Supplemental Methods, Figures, Tables, and References

Supplemental Methods

Reagents and antibodies

RPMI 1640 medium was purchased from Welgene. Fetal bovine serum (FBS) was purchased from Gibco Life Technologies. Magnetic beads coated with CD14 Ab (catalog 130-050-201) were from Miltenyi Biotec. Cycloheximide (CHX), sodium nitroprusside (SNP), thapsigargin (TG), lipopolysaccharide (LPS), and $CoCl_2$ were purchased from Sigma-Aldrich. Recombinant human M-CSF, recombinant mouse CCL2, recombinant human CCL2, neutralizing mouse anti-CCL2 Ab (clone 123616), neutralizing human anti-CCL2 Ab (clone 23007), recombinant human IL-4, recombinant human IFN-γ, and peridinin chlorophyll protein complex (PerCP)-conjugated Ab to OLR1 (human, clone 331212) were from R&D Systems. Phycoerythrin (PE)-conjugated Ab to F4/80 (mouse, clone BM8) or to CD14 (human, clone 61D3), fluorescein isothiocyanate (FITC)-conjugated Ab to CCL2 (mouse, clone 2H5), allophycocyanin (APC)-conjugated Ab to CCL2 (human, clone 5D3-F7) or to CD44 (human, clone, IM7) were from eBioscience. FITC-conjugated Annexin V was from Santa Cruz Biotechnology. Anti-Bcl-2 (catalog 2876), Bax (clone D3R2M) and cleaved caspase-8 (clone D5B2) Ab were from Cell Signaling Technology. Anti-β-actin Ab (clone AC-74) was from Sigma-Aldrich. SYBR Premix was from BioRad. Anti-NFAT5 Ab was generated as described previously (1). A lenti-viral vector expressing NFAT5 shRNA was obtained from Santa Cruz Biotechnology.

Generation of NFAT5-deficient cells

Briefly, RAW 264.7 macrophages were seeded in 12 well plates and then

transduced with NFAT5 shRNA-harboring lenti-viral particles (Santa Cruz Biotechnology) in the presence of Polybrene (5 μg/ml). Scrambled shRNA was used as a control. For NFAT5 decoy, NFAT5 consensus sequence (TGGAAAATTACCG) with three tandem repeats was transferred into the pEGFP-N1 vector (Clontech) and pDsRed-Express-N1 vector (Clontech) from which CMV promoter had been removed by *Ase*I and *Bam*HI restriction enzymes. RAW 264.7 macrophages were seeded in 12 well plates and then transfected with GFP-fused NFAT5 consensus sequence (5 μg) using Lipofectamine. After 3 days, the cells transfected with shRNAs or transduced with NFAT5 decoy were reseeded to less than 20 to 25% confluence, and then selected by treating 125 μg/ml geneticin (Invitrogen) for 3 weeks. Like NFAT5-deficient RAW 264.7 macrophages, NFAT5-deficient CD14 (+) cells were generated by transduction of NFAT5 shRNA-harboring lenti-viral particles. Briefly, CD14 (+) cells isolated from human peripheral blood mononuclear cells (PBMCs) were seeded in 6 well plates and then transduced with NFAT5 shRNA-harboring lenti-viral particles (Santa Cruz Biotechnology) or control shRNA (Santa Cruz Biotechnology) in the presence of Polybrene (5 μg/ml) for 24 hours. The cells were additionally incubated for 24-48 hours under normal culture conditions before being used for experiments. The transfection rate was determined by fluorescence microscopy and the level of knock-down was determined by immunoblotting for NFAT5 levels.

Western blot analysis for NFAT5, Bcl-2, Bax, and cleaved caspase-8

To detect the expression of NFAT5, cell lysates were separated on 8% sodium dodecyl sulfate- polyacrylamide gel electrophoresis, followed by transfer to a nitrocellulose membrane (BioRad). The membranes were blocked for at least 1 hour at

room temperature in blocking buffer containing 5 % nonfat dry milk (BD Biosciences) in T-TBS. Membranes were then incubated with Ab to NFAT5 (1) diluted 1:1,000 in blocking buffer overnight at 4°C. To detect Bcl-2, Bax and cleaved caspase-8, cell lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were then incubated with anti-Bcl-2 Ab (1:1,000, Cell Signaling Technology), anti-Bax Ab (1:1,000, Cell Signaling Technology) or anti-cleaved caspase-8 Ab (1:1000, Cell Signaling Technology) in 5% (w/v) BSA in T-TBS overnight at 4°C. An anti-β actin Ab (Sigma-Aldrich) diluted 1:10,000 in blocking buffer was used as an internal control. After washing in washing buffer (T-TBS), the membranes were incubated for 2 hours at room temperature with a peroxidase-conjugated goat anti-rabbit Ab (Santa Cruz Biotechnology) diluted 1:1,000 in blocking buffer. The membranes were then washed three times in T-TBS, and exposed to a chemiluminescent (ECL) kit (KPL Inc.). The chemiluminescence signal was detected using a Luminescent Image Analyzer LAS-4000 (Fuji Film). Bands were quantified using Image J software (http://rsbweb.nih.gov/ij/).

Immunofluorescence staining for NFAT5 and CCL2 expression in RA synovium

Rheumatoid synovia (n=4) were obtained form 3 RA patients, immediately fixed in cold acetone for 10 minutes at -20°C, and blocked with 1 % BSA for 30 minutes. All patients were female and positive for both the circulating rheumatoid factor and anti-cyclic citrullinated antibody. The tissue sections were then incubated with rabbit anti-human NFAT5 Ab overnight at 4°C. As a negative control, the sections were incubated with isotype-control Ab. After the incubation, bound primary Ab was reacted

with Alexa Fluor 488-conjugated Ab for 1 hour and then stained again with Alexa Fluor 647-conjugated anti-human CCL2 Ab and Alexa Fluor 594-conjugated anti-human CD14 Ab for 1 hour. After washing in PBS, the coverslips were mounted on glass slides with ProLong Gold Antifade Reagent (Life Technologies). Image J software was used to determine the CCL2 expression in NFAT5 (+) or (–) macrophages. Briefly, an outline was drawn around the CD14 (+) cell area, which could be divided into two areas according to the NFAT5 positivity. In the region of interest (ROI), the percentage of CCL2 (+) or (–) cells was then assessed, and the entire area of synovial tissue section was examined. The intensity of a signal from the control slide labeled with isotype Ab was comparable to the intensity of the background in the experimental samples.

Generation of bone marrow-derived macrophages (BMDMs)

Bone marrow-derived macrophages (BMDMs) were cultured as previously described (2). Briefly, bone marrow (BM) cells collected from femurs and tibias of NFAT5 (+/+) and NFAT5 (+/–) mice were cultured in BM-differentiation media, which is IMDM (Gibco Life Technologies) supplemented with 10% FBS (Gibco Life Technologies), 30% L929-cell conditioned medium, 100 units/ml of penicillin-streptomycin (Gibco Life Technologies), and MEM NEAA (Gibco Life Technologies). After 3 days, the media were replaced with fresh BM-differentiation media. On day 7, the differentiated BMDMs were used for experiments.

Induction of mBSA/IL-1-induced arthritis

To investigate direct effect of NFAT5 in macrophages on the progression of chronic arthritis, splenic macrophages ($5X10^5$ cells/mouse) of wild-type NFAT5 (+/+) or

NFAT5 (+/–) mice were injected intra-articularly with mBSA (200 μg) to investigate the effect of NFAT5 levels in macrophages on the progression of chronic arthritis; the splenic macrophages were isolated using anti-F4/80 microbeads (catalog 8802-6863) 24 hours after intraperitoneal injection of LPS (10 mg/kg) into the mice. The mice were sacrificed on day 7 following mBSA administration. In some experiments, to determine the role of NFAT5-CCL2 axis in macrophage infiltration and arthritis progression *in vivo,* recombinant mouse CCL2 (2 μg, R&D Systems) was injected into one of the knee joints of NFAT5 (+/–) mice on days 1, 3 and 5.

The knee joints of arthritic mice were excised, fixed in neutral buffered formalin (Sigma-Aldrich), decalcified, and paraffinized. Immunohistochemical staining of the joint tissues was performed using the Macrophage Marker (RM0029-11H3) Ab (Santa Cruz Biotechnology), anti-cleaved caspase-3 Ab (clone D3E9, Cell Signaling Technology), and anti-CCL2 Ab (ab25124, Abcam) overnight at 4^{o}C. Each slide was then incubated with biotinylated anti-rat IgG (Vector Laboratories) or anti-rabbit IgG (Vector Laboratories) followed by treatment with 3’3-diaminobenzidine tetrahydrochloride (Vector Laboratories). The slides were counterstained with haematoxylin (Sigma-Aldrich). The experimental procedures were approved by the Institutional Animal Care and Use Committee of the Catholic University of Korea (IACUC). Each experiment was performed multiple times using different donor cells.

Histological assessment of arthritis

Arthritis was assessed by histological examination as described previously with some modifications (3, 4). Knee joints were exposed by removing the overlying skin and were subsequently excised. Limbs were fixed in 10% formalin solution (Sigma-

Aldrich) overnight and decalcified in 5% formic acid for 10~14 days. A transverse cut was made when the bones were fully decalcified and processed for paraffin embedding. Tissues were cut into 4 μm sections and stained with hematoxylin and eosin (Sigma-Aldrich) to assess joint pathology. The degree of synovial hyperplasia, inflammation, and bone destruction in the joints were determined using a standard scoring protocol (1=weak, 2=moderate, and 3=severe) (5). Synovial hyperplasia has been defined as the increased number of fibroblast-like synoviocytes in the lining layer of the synovium (3, 4). The severity of inflammation was graded according to the extent of inflammatory cell infiltration into the infra-patellar fat pads, joint capsule, and the area adjacent to the periosteal sheath. Evaluation of bone destruction was based on loss of cartilage matrix, disruption and loss of cartilage surface, and the extent and depth of the subchondral bone erosion.

The histological severity of cartilage destruction, was also assessed by loss of Safranin O staining (6) using a scale of 0 to 6, where 0=normal cartilage, with a perfectly regular surface and homogeneous staining, 1=slight loss of staining in the most superficial part of the articular cartilage but an intact surface, 2=the same as grade 1 but with mild surface irregularities of the articular surface, 3=moderate to severe articular surface irregularities associated with clefts in the superficial half of the cartilage thickness, 4=clefts extending deep into the deep half of the cartilage or severe surface irregularities associated with loss of staining, 5=complete loss of non-calcified cartilage without damage to calcified cartilage, and 6=erosion of calcified cartilage and exposure of subchondral bone. Scoring was based on the most severe histological changes observed within each cartilage section.

Statistical analysis

The DEGs were identified based on an integrative statistical method reported previously (7, 8). Briefly, 1) two independent tests, the T-test and the $\log_2$ median-ratio test, were performed. 2) For each test, an empirical distribution of the null hypothesis that the means of the genes are not different was estimated by random permutations of the samples; 3) for each gene, *P*-value was computed by performing a two-tailed test using the empirical distributions; and 4) the false discovery rate (FDR) was computed by combining the two sets of *P*-values from the individual tests using Stouffer's method (7, 8). The DEGs were selected with FDR < 0.05 and fold change ≥ 1.5. To determine the cutoff value of fold change, we computed fold changes of randomly permuted samples, fitted a Gaussian distribution to the random fold changes, and then calculated the 2.5 percentile (i.e. the level of significance α=0.05 for a two-tailed test), resulting in 1.5. Finally, to identify cellular processes enriched by the DEGs, functional enrichment analysis was performed using the DAVID software (8, 9). The cellular processes enriched by the DEGs were identified as the GO biological processes with *P*-values <0.05. Data are expressed as the mean ± standard deviation (SD). Comparisons of the numerical data between groups were performed by the paired or unpaired Mann-Whitney *U* test. *P*-values <0.05 were considered statistically significant.

Supplemental Figures

Supplemental Figure S1. Expression of Bcl-2, Bax, and cleaved caspase-8 in NFAT5-deficient macrophages. (**A-D**) NFAT5 knock down (KD) and control RAW 264.7 macrophages were cultured overnight; the media were then changed to fresh RPMI 1640 in the presence (**A**, **C** and **D**) or absence (**B**) of 10% FBS. The cells were then treated with 1 mM SNP (**A**), 0.5 μg/ml CHX (**C**) or 100 nM TG (**D**). Protein lysates were collected at the indicated time points to evaluate the expressions of anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax or cleaved caspase-8 by Western blot analysis. Detection of β-actin was served as a loading control. Data are the representative of three independent experiments.

Supplemental Figure S2. Increased susceptibility to apoptotic death in NFAT5-deficient primary macrophages. Thioglycollate peritoneal macrophages ($1X10^5$ cells) from wild-type NFAT5 (+/+) versus NFAT5 (+/–) mice were cultured in RPMI 1640 containing 10% FBS in the presence of SNP (150 μM) for 12 hours or with TG (125 nM) and CHX (0.12 μg/ml) for 24 hours. The degree of apoptotic death was determined by flow cytometry for Annexin V and PI. Data are representative of two independent experiments.

Supplemental Figure S3. NFAT5 regulates the survival and proliferation of bone marrow-derived macrophages (BMDMs). (**A**) Increased cell death in BMDMs from NFAT5 (+/–) mice. The degree of apoptosis was assessed in BMDMs from wild-type NFAT5 (+/+) versus NFAT5 (+/–) mice for 24 hours after treatment with CHX (0.16 μg/ml), SNP (16 mM) or TG (500 nM) using the MTT assay. Data are the mean and SD

of three independent experiments. *P<0.05 versus NFAT5 (+/+) mice. (**B** and **C**) Decreased proliferation of BMDMs by NFAT5 deficiency. BMDMs of NFAT5 (+/+) versus NFAT5 (+/–) mice were cultured in IMDM supplemented with 10% FBS and 30% L929-cell conditioned medium for 8 days. Cell proliferation was determined by trypan blue exclusion (**B**) and MTT assay (C). Data are the mean and SD of three independent experiments. *P<0.05 versus NFAT5 (+/+) mice.

Supplemental Figure S4. (**A**) CCL2-centered sub-network (left) illustrates interactions between *CCL2* and its first interactors in CD14 (+) macrophages in RA synovial fluid (RA-SF). Node color and size represent the fold change and degree, respectively. Edge thickness represents edge betweenness centrality. Cytoscape version 3.2 was used to visualize this network. (**B**). CD44 and OLR1 expression in CCL2 (+) versus CCL2 (–) macrophages in synovial CD14 (+) cells of RA patients (n=6), as assessed by flow cytometry. Samples from the same patients are connected by lines. Mφ=macrophages. *P<0.05.

Supplemental Figure S5. Immunofluorescent staining for NFAT5 and CCL2 in RA synovial tissue using Alexa594-conjugated anti-CD14 (green), anti-NFAT5 Ab (red), and Alexa647-conjugated anti-CCL2 Ab (orange). A representative is shown in the left panel. In the right panel, CCL2 positivity was estimated in NFAT5 (+) versus NFAT5 (–) macrophages using the ImageJ program and is presented as the mean (±SD) percentage of four different RA synovia from three RA patients. *P<0.05, **P<0.01.

Supplemental Figure S6. CCL2 expression levels in NFAT5-deficient RAW 264.7 macrophages, RA peripheral CD14 (+) cells, and RA synovial CD14 (+) cells. (**A**) Decreased CCL2 production by NFAT5-deficient RAW 264.7 macrophages. NFAT5-deficient RAW 264.7 cells ($1X10^5$ cells) were generated by stable transfection of NFAT5 shRNA (NFAT5 KD) or by stable transduction of the NFAT5 decoy. The cells were stimulated with LPS (10 and 100 ng/ml) for 12 hours and CCL2 concentrations in the culture supernatants were assessed by ELISA. Data are mean±SD of three independent experiments. **P*<0.01, ***P*<0.001. (**B**) Comparison of CCL2 expression in CD14 (+) cells between the peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) of RA patients obtained simultaneously (n=5). Samples from the same patients are connected by lines. **P*<0.05

Supplemental Tables

Supplemental Table S1. Sequences of the gene specific primers used for real-time PCR.

Supplemental Table S2. *CCL2* and its first interactors are listed with their fold changes and the degree of interaction. The genes were ranked by their degree of interaction and were chosen from a network consisting of the DEGs in RA-SF macrophages.

Supplemental References

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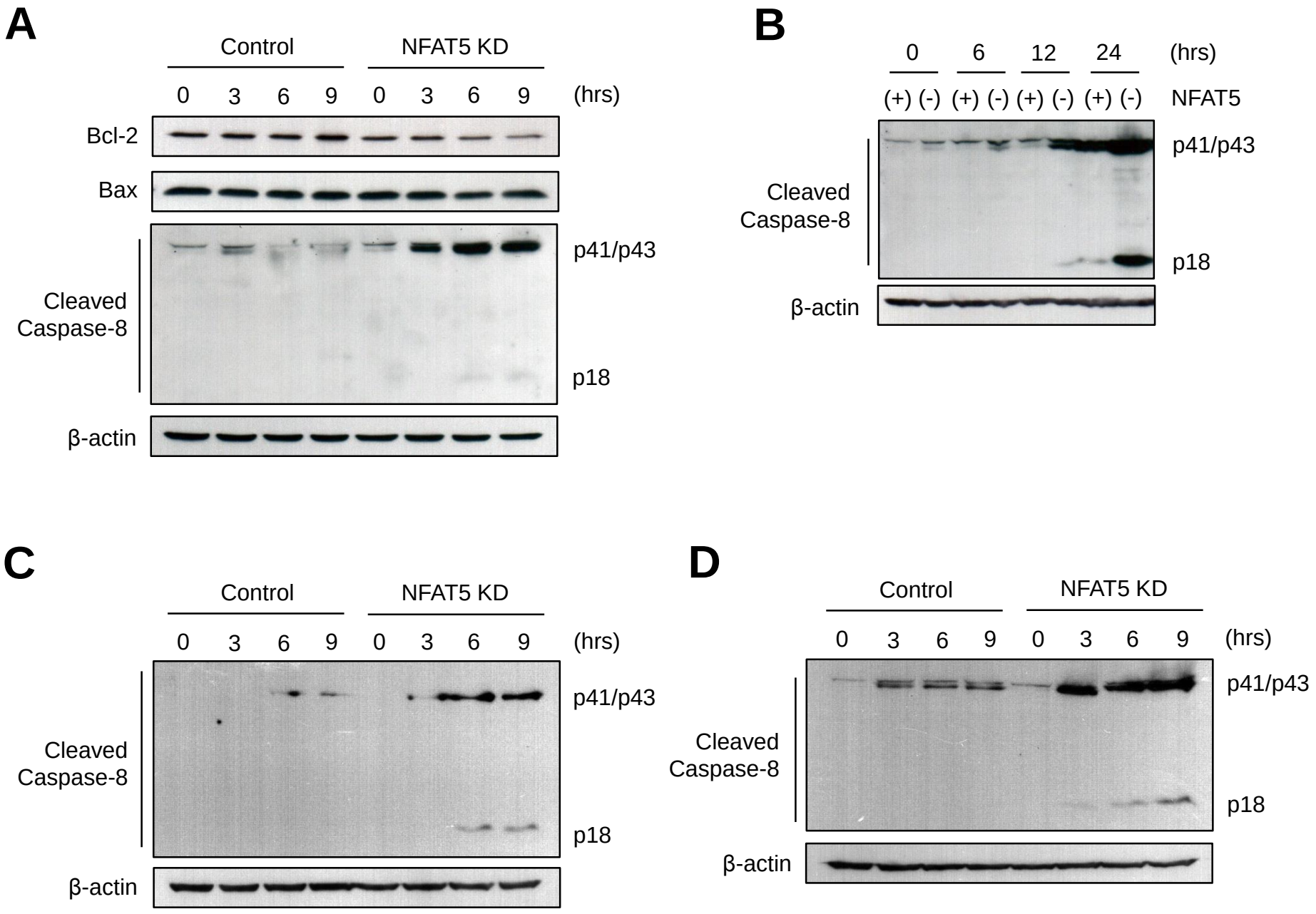
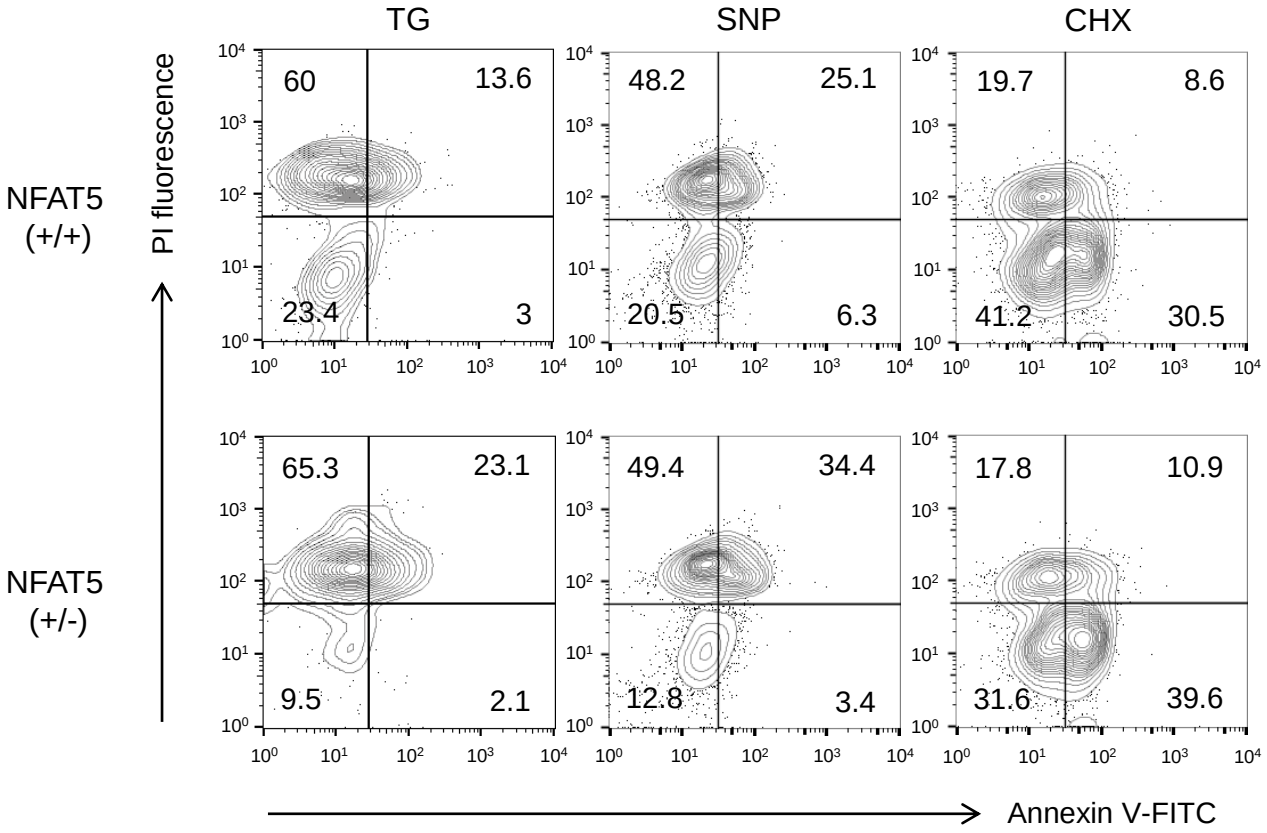
ysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 2009;4(1):44-57.

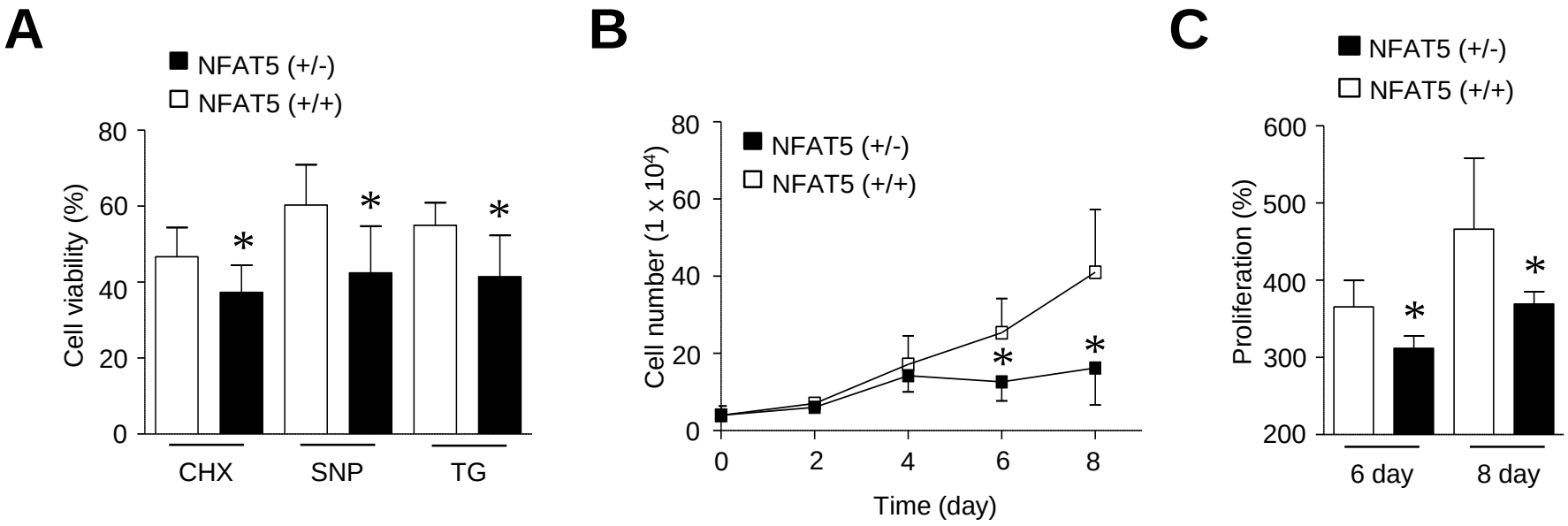
Supplemental Figure 1



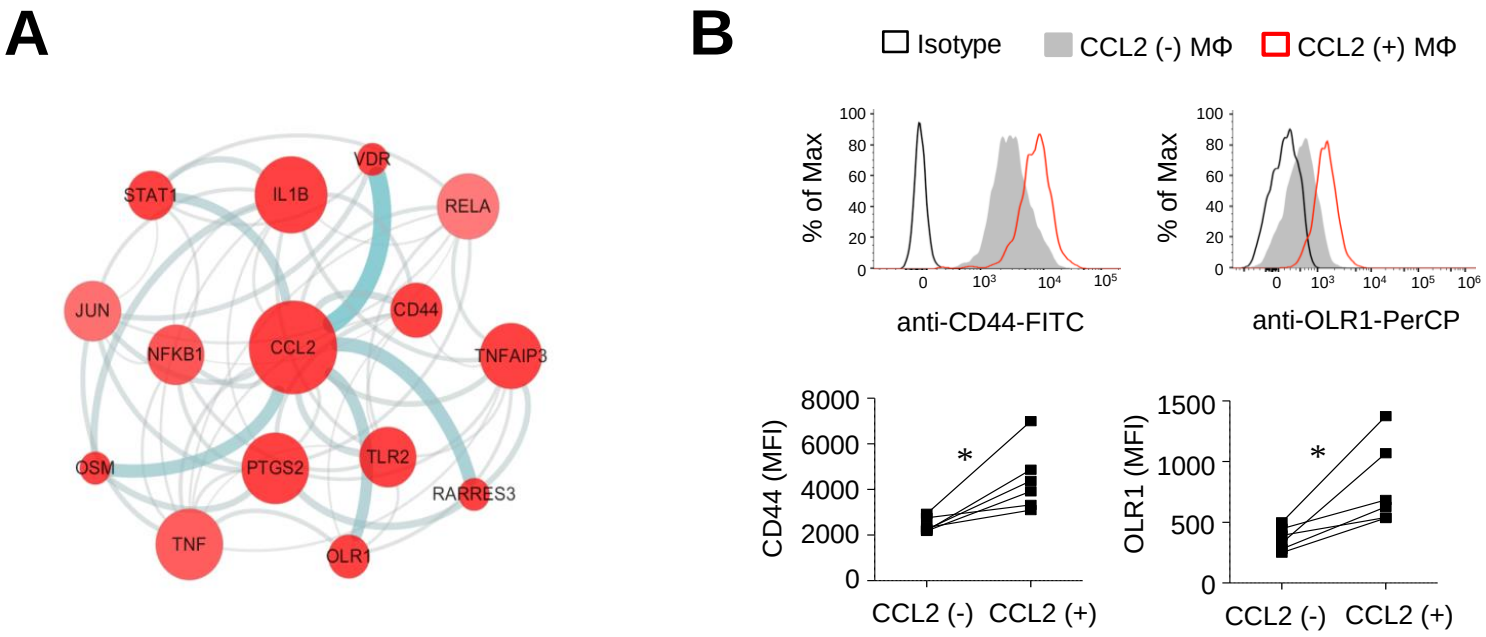
Supplemental Figure 2



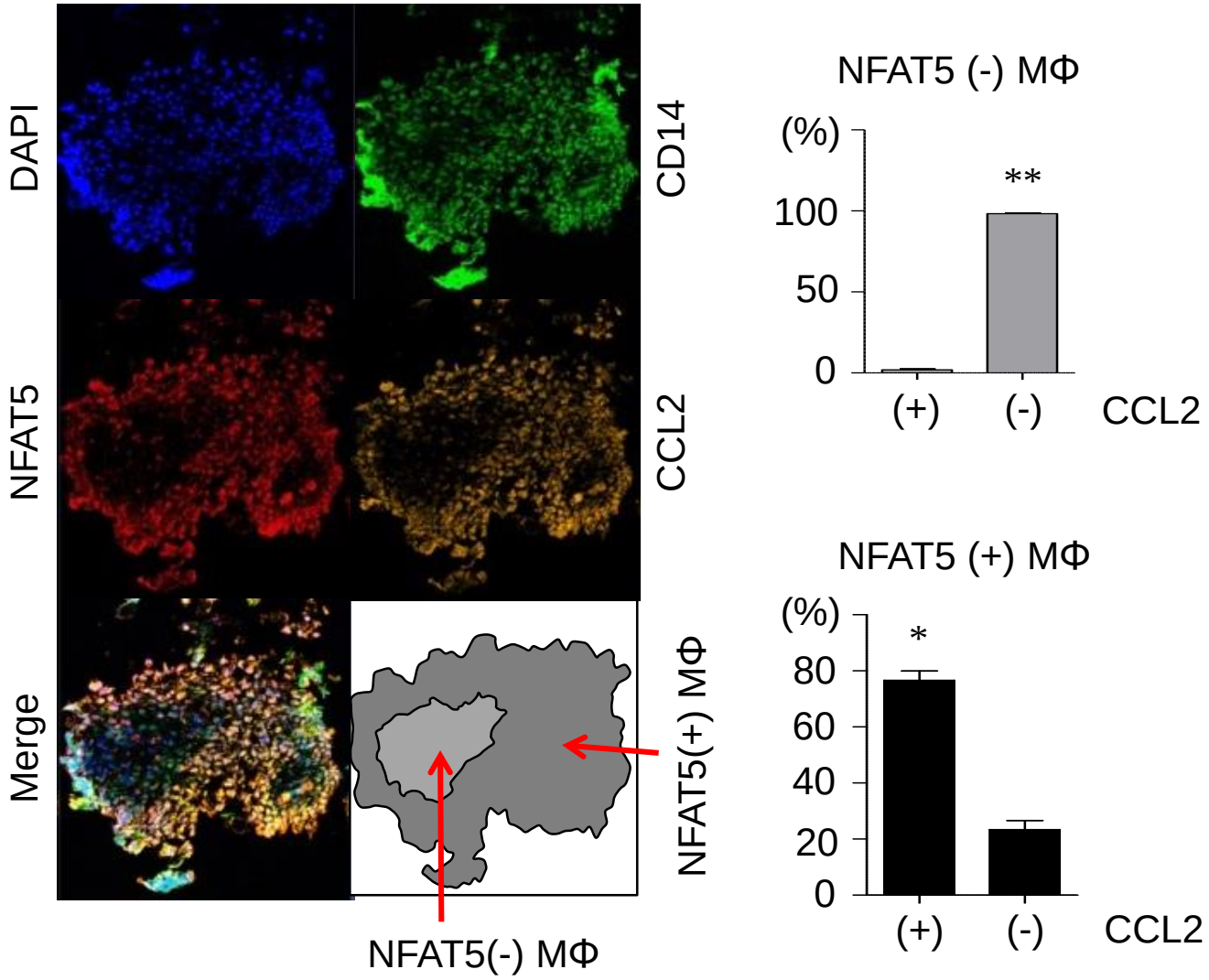
Supplemental Figure 3



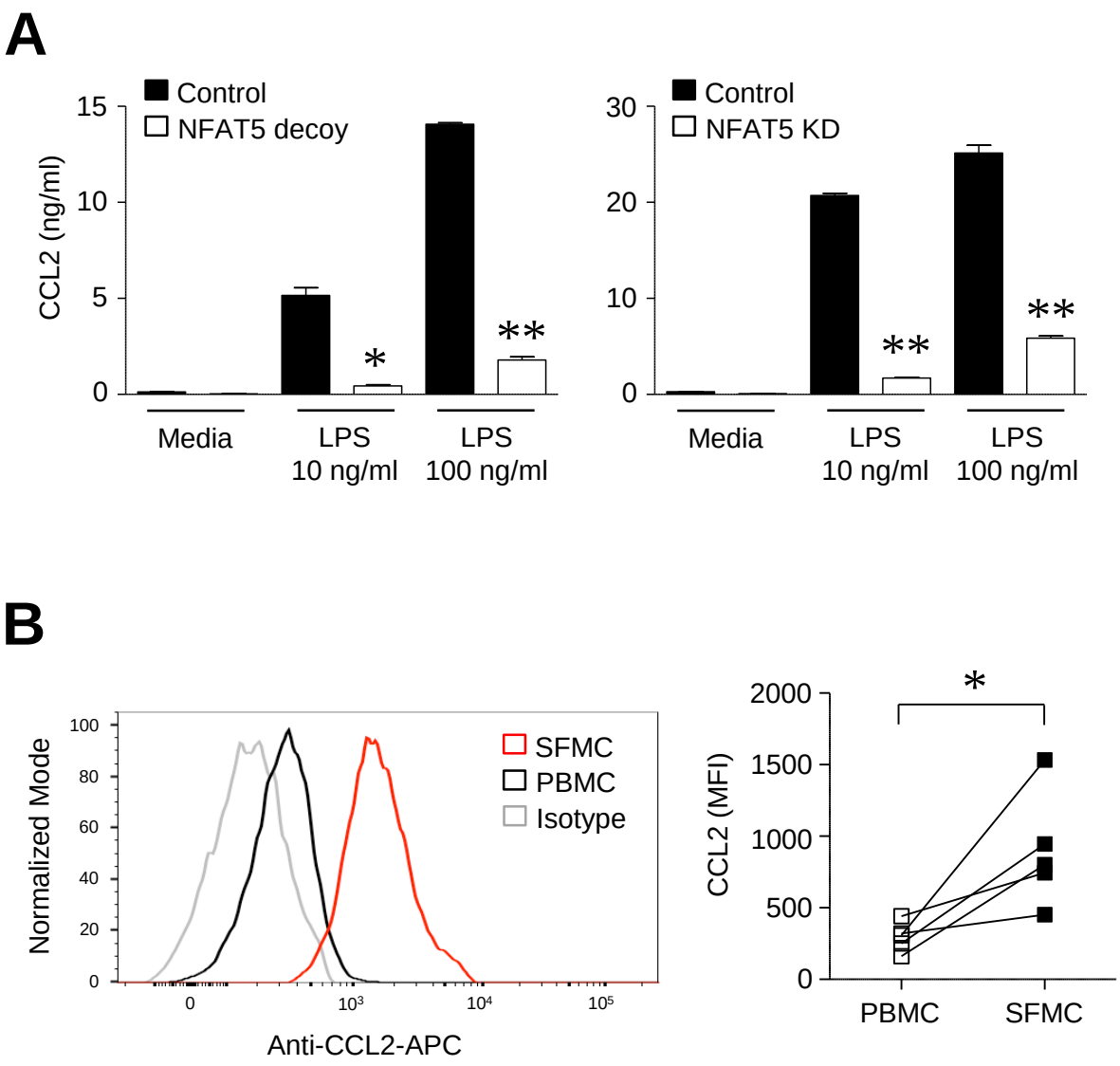
Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6



Supplemental Table 1

Origin	Gene	Forward	Reverse
Mouse	*Cd74*	ATTTGCTGCCTTCTCCTCAA	TGAGCAAGGAACCTGAAAGG
	Sod2	CACCACACCCTGCTCTTTTT	TTCAATTCCCAGCAAACACA
	Ccl2	CAGCAAGATGATCCCAATGA	TCTGGACCCATTCCTTCTTG
	Bop1	GTGGCAGCTATGACAGCAAA	CGGATGCAAAGAGTGGGTAT
	Cdkn2d	TCCATTGAAGAAGGGAGTGG	CACCAAAAGGGGTGAGAAAA
	Ran	AGGTTGTGCAGGAGAGGAAA	CGGAATGGCTTGCTGTAAAT
	Nfat5	CAGAGCTGCAGTATGTG	CCTCTGCTTTGGATTTCG
	Stk3	GCAAAACGCAACACTGTAATAGG	AGCCCTCATCGGATGTATATCAG
	Fas	GCGGGTTCGTGAAACTGATAA	GCAAAATGGGCCTCCTTGATA
	Dap3	TCCAGAAGTTGGACCCGAG	CTTCACCTGCATCATGTACCG
	Tiam1	GAAGATGGAGTAATACTGGTCCC	CAGAAGGCACTGTAGAGCTTG
	Gapdh	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT
Human	*CD74*	ACAAACCAAGTCGGAACAGC	TAGCTTTTGGCCACTTCCTG
	SOD2	TGTCACCCAGTGGTTTTTGT	TTGCCTTTACTGTGCAGGTG
	CCL2	AAGCAGAAGTGGGTTCAGGA	GGGGAAAGCTAGGGGAAAAT
	BOP1	CAAGAAGCTGATGCCCAACT	CAAACCACACCAGCTTGCTA
	CDKN2D	TCTTGTTTCTCCTGCCCACT	TGTCCAACACACCAAAAGGA
	RAN	TGTGTGGCAACAAAGTGGAT	TTCCTAGCAAGCCAGAGGAA
	NFAT5	CCAGAAGTCATTTGCCTGGT	GATTCCAAGCCCACTCTTCA
	STK3	CGATGTTGGAATCCGACTTGG	GTCTTTGTACTTGTGGTGAGGTT
	FAS	AGATTGTGTGATGAAGGACATGG	TGTTGCTGGTGAGTGTGCATT
	DAP3	CTTGCCTGATGGTAAGGAAACC	TGCAGAAGATCCCGACAATTTTT
	APAF1	GTCACCATACATGGAATGGCA	CTGATCCAACCGTGTGCAAA
	TIAM1	ATGACGCTACATATTTGGCTGAG	ACCCAAGATTTCTTCGTTGCTT
	GAPDH	CAGCCTCAAGATCATCAGCA	TGTGGTCATGAGTCCTTCCA

Supplemental Table 2

Symbol	Log2 Fold Change	Degree
RELA	0.69	36
NFKB1	0.88	31
TNF	0.84	31
IL1B	1.82	27
TNFAIP3	1.72	26
STAT1	1.91	25
JUN	0.74	23
PTGS2	1.15	20
CD44	1.40	17
CCL2	**1.22**	**14**
TLR2	1.11	14
VDR	1.05	9
OLR1	2.77	6