Identification of mechanism and role of succinate on colitis and immune cells

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This certifies that the Master's Thesis
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I Seul Park

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ABSTRACT

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Inflammatory bowel disease (IBD) is a chronic and inappropriate inflammatory response of the gastrointestinal tract. Succinate, well known as a metabolic intermediate of the tricarboxylic acid cycle, increases in the inflammatory lesion of IBD patients. Succinate can act as a signaling ligand binding to succinate receptor 1 (SUCNR1). Macrophage is an important innate cell of immunopathology in autoimmune diseases such as IBD because intestinal macrophage promotes or inhibits IBD pathogenesis according to M1 and M2 polarized phenotype. Macrophage can be divided into two phenotypes, M1 and M2. It is known that M1 macrophage makes pro-inflammatory cytokine that can induce inflammation, and M2 macrophage makes anti-inflammation cytokine that can promote wound healing.

We found that *Sucnr1*-deficient mice were protected against dextran sulfate sodium (DSS)-induced colitis compared to wild type mice. Administration of DSS to *Sucnr1*-deficient mice showed the attenuated a loss of body weight, a shortening in colon length, and an increase of the disease activity index. Moreover, bone-marrow-derived macrophages from *Sucnr1*-deficient mice showed lower pro-inflammatory markers.

On the other hand, macrophages treated with succinate showed the characteristic of anti-inflammatory macrophages with increased CD206. This is consistent with the alleviation of DSS-induced colitis in recipient mice receiving macrophages from succinate pretreatment peritoneal cavity cells (PCCs) during transfer experiments. Transfer of peritoneal cavity macrophage was conducted to determine the difference of the macrophage's role between WT and *Sucnr1*-deficient.

These conflicting results may be due to the complex network of the immune system, and further research is needed depending on the cell types. The results of the microarray showed that some genes related to the immune mechanism were downregulated. It would be valuable to study the relationship between immune cells and Sucnr1 as a further study. Therefore, I propose that SUCNR1 signaling exacerbates colitis in the DSS-induced colitis model, and SUCNR1 might be a potential target for IBD treatment.

Key words: inflammatory bowel disease (IBD), metabolite, succinate, GPR1 (SUCNR1), macrophage

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I. INTRODUCTION

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic and inappropriate inflammation disorder that occurs in the gastrointestinal tract.[1] CD is observed in any part of the digestive tract, from the mouth to the anus,[2] while UC is observed in the large intestine.[3] IBD is a disease with a high incidence in industrialized countries.[4] Recently, the incidence of IBD has increased not only in South Korea but also worldwide. In the United States, the number rose from about 1.8 million in 1999 to about 3 million in 2015.[5,6] In Korea, according to National health insurance statistics, the patient number increased from 43,000 in 2012 to about 62,000. Although the pathogenesis of IBD is incompletely understood, it is considered that there are interactions between genetic factors, microbiota, and immune system.[7] Mutations of genes, such as *NOD2* and *ATG16L1*, are known as the pathogenesis of IBD, and microbiota dysbiosis is observed in IBD patients. Also, various immune cells such as Th17 are involved as immune system factor.[8-10]

Succinate concentrations were increased in the inflammatory lesion and serum of IBD patients[11-13], also succinate levels in the fecal samples were 4 times higher compared with healthy control.[11] Furthermore, succinate levels in

the breast milk of IBD patients were higher than healthy mothers at 3 and 6 months postpartum.[14] Increased succinate levels in human IBD samples correlate with dextran sulfate sodium (DSS)-induced colitis model.[15] However, succinate levels in urine were different from serum and plasma metabolites. A few metabolites such as citrate, succinate, and methanol were decreased in IBD patients as compared with controls.[3] Based on the results that succinate is accumulated in IBD patients, I decided to focus on the function of succinate in colitis.

Succinate was purified by a German chemist in 1546, and it became a topic in biologic studies.[16] The role of succinate is not only involved in metabolites but also acts as alarmins and signal molecules: (1) Succinate is an intermediate metabolite of the tricarboxylic acid cycle (TCA cycle) in mitochondria. It is converted to fumarate by the enzyme succinate dehydrogenase and plays an important role in electron delivery in the respiratory chain.[16] (2) Succinate is a kind of alarmins, which are endogenous danger signals, and it is released during injury and inflammatory events. However, it is not completely understood how this immune sensing pathway contributes to the development of inflammatory responses.[17] (3) As a signal molecule, succinate can promote angiogenesis, and acts as an epigenetic hacker to inhibit DNA.[16]

G-Protein Coupled Receptor 91 (GPR91), known as orphan G-protein coupled receptor or succinate receptor 1 (SUCNR1), was found to be a succinate receptor. SUCNR1 is activated by succinate[18], and SUCNR1 is known to go through the G_i/G_o pathway and the G_q pathway.[18,19] Although SUCNR1 is expressed on many cell types, the mechanism of how succinate regulates metabolism and mitochondrial stress has not yet been revealed.[20] Therefore, succinate and its receptor, as a signal molecule, was used to investigate the effect on inflammation.

Macrophage which is an innate immune cell is involved in not only the primary response to the pathogen but also the coordination with the adaptive immune response, tissue homeostasis, and resolution.[21] There are 2 types of immune responses: Innate and adaptive. Innate immunities begin a rapid and effective response against infection or injury and result in appropriate responses such as tolerance or inflammatory response. Inflammatory monocytes are also recruited by infection or injury sites and differentiated into macrophages.[22,23] These reactions are beneficial initially, but subsequent inflammatory reactions are accompanied by tissue damage. Therefore, if it is not controlled, chronic inflammatory disease occurs.[24,25] M1 macrophage, called the 'classical macrophage', produces pro-inflammatory cytokines (i.e. IL-6, IL-1β, IFN-γ) that induce type 1 response and acute inflammation. On the other hand, M2 macrophage is called an 'alternative macrophage' and secretes anti-inflammatory cytokine (i.e. IL-4, IL-10) to promote type 2 response and tissue repair.[1,23,26] The macrophage is an important immune cell of immunopathology in autoimmune diseases such as IBD[24] because intestinal macrophage promotes or inhibits IBD pathogenesis according to M1 and M2 polarized phenotype.

There are 2 central objectives of this study: (1) the observation of phenotype in *Sucnr1*-deficient mouse and investigation of sensitivities on colitis, (2) to determine the effect of succinate on the macrophage.

II. MATERIALS AND METHODS

1. Cell culture

A. THP-1

The human monocytic cell line THP-1 was grown at 37°C in 5% CO_2 in RPMI 1640 media (HyClone™, LOGAN, UT, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Younginfrontier, Seoul, Korea) and 1% penicillin-streptomycin solution (GenDEPOT, Katy, TX, USA). THP-1 monocytes were differentiated into macrophages by 24 hours incubation with 150 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, USA) in conditioned RPMI 1640. Macrophages were polarized to M1 macrophages by incubation with 20 ng/ml of recombinant human interferon-gamma (IFN-γ) (*E. coli*-derived) (PromoCell, Heidelberg. Germany) and 100 ng/ml of lipopolysaccharides (LPS) (Sigma-Aldrich, St. Louis, MO, USA). For polarization to M2 macrophages, macrophages were incubated with 20 ng/ml of recombinant human interleukin 4 (IL-4) (*E. coli*-derived) (PromoCell, Heidelberg, Germany) and 20 ng/ml of recombinant human interleukin 13 (IL-13) (PromoCell, Heidelberg. Germany).

B. Bone marrow-derived macrophage (BMDM)

BMDMs were isolated from adult C57BL/6 mice or *Sucnr1*-deficient mice. After euthanasia, hind legs (femurs and tibia) were removed and stripped of muscles. Bone marrow cells were flushed out of the hind legs using 5 ml of Dulbecco's phosphate-buffered saline (DPBS) (GenDEPOT, Katy, TX, USA) into 100 mm Petri dishes with a 26-gauge needle. Red blood cells were lysed in RBC lysis buffer. To eliminate adherent cells, single-cell suspensions of bone marrow precursors were incubated in complete

Dulbecco's Modified Eagle Medium (DMEM) (HyClone™, LOGAN, UT, USA) containing 10% FBS and 1% penicillin-streptomycin (GenDEPOT, Katy, TX, USA) for 4 hours at 37°C. After 4 hours, the supernatants were collected and resuspended in complete DMEM containing 20% L-929 cells supernatant, which secrete macrophage colony-stimulating factor (M-CSF) required for monocyte differentiation into macrophages. BMDMs were differentiated with L-929 conditioned media in Petri dishes for 6 days.

On day 6, BMDMs were mechanically removed from the petri dish using the scraper. To promote polarization into M1 or M2 macrophages, BMDMs were incubated with 20 ng/ml of recombinant mouse interferon-γ (Ifn-γ) (Gibco®, Grand Island, NY, USA) and 100 ng/ml of LPS or with 20 ng/ml of recombinant mouse interleukin 4 (Il-4) (Gibco®, Grand Island, NY, USA) and 20 ng/ml of recombinant mouse interleukin 13 (Il-13) (Gibco®, Grand Island, NY, USA), respectively, for 24 hours, in the presence or absence of succinate (200 μM).

C. L-929 conditioned media

L-929 cells, which produce macrophage colony-stimulation factor (M-CSF), conditioned medium was used for differentiation of bone marrow-derived macrophages (BMDMs). L-929 cells were resuspended in DMEM supplemented with 10% fetal bovine serum (YounginFrontier, Seoul, Korea) and 1% penicillin-streptomycin solution (GenDEPOT, Katy, TX, USA) and incubated at 37°C in a 5% CO_2 atmosphere for 5 days. At confluence, supernatants were filtered through a 0.45 μm filter (Pall, Washington, NY, USA) and stored 10 ml aliquots frozen at -20°C. BMDMs were incubated in 20% (vol/vol) L-929 conditioned media.

D. Succinate solutions

To prepare succinate stock, succinate (Sigma-Aldrich, Missouri, USA) was dissolved in 20 ml of DPBS and 240 ml of distilled water. To make a neutral solution (pH 7.4) for experiments, a succinate solution was titrated with sodium hydroxide (NaOH).

2. Mouse model

A. Wild type mouse

Eight-week-old or weighted 20–25 g C57BL/6 (Orient, Seongnam, South Korea) mice were used. The mice were maintained in a 12 hours light/ 12 hours dark cycle at 22°C under specific pathogen-free (SPF) conditions. All experiments were performed under the approval of the Yonsei University Institutional Animal Care and Use Committee (IACUC) and Accreditation of Laboratory Animal Care International (AAALAC).

B. Knock out mouse

Sucnr1-deficient mice of the C57BL/6J background were generated from ToolGen (Seoul, South Korea). The schematic diagram of the knockout (KO) strategy for *Sucnr1*-deficient was shown in Figure 1.

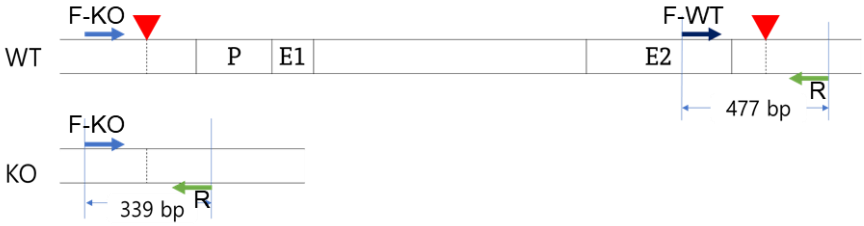


Figure 1. Strategy for *Sucnr1*-deficient. F-KO, KO Forward primer; F-WT, WT Forward primer; and R, Reverse common primer. The red triangle (▼) is the site of cleavage for deletion.

The deletion of *Sucnr1* was verified by genotyping PCR and DNA sequencing. The primers used to PCR were followed; WT forward (CAT TGG TCA GGT CGA TAA GCG), KO forward (ACA GCC TTT CAG CAG CAC AC), and common reverse (GGC AGC ACA ACC ATC AGA GA). PCR condition was followed; 95°C, 3 minutes; 34 cycles at 95°C, 30 seconds; 65°C, 30 seconds; 72°C, 60 seconds; and 72°C, 5 minutes; a final hold at 4°C. The amplificon of PCR product was checked by 2% gel electrophoresis (WT; 477 bp, KO; 339 bp).

C. Dextran sulfate sodium (DSS)-induced colitis mouse

For the induction of chemical-induced colitis, mice were administered 2% (wt/vol) DSS (MP Bio, California, USA) in drinking water for 7 days. Mice were monitored daily about bodyweight loss, stool consistency, and bleeding in the stool or at the anus. Mice were sacrificed on day 8. After sacrifice mice, entire colons, from the cecum to the anus, were removed and measured the entire colon length. The parts of the colon were cut into 3–4 pieces and stored at -70°C for subsequent RNA isolation, Western blot analysis. For histological analysis, segments of distal colons were fixed in 10% formalin (Biosesang, Seongnam, Korea) overnight at 4°C.

D. Transfer experiments

To conduct macrophage transfer experiments, mice were intraperitoneally injected for 3 days with succinate or DPBS before isolated. Peritoneal cavity cells (PCCs) were isolated from WT or *Sucnr1*-deficient mice. Macrophages were isolated using BD FACS Aria II (BD Biosciences, CA, USA) and identified by expression of $F4/80^{+}$. 2×10^{5} isolated cells were intraperitoneally injected in 200 μl Hank's balanced salt solution (HBSS) (ThermoFisher, Carlsbad, CA, USA) on day 0 treated with 2% (wt/vol) DSS until day 6. These mice were sacrificed on day 8.

3. Evaluation of inflammation

A. Disease activity index (DAI)

Mice were monitored daily about the average body weight loss, stool consistency, and bleeding in the stool or at the anus. Based on these findings, the DAI scores, which ranged from 0 to 4, were calculated. The parameters outlined in the following table.

Table 1. DAI scoring system

Score	***A. Body weight change***	***B. Stool consistency***	***C. Bleeding***
0	none	normal	normal
1	1~5%		
2	5~10%	loose	slightly bleeding
3	10~20%		
4	>20%	diarrhea	bloody

* Weight loss was calculated to day 0 as 100%.

* The calculated DAI score = (A+B+C)/3.

B. Periodic acid-Schiff (PAS) staining score

Colon segments were embedded in paraffin and stained periodic acid-Schiff (PAS) staining. Images were obtained using a light microscope (Olympus BX41; Olympus Optical, Tokyo, Japan). Quantification with integrated density (IntDen) of goblet cells of colon tissue from mice were assessed by software Image J (National Institutes of Health, Bethesda, MD, USA). Histopathologic scores were calculated in the following table.[27]

Table 2. Scoring scheme for colonic inflammation

A. Inflammatory cell infiltrate		
Severity	*Extent*	***Score***
Mild	Mucosa	1
Moderate	Mucosa & submucosa	2
Marked	Transmural	3

B. Intestinal architecture		
Epithelial changes	*Mucosal architecture*	***Score***
Focal erosions		1
Erosions	± Focal ulceration	2
	Extended ulceration ± granulation tissue ± pseudopolyps	3

* The histopathological score (0–6)= A + B.

4. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) and Ribospin™ II (GeneAll, Seoul, Korea) as per the manufacturer's instruction. 500 ng – 1 μg of RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; ThermoFisher Inc., Carlsbad, USA), according to the manufacturer's protocol. qPCR was carried out using Power SYBR™ Green PCR Master Mix (Applied Biosystems; ThermoFisher Inc., Carlsbad, USA), and the primer on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). β-actin was used as the endogenous control gene for normalization.

The thermal cycles were: 95°C for 10 minutes for holding; 45 cycles at 95°C for 15 seconds; 60°C for 30 seconds; 72°C for 40 seconds for cycling stage; a final hold at 4°C.

Quantitative analysis was performed using the relative comparative method using the following equation: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$ and relative gene expression = $2^{-\Delta Ct}$

Primers used for real-time PCR were as follows table:

Table 3. Primers used for real-time RT-PCR

Organism: Human		
Gene	**Primer 1 (5′-3′)**	**Primer 2 (5′-3′)**
ACTB	CTCTTCCAGCCTTCCTTCCTG	CAGCACTGTGTTGGCGTACAG
TNF	ATCTTCTCGAACCCCGAGTG	GGGTTTGCTACAACATGGGC
IL1B	AGCTACGAATCTCCGACCAC	CGTTATCCCATGTGTCGAAGAA
CD274	TGGCATTTGCTGAACGCATTT	TGCAGCCAGGTCTAATTGTTTT
TGFβ	AAGGACCTCGGCTGGAAGTG	CCGGGTTATGCTGGTTGTA
IL10	AGGGAAGAAATCGATGACAGC	TCAAGGCGCATGTGAACTC
MRC1	TTCGGACACCCATCGGAATTT	CACAAGCGCTGCGTGGAT

Table 4. Primers used for real-time RT-PCR

Organism: Mouse		
Gene	**Primer 1 (5′-3′)**	**Primer 2 (5′-3′)**
Actb	AGTGTGACGTTGACATCCGT	TGCTAGGAGCCAGAGCAGTA
Sucnr1	TCACTGTGGTGTTTGGCTACCT	CCCTTATCATTGGCATAACTCTTTATC
Tnf	CAAAGGGAGAGTGGTCAGGT	ATTGCACCTCAGGGAAGAGT
Nos2	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
Tgfβ	TAATGGTGGACCGCAACAACGC	GACGGAATACAGGGCTTTCG
Atg5	TGTGCTTCGAGATGTGTGGTT	GTCAAATAGCTGACTCTTGGCAA
Becn1	ATGGAGGGGTCTAAGGCGTC	TCCTCTCCTGAGTTAGCCTCT
Il1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Slc26a6	GCTTCCATAGCCTCATCCTG	TTCAATCTCCCGGAATCAC
Cd274	GCTCCAAAGGACTTGTACGTG	TGATCTGAAGGGCAGCATTTC
Il10	TGAATTCCCTGGGTGAGAAG	TCACTCTTCACCTGCTCCACT
Mrc1	CAGGTGTGGGCTCAGGTAGT	TGTGGTGAGCTGAAAGGTGA

5. Enzyme-linked immunosorbent assay (ELISA)

The concentration of Il-10 in the culture medium of BMDMs was measured by mouse ELISA kit (BioLegend, San Diego, CA, USA) according to manufacturer's instruction. The supernatants were harvested and stored at -70°C. The optical density at 450 nm was detected using a VersaMax ELISA Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) and analyzed with SoftMax Pro 6.3 (Molecular Devices).

6. Microarray

Colon tissue was incubated in RNAlater RNA stabilization reagent (QIAGEN, Netherlands) overnight at 4°C and total RNA was extracted from the colon tissue of WT and Sucnr1-deficient mice using TRizol Reagent according to manufacturer's instruction. RNA quality was confirmed by 1% denaturing agarose gel electrophoresis and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and Quantus Fluorometer (Thermo Fisher, Waltham, MA, USA). The agarose gel components are as follows: 0.5 g agarose (Bio-Rad, CA, USA) + 72 ml distilled water + 10 ml 10× MOPS buffer (Biosesang, Korea) + 18 ml Formalin (Biosesang, Korea). Microarray was conducted with GeneChip® Mouse Gene 2.0 ST Array (Applied Biosystems™, MA, USA) by Macrogen (Macrogen, Seoul, Korea). Data were normalized using Affymetrix Power Tools (APT) software. Differentially Expressed Gene (DEG) was defined by |fold changes (f.c.)| $\geq$ 1.5 with False Discovery Rate (FDR) < 0.05. Gene-Enrichment and Functional Annotation analysis were performed using g:Profiler tool (https://biit.cs.ut.ee/gprofiler/gost) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway.

7. Flow cytometry

Immune cells isolated from spleen or peritoneal cavity cells were stained fluorophore-conjugated antibodies in the following tables. The cells were quantitated with a FACSVerse analyzer (BD Biosciences, CA, USA) with BD FACSuite (BD Biosciences, CA, USA) and analyzed using FlowJo software (BD Biosciences, CA, USA).

Table 5. The list of antibodies used for peritoneal cavity cells

Antigen	Clone	Fluorophore	Supplier
CD11b	M1/70	eFluor® 450	eBioscience
CD206	C068C2	FITC	Biolegend
TLR4	UT41	PE	eBioscience
F4/80	BM8	PerCP-Cyanine5.5	eBioscience
IL-10	JES5-16E3	APC	BD bioscience

Table 6. The list of antibodies used for splenocytes

Antigen	Clone	Fluorophore	Supplier
GATA3	L50-823	BV421	BD bioscience
CD3	500A2	V500	BD bioscience
CD4	GK1.5	FITC	eBioscience
Foxp3	150D/E4	PE	eBioscience
CD25	PC61.5	PerCP-Cyanine5.5	eBioscience
T-bet	eBio4B10	PE-Cy7	eBioscience
RORγt	B2D	APC	eBioscience

8. Reagents

RPMI 1640 media (HyClone™, LOGAN, UT, USA)
Dulbecco's Modified Eagle Medium (HyClone™, LOGAN, UT, USA)
Dulbecco's phosphate-buffered saline (GenDEPOT, Katy, TX, USA)
Hank's Balanced Salt Solution (ThermoFisher, CA, USA)
Fetal bovine serum (Younginfrontier, Seoul, Korea)
Penicillin-streptomycin solution (GenDEPOT, Katy, TX, USA)
Lipopolysaccharide (LPS) (Sigma-Aldrich, MO, USA)
Phorbol myristate acetate (PMA) (Sigma-Aldrich, MO, USA)
Recombinant human interferon gamma (PromoCell, Heidelberg, Germany)
Recombinant human interleukin 4 (PromoCell, Heidelberg, Germany)
Recombinant human interleukin 13 (PromoCell, Heidelberg, Germany)
Recombinant mouse interferon-γ (Gibco®, Grand Island, NY, USA)
Recombinant mouse interleukin 13 (Gibco®, Grand Island, NY, USA)
Recombinant mouse interleukin 4 (Gibco®, Grand Island, NY, USA)
RBC Lysis Buffer (10X) (BioLegend, San Diego, CA, USA)
Succinate (Sigma-Aldrich, MO, USA)
Dextran Sulfate Sodium Salt (DSS) (MP Bio, California, USA)
Power SYBR™ Green PCR Master Mix (Applied biosystems, MA, USA)
100 bp DNA Ladder (Bioneer, Daejeon, Korea)
Certified Molecular Biology Agarose (Bio-Rad, CA, USA)
ChamelGreen I Nucleic acid gel stain (Dawinbio, Hanam, Korea)
10× TBE (iCell, Hanam, Korea)
RNAlater RNA Stabilization Reagent (QIAGEN, Venlo, Netherlands)
TRIzol reagent (Invitrogen, Carlsbad, USA)
Ribospin™ II (GeneAll, Seoul, Korea)
High Capacity cDNA Reverse Transcription Kit (ThermoFisher, CA, USA)
Power SYBR™ Green PCR Master Mix (ThermoFisher, CA, USA)

ELISA MAX™ Deluxe Set Mouse IL-10 (BioLegend, San Diego, CA, USA)
Stop Solution (BioLegend, San Diego, CA, USA)
10× Mops buffer (Biosesang, Seongnam, Korea)
10% Neutral Buffered Formalin (Biosesang, Seongnam, Korea)
Diethyl pyrocarbonate (DPEC) (Biosesang, Seongnam, Korea)

9. Analysis

The results were examined using GraphPad Prism Software (GraphPad Inc., San Diego, CA, USA). Statistical significance was evaluated either by unpaired student's *t*-test or analysis of variance (ANOVA). Experimental results were expressed as mean values and standard error of the mean (S.E.M). The results that P-value < 0.05 were regarded to be significant.

III. RESULTS

1. The effect of succinate on THP-1

To investigate the effect of succinate on the macrophage, THP-1 cells were used as an *in vitro* model for human monocyte. THP-1 were differentiated into macrophage using 150 nM of phorbol 12-myristate 13-acetate (PMA) for 24 hours and incubated with LPS (100 ng/ml) + IFN-γ (20 ng/ml) and IL-4 (20 ng/ml) + IL-13 (20 ng/ml) for 4 hours to polarization into M1 or M2, respectively. The succinate was treated simultaneously with polarization and then harvest RNA.

Polarization into M1 macrophages, the gene expressions of pro-inflammatory cytokine *(TNFα, IL1β)* were increased by administration with succinate. Administration with succinate to M2 polarization macrophages, the expressions of anti-inflammatory cytokines (*TGFβ, IL10*) were reduced. Interestingly, the expression of *CD206* in M2 macrophages was highly increased by succinate treatment (Figure 2).

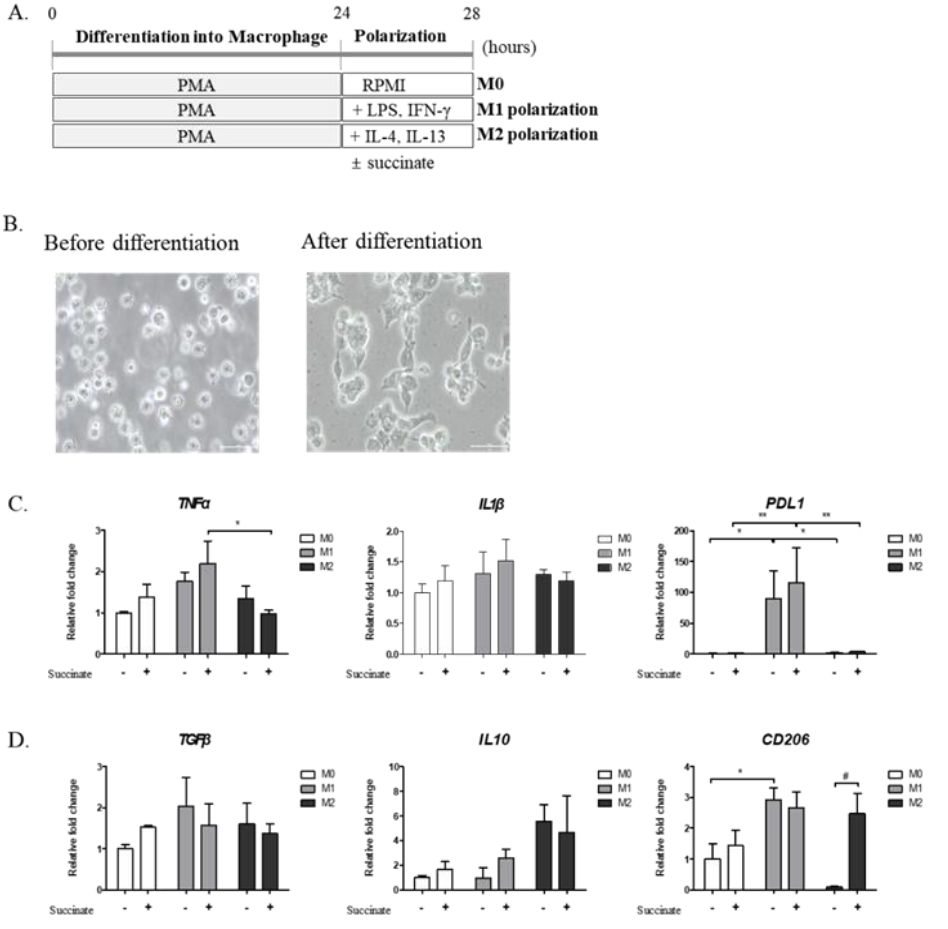


Figure 2. Gene expression after treatment of succinate in THP-1 cells. (A) Schematic representation of the differentiation and polarization protocol. After treatment with 150 nM PMA for 24 hours, differentiated THP-1 cells were incubated with cytokine to polarize into M1 or M2 macrophages, using LPS+IFN-γ or IL-4+IL-13, respectively. (B) Morphology of THP-1 cells by light microscopy. Magnification, ×200. (C) Effects of succinate (1 mM) on proinflammatory factors. (D) Effects of succinate (1 mM) on anti-inflammatory factors. Bars in graphs represent mean ± S.E.M. Significant differences in relation to polarization are shown by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ and relation to succinate treat shown by $\#p < 0.05$.

2. Generation and genotyping of *Sucnr1*-deficient mice

To elucidate the role of Sucnr1, we established *Sucnr1*-deficient mice. To define knockout, ear punching was performed and examined PCR genotyping. When performed 2% agarose gel electrophoresis after PCR genotyping, wild type (477 bp), KO (339 bp) bands for *Sucnr1* genes were located at the correct size (Figure 3-A). It was also confirmed through DNA sequencing using BLAST on NCBI (Figure 3-B).

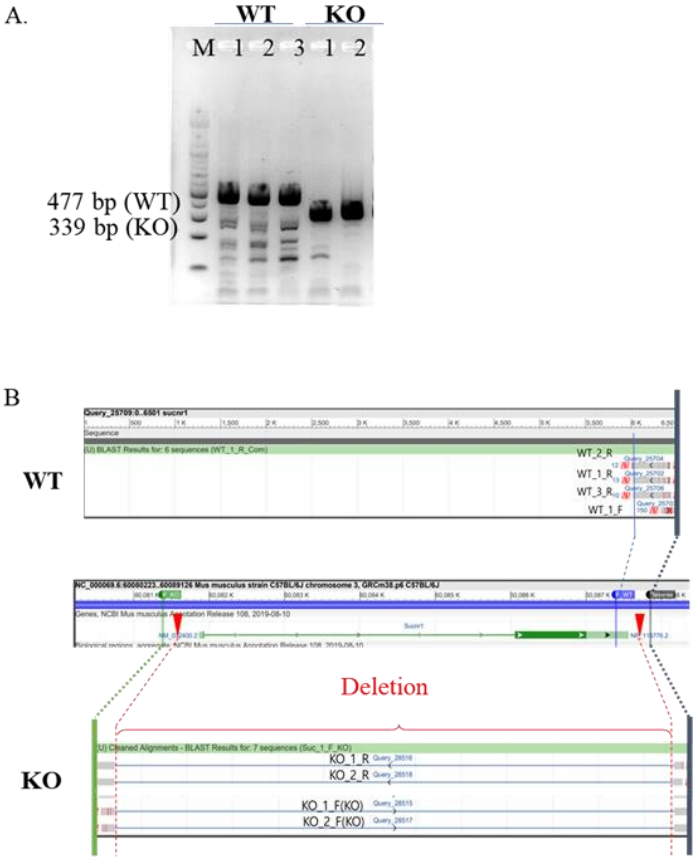


Figure 3. Identification of *Sucnr1*-deficient mice. (A) 2% agarose gel electrophoresis for genotyping PCR product. (B) Analysis of DNA sequencing of PCR products. Sequencing alignment was analyzed by BLAST on NCBI. WT, wild type; KO, *Sucnr1*-deficient.

3. The effect of succinate receptor 1 (Sucnr1) on the phenotypes of BMDMs

To investigate the effect of succinate on primary monocytes, mouse BMDMs were used. BMDMs were differentiated by L-929 conditioned media (LCM) for 6 days, and M1 and M2 macrophages polarized by LPS (100 ng/ml) + Ifn-γ (20 ng/ml) and Il-4 (20 ng/ml) + Il-13 (20 ng/ml), respectively. On day 7, the morphology of macrophages was observed under the microscope (Figure 4-A). The morphology of M1-polarized macrophages displays a round shape, while the morphology of M2-polarized macrophages displays an elongated and spindle-like cell. However, no significant morphologic change was shown after the treatment of succinate (Figure 4-B, C).

Although there was no significant change in morphology, RT-qPCR was performed to investigate the change in mRNA expression. On day 7, polarized macrophages were harvest and RT-qPCR was done. Polarization to M1 macrophages, the expression of M1 marker (*iNos*), and proinflammatory cytokine (*Tnfα, Il1β*) were reduced in *Sucnr1*-deficient BMDM. Polarization to M2 macrophages, the expression of M2 surface marker (*Cd206*) in *Sucnr1*-deficient BMDM was significantly higher than WT BMDM. The expression of *Cd206* was higher in *Sucnr1*-deficient BMDM when untreated BMDM as M0 macrophages. The genes related to autophagy (*Atg5, Beclin1*) in all populations (M0, M1, M2) were increased in *Sucnr1*-deficient BMDM (Figure 5). The mRNA expression of *Il10* was decreased in all populations in *Sucnr1*-deficient BMDM. Similarly, the concentration of Il-10 measured in BMDM media was also reduced at *Sucnr1*-deficient BMDM (Figure 6).

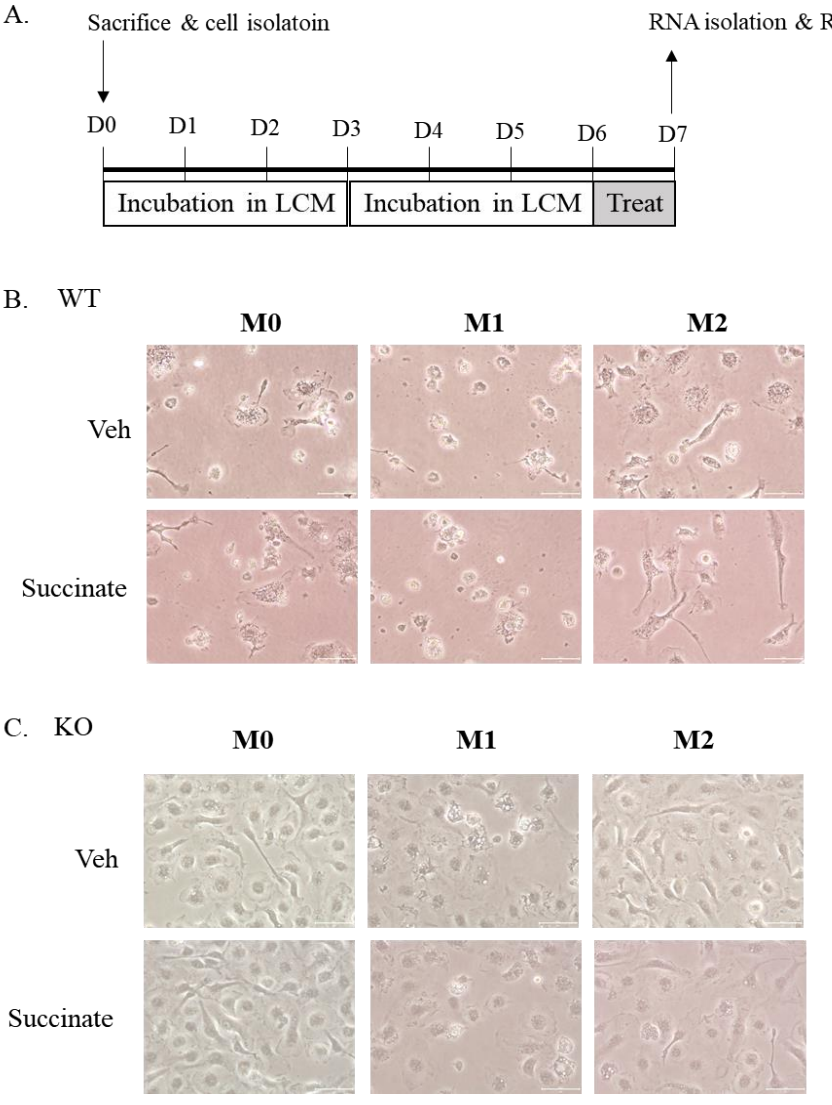


Figure 4. Differentiation and polarization of macrophages. (A) Experiment schedule of BMDM cultures. BMDMs were differentiated in L-929 conditioned media (LCM) for 6 days, and polarized by LPS (100 ng/ml) + Ifn-γ (20 ng/ml) to induce M1 and Il-4 (20 ng/ml) + Il-13 (20 ng/ml) to induce M2 for 24 hours. Each group divided into succinate treatment or not. Morphology of WT BMDM (B) and *Sucnr1*-deficient BMDM (C) were captured at ×200 magnification. M1 macrophages were round and M2 macrophages were spindle-like cells. No significant morphologic change was shown between WT and KO.

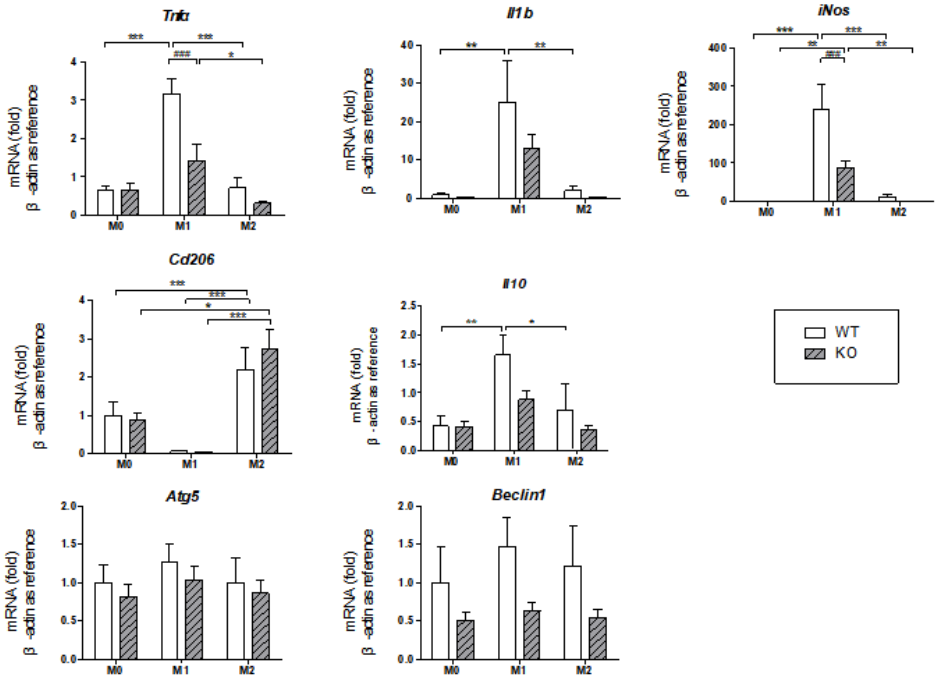


Figure 5. Gene expression respond to polarization. BMDMs were obtained from WT and *Sucnr1*-deficient mice and polarized towards M1 or M2 macrophages. Graphs show fold change of mRNA expression. (A) Pro-inflammatory factors, (B) Anti-inflammatory factors, and (C) Autophagy factors. Bars in graphs represent mean ± S.E.M. Significant differences in relation to polarization are shown by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ and significant differences from the respective group of WT BMDMs are shown by $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$.

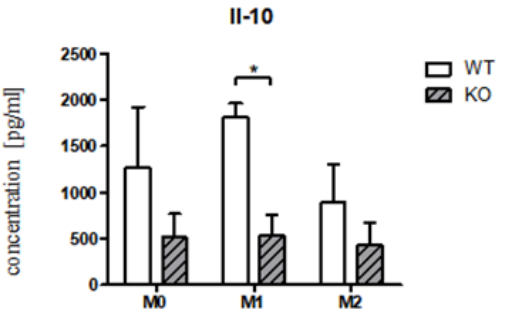


Figure 6. Concentration if Il-10 in media. ELISA assay results of Il-10 secreted by BMDMs. Bars in graphs represent mean ± S.E.M. Significant differences from WT BMDMs were shown by $*p < 0.05$.

4. Colitis was attenuated in *Sucnr1*-deficient mice.

To investigate the role of Sucnr1 in colitis, *Sucnr1*-deficient (KO) mice and wild type (WT) mice were used to investigate susceptibility to DSS-induced colitis. All mice were randomly divided into the following groups (Figure 7): water drinking WT group, water drinking KO group, DSS + WT group, DSS + KO group, and DSS + WT + intraperitoneal injected with succinate group. Colitis was induced by administration of 2% DSS in drinking water for 7 days followed by normal water for 2 days.

A.

	Condition	*Genotype*	*Injection*	*n*
Group 1	Water	WT	-	8
Group 2	Water	KO	-	4
Group 3	2% DSS	WT	-	8
Group 4	2% DSS	KO	-	9
Group 5	2% DSS	WT	succinate	3

B.

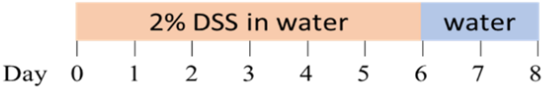
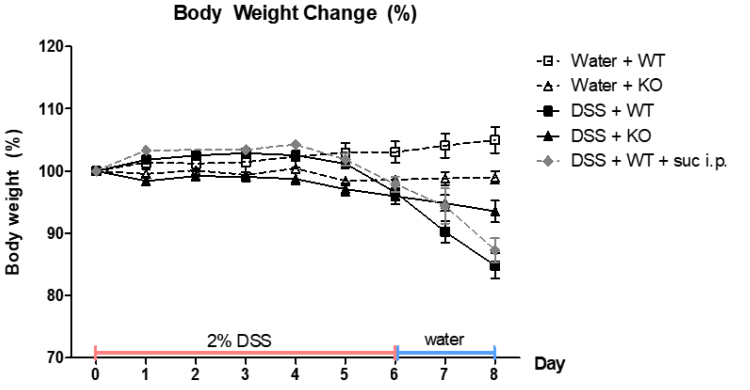


Figure 7. Scheme of the experimental design. (A) Experimental groups. WT, wild type; KO, *Sucnr1*-deficient. (B) Schedule for the experiment.

Changes in body weight and DAI score during the experiment were shown in Figure 8. DSS + KO group showed a reduction of body weight loss, DAI score severity compared to DSS + WT group. DSS-induced colitis in *Sucnr1*-deficient mice was also shown a protective effect in colon length shortening and histopathology in Figure 9.

To compare the effect of Sucnr1 on the DSS-induced colitis model, I performed periodic acid-Schiff (PAS) staining and histopathological scoring to the quantification of colitis. The PAS staining result and the histopathological score of the colon showed that inflammation decreased significantly in the DSS+KO group compared with the DSS+WT group (Figure 10). Colon tissue pieces from distal parts of the colon were used to compare mRNA expression by RT-qPCR (Figure 11). *Sucnr1* (as known as *GPR91*) was significantly increased in DSS-induced colitis. Also, in *Sucnr1*-deficient mice, *Muc2* expression level was higher than DSS-induced colitis in WT, whereas inflammatory factors such as *Tnfα*, *Il1β*, and *Il15* were decreased. The results suggest that *Sucnr1*-deficient can reduce colitis.

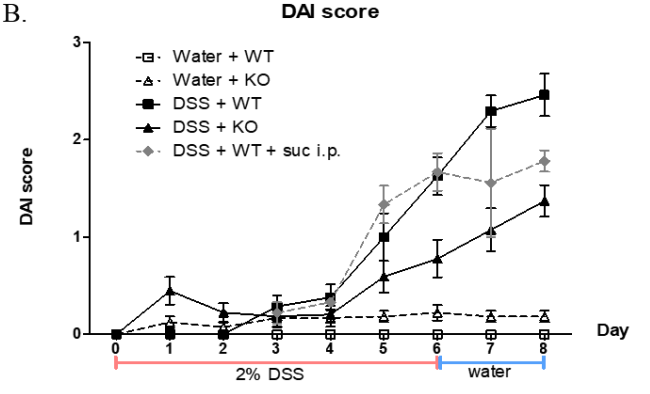
A.



2-way ANOVA compare to Water+WT group

Group \ Day	0	1	2	3	4	5	6	7	8
Water + WT									
Water + KO	ns	ns	ns	ns	ns	ns	ns	ns	ns
DSS + WT	ns	ns	ns	ns	ns	ns	*	***	***
DSS + KO	ns	ns	ns	ns	ns	**	**	**	***
DSS + WT + suc	ns	ns	ns	ns	ns	ns	ns	*	***

B.

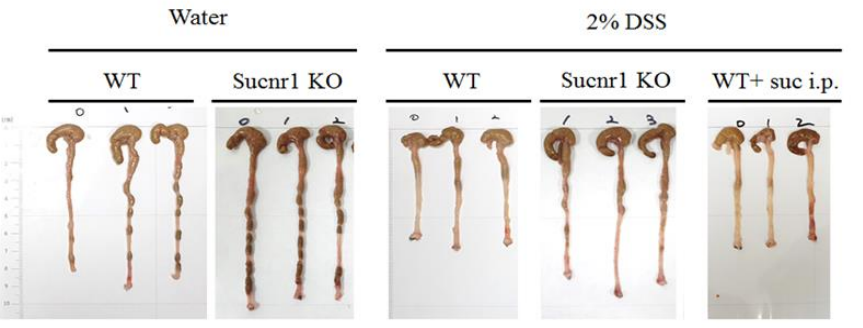


2-way ANOVA compare to Water+WT group

Group \ Day	0	1	2	3	4	5	6	7	8
Water + WT									
Water + KO	ns	ns	ns	ns	ns	ns	ns	ns	ns
DSS + WT	ns	ns	ns	**	ns	**	***	***	***
DSS + KO	ns	ns	ns	ns	ns	ns	*	**	***
DSS + WT + suc	ns	ns	ns	**	ns	**	***	**	***

Figure 8. Severity of colitis was attenuated in *Sucnr1*-deficient mice. (A) Bodyweight change (%) and (B) disease activity index (DAI) scores during the administration of 2% DSS in drinking water followed by normal water. Bars in graphs represent mean ± S.E.M. Significant differences from Water+WT were shown by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

A.



B.

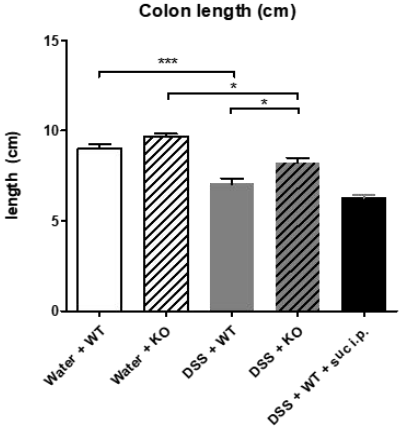


Figure 9. Colon morphology and colon length. (A) Representative images of colons from WT and *Sucnr1*-deficient mice after sacrificed. (B) Measurement of colon length. Bars in graphs represent mean ± S.E.M. Significant difference from Water+WT was shown by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

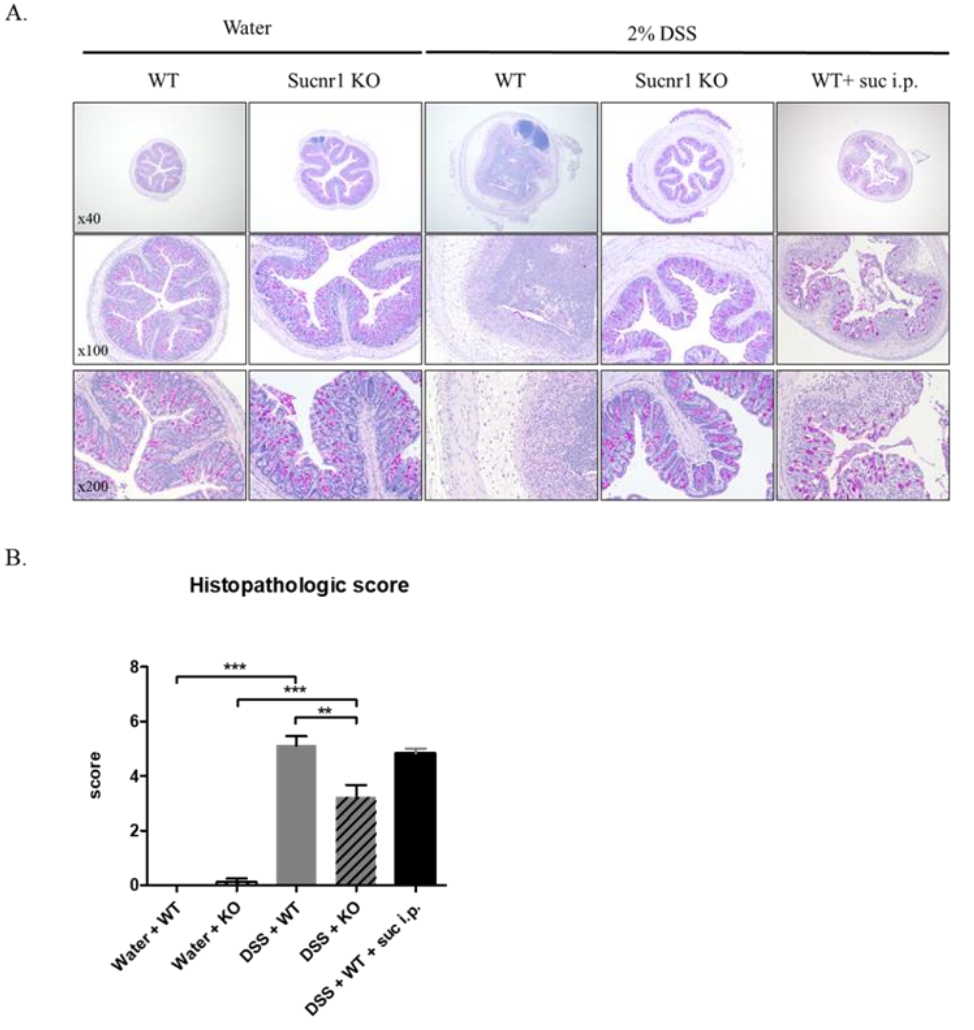


Figure 10. PAS staining and quantification of colitis. (A) Section from colon tissue stained with PAS. Original magnification, ×40, ×100, and ×200. (B) Histopathologic scores of colon tissue from mice. Bars in graphs represent mean ± S.E.M. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with the Water+WT group.

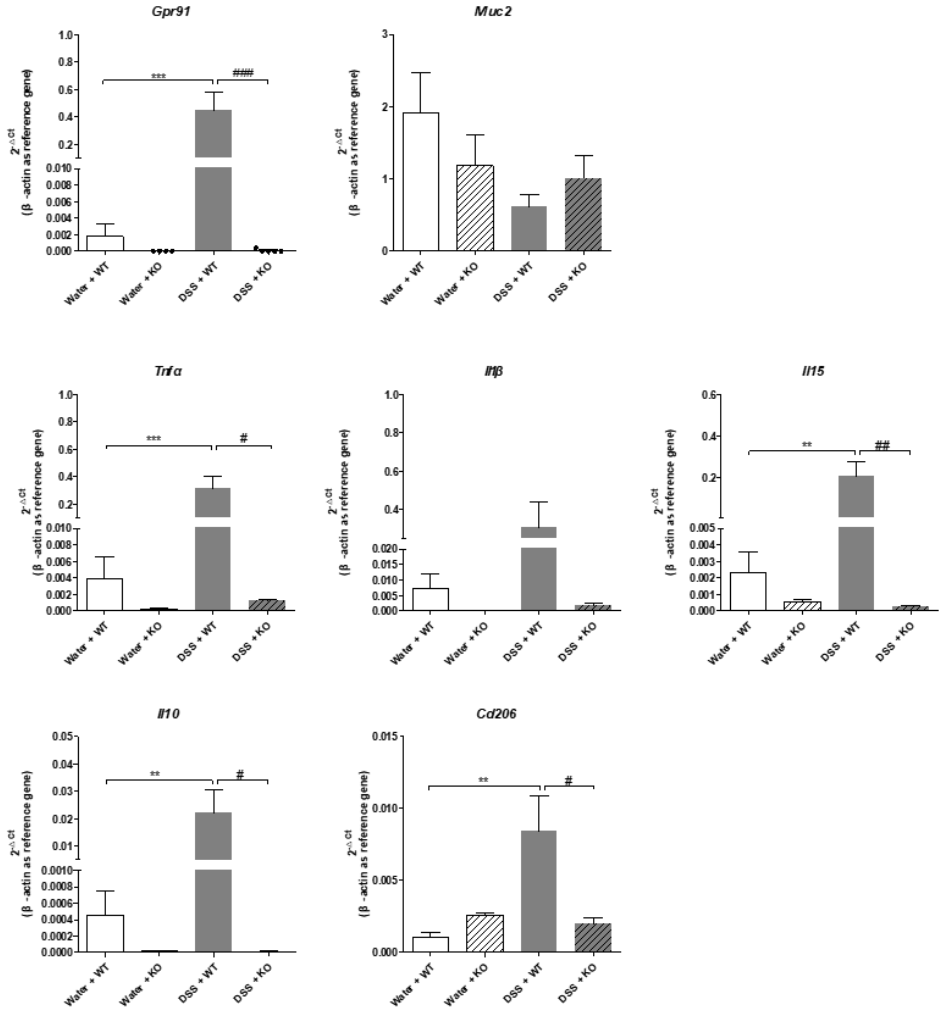


Figure 11. mRNA expression in colon tissues. Bars in graphs represent mean ± S.E.M. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 compared within WT and #*p* < 0.05, ##*p* < 0.01 compared within DSS-induced colitis.

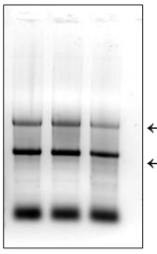
5. Microarray comparison of gene expression changes between WT and *Sucnr1*-deficient

To confirm the quality of RNA extracted from mice colon tissue (n=3), RNA electrophoresis was performed and 18S and 28S rRNA bands were visible. To measure RNA integrity and calculate an RNA Integrity Number (RIN),[28] 2100 Bioanalyzer was used. RNA degradation did not occur because 18S and 28S rRNA bands were detected by RNA electrophoresis and RIN > 7 was calculated by 2100 Bioanalyzer (Figure 12).

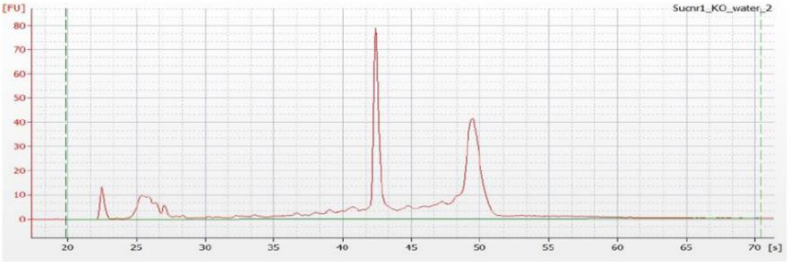
Next step, to identify gene expression changes between WT and KO, microarray analysis was performed. The Volume plot of expression level indicates the probe with the expression difference between two groups depending on the volume (strength) of the signal. The top five probes with high volume while satisfying the significant cut-off were marked with red dots (Figure 13). Among them, *Muc2* was downregulated in *Sucnr1*-deficient (Figure 13-B), consistent with the results of the mRNA expression data (Figure 11). Furthermore, downregulated genes with fold change < -10 (fc < -10) in *Sucnr1*-deficient was extracted and visualized using g:Profiler.[29] g:GOSt was used to perform functional enrichment analysis. 9 genes were downregulated with fc < -10 in *Sucnr1*-deficient (Figure 14). Upregulated genes with fold change > 10 were shown in Table 7.

To analyze how enriched the DEG set of a KEGG pathway was performed (Table 8). Pathways with raw *p*-value were under 0.05 were selected, and then visualized the value of false discovery rate (FDR) on the heat map and number of unique genes (Number of SigGenes) among the DEG on the bar charts. The high number of the unique genes associated KEGG pathways were followed: metabolic pathways, olfactory transduction, pancreatic secretion and pathways in cancer.

A. Mouse colon tissue



B.



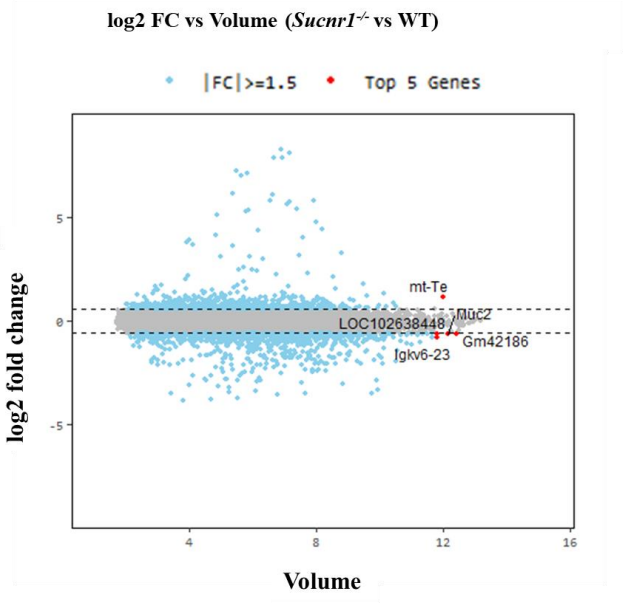
C.

Sample Name	Purity (A260/A280)	Purity (A260/A230)	Conc. (ng/ul)	Volume (ul)	Total Amount	RIN	rRNA ratio
Wild type	1.83	2.19	729.1	20	14.58	7.5	1.3
Sucnr1$^{-/-}$	1.67	2.21	297.4	17	5.06	8.7	1.2

* RIN (RNA integrity number) was used to check total RNA integrity

Figure 12. Confirm the quality of RNA before microarray. (A) RNA electrophoresis results with 18S and 28S rRNA bands. Representative gel images of RNA from *Sucnr1*-deficient. (B) Representative image of electropherogram from *Sucnr1*-deficient samples. X-axis, size of ribosomal RNA peak; Y-axis, the fluorescence of the peak. (C) RNA integrity result from 2100 Bioanalyzer.

A.



B. **Top 5 probes with high volume with significant**

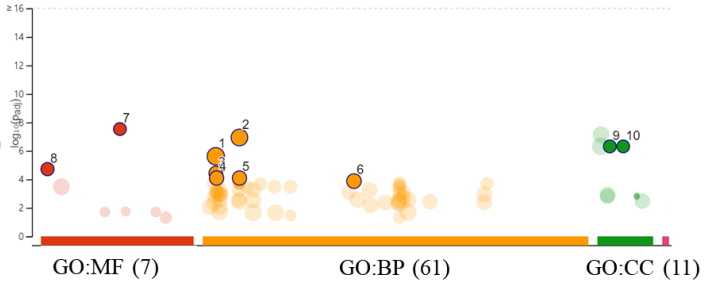
ProbeID	Gene_Symbol	mRNA Accession	*Sucnr1*$^{-/-}$/WT .fc	*Sucnr1*$^{-/-}$/WT . volume	N_WT	N_*Sucnr1*$^{-/-}$
17532651	mt-Te	ENSMUST00000082420	2.231664262	11.99472072	11.42963	12.58775
17484991	Muc2	NM_023566	-1.536640837	12.15468024	12.46852	11.84874
17358223	LOC102638448	XR_877772	-1.53725873	11.82040097	12.13465	11.51429
17314435	Gm42186	XR_880674	-1.54573178	12.42794526	12.74606	12.11777
17459423	Igkv6-23	BC091754	-1.761213734	11.81403208	12.22937	11.4128

Figure 13. Volume plot of the expression level of *Sucnr1*-deficient compared to WT mice. (A) Top 5 probes with the significant and high volume of signals were marked at Volume plot. Volume is defined as sqrt (control.mean × test.mean, log2 normalized value). X-axis, Volume; Y-axis, log2 FC. (B) Data on the top 5 probes shown in (A).

A. Downregulated genes with fc < -10

ProbeID	Gene_Symbol	mRNA Accession	*Sucnr1*$^{-/-}$/WT .fc	*Sucnr1*$^{-/-}$/WT .volume	N_WT	N_*Sucnr1*$^{-/-}$
17284667	Ighv1-85	OTTMUST00000131544	-14.539240	3.780466	6.175990	2.314110
17284660	Ighm	AF045497	-13.774041	4.656484	6.918100	3.134220
17467480	Igkv12-46	OTTMUST00000132847	-13.384395	5.507491	7.687940	3.945460
17467486	Igkv12-44	OTTMUST00000132883	-11.876518	6.775211	8.791430	5.221390
17459324	Igkv1-117	OTTMUST00000131864	-11.384271	7.665422	9.618130	6.109160
17214025	Gm25360	ENSMUST00000178786	-11.210665	9.730198	11.628550	8.141750
17284652	Ighv1-78	OTTMUST00000131426	-11.192574	3.408206	5.569930	2.085460
17467537	Igkv8-21	OTTMUST00000133067	-10.946138	5.264080	7.266050	3.813700
17284512	Ighv1-9	OTTMUST00000130801	-10.415643	6.069632	7.990950	4.610270

B.



C.

ID	Source	Term ID	Term Name	P_{adj}
1	GO:BP	GO:0002376	Immune system process	2.399×10^{-6}
2	GO:BP	GO:0006955	Immune response	1.144×10^{-7}
3	GO:BP	GO:0002377	Immunoglobulin production	3.669×10^{-5}
4	GO:BP	GO:0002440	Production of molecular mediator of immune response	8.052×10^{-5}
5	GO:BP	GO:0006959	Humoral immune response	8.052×10^{-5}
6	GO:BP	GO:0042742	Defense response to bacterium	1.325×10^{-4}
7	GO:MF	GO:0034987	Immunoglobulin receptor binding	2.936×10^{-8}
8	GO:MF	GO:0003823	Antigen binding	1.909×10^{-5}
9	GO:CC	GO:0019814	Immunoglobulin complex	4.839×10^{-7}
10	GO:CC	GO:0042571	Immunoglobulin complex, circulating	4.839×10^{-7}

Figure 14. Downregulated genes in *Sucnr1*-deficient compared to WT mice. (A) List of downregulated genes with fc < -10. (B) Annotation and visualization of gene ontology (GO) with g:Profiler. X- axis, grouped with functional terms; Y-axis *p*-value. MF, molecular function; BP, biological process; CC, cellular component. (C) List of highlighted plots on (B).

Table 7. Upregulated genes with fc > 10

Probe ID	Gene_ Symbol	mRNA Accession	*Sucnr1*$^{-/-}$/WT .fc	*Sucnr1*$^{-/-}$/WT .volume	N_ WT	N_ *Sucnr1*$^{-/-}$
17409540	Amy2a5	NM_001042711	278.292844	7.137846	4.151610	12.272070
17360751	Pnlip	NM_026925	142.873050	5.806288	3.241580	10.400170
17404350	Cpb1	NM_029706	130.734853	5.614456	3.108880	10.139380
17459769	Reg1	NM_009042	70.118343	5.372132	3.119550	9.251270
17513149	Ctrb1	NM_025583	57.332449	7.904055	5.505760	11.347040
17456918	Cpa1	NM_025350	54.138762	7.162593	4.840360	10.598950
17360770	Pnliprp1	NM_018874	50.500298	7.081582	4.796680	10.454900
17383381	Cel	NM_009885	42.218693	7.376434	5.155110	10.554920
17543785	Xist	NR_001463	41.357297	5.865359	3.765690	9.135760
17306098	Rnase1	NM_011271	39.801475	5.790518	3.713790	9.028540
17459760	Reg2	NM_009043	35.242638	4.847315	2.916670	8.055920
17512544	Ctrl	NM_023182	27.103364	7.997708	5.964180	10.724580
17457753	Gm5409	NM_001003664	22.036298	8.186941	6.254550	10.716360
17456868	Cpa2	NM_001024698	20.387458	6.156358	4.354400	8.704010
17441160	Pla2g1b	NM_011107	17.469180	4.817508	3.177420	7.304160
17432320	Cela2a	NM_007919	16.661080	7.585507	5.823030	9.881440
17409502	Amy2b	NM_001190403	14.860904	3.974248	2.478700	6.372150
17246386	Olfr764-ps1	XM_887185	14.178864	3.890894	2.422830	6.248500
17405955	Serpini2	NM_026460	13.104315	4.104242	2.648400	6.360370
17495622	Gp2	NM_025989	12.348381	5.367111	3.851970	7.478220

* Probe ID, Affymetrix mRNA probe ID; fc, fold change; N_sample name, normalized signal.

Table 8. KEGG pathway with *p*-value < 0.05

MapID	MapName	FDR	Number_of_SigGenes
00140	Steroid hormone biosynthesis		5
00190	Oxidative phosphorylation		9
00240	Pyrimidine metabolism		6
00280	Valine, leucine and isoleucine degradation		5
00330	Arginine and proline metabolism		6
00480	Glutathione metabolism		11
00561	Glycerolipid metabolism		7
00590	Arachidonic acid metabolism		6
00830	Retinol metabolism		5
00980	Metabolism of xenobiotics by cytochrome P		4
00983	Drug metabolism - other enzymes		7
01100	Metabolic pathways		70
01524	Platinum drug resistance		8
03008	Ribosome biogenesis in eukaryotes		6
03010	Ribosome		13
03013	RNA transport		7
03018	RNA degradation		5
03030	DNA replication		9
03320	PPAR signaling pathway		6
03420	Nucleotide excision repair		5
03430	Mismatch repair		6
03440	Homologous recombination		5
03460	Fanconi anemia pathway		4
04024	cAMP signaling pathway		9
04066	HIF-1 signaling pathway		10
04080	Neuroactive ligand-receptor interaction		13
04110	Cell cycle		7
04114	Oocyte meiosis		7
04141	Protein processing in endoplasmic reticulum		10
04144	Endocytosis		8
04150	mTOR signaling pathway		8
04151	PI3K-Akt signaling pathway		11
04217	Necroptosis		7
04261	Adrenergic signaling in cardiomyocytes		6
04714	Thermogenesis		13
04722	Neurotrophin signaling pathway		6

04740	Olfactory transduction	36
04915	Estrogen signaling pathway	6
04923	Regulation of lipolysis in adipocytes	4
04924	Renin secretion	7
04972	Pancreatic secretion	19
04974	Protein digestion and absorption	12
04975	Fat digestion and absorption	9
04976	Bile secretion	5
04978	Mineral absorption	5
05010	Alzheimer disease	7
05012	Parkinson disease	6
05016	Huntington disease	9
05034	Alcoholism	10
05160	Hepatitis C	6
05162	Measles	7
05164	Influenza A	6
05168	Herpes simplex virus 1 infection	12
05200	Pathways in cancer	19
05202	Transcriptional misregulation in cancer	9
05203	Viral carcinogenesis	8
05204	Chemical carcinogenesis	8
05210	Colorectal cancer	5
05322	Systemic lupus erythematosus	12
05323	Rheumatoid arthritis	6
05418	Fluid shear stress and atherosclerosis	9

* Map ID, KEGG pathway map ID; FDR, *p*-value calibrated with the false discovery rate (FDR); Number_of_SigGenes, the number of a unique genes (Number of SigGenes) among the DEG.

* Heat map with FDR:

6. Succinate-pretreated PCCs reduces the severity of DSS-induced colitis

To investigate the effect of succinate on peritoneal cavity cells (PCCs), WT mice were injected every 4 days with succinate into intraperitoneal. On day 4, peritoneal cavity cells were isolated and analyzed by flow cytometry. $F4/80^{+}$ $Cd11b^{+}$ $Cd206^{+}$ cells were increased in mice injected with succinate in figure 15.

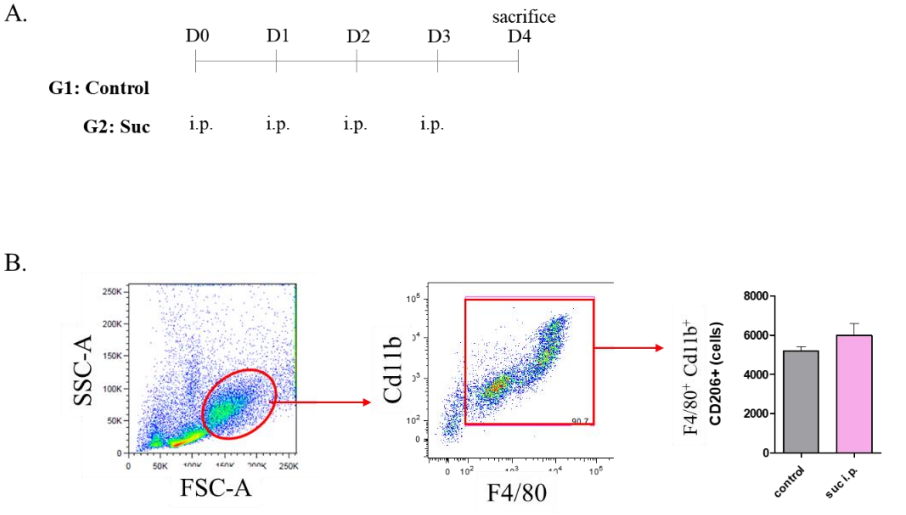
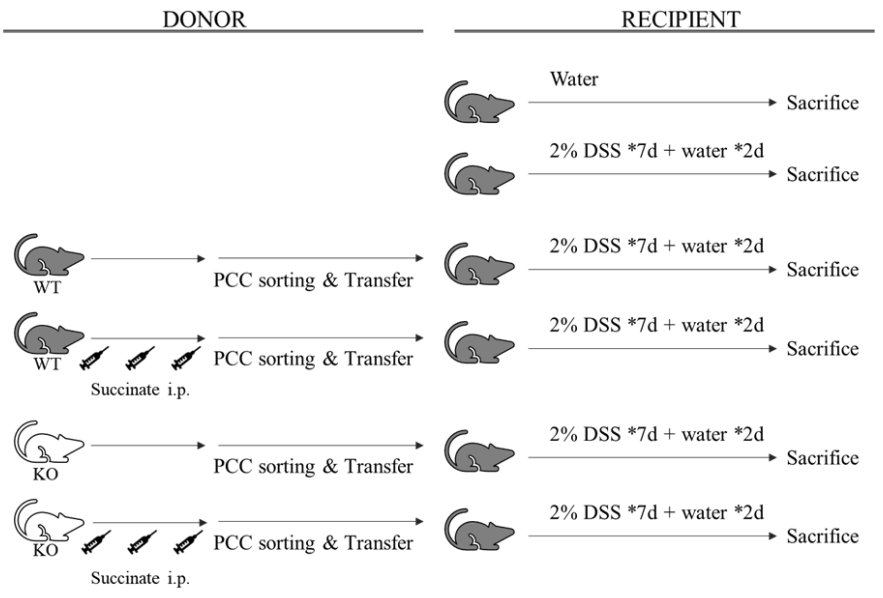


Figure 15. Succinate-pretreatment on peritoneal macrophages. (A) Timetable of the experiment. Control, injected with vehicle; Suc, injected with 40 mM of succinate into intraperitoneal. (B) Gating strategy and cell number of $F4/80^{+}$ $Cd11b^{+}$ $Cd206^{+}$.

To find out the difference of the peritoneal macrophages between WT and *Sucnr1*-deficient, and the effect of the succinate treatment, PCC transfer experiment was performed. The experimental design was shown in Figure 16. $F4/80^+$ macrophages were isolated from peritoneal cavity cells through FACS sorting and 2×10^5 of isolated macrophages in 200 μl HBSS were transferred into WT recipient mice and induced colitis using DSS. These mice were sacrificed on day 8.

Although not statistically significant, recipients of succinate-pretreatment PCCs from both WT (Suc-treat WT PCC) and *Sucnr1*-deficient (Suc-treat KO PCC), showed the attenuated a loss of body weight, severity of DAI and shortening in colon length (Figure 17). Histopathological score and goblet cell Integrated Density were reduced in recipients of succinate-pretreatment PCCs groups (Figure 18). Colitis was alleviated when receiving the succinate-pretreatment PCCs, as the frequency of $CD206^+$ was increased when injected succinate intraperitoneal injection into WT mice (Figure 15).

A.



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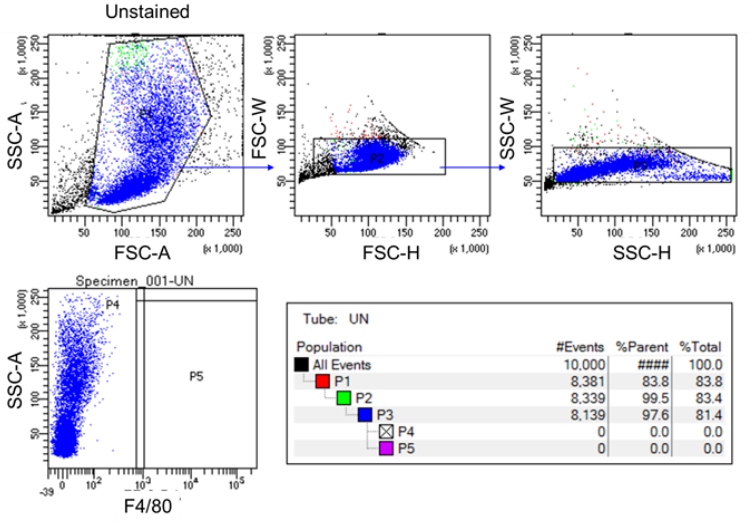
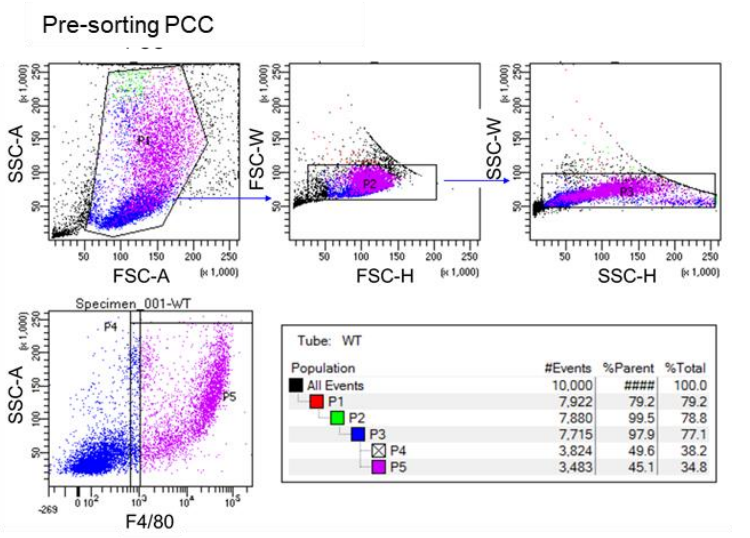


C.



D.

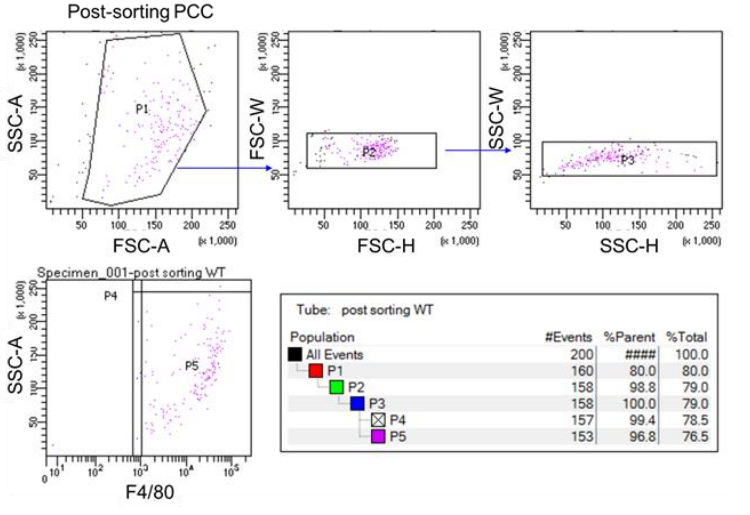


Figure 16. Experimental design of the peritoneal cavity cell transfer. (A) Experimental design of the experiment. Wild type (WT) and *Sucnr1*-deficient (KO) mice were used for donor mice, and each group divided into succinate pretreatment or not. And F4/80^{+} cells were collected through FACS sorting and transferred to WT recipients followed by DSS-induced colitis. (B–D) Gating Strategy of F4/80^{+} peritoneal macrophage. (B) unstained PCC; (C) pre-sorting PCC; (D) post-sorting PCC.

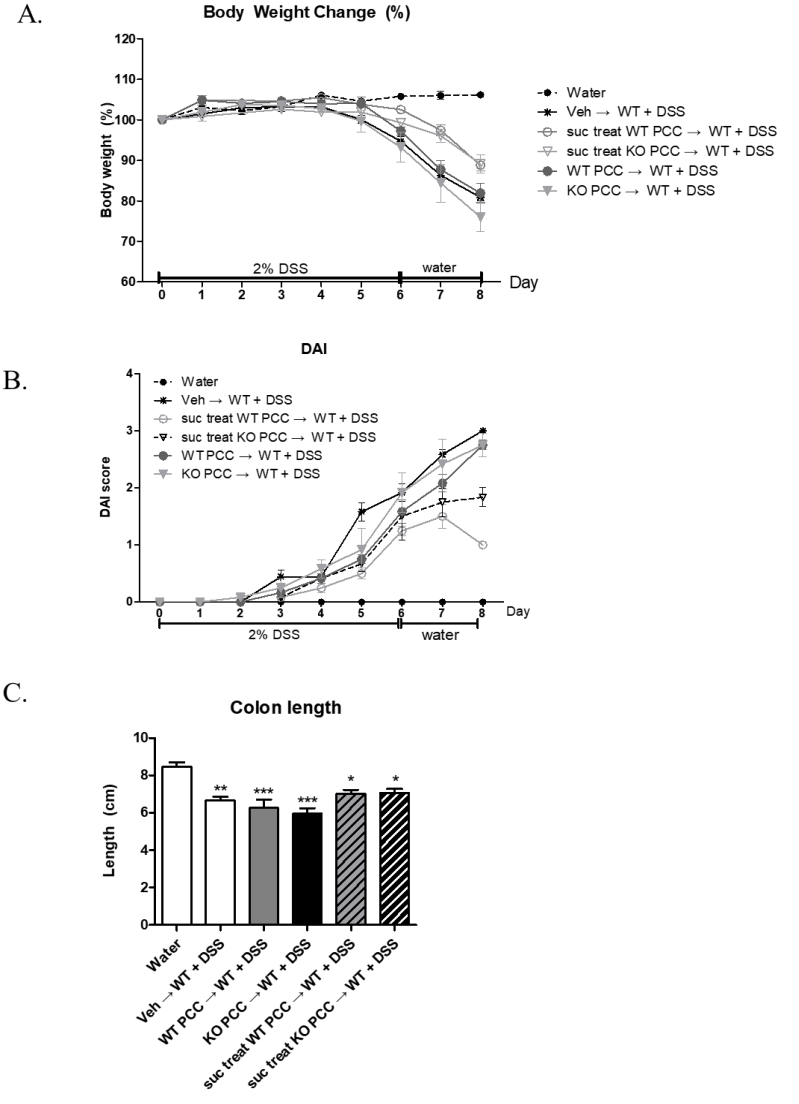


Figure 17. Recipients of succinate-treated PCCs were reduced DSS-induced colitis. (A) Bodyweight changes of mice. (B) Disease activity index (DAI). (C) Colon length. Veh, administered with the vehicle; WT PCC→ WT+DSS, recipients of WT PCC; KO PCC→WT+DSS, recipients of *Sucnr1*-deficient PCC; Suc-treat WT PCC→ WT +DSS, recipients of WT PCC treated succinate; Suc-treat KO PCC→ WT +DSS, recipients of *Sucnr1*-deficient PCC treated succinate. Bars in graphs represent mean ± S.E.M. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 compared with the Water group.

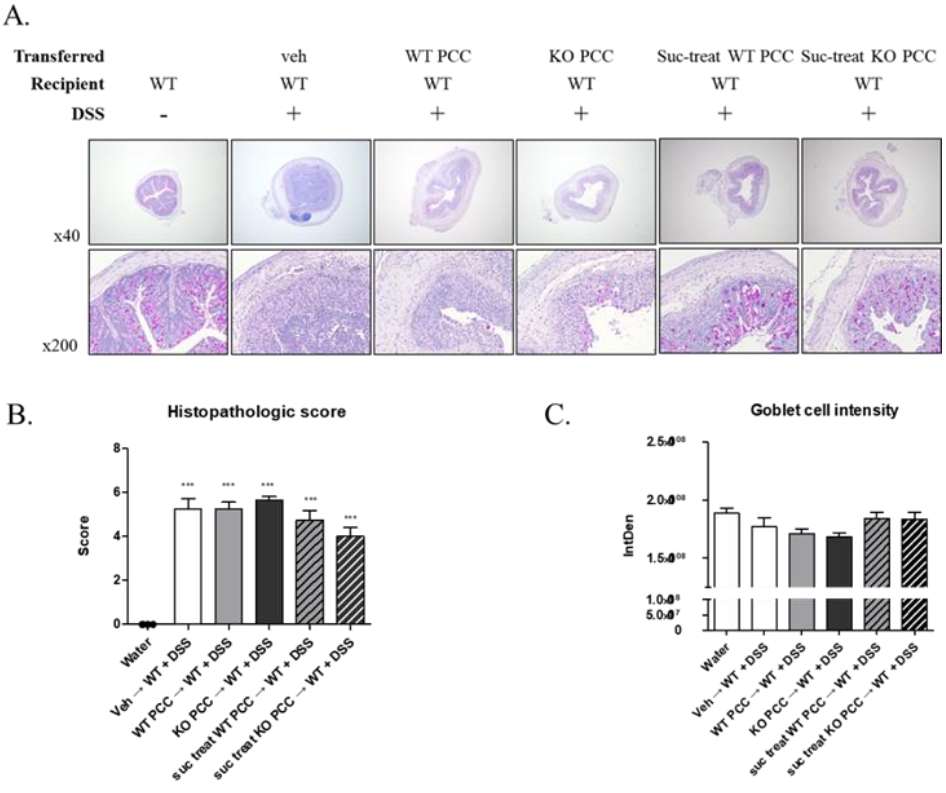


Figure 18. Quantification of colitis after PCC transferred. (A) Section from colon tissue stained with PAS. Original magnification, ×40, and ×200. (B) Histopathologic scores of colons from mice. (C) Quantification of the area of goblet cells. Bars in graphs represent mean ± S.E.M. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with the Water group.

IV. DISCUSSION

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic and inappropriate inflammation disorder that occurs in the gastrointestinal (GI) tract.[1] Recently, the incidence of IBD has increased not only in South Korea but also worldwide. Although the exact pathogenesis of IBD remains unknown, recent studies show that genetic factors, environmental factors, intestinal microbiota, and immune response factors contribute to pathogenesis.[30]

Macrophage which is an innate immune cell is involved in not only the primary response to the pathogen but also the coordination with the adaptive immune response, tissue homeostasis, and resolution.[21] Metabolic switching is involved in the reprogramming of macrophage. Monocytes are recruited and differentiated into macrophages where injury or infection sites. Proinflammatory macrophages produce TNF-α, IL-1β, and NO to promote inflammatory response.[31] In homeostasis, macrophages contribute to wound healing by inducing apoptosis or switching to an anti-inflammatory phenotype. However, in chronic inflammatory disease, proper switching does not occur, resulting in tissue damage.[32] Therefore, the proper switching between M1 and M2 is critical for homeostasis.

Succinate concentrations were increased in the inflammatory lesion of IBD patients and IBD models.[11,14,15] The role of succinate is not only involved in metabolites but also acts as alarmins and signal molecules.[16] There are two sources of succinate in our body; host-derived succinate and microbe-derived succinate.[11] First, host-derived succinate is an intermediate in the TCA cycle. Succinate is produced in the mitochondria of host cells and involved in the mitochondrial respiratory chain.[33] Second, succinate is the metabolite of microbial fermentation. For example, succinate producing bacteria[33] are as

follow; *Bacteroides fragilis*,[34] *Bacteroides vulgatus*,[35] *Faecalibacterium prausnitzii*,[36] *Paraprevotella clara*,[37] *Succinivibrio dextrinosolvens*,[38] etc. However, it is not clear whether the increased succinate levels in the sample of IBD patients was produced by host-derived or by microbiota-derived.

Recent studies explain the role of extracellular succinate as a signal molecule.[16] Succinate binds to succinate receptor1 (SUCNR1, GPR91), which is a kind of G protein-coupled receptor, in the plasma membrane. Related pathologies of SUCNR1 signaling include hypertension, diabetic nephropathy, retinal angiogenesis, rheumatoid arthritis (RA),[39] diabetic nephropathy, age-related macular degeneration (AMD),[40] and renovascular hypertension.[41,42] Especially, there are some studies that SUCNR1 as a drug target in the immune system. It is known that SUCNR1 boost inflammatory response in synergy with TLR in myeloid cells. Especially, SUCNR1 expressed on the immature dendritic cells is downregulated during dendritic cell maturation.[41] In rheumatoid arthritis and obesity, succinate-SUCNR1 signaling exacerbates and sustaining the inflammation.[11] There is a study about human B cell subset and succinate. In this study, succinate synergized with IL-10 and could promote Ig secretion by naïve B cell activations.[42]

From an immunological point of view, there are conflicting studies on whether succinate is a pro-inflammatory effector or anti-inflammatory effector. Succinate is mainly known as a pro-inflammatory factor in response to tissue damage or metabolic stress, but there is also research showing an anti-inflammatory response. First, some results indicate succinate-SUCNR1 signaling as a pro-inflammatory response. SUCNR1 expression was higher in intestinal tissue from CD patients compared to controls, and they show that *Sucnr1*-deficient mice have a protective effect in the TNBS-colitis model.[12] On the contrary, some results indicate succinate-SUCNR1 signaling as an anti-inflammatory response. A study on a myeloid-specific *Sucnr1* knockout

shows that adipose-tissue-resident macrophages represent more pro-inflammatory phenotypes and that SUCNR1 is required in the anti-inflammatory program.[43] In another study, inflammatory mononuclear phagocytes release succinate, which activates SUCNR1 on neural stem cells, leading them to secrete prostaglandin E2 and inducing the resolution of inflammation.[44] In the case of the intestine, the three majors luminal short-chain fatty acids—butyrate, propionate, and acetate— have a well-established anti-inflammatory function in IBD through their respective GPCR. Therefore, the interactions between the host and microbiome must also be considered to fully understand the role of succinate on intestinal inflammation.[11]

To investigate the role of succinate as a signal ligand, *Sucnr1*-deficient mice were used. After confirming through genotyping and sequencing, the experiments were conducted. After BMDM was obtained and differentiation and polarization, the changes in morphology were compared. These results are consistent with the results of previous studies done by Sridharan et al.[45] Because there were no differences in morphology due to succinate treatment, the expression of mRNA was confirmed through RT-qPCR. In M1 polarization, proinflammatory factors decreased in *Sucnr1*-deficient mice. *Cd206*, also known as M2 marker, increased in BMDM from *Sucnr1*-deficient. This result matches in the previous study that higher mRNA levels of *Cd206* in colon tissue from *Sucnr1*-deficient were detected compared with WT mice.[12]

Next, in vivo experiments were conducted to identify the effect of succinate-Sucnr1 signaling in colitis. The severity of colitis was more attenuated in *Sucnr1*-deficient mice compared to WT mice. This result supports the argument that *Sucnr1*-deficient mice are protected from TNBS-induced colitis.[12] Transfer of peritoneal cavity macrophage was conducted to

determine the difference of the macrophage's role between WT and *Sucnr1*-deficient and colitis was alleviated when receiving the succinate pre-treatment PCC. Therefore, PCC transfer,[26,46-48] confirmed in previous study[48] that the immune response did not occur, to find out the role of resident macrophage on colitis. Donor mice divided into WT and *Sucnr1*-deficient and each of them into succinate treated or untreated groups. Although the recipients of WT-PCC and veh group were similar severity, the recipients of *Sucnr1*-deficient PCC were exaggerated colitis. Surprisingly, the recipients of succinate-pretreatment PCC were attenuated colitis. It is consistent with the result that Cd206 was increased when the administration of succinate through intraperitoneal injection in WT mice. It can be assumed that CD206 was increased when treatment succinate, leading to M2 polarization. RAW 264.7 macrophages with LPS treatment, mimicking the inflammatory situation, the lysosomes stained with LysoTrakcer Red were reduced by succinate treatment. (Data not shown) However, there is a limitation of early-phase reaction within 2 hours of LPS treatment, so further studies such as LPS concentration and time regulation are needed.

In summary, Sucnr1 signaling mediates the activation in the colitis model, but *Sucnr1*-deficient macrophages reduced the pro-inflammatory cytokine and succinate treat macrophage shows M2-like cell. The opposite of the results of DSS-induced colitis in *Sucnr1*-deficient mice and the results of macrophage transfer treated with succinate is thought to involve a complex network that functions other than succinate-Sucnr1 signaling. The opposite of the results is thought to involve a complex network of the immune system. Furthermore, some genes related to the immune mechanism were downregulated in *Sucnr1*-deficient mice as shown in microarray analysis. Therefore, it would be worth to study the relationship between immune cells and Sucnr1 as further research.

V. CONCLUSION

In this investigation, DSS-induced colitis was reduced in *Sucnr1*-deficient mice, but macrophages with succinate treatment induce M2-like macrophages. Therefore, SUCNR1, which can regulate signaling, is an attractive therapeutic drug target. Further studies of the succinate-SUCNR1 signaling effect on inflammation are required according to cell type, such as innate lymphoid cell, innate epithelial cell, innate immune cell, adaptive immune cell.

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ABSTRACT (IN KOREAN)

숙신산이 대장염과 대식세포에 미치는 영향 및 기전

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대사체 연구에서 염증성 장질환 환자의 대변 검체나 점막의 염증부위에서 숙신산의 농도가 높다는 보고가 있다. 숙신산은 대사 중간체일 뿐 아니라 succinate receptor 1 (SUCNR1)이라는 숙신산 수용체에 결합해 신호전달 경로를 유도하는 라이간드의 역할도 가능하다. 장내 항상성을 유지하는 데 있어 면역세포가 중요한 핵심 인자이기에, 숙신산과 대장염, 면역세포의 관계에 대해 연구하였다. 수용체가 대장염에 미치는 영향을 알아보기 위해 SUCNR1 전신 결손 쥐에서 dextran sulfate sodium (DSS)로 대장염을 유도했다. 그 결과 SUCNR1이 결손된 그룹에서 체중감소, 대장 길이 짧아짐, 질병 활성도 점수의 완화 등 대장염이 완화되는 결과를 보였다. 반면, 숙신산이 처리된 대식세포는 CD206이 증가하는 항염증성 대식세포의 특징을 보였다. 이는 복강 대식세포 이식 실험 시, 복강에 숙신산을 미리 처리한 공여 쥐의 대식세포를 받은 수혜 쥐에서 DSS 유도 대장염이 완화되는 결과와 일치한다. 서로 상반된 결과가 나온 것은 면역계의 복잡한 관계망 때문일 것이라 보이며 세포 종류에 따른 추가 연구가 필요할 것이다. 특히 마이크로어레이 결과에서 숙신산 수용체가 결손했을 때 야생형 쥐에 비해, 면역 기전 관련된 인자들이 하향조절 되었다. 따라서 숙신산 수용체와 면역 세포와의 관계에 대한 추가 연구가 필요 하다.

핵심되는 말 : 염증성 장질환, 숙신산, 숙신산수용체1, 대식세포