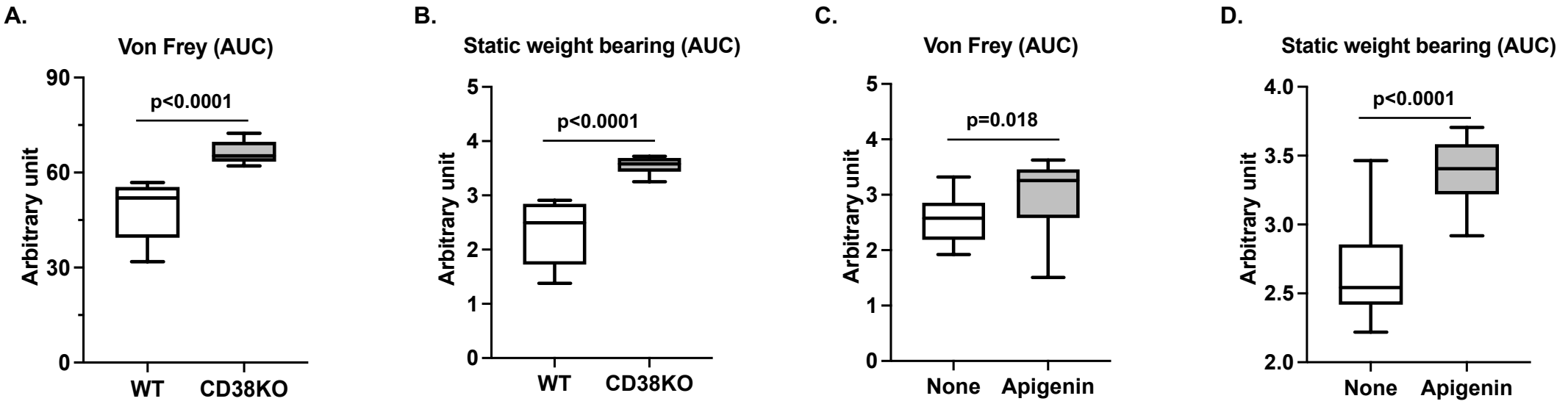
Suppl Figure 4. Increased CD38 expression in the synovial macrophages in WT but not CD38KO or apigenin-treated WT mice at 2 weeks post-DMM surgery. Knee sections of WT-sham, WT-DMM, CD38KO-DMM, WT/Api-DMM mice at 2 weeks after DMM surgery were subjected to H&E staining and fluorescence IHC analysis for expression of CD38 (red) and F4/80 (green). DAPI staining (nuclear stain) was included (blue). Representative H&E images of the synovium in the medial femur (MF) and medial tibia (MT) regions and immunostaining images of the synovium (Syn) in the MT region were shown (n=4/group).

Suppl Figure 5.



Suppl Figure 5. Increased CD38 expression in the synovial macrophages in WT but not CD38KO or apigenin-treated WT mice at 10 weeks post-DMM surgery. WT-DMM, CD38KO-DMM, and WT/Api-DMM mice at 10 weeks after DMM surgery were subjected to fluorescence IHC analysis for expression of CD38 (red) and F4/80 (green). DAPI staining was included (blue). Representative immunostaining images of the synovium (Syn) in the MT compartment were shown (n=4/group).

Suppl Figure 6.



Suppl Figure 6. Mice with CD38 deficiency exhibited less pain associated with OA post-DMM surgery. The time course data of Von Frey and weight bearing tests in Figure 6 were presented in the format of Area under curve (AUC) in box plots (Min to Max). For data in A and B, n=10/group. For data in C and D, n=9/group. Statistical analysis was conducted using unpaired Student t-test. p values with significance were indicated in the figures.