의학박사 학위논문

The Role of Macrophage Ninjurin1 on Mouse Colitis

마우스 대장염에서 대식세포 Ninjurin1의 기능 연구

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정현진

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Abstract

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The incidence of inflammatory bowel disease (IBD) has been increasing, and IBD has become an emerging disease that requires greater elucidation of its pathogenesis. In common, IBD leads to complications like colon cancer, which is a life-threatening problem. IBD was considered as a growing disease only in western world in the past. However, recent studies report a significance rise in the number of cases of IBD in Asian countries. Especially, there has been a twentyfold increase in the number of patients with IBD over the last fourteen years in Korea. IBD is characterized by the dysregulated immune system, which means that it is an immune-mediated disease. Immune cells in the gut including macrophages, T cells, B cells, and dendritic cells are involved in contributing IBD. These immune cells induce inflammation via secreting pro-inflammatory cytokines and chemokines. Especially, macrophages are able to regulate IBD

development by changing their polarized phenotype.

Nerve injury-induced protein 1 (Ninjurin1, Ninj1) is a cell-surface adhesion molecule that regulates cell migration and attachment. Several studies revealed that Ninjurin1 promoted macrophage migration capacity. However, the precise function of Ninjurin1 has not yet been clearly defined.

Herein, I demonstrate that Ninjurin1 protein expression is increased in a dextran sodium sulfate (DSS)-induced colitis model, which is confirmed in IBD patients. To directly assess whether Ninjurin1 plays an important role in colitis, I introduced WT and Ninjurin1-deficient mice (KO) mice to 1.5% and 2.5% DSS and evaluated colitis incidence. Ninjurin1 KO mice showed significantly less body weight loss and longer colon length when compared with WT mice. I also performed hematoxylin and eosin staining of colon tissue sections, which revealed that less crypt destruction, surface epithelial erosion, and reduced submucosal edema in DSS-treated Ninjurin1 KO mice compared to DSS-treated WT mice. No such differences in colon tissue histology were shown in control mice of either genotype. Likewise, relatively intact mucosa and larger number of mucin-containing goblet cells were remained in DSS-treated Ninjurin1 KO mice compared to WT mice, which were assessed by Ki-67 and Alcian blue staining, respectively.

Various immune cells infiltrate into the site of inflammation during the development of colitis. To study what type of immune cell made a difference in colitis incidence according to Ninjurin1 expression, I investigated Ninjurin1 expression pattern on lymphocytes and myeloid cells. Myeloid cells showed the

highest expression of Ninjurin1 among the various immune cells. Interestingly, Ninjurin1 does not regulate migration capacity of macrophages during development of experimental colitis. Next, I examined activation of macrophages under inflammatory conditions. Cytokines and chemokines secreted by activated macrophages play critical roles at the site of inflammation. I evaluated gene and protein expression of cytokines and chemokines in the presence or absence of Ninjurin1 in macrophages. Compared to WT cells, Ninjurin1 KO macrophages expressed less amount of cytokines and chemokines.

To validate that Ninjurin1 on macrophages affects the intestinal inflammation, I generated mice with a conditional deletion of Ninjurin1 in macrophages (Ninjurin1$^{fl/fl}$; Lyz2-Cre^{+}). Myeloid specific Ninjurin1-deficient mice exhibited significantly attenuated body weight loss, shortening of colon length, intestinal inflammation, and lesser pathological lesions than WT mice. Furthermore, DSS-treated myeloid specific Ninjurin1-deficient mice expressed less amount of pro-inflammatory cytokines compared to DSS-treated WT mice.

During the development of colitis, the Ninjurin1 expressing macrophages secrete more cytokines and chemokines by regulating PKCδ/θ-STAT1 activation. Colon tissues from DSS-treated WT mice expressed activated PKCδ/θ compared with those from DSS-treated Ninjurin1 KO mice. Pharmacological and genetic inhibition of PKCδ/θ-STAT1 pathway led to reduced production of pro-inflammatory cytokines in WT macrophages.

In summary, I suggest that Ninjurin1 in macrophages has a pivotal function in

colon inflammation.

Keywords: Nerve injury-induced protein 1 (Ninjurin1), Colitis, Macrophage, Protein Kinase C δ/θ (PKC δ/θ), Inflammatory bowel disease (IBD)

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Abbreviation

Arg-1	Arginase 1
BAFF	B cell-activating factor
BMDM	Bone marrow derived macrophage
CCL2	C-C Motif Chemokine Ligand 2
CCL17	C-C Motif Chemokine Ligand 17
CCL12	C-C Motif Chemokine Ligand 12
CD	Crohn's disease
CD3	Cluster of differentiation molecule 3
CD4	Cluster of differentiation molecule 4
CD8	Cluster of differentiation molecule 8
CD11b	Cluster of differentiation molecule 11b
CD28	Cluster of differentiation molecule 28
CD43	Cluster of differentiation molecule 43
CRISPR	Clustered regularly interspaced short palindromic repeats
CXCL1	C-X-C Motif Chemokine Ligand 1
CXCL12	C-X-C Motif Chemokine Ligand 12

DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DSS	Dextran sodium sulfate
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
Fizz-1	found in inflammatory zone 1
GEO	Gene expression omnibus
H&E	Hematoxylin and Eosin
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IL1α	Interleukin 1 alpha
IL1β	Interleukin 1 beta
IL2	Interleukin 2
IL4	Interleukin 4
IL6	Interleukin 6
KO	Knock-out
LPS	Lipopolysaccharides
Lyz2	Lysozyme 2 gene

MFI	Mean fluorescent intensity
NFκB	Nuclear factor kappa B
Ninjurin	Nerve injury induced protein
Ninj1	Nerve injury induced protein 1
PBMC	Peripheral blood mononuclear cell
PKC	Protein kinase C
Rott	Rottlerin
STAT1	Signal transducer and activator of transcription 1
TAM	Tumor associated macrophage
TLR	Toll like receptor
TNFα	Tumor necrosis factor α
UC	Ulcerative colitis
WT	Wild type
Ym-1	Chitinase 3-like 3
7AAD	7-amino-actinomycin D

I. Introduction

Since Nerve injury-induced protein 1 (Ninjurin1, Ninj1) was first identified in sciatic nerve of rats, a variety of studies about the function and structure of Ninjurin1 has been conducted (Araki and Milbrandt, 1996). It was initially discovered that expression of Ninjurin1 was induced in Schwann cells in response to nerve injury (Araki and Milbrandt, 1996). The gene encoding Ninjurin1 is located on chromosome 9 in human and chromosome 13 in mouse. Ninjurin1 is composed of 152 amino acids (aa) and associated with plasma membrane (Figure 1). Ninjurin1 contains N-terminal (1-80 aa) and C terminal extracellular domains (142-152 aa), two transmembrane domains (81-101, 121-141 aa) and cytoplasmic domains (102-120 aa). In N-terminal domain, a novel adhesion motif is located between amino acids Ala^{26} and Val^{37}. Ninjurin1 mediates cell to cell and cell to matrix adhesion with this adhesion domain via homophilic binding (Araki et al., 1997; Lee et al., 2009). The sequence of Ninjurin1 in vertebrates is highly conserved (Bae et al., 2017). In drosophila, N-terminal domain of NinjurinA contains 98% homology with mouse Ninjurin1 and cleavage of the ectodomain of NinjurinA led to loss of cell adhesion (Zhang et al., 2006).

The tumor suppressor protein p53 has been discovered to regulate Ninjurin1 expression, which is the only known pathway that can regulate Ninjurin1 expression until now. p53 increases Ninjurin1 expression via binding to the p53 response element in the Ninjurin1 promoter (Cho et al., 2013; Kannan et al., 2001). p21, one of the p53

target genes, does not affect Ninjurin1 expression; however, Ninjurin1 increases p21 expression (Cho et al., 2013; Toyama et al., 2004).

Post-translational modification of Ninjurin1 has been also identified. In living cells, Ninjurin1 forms homomeric protein complex, which is mediated by N-glycosylation at Asn^{60} in its N-terminal domains (Bae et al., 2017; Kny et al., 2019). Another modification of Ninjurin1 is assessed by matrix metalloproteinase (MMP) 9. MMP9 cleaves Ninjurin1 between Leu^{56} and Leu^{57}, which liberates N-terminal ectodomain of Ninjurin1 (Ahn et al., 2012). This soluble Ninjurin1 may act as chemoattractant because it has similar structure with chemokines.

Ninjurin2 was identified to bear significant homology to Ninjurin1 (about 50% amino acid sequence identity) (Figure 2) (Araki and Milbrandt, 2000). Ninjurin2 is also a cellular surface adhesion molecule; however, Ninjurin1 and Ninjurin2 do not share the sequence of adhesion motif (Figure 2). Transmembrane domains of Ninjurin1 and Ninjurin2 are nearly conserved (Figure 2). Expression of Ninjurin2 is also increased in Schwann cells in the injured nerve. Several studies indicated that Ninjurin2 was associated with increased risk of ischemic stroke, endothelial inflammation, colorectal cancer cell growth, and multiple sclerosis (Bis et al., 2014; Li et al., 2019; Noroozi et al., 2019; Wang et al., 2017).

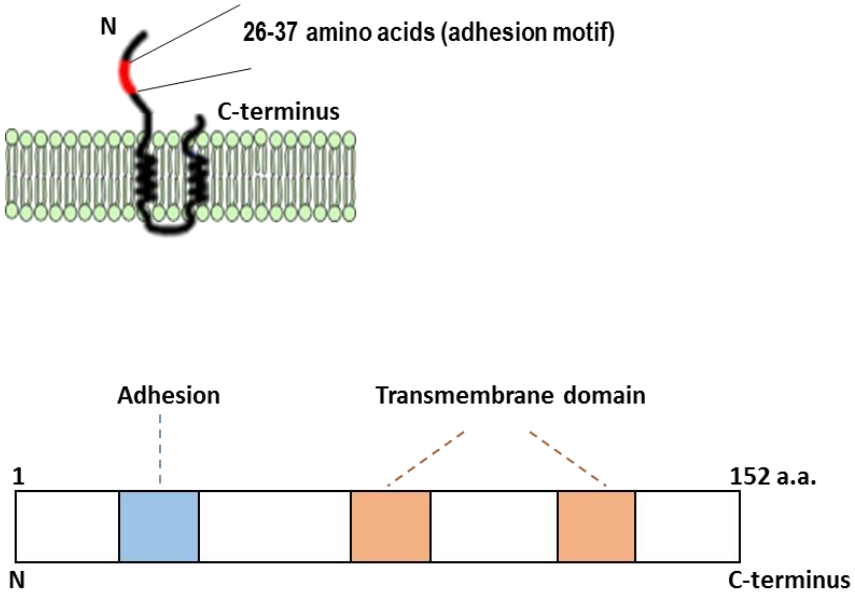

Figure 1. The structure of human Ninjurins.

Schematic illustration of Ninjurin1 structure. Ninjurin1 contains N-terminal, C-terminal, intracellular domain, and two transmembrane domains.

Figure 2. The sequences of human Ninjurins.

Alignment of human Ninjurin1 and Ninjurin2. Identical amino acids are boxed. Adhesion motif is indicated with dotted lines and transmembrane domains are underlined.

Ninjurin1 in inflammatory conditions

It has been shown that Ninjurin1 is basally expressed in endothelial cells, fibroblasts, myeloid cells, and several organs such as kidney, liver, and thymus (Ahn et al., 2009; Araki et al., 1997; Ifergan et al., 2011). Under inflammatory conditions, Ninjurin1 expression has been known to be induced. In keratinocytes and dermal fibroblasts, Ninjurin1 is increased by ionizing radiation (Koike et al., 2008). Hypoxia induces Ninjurin1 gene expression in capillary pericytes, and Ninjurin1 negatively modulates angiogenic effect of pericytes (Matsuki et al., 2015). Furthermore, Ninjurin1 expression in microvessels is increased in mouse ischemic hindlimb ischemia (Minoshima et al., 2018).

According to previous studies, macrophage is one of the cells highly expressing Ninjurin1 expression that is significantly increased under inflammatory conditions (Ahn et al., 2014b; Choi et al., 2018; Ifergan et al., 2011; Jennewein et al., 2015; Lee et al., 2016). Thus, the role of Ninjurin1 has been studied most intensively in macrophages.

Ninjurin1 on macrophages regulate its migration capacity. Ninjurin1 induces infiltration of myeloid lineage cells across blood-brain barrier, which leads to disease activity of experimental autoimmune encephalomyelitis (Ifergan et al., 2011). Ninjurin1 on macrophages especially regulate protrusive membrane dynamics and consequently modulate transendothelial migration of macrophages (Ahn et al., 2014a). Moreover, Ninjurin1 inhibition reduces susceptibility to systemic

inflammation, liver damage, and pulmonary inflammation in septic mice by mediating migration of leukocyte including macrophages (Jennewein et al., 2015).

In addition, Ninjurin1 on macrophages induces the activation under inflammatory conditions. Ninjurin1 increases production of pro-inflammatory cytokines such as IL-6 and TNFα upon LPS treatment, and inhibition of Ninjurin1 with blocking peptides results in reduced mRNA expression of pro-inflammatory cytokines (Jennewein et al., 2015). Ninjurin1 modulates this macrophage activation through regulating Toll-like receptor 4 (TLR4)-p38-AP-1 dependent pathway (Jennewein et al., 2015). In addition, Shin and the colleagues reported that LPS directly binds to 81-100 aa region of Ninjurin1 (Shin et al., 2016). A previous study reported that Ninjurin1-deficient mice show a mild lung pulmonary fibrosis phenotype associated with interaction between macrophages and alveolar epithelial cells (Choi et al., 2018). In this study of lung fibrosis and Ninjurin1, it is demonstrated that macrophage activation, caused by binding with alveolar epithelial cells, is a factor determining the phenotypic difference between bleomycin-treated WT and Ninjurin1 KO mice (Choi et al., 2018).

Ninjurin1 in cancers

Recently, several studies investigate that Ninjurin1 is related to progression and prognosis of tumors. Ninjurin1 is highly expressed in hepatocellular carcinoma, acute lymphoblastic leukemia, urothelial bladder cancer, and circulating tumor cells from locally advanced prostate cancer (Chen et al., 2001; Kim et al., 2001; Mhawech-Fauceglia et al., 2009; Park et al., 2017). These studies suggest Ninjurin1 has an oncogenic function.

p53, the regulator of Ninjurin1 expression, is one of the most well-known tumor suppressor and it controls cell cycle arrest and apoptosis of cancer cells. Ninjurin1 modulates p53 mRNA translation, which makes a feedback loop between Ninjurin1 and p53 (Cho et al., 2013). Ninjurin1 deficiency increases p53 expression, which results in increased cellular senescence, cell survival, and radiosensitivity (Cho et al., 2013).

In addition, Ninjurin1 regulates migration of cancer cells. Ninjurin1 inhibition increased migration and invasion of human lung cancer cells via activating IL6/STAT3 signaling and increasing ICAM1 expression, which means that Ninjurin1 functions as a metastasis suppressor gene (Jang et al., 2016). On the other hand, Ninjurin1 in prostate cancer cells enhances cell motility (Park et al., 2017). Overexpression of Ninjurin1 with adenovirus significantly increases invasion and migration of prostate cancer cells (Park et al., 2017).

The tumor mass is comprised of not only proliferating cancer cells but also a variety

of different molecules such as infiltrating immune cells. The function of Ninjurin1 has been studied in tumor-associated macrophage (TAM) as well as cancer cells. Approximately half of tumor mass consist of TAMs, which helps tumor cells grow (Vinogradov et al., 2014). Therefore, infiltration of TAMs to tumor mass is important for tumor growth and metastasis. Ninjurin1 overexpression on macrophages reduces colon cancer incidence by repressing infiltration of TAM to tumors (Woo et al., 2016). In addition, this migration and invasion of TAM are regulated by Ninjurin1-suppressed focal adhesion kinase signaling (Woo et al., 2016). This result shows the opposite role of Ninjurin1 in macrophages under inflammatory conditions and TAMs.

Inflammatory bowel disease

Inflammatory bowel diseases (IBD) is a global problem that has been emerging in recent years. Incidence of prevalence of IBD vary depending on the geographic region. In the past decade, IBD was regarded as a disease of westernized countries. However, nowadays, the incidence of IBD is greatly increasing in other countries. Especially, in South Korea, the recent incidence of IBD has been the highest in East Asia, and the economic burden of IBD is significantly increasing (Jung et al., 2017b).

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), are inflammatory disorders that affect the gastrointestinal tract. CD occurs anywhere in gastrointestinal tract and there are non-inflamed areas mixed in between inflamed parts. UC is limited to colon and the inflammation is continuous. Moreover, CD affects in all the layers of the gut and UC occurs only the inner most lining of colon.

Another disorder of gastrointestinal tract is irritable bowel syndromes (IBS), which has been used interchangeably with IBD because they have some similar symptoms such as abdominal pain and diarrhea. However, these two are not the same disorder (Figure 3). The major difference between IBS and IBD is inflammation in gastrointestinal tract. IBS is a non-inflammatory condition and does not cause permanent intestinal damage. On the other hand, IBD is an inflammatory condition that requires treatment with drugs and surgery.

Development of IBD has been associated with environmental and genetic factors as well as immune responses (Figure 4). A recent study reports that IBD involves the

differential expression of genes that regulate inflammation and tissue remodeling (Wu et al., 2008). Based on the therapeutic effect of immunosuppressive drugs, dysregulation of the immune system has been implicated in IBD (Deusch and Reich, 1992). Even though previous studies have reported several factors involved in IBD, the epidemiology is still incompletely described.

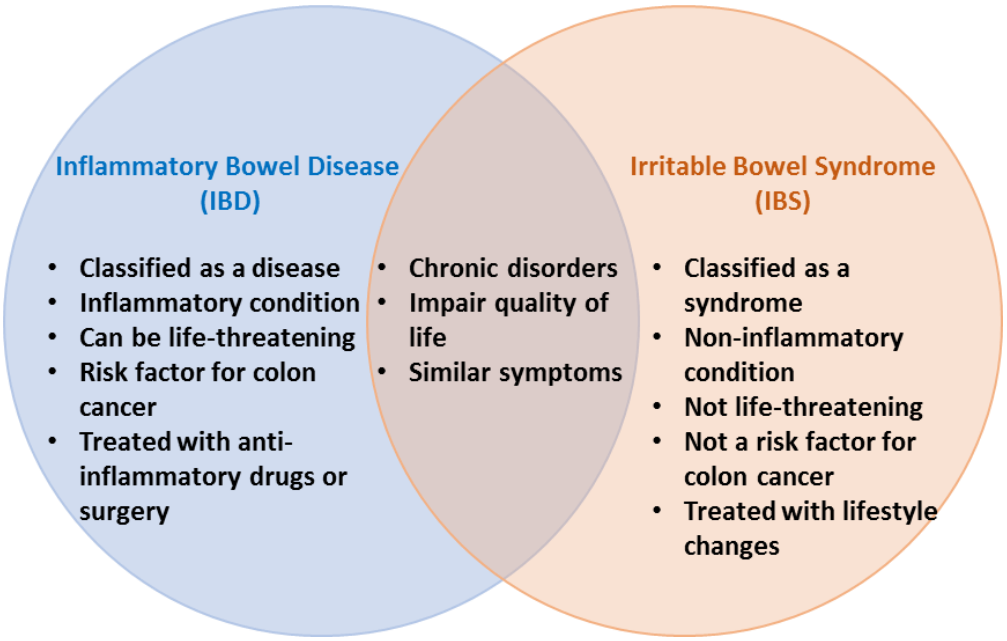

Figure 3. Difference between IBD and IBS

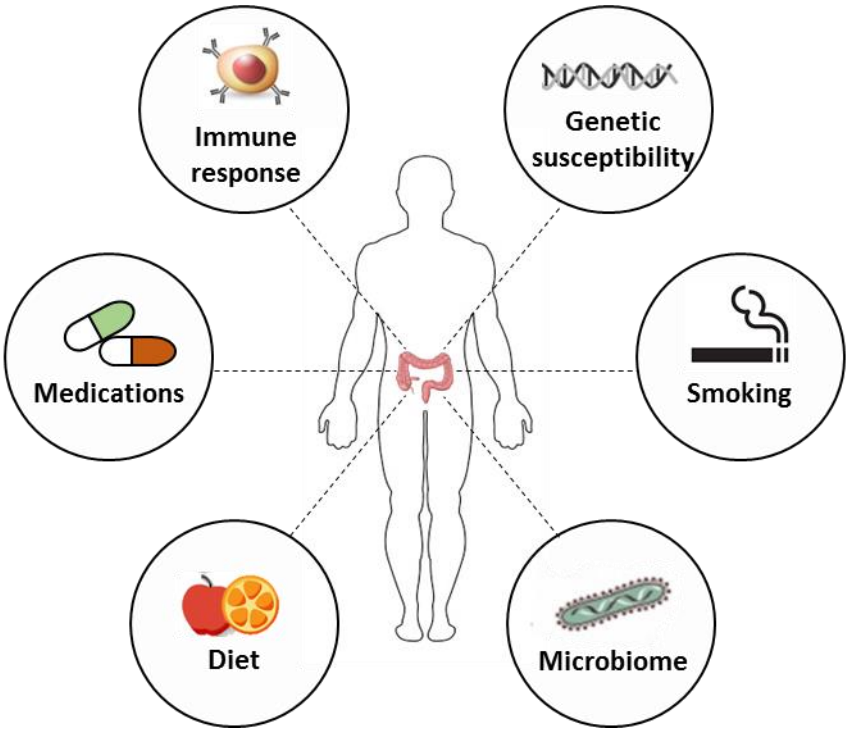

Figure 4. Several factors that potentially trigger IBD.

Macrophages in inflammatory conditions

Dysregulated homeostasis of immune cells has been associated with intestinal inflammation (Xu et al., 2014). Of the several immune cells that regulate inflammatory responses, macrophages are important during the development of colitis (Lv et al., 2019; Tamoutounour et al., 2012). Toll-like receptor 4 (TLR4), NF-κB, p38, and Protein Kinase C (PKC) have been reported to be activated to induce the inflammatory signaling cascade during colitis (Figure 5) (Xu et al., 2016). After this complex cascade is triggered, macrophages produce pro-inflammatory cytokines and chemokines to amplify the inflammatory signal and to recruit leukocytes to the lesion site. The pro-inflammatory cytokines and chemokines produced by activated macrophages have a pivotal role in the development of IBD (Murakami et al., 2001).

The production of cytokines and chemokines by monocytes and macrophages at the lesion site is important for the development of inflammation (Arango Duque and Descoteaux, 2014). Several articles demonstrate that IL1β is essential for regulating IBD, and elevated amounts of IL1β have been detected in IBD patients as compared to healthy subjects (Ludwiczek et al., 2004; Mazzucchelli et al., 1996). An animal experiment study reported that treatment with IL1β antagonist significantly diminishes intestinal inflammation (Cominelli et al., 1990). Moreover, IL1β is a master regulator of the inflammatory response, and secretion of IL1β results in upregulation of other pro-inflammatory cytokines and chemokines such as IL6, TNFα, and CCL2 (Li et al., 2010; Su et al., 2009). Therefore, IL1β secretion by activated macrophages is an important factor which drives the intestinal inflammation.

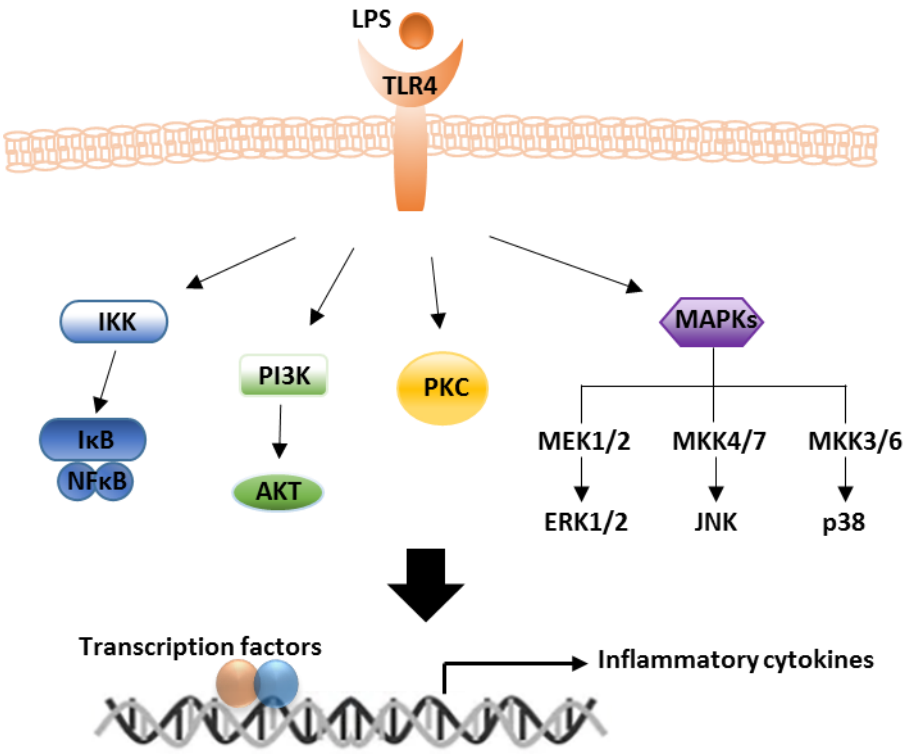

Figure 5. LPS/TLR4 signal transduction pathway

Ninjurin1 and inflammatory signals

The function of Ninjurin1 during inflammation was mainly observed in the nervous system, and Ninjurin1 expression is induced under central nervous system inflammatory conditions. Ninjurin1 knock-out (KO) mice are less susceptible to experimental autoimmune encephalomyelitis (EAE) than wild-type (WT) mice (Ahn et al., 2014b). Recent studies reveal that Ninjurin1 regulates several inflammatory diseases outside of the nervous system. Ninjurin1 inhibition reduces susceptibility to systemic inflammation, liver damage, and pulmonary inflammation in septic mice (Jennewein et al., 2015). In addition, a previous study reported that Ninjurin1-deficient mice show a mild lung pulmonary fibrosis phenotype associated with interaction between macrophages and alveolar epithelial cells (Choi et al., 2018). Macrophage infiltration has been shown to be crucial in the regulation of progression of lung fibrosis (Wynn and Vannella, 2016; Yao et al., 2016). However, in a study of lung fibrosis and Ninjurin1, it was demonstrated that the numbers of infiltrating macrophages in bronchoalveolar lavage fluids of bleomycin-treated WT and Ninjurin1 KO mice for lung fibrosis were not different, which means macrophage infiltration is not a factor determining the phenotypic difference between bleomycin-treated WT and Ninjurin1 KO mice (Choi et al., 2018). In colitis, an inflammatory disorder, macrophage infiltration is also vital for pathogenesis; however, Ninjurin1 function and macrophage infiltration in the development of colitis have not yet been studied.

Ninjurin1 is known to be a target of p53 and is increased by DNA damage (Cho et al.,

2013; Yang et al., 2017). And it modulates p53 mRNA translation, which makes a positive feedback loop between Ninjurin1 and p53. Another paper shows that Ninjurin1 is reported to modulate TLR4 signaling cascade, which results in increased pathogenesis in septic mice (Jennewein et al., 2015). Abundant studies demonstrate that lipopolysaccharides (LPS) activate TLR families and PKC isoforms (Cuschieri et al., 2006; Huang et al., 2009; Kim et al., 2015; Koff et al., 2006; Slomiany and Slomiany, 2018). PKC isoforms are categorized into three groups: conventional (α, βI, βII, γ), atypical (ζ, λ/ι), and novel (δ, ε, η, θ) isoforms based on activation requirements. PKC activation under inflammatory conditions increases the production of cytokines and chemokines in immune cells. Among the several isoforms, PKCδ/θ contributes to the secretion of cytokines and chemokines in animal models, and is therefore related to inflammatory diseases (Bhatt et al., 2010; Kontny et al., 2000). In the cecal ligation and puncture rat model for sepsis, TLR signaling through PKCδ activation increases the sepsis-induced lung injury, which is evidenced by detecting levels of chemokines in the lungs (Kilpatrick et al., 2011). PKCδ KO mice exhibit reduced production of cytokines in a lung injury mouse model (Shukla et al., 2007). Moreover, PKCθ inhibition provides protection to mice from experimental colitis (Zanin-Zhorov et al., 2010).

The purpose of this study

Ninjurin1 is closely associated with a wide range of pathophysiological conditions especially related to inflammation (Figure 6). Induction of Ninjurin1 expression in tissues and cells is commonly observed. Even though studies about Ninjurin1 have been conducted for several years, underlying mechanism of Ninjurin1 showing how Ninjurin1 modulates inflammatory response, associated with development of progression of diseases, has been poorly understood.

In this study, I investigated the role of Ninjurin1 in the pathogenesis of colitis by examining Ninjurin1-deficient mice treated with dextran sodium sulfate (DSS). Loss of Ninjurin1 alleviated the DSS-induced colitis. Mice harboring Ninjurin1-deficient macrophages also dampened the level of inflammation against colitis, which was associated with PKCδ/θ activation. Therefore, my results show Ninjurin1 has a critical function in macrophages in colonic inflammation.

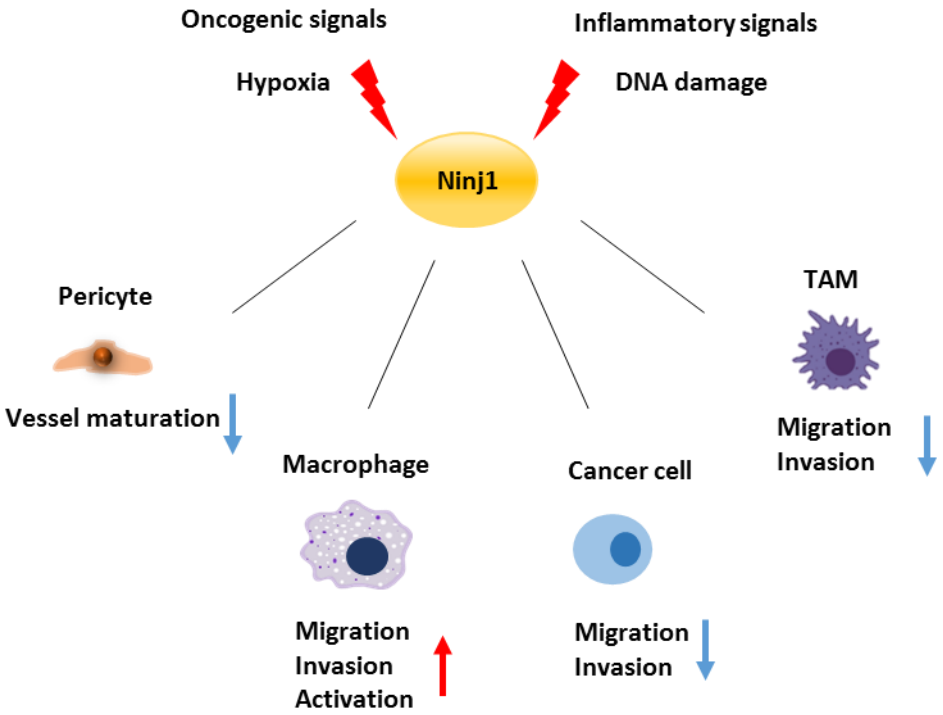

Figure 6. The role of Ninjurin1.

Ninjurin1 is regulated by several cellular stress signals. Ninjurin1 acts as a modulator in cellular process involved in vessel maturation, migration, invasion, and immune cell activation. Tumor-associated macrophages (TAM).

II. Materials and Methods

1. Mice and experimental colitis

All animal experiments were conducted in accordance with the protocols approved by the Institutional Animal Care and Usage Committee. Mice were housed under 12 h light and dark cycles at 22 °C, and provided with food and water *ad libitum*. Ninjurin1 KO C57BL/6 mice were generated as previously described (Choi et al., 2018). To generate myeloid-specific Ninjurin1-deficient mice, Ninjurin1$^{fl/fl}$ mice were bred with heterozygous Lyz2-cre+/- mice. Male and female (9 to 11-weeks-old) mice were randomly assigned to experimental groups. Colitis was induced in mice by administering 1.5% or 2.5% (w/v) DSS (36-50 kDa) (MP Biomedicals, OH, USA) in drinking water for 8 days. Mice were weighed every 2~3 days after initiation of DSS treatment, and subsequently sacrificed on day 8 after initiation of treatment (Figure 7). Animals were euthanized by carbon dioxide inhalation, and the colon tissues were harvested to determine the colitis incidence. For blocking peptide treatment, Ninjurin126-37 peptide (1 mg/kg) was diluted in 0.9% NaCl and administered intravenously immediately before initiation of DSS treatment. All animal experiments were conducted in accordance with the protocols approved by the Institutional Animal Care and Usage Committee in Korea (GIACUC-R2016005, GIACUC-R2017014, GIACUC-R2018006).

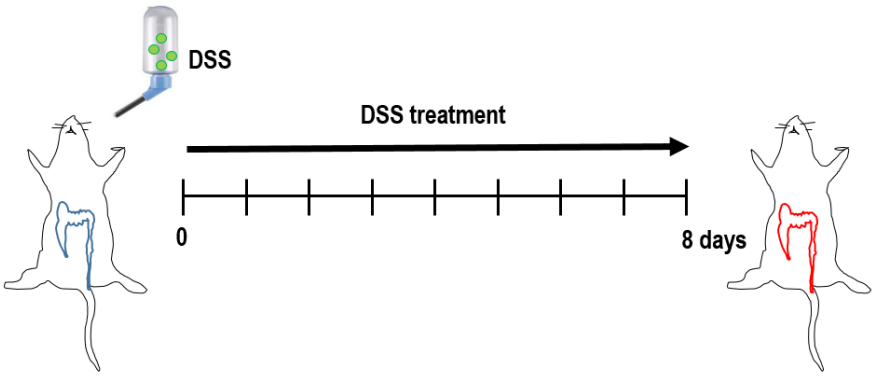

Figure 7. *In vivo* experimental schedule for DSS-induced colitis.

C57BL/6 WT, Ninjurin1 KO, Ninjurin1$^{fl/fl}$, and Ninjurin1$^{fl/fl}$; Lyz2-Cre+ mice were treated with 1.5% or 2.5% (w/v) DSS in drinking water for 8 days.

2. Cell lines

All cell lines and primary cells were incubated at 37 °C in the humidified chamber with 5% CO_2. Raw 264.7 cells were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). Raw264.7 cells were cultured in DMEM supplemented with 10% FBS (Welgene, Daegu, Korea) and streptomycin (100 μg/ml)/penicillin (100 units/ml). Using CRISPR Cas9, Ninjurin1-deficient cells were generated as previously described (Choi et al., 2018). Briefly, Raw 264.7 cells were transfected with CRISPR Cas9 lentiviral expression vectors targeting Ninjurin1, purchased from transOMIC Technologies (Hunsville, AL, USA). Using SONY cell sorter SH800Z, the transfected cells were sorted (SONY, Tokyo, Japan). In the current study, Ninjurin1-expressing cells are denoted as WT cells, while Cas9-treated Ninjurin1 KO cells are referred to as Ninjurin1 KO cells. The WT and Ninjurin1 KO Raw264.7 cells were treated with LPS (Sigma-Aldrich, St Louis, MO, USA) or rottlerin (rott; Santa Cruz Biotechnology, CA, USA). Human monocytic cell line (THP-1) was purchased from ATCC and cultured in RPMI 1640 supplemented with 10% FBS and streptomycin (100 μg/ml)/penicillin (100 units/ml). THP-1 cells were transfected with adenovirus expressing human Ninjurin1 (Ad-Ninjurin1) or empty vector (Ad-EV), as previously described (Jang et al., 2016). Human colon carcinoma cell line (Caco2) was purchased from the Korea Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10% FBS and streptomycin (100 μg/ml)/penicillin (100 units/ml).

3. Immunoblotting

As previously described, Western blot analysis was performed against anti-Ninjurin1 (Abclon, Seoul, Korea), anti-pSTAT6, anti-pPKCδ/θ and anti-pSTAT1 (Cell Signaling Technology, MA, USA), anti-actin, and anti-GAPDH (Millipore, Schwalbach, Germany) (Jung et al., 2019). Briefly, cell or tissue lysates were prepared with modified RIPA buffer containing 20 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% triton x-100, 1mM sodium fluoride, and 1 mM sodium vanadate. Equivalent amounts of protein were resolved in sodium dodecyl sulfate-polyacrylamide gels. The proteins were transferred to polyvinylidene fluoride membrane. The blot was blocked in 3% skim milk and washed with TBST (0.1% Tween 20). After the primary antibodies were applied overnight at 4 °C, HRP-conjugated secondary antibodies were conjuagated for 1 h at room temperature (RT). The resultant protein-antibody complexes were analyzed using chemiluminescence Western blotting detection reagents (Abclon).

4. Assessment of inflammation in colon

Entire colons were fixed overnight in 10% neutral buffered formalin, and subsequently embedded in paraffin. Histology was evaluated after longitudinal sections of colons (swiss-roll) were stained with Hematoxylin and eosin staining (H&E). Histopathological evaluation assigned scores ranging from 0 to 3 for submucosal edema (0–3), surface epithelial erosion (0-3), and crypt damage (0-3), for a combined total score of 9; submucosal edema: 0 = no significant edema, 1 = rare

areas of submucosal edema, 2 = occasional areas of mild submucosal edema, 3 = frequent areas of marked submucosal edema; surface epithelial erosion: 0 = none, 1 = rare small breaches in epithelium, 2 = frequent small breaches in epithelium, 3 = extensive areas lacking surface epithelium; crypt damage: 0 = none, 1 = some crypt damage, 2 = large areas without crypts, 3 = no crypts (Table 2) (Fodil et al., 2017).

5. Immunofluorescence

For immunofluorescence assessment, colon sections were deparaffinized, hydrated with distilled water. Antigen retrieval was performed by heating the sections in 10 mM citrate solution supplemented with 0.1% Tween 20 using microwave. Then, the sections were incubated in a blocking solution (Life Technologies, MD, USA) for 1 h at RT. The primary antibody against Ninjurin1 (Abclon) was diluted 1:300 and applied to the section-containing slides at RT for 1 h. The bound primary antibodies were detected using 1:300 AlexaFluore568. To assess co-localization of Ninjurin1 and F4/80, 1:500 rat F4/80-FITC (eBioscience, CA, USA), was incubated with the slides, overnight at 4°C, followed by staining with Ninjurin1 antibodies. The nucleus was stained, and the slides were mounted with Vectashield mounting medium (Vector Laboratories, CA, USA). The slides were analyzed under a confocal microscope (Nikon Instruments Inc., New York, USA).

6. Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections of colons from DSS-treated WT and Ninjurin1 KO mice were deparaffinized and rehydrated prior to ki-67 staining

(Abcam, MA, USA). Slides were incubated with antibody against ki-67 for 1 h at RT, followed by incubation with the secondary antibodies for 1 h at RT. Finally, the slides were incubated with DAB substrate (DAKO, CA, USA) and counterstained with hematoxylin. For Alcian blue staining, the slides were stained in Alcian blue solution for 30 min and subsequently washed with distilled water. Counterstaining was conducted with nuclear fast red solution for 5 min, after which the slides were washed and mounted.

7. Purification and activation of lymphocytes

A single cell suspension was isolated from the spleen of C57BL/6 mice. CD4+ and CD8+ T cells were purified using CD4 and CD8 microbeads, in accordance with the manufacturer's instructions (Miltenyi Biotech, Gladbach, Germany). B cells were purified using CD43 microbeads with MACS kit. CD4+ and CD8+ T cells were activated with plate-bound anti-CD3 (5 μg/mL) and soluble anti-CD28 (5 μg/mL) supplemented with IL-2, and cultured for 3 days. B cells were stimulated with BAFF (50 ng/mL) and LPS (10 μg/mL) for 3 days.

8. RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR)/real-time quantitative PCR (qRT-PCR) analysis

Total RNA was isolated from colon tissues and cells using the TRIzol reagent (Invitrogen, CA, USA). Briefly, 1 ml of TRIzol was applied to cells or tissues, and the solution was mixed vigorously. Chloroform was added to each sample and the

mixtures were centrifuged at 12,000 g for 20 min at 4 °C. The upper layer of solution was transferred to a new tube and mixed with isopropanol. After the samples were centrifuged at 12,000 g for 20 min at 4 °C, pellet was washed with 70% EtOH at 12,000 g for 10 min at 4 °C two times. The supernatant was discarded and the pellet was dried. DEPC-treated distilled water was added to dissolve RNA. Using a synthesis kit, PrimeScript RT reagent, the extracted RNA (1~2 μg) was used as a template for reverse transcription to synthesize cDNA according to the manufacturer's instructions (Takara, Kyoto, Japan). Each RNA sample was mixed with oligo DT and incubated at 65 °C for 5 min. After 1 min of incubation at 4 °C, the mixture of reverse transcriptase, Rnase inhibitor, dNTP, and reaction buffer was transferred to each sample and incubated at 42 °C for 1 h. To inactivate the enzymatic reaction, the samples were incubated at 95 °C for 5 min, followed by cooling on ice. The RT-PCR products were analyzed on 1.5% agarose gel in Tris-acetate/ethylenediaminetetraacetic acid buffer. qRT-PCR was performed with the Stratagene Mx3000P QPCR System (Agilent Technologies, La Jolla, CA, USA). The primers for PCR are indicated in Table **1**.

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Genes	Species	Direction	Primers (5' → 3')
Ninj1	Mouse	Forward	GAGTATGAGCTCAACGGCGA
		Reverse	TGACCAGGAAGATGAGCAGC
IL1β	Mouse	Forward	GCCTTGGGCCTCAAAGGAAAGAATC
		Reverse	GGAAGACACAGATTCCATGGTGAAG
CCL2	Mouse	Forward	TCCCAATGAGTAGGCTGGAGAGC
		Reverse	CAGAAGTGCTTGAGGTGGTTGTG
IL1α	Mouse	Forward	GATGACCTGCAGTCCATAACC
		Reverse	CTGGCAACTCCTTCAGCAAC
IL6	Mouse	Forward	GAGGATACCACTCCCAACAGACC
		Reverse	AAGTGCATCATCGTTGTTCATACA
TNFα	Mouse	Forward	ATAGCTCCCAGAAAAGCAAGC
		Reverse	CACCCCGAAGTTCAGTAGACA
Arg-1	Mouse	Forward	CAGAAGAATGGAAGAGTCAG
		Reverse	CAGATATGCAGGGAGTCACC
Ym-1	Mouse	Forward	AGAAGGGAGTTTCAAACCTGG
		Reverse	TGTTTGTCCTTAGGAGGGC
Fizz-1	Mouse	Forward	TCCAGCTGATGGTCCCAGTGAATA
		Reverse	GGCAGTGGTCCAGTCAAC
Actin	Mouse	Forward	TGGAATCCTGTGGCATCCATGAAAC
		Reverse	TAAAACGCAGCTCAGTAACAGTCCG
GAPDH	Mouse	Forward	AAGGGCATCTTGGGCTACACT
		Reverse	TACTCCTTGGAGGCCATGTAGG
Ninj1	Human	Forward	CAAGCTGGACTTCCTCAACAA
		Reverse	CATGTCCATCAAGGGCTTCT
IL1β	Human	Forward	AGCTGTACCCAGAGAGTCC
		Reverse	ACCAAATGTGGCCGTGGTTT
IL6	Human	Forward	AGACAGCCACTCACCTCTTCA
		Reverse	CACCAGGCAAGTCTCCTCATT

Table 1. Primers for RT- and qRT-PCR

9. Cell isolation from colons

Colons were dissected from mice and washed with cold phosphate-buffered saline (PBS). Colons were then cut into several small pieces, inverted, and incubated at 37°C in RPMI supplemented with 0.015% DTT and 1 μM EDTA for 30 min with gentle shaking. The remaining tissue was incubated at 37°C in RPMI supplemented with 1.5 mg/mL collagenase and 0.5 mg/mL dispase, with gentle shaking. The supernatant containing the lamina propria cells was passed through a 70 μm strainer and stained for use in flow cytometric analysis.

10. Flow cytometry analysis

Lamina propria cells were stained with fluorochrome-conjugated antibodies against CD45.2, CD11b and F4/80 (BD Bioscience, CA, USA). After fixed with 4% paraformaldehyde, the cells were stained with rabbit anti-Ninjurin1 followed by FITC-conjugated secondary antibody. A FACS Canto II instrument (BD Biosciences) was used to acquire the results, which were analyzed by FlowJo V10 software.

11. Peritoneal macrophage isolation

Peritoneal macrophages were harvested from WT and Ninjurin1 KO mice. After the abdominal skin of mouse was removed, 3 ml of PBS was injected to the peritoneal cavity using a syringe and aspirated PBS from peritoneum. This step was repeated two more times. The solutions containing peritoneal cells were centrifuged for 5 min at 500 g. The peritoneal cells were resuspended in DMEM supplemented with 10% FBS and streptomycin (100 μg/ml)/penicillin (100 units/ml). The cells were incubated

at CO_2 incubator for 40 min in cell culture plates. After non-adherent cells were washed with PBS three times, the adherent cells were collected and prepared for further experiments.

12. Bone marrow-derived macrophage (BMDM) isolation and culture

After mice were euthanized by carbon dioxide inhalation, femurs of mice were excised. Cells were flushed from the femur of mice and incubated in RPMI1640 medium (Welgene) supplemented with 10% FBS, 30 ng/mL of mouse colony-stimulating factor (Miltenyi Biotec), and streptomycin (100 μg/ml)/penicillin (100 units/ml). After 5 days, adherent cells were harvested with cell dissociation solution (Sigma-Aldrich), and cultured in new plates for use in further experiments. The adherent cells were stained with antibodies against CD11b (BD Biosciences) and F4/80 (eBioscience) and analyzed by FACS Calibur (BD Biosciences). Over 90% adherent cells were positive for CD11b and F4/80. To stimulate BMDM, 0.5 μg/mL of LPS was applied for 2 h, or 10 ng/mL of IL4 (Peprotech, London, UK) was applied for 12 h.

13. Analysis of tight junction protein complex in vitro

Coverslips were sterilized and placed in 24-well cell culture plates. Caco2 cells were seeded on sterile coverslips at 90~100% confluency, and allowed to differentiate for an additional 10 days. Every 2~3 days, cell culture media were changed. The differentiated cell monolayers were transfected with adenovirus expressing human

Ninjurin1 (Ad-Ninjurin1) or empty vector (Ad-EV), with or without 5% DSS, as previously described (Jang et al., 2016). The cells were fixed and incubated with ZO-1 antibody, as the protocol published in a previous report (Jung et al., 2017a). Briefly, cells were incubated with MeOH/acetone for 20 min at -20 ℃ and blocked with blocking solution (Life technologies) for 1 h at RT. Fixed cells were then reacted with anti-ZO-1 antibody (Thermo Fisher Scientific, MA, USA) overnight at 4 ℃. FITC-conjugated secondary antibody (Santa Cruz Biotechnology) was applied for 2 h at RT in the dark. The nucleus was stained and mounted with Vectashiled mounting medium (Vector Laboratories).

14. Microarray analysis

RNA was isolated from peritoneal macrophages extracted from WT and Ninjurin1 KO mice. RNA from each sample was synthesized to make cDNA by using the GeneChip Whole Transcript amplification kit, according to the manufacturer's protocol. The cDNA was hybridized with the Affymetrix Mouse Gene ST 2.0 arrays and analyzed by Macrogen (Seoul, Korea). Data were normalized by applying the multi-average (RMA) method, and statistical significance was calculated by applying the LPE test. The raw data were uploaded in NCBI (GSE142544).

15. Mouse cytokine array

WT and Ninjurin1 KO Raw264.7 cells were cultured with 0.5 μg/mL of LPS for 12 h. Approximately 1 mL of media was collected from each group and centrifuged to remove particulates. The secreted cytokines and chemokines in media were assessed

by using a mouse cytokine array according to the manufacturer's instructions (R&D Systems, MN, USA). Briefly, nitrocellulose membranes containing 40 different capture antibodies were blocked for 1 h at RT with gentle shaking. Antibody cocktail buffer was applied to the membranes for 1 h at RT. After discard the antibody buffer, the membranes were incubated with the collected media from Raw 264.7 cells overnight at 4 °C with gentle shaking. Each membrane was washed three times and Streptavidin-HRP solution was applied. After washing three times, membranes were incubated with chemiluminescent reagent mixtures. The pixel density of each spot of the array was determined by using Image J software.

16. Colon explant and ELISA

Approximately 1 cm of colon tissue was excised and washed several times with PBS supplemented with penicillin and streptomycin. The tissue was then incubated in RPMI 1640 media in 24-well plates at 37°C for 24 h. The supernatant was collected and centrifuged to remove debris. Using a DuoSet mouse IL1β and IL6 ELISA, the supernatants were analyzed for IL1β and IL6 content according to the manufacturer's instructions (R&D Systems). Briefly, PBS containing capture antibodies was transferred to 96-well microplate overnight at RT to coat the plate. After washed with 0.05% Tween 20 in PBS three times, the plates were blocked at RT for 1 h. The colon explants were added to the plates for 2 h at RT. The plates were washed and incubated with solution containing detection antibodies. Streptavidin-HRP solution and substrate solution were applied sequentially. 2 N H_2SO_4 was added to the plates to stop the reaction. Using a microplate reader (Multiskan GO Microplate

Spectrophotometer, Thermo Fisher Scientific, Vantaa, Finland), the optical density of each well was determined.

17. Human NF-κB Pathway Array

THP-1 cells were cultured with 10 nM of phorbol 12-myristate 13-acetate overnight. The cells were incubated with 0.1 μg/ml of LPS for 2 h. Cells were lysed with modified RIPA buffer and the same protein amount was utilized. NF-κB-related proteins were investigated using human NF-κB pathway array following the manufacturer's instructions (ARY029; R&D Systems). Nitrocellulose membranes containing 45 different capture antibodies were blocked for 1 h at RT with gentle shaking. The membranes were incubated with the prepared THP-1 cell lysates overnight at 4 °C on a rocking platform. After washing three times, each membrane was incubated with solution containing detection antibodies for 1 h at RT. Streptavidin-HRP solution was applied. After washing three times, membranes were incubated with chemiluminescent reagent mixtures.

18. RNA interference

Small interfering RNA (siRNA) duplexes against PKCδ was synthesized and purified by Bioneer (Daejeon, Korea). The sequences of siRNA targeting PKCδ were siRNA1 (5′-GAUGAAGGAGGCGCUCAGTT-3′) and siRNA2 (5′-GGCUGAGUUCUGGCUGGACTT-3′) (Yoshida et al., 2003). For siRNA treatment, duplexed siRNA was introduced into WT and Ninjurin1 KO Raw264.7 cells using Mirus TransIT-X2 (Mirus Bio, WI, USA). Cells were lysed with modified RIPA

buffer for western blot analysis at 48 h after transfection.

19. Statistical analysis

Results are presented as the mean ± SD or mean ± SEM. When comparing two groups, statistical significance was determined by applying the two-tailed Student's t-test. Ninjurin1 gene expression data were retrieved from the microarray data matrices uploaded on GEO by the original authors (GSE1710, GSE22307, and GSE3365). R scripting was applied to obtain the expression values of Ninjurin1 from data matrices. Welch's T test was utilized to compare Ninjurin1 gene expressions in different specimens. Testing was performed using the SPSS 20 software (IBM Corporation, NY, USA). Significance of difference p-values are represented in text and figures as * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.005$.

III. Results

1. Ninjurin1 expression increases under intestinal inflammatory conditions

Since Ninjurin proteins are reported to play a crucial role in immune responses, I analyzed the expression of Ninjurin1 and Ninjurin2 genes in an IBD patient cohort and in a mouse model of colitis. By analyzing the publicly published mRNA expression profiles (Gene expression omnibus, GEO), I determined that Ninjurin1 mRNA expression was greater in colon tissues from patients with CD (n = 10) and UC (n = 10), as compared to normal controls (n = 11) (Figure 8A) (Costello et al., 2005). Moreover, Ninjurin1 mRNA expression was upregulated in the colon of DSS-treated mice, when compared to untreated (normal) mice (Figure 8B) (Fang et al., 2011). However, Ninjurin2 mRNA expression was not different between normal controls and colon tissues from patients with CD and UC (Figure 9A). In parallel to this result, DSS-treated mice did not show changed expression level in Ninjurin2 compared to untreated mice (Figure 9B). These results indicate that only Ninjurin1 expression is related to colitis incidence.

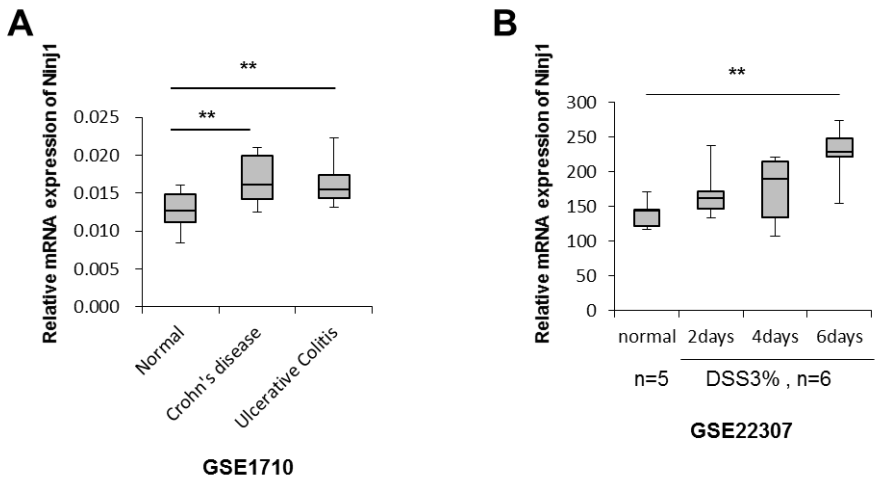


Figure 8. Ninjurin1 mRNA expression in colon tissues.

(A) Analysis of Ninjurin1 expression in colon tissues from UC and CD patients. The gene expression data (GSE1710) were obtained from the GEO database. ** $p < 0.01$, Welch's T test. (B) Analysis of Ninjurin1 expression in colon tissues from mice. The gene expression data (GSE22307) were obtained from the GEO database. ** $p < 0.01$, Welch's T test.

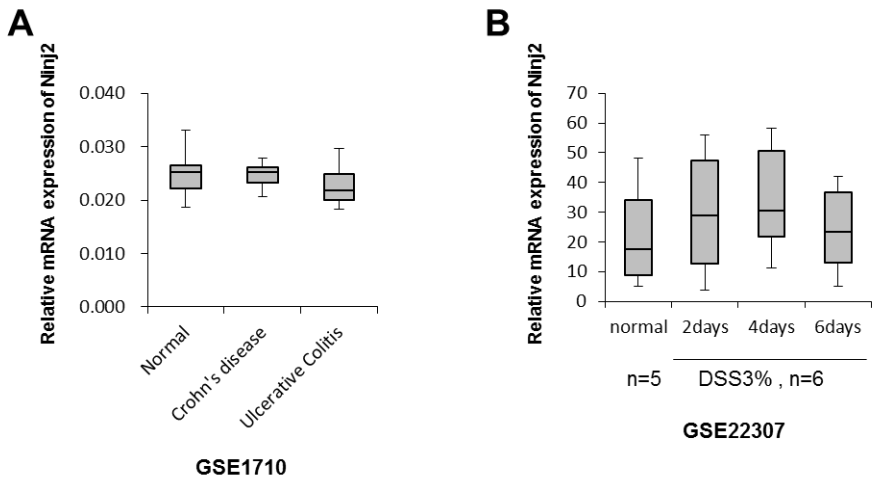


Figure 9. Ninjurin2 mRNA expression in colon tissues.

(A) Analysis of Ninjurin2 expression in colon tissues from UC and CD patients. The gene expression data (GSE1710) were obtained from the GEO database. (B) Analysis of Ninjurin2 expression in colon tissues from mice. The gene expression data (GSE22307) were obtained from the GEO database.

2. Ninjurin1 expression increases in colon tissues of DSS-treated mice

I further validated Ninjurin1 expression in mice colon tissues by western blot analysis. I treated 1.5% DSS to mice for 8days and sacrificed to assess Ninjurin1 expression in colon tissues. As expected, Ninjurin1 in colons of DSS-treated mice show increased expression levels as compared to untreated mice (Figure 10). Moreover, immunofluorescence staining revealed that the number of Ninjurin1^{+} cells significantly increased in colon tissues of DSS-treated mice (Figure 11) compared to control mice. Interestingly, Ninjurin1 expression in inflamed colon was limited to non-epithelial cells, and cells expressing Ninjurin1 were mainly localized in the submucosa (Figure 11). Surface epithelial cells which are linked by tight junction proteins did not express Ninjurin1. These findings suggest that during colitis, there is increased Ninjurin1 expression in cells at the sites of inflammation.

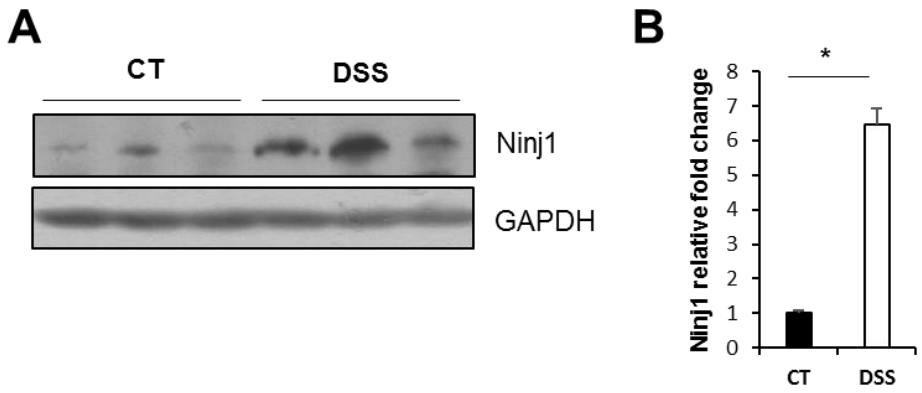


Figure 10. Immunoblot analysis of Ninjurin1 in DSS-treated colon tissues.

(A) Ninjurin1 and GAPDH performed on colonic tissues extracted from control (CT) and 1.5% DSS-treated mice; n = 3 per group. Similar results were observed in three independent experiments. (B) Densitometry represents relative protein levels of Ninjurin1. Values are mean ± SD. * $p < 0.05$, Student's t-test.

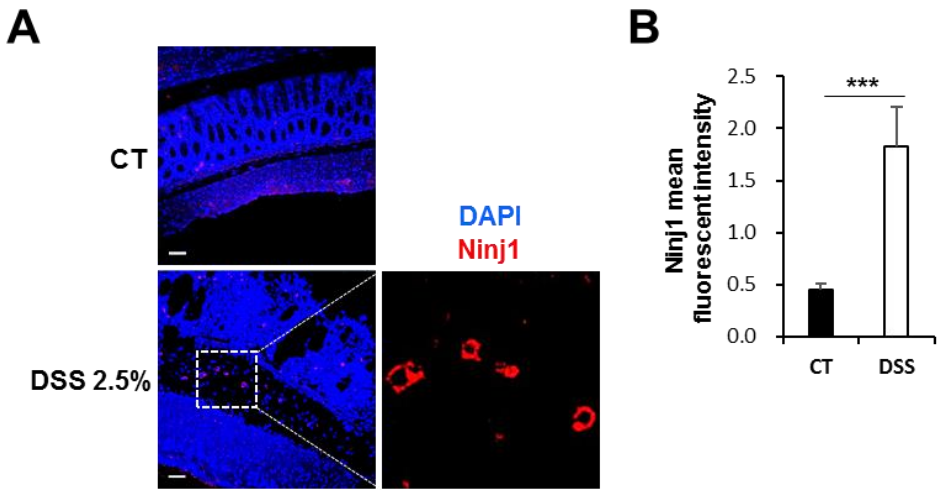


Figure 11. Immunofluorescence staining of Ninjurin1 in colon tissue sections harvested from mice.

(A) Representative images are shown. Scale bars, 50 μm. Boxed area is magnified on right. (B) The quantification of Ninjurin1 immunofluorescence is presented as the mean fluorescence intensity; $n = 3$ per group. Values are mean ± SD. *** $p < 0.005$, Student's t-test. CT, control.

3. Ninjurin1 deficiency alleviates experimental colitis

To assess whether Ninjurin1 has a functional role in colitis, WT and Ninjurin1 KO mice were administered 1.5% or 2.5% DSS for 8 days, followed by determining the colitis incidences. Ninjurin1 KO mice were generated by deletion of exon1 of Ninjurin1 (Figure 12). Ninjurin1 KO mice exhibit lesser body weight loss (Figure 13) and a considerably longer colon length (Figure 13 and Figure 14A, B) as compared to WT mice. H&E analysis reveal less epithelial erosion, crypt destruction, and submucosal edema in the colon of DSS-treated Ninjurin1 KO mice than DSS-treated WT mice (Figure 15A). Histopathological assessments associated with colitis were quantified as described in Table 2. The tissue phenotypes associated with colitis were also quantified, and results reveal reduced colitis incidence in DSS-treated Ninjurin1 KO mice (Figure 15B). Untreated WT and Ninjurin1 KO mice did not display significant differences in tissue histology features (Figure 15). Ki-67 and Alcian blue staining were performed to confirm the severity of colitis incidence in Ninjurin1 KO mice. DSS-treated Ninjurin1 KO mice had relatively intact epithelia and a greater number of proliferating cells and mucin-containing goblet cells, as compared to DSS-treated WT mice (Figure 16). Taken together, these results indicate that Ninjurin1 aggravates experimental colitis.

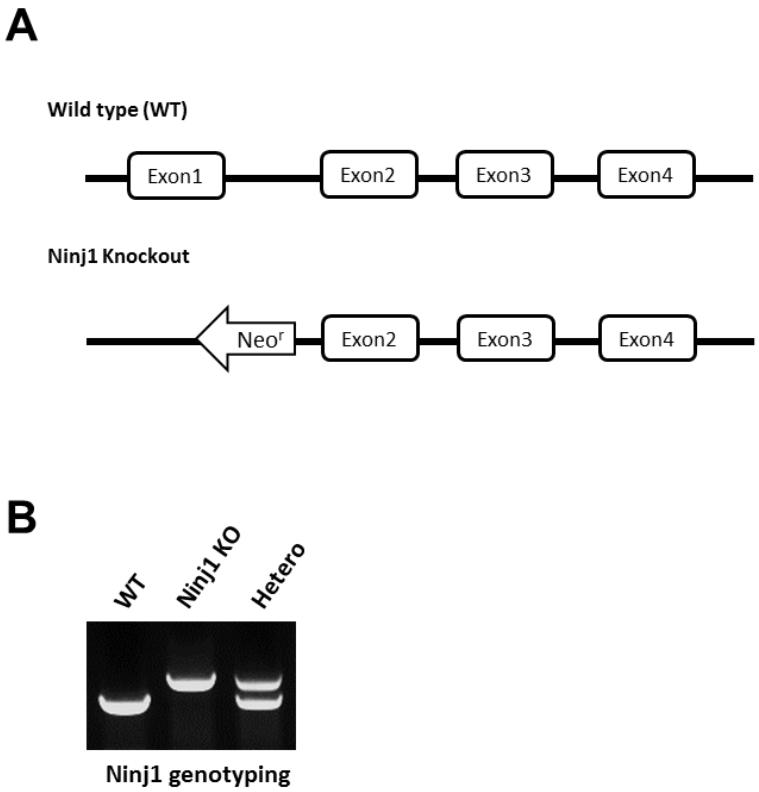


Figure 12. Schematic diagram and confirmation of Ninjurin1 deficient mice.

(A) Gene structure of WT and Ninjurin1 KO mice. (B) Mouse genotype was verified using PCR of genomic tail DNA.

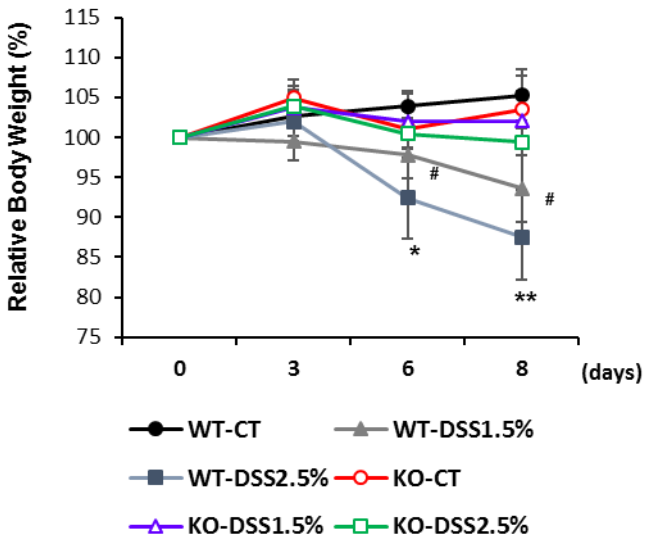


Figure 13. Body weight loss of WT and Ninjurin1 knock-out (KO) mice were treated with DSS to induce experimental colitis.

WT and Ninjurin1 KO mice were administered 1.5% or 2.5% DSS for 8 days. Bodyweight loss is presented as a percentage of the initial weight ± SD; n = 5 per group. Student's t-test comparing 1.5% DSS treated WT mice and 1.5% DSS treated Ninjurin1 KO mice, $^{\#}$ $p < 0.05$, and comparing 2.5% DSS treated WT mice and 2.5% DSS treated Ninjurin1 KO mice, * $p < 0.05$, ** $p < 0.01$. Similar results were observed in four independent experiments. CT, control

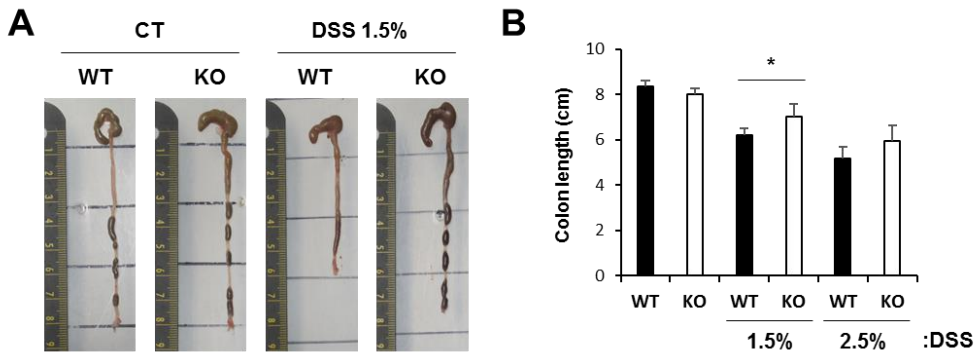


Figure 14. Colon length of DSS treated mice.

(A) Mice were sacrificed on day 8 after initiation of DSS treatment. Representative images of colons from WT and Ninjurin1 KO mice are presented. (B) Colon lengths were quantified (mean ± SD). * $p < 0.05$, Student's t-test. CT, control.

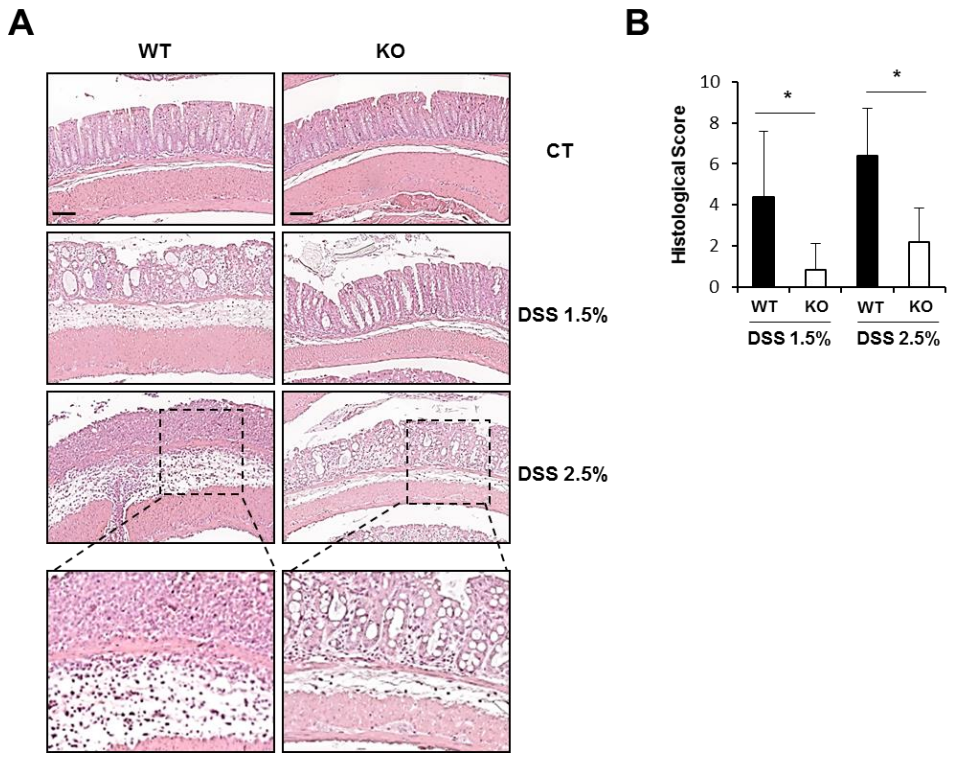


Figure 15. Histological analysis of colon tissues (H&E staining).

(A) H&E staining of colon sections was performed, and representative images are presented. Scale bars, 50 μm. Boxed areas are magnified below. (B) Submucosal edema, surface epithelial erosion, and crypt damage were evaluated and quantified by histology scores (0–9). Data are presented as mean ± SD. * $p < 0.05$, Student's t-test. CT, control.

Feature score	Score	Description
Submucosal edema	0	No significant edema
	1	Rare areas of submucosal edema
	2	Occasional areas of mild submucosal edema
	3	Frequent areas of marked submucosal edema
Surface epithelial erosion	0	None
	1	Rare small breaches in epithelium
	2	Frequent small breaches in epithelium
	3	Extensive areas lacking surface epithelium
Crypt damage	0	None
	1	Some crypt damage
	2	Large areas without crypts
	3	No crypts

Table 2. Histological scoring of colitis (Fodil et al., 2017)**.**

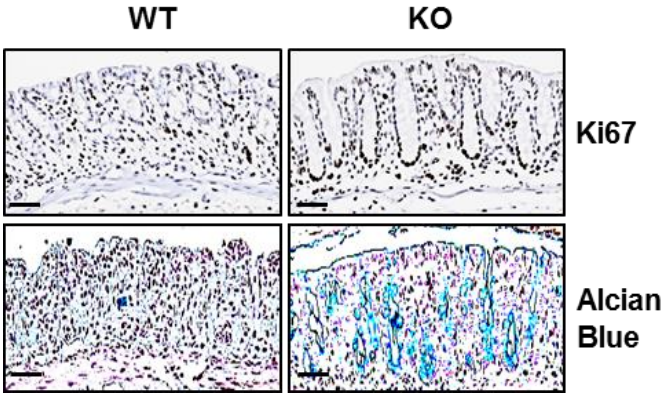


Figure 16. Histological analysis of colon tissues (Ki67 and Alcian blue staining).

Ki67 staining of colon sections from 1.5% DSS-treated WT and Ninjurin1 KO mice were visualized for crypt regeneration. Scale bars, 50 μm (upper panel). Alcian blue staining of colon sections from 1.5% DSS-treated WT and Ninjurin1 KO mice indicate the epithelial integrity. Scale bars, 50 μm (lower panel).

4. Ninjurin1 is highly expressed on cells of myeloid lineage

Among the immune cells, myeloid cells have been reported to predominantly express Ninjurin1 during experimental stimulation (Ahn et al., 2009; Ifergan et al., 2011). A previous report indicates that Ninjurin1 is mainly expressed in myeloid cells isolated from human peripheral blood leukocytes, whereas B and T lymphocytes express relatively low levels of Ninjurin1 (Ifergan et al., 2011). To define the immune compartment expressing Ninjurin1 and modulating colonic inflammation, I evaluated Ninjurin1 expression in BMDMs, T cells, and B cells. In the naïve state, Ninjurin1 is highly expressed in BMDMs, as compared to the lymphoid populations (including $CD4^+$ and $CD8^+$ T cells, and B cells) (Figure 17, upper). During inflammation, leukocytes are differentiated and activated to function appropriately. In the activated state, macrophages reveal highest expression of Ninjurin1, compared to other lymphocytes (Figure 17, lower). In both naïve and activated states, Ninjurin1 is expressed by myeloid cells in peripheral blood leukocytes.

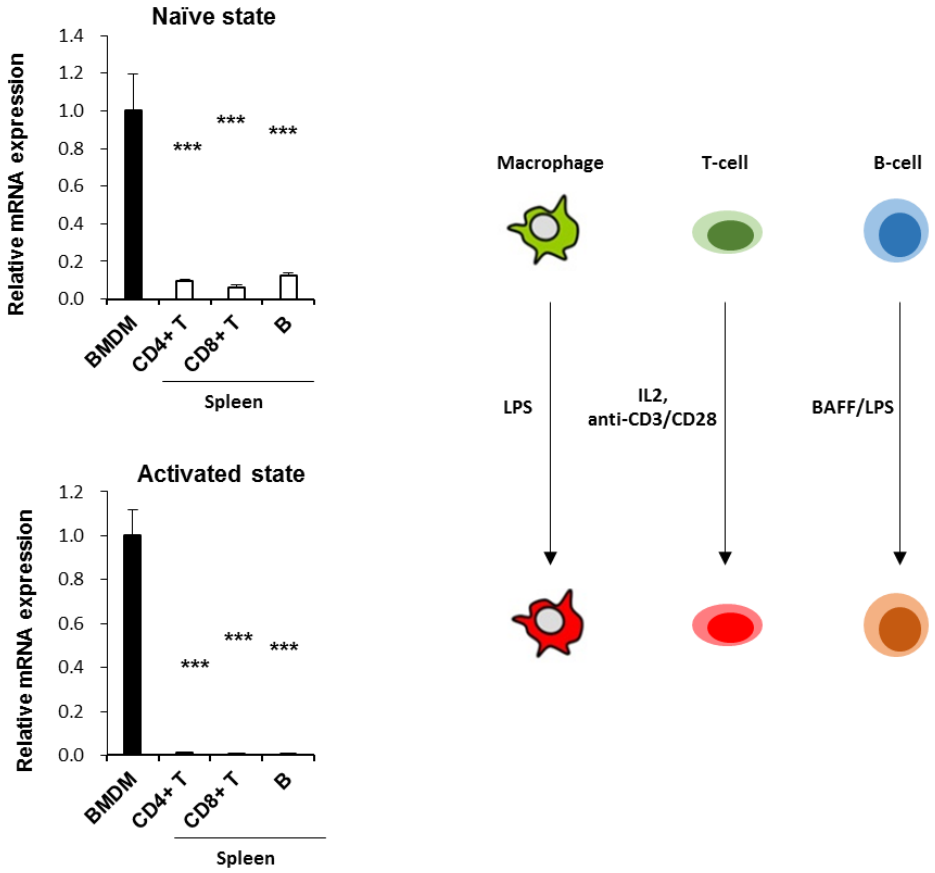


Figure 17. mRNA expression of Ninjurin1 in the indicated leukocytes and macrophages was analyzed by qRT-PCR.

Bone marrow-derived macrophages (BMDMs) were cultivated from bone marrow cells, and $CD4^+$ and $CD8^+$ T cells and B cells were isolated from spleen using MACS (upper panel). Ninjurin1 expression was determined using lipopolysaccharide (LPS)-treated BMDMs, anti-CD3/anti-CD28-activated $CD4^+$ and $CD8^+$ T cells, and LPS/BAFF-activated B cells (lower panel); $n = 3$ per group. Data represent mean ± SEM. *** $p < 0.005$ compared to the BMDM group, Student's t-test.

5. Macrophages during colitis development show increased expression of Ninjurin1

To research further information on the expression pattern of Ninjurin1 in various immune cells under inflammatory conditions, I investigated alteration of Ninjurin1 protein expression on lymphocytes and myeloid cells during development of experimental colitis. Lymphocytes and myeloid cells were extracted from mesenteric lymph node and spleen of untreated and DSS-treated mice. Ninjurin1 expressions on $CD19^+$ B cells, $CD4^+$, and $CD8^+$ T cells extracted from mesenteric lymph node were not altered with DSS treatment (Figure 18 upper). In contrast to lymphocytes, expression of Ninjurin1 was significantly upregulated on $CD11b^+$ myeloid cells (Figure 18 lower). Macrophages, monocytes ($CD11b^+Gr\text{-}1^{low}$ and $CD11b^+ Gr\text{-}1^{int}$), and neutrophils ($CD11b^+Gr\text{-}1^{hi}$) showed increased Ninjurin1 expression pattern under inflammatory conditions. The lymphocytes and myeloid cells extracted from spleen showed increased Ninjurin1 expression from DSS-treated mice compared to that from untreated mice (Figure 19). Moreover, in accordance with Figure 17, macrophages and monocytes from DSS-treated mice showed the strongest expression of Ninjurin1 compared with lymphocytes and neutrophils (Figure 18 and Figure 19). To gain further information about Ninjurin1 expression in macrophages, I extracted peritoneal and colonic macrophages from untreated and DSS-treated mice. I observed that mice treated with DSS showed increased Ninjurin1 expression in peritoneal macrophages (Figure 20). Furthermore, a FACS analysis revealed that the colonic macrophages extracted from lamina propria of DSS-treated WT mice exhibited

enhanced Ninjurin1 expression compared to those from untreated mice (Figure 21). Therefore, these results suggest that macrophages are responsible for expressing Ninjurin1 in colon tissues, which exhibit increased Ninjurin1 during colitis.

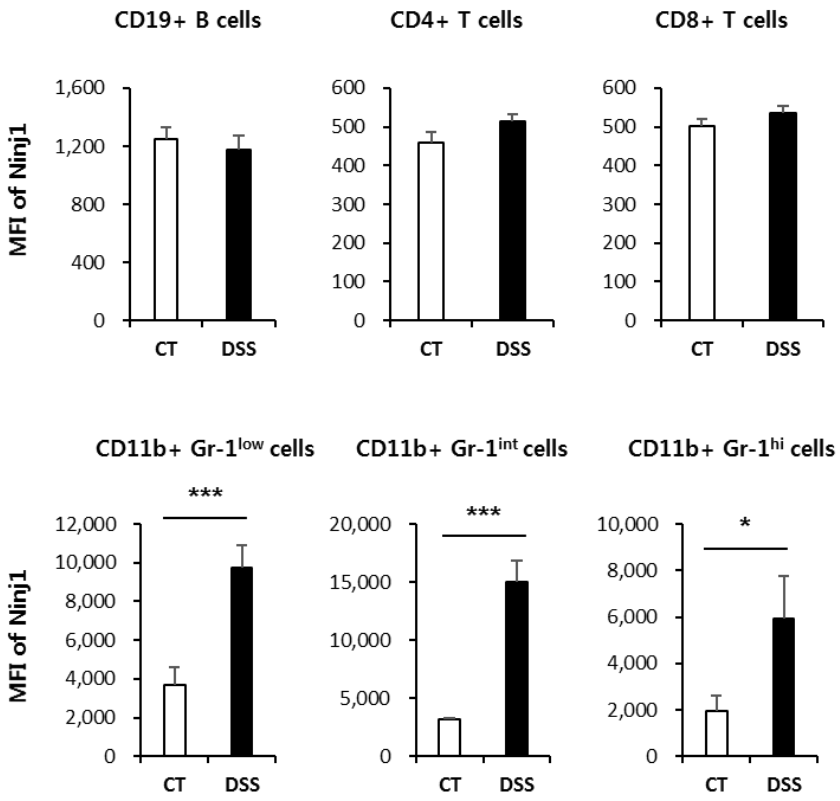


Figure 18. Ninjurin1 expression on lymphocytes and myeloid cells in mesenteric lymph node of mice.

Single cell suspensions were isolated from the mesenteric lymph node of 1.5% DSS-treated mice or control mice, and stained with surface markers. Shown are the representative FACS histograms for Ninjurin1 expression with mean fluorescence intensities (MFIs). * $p < 0.05$, *** $p < 0.005$ compared to the CT group.

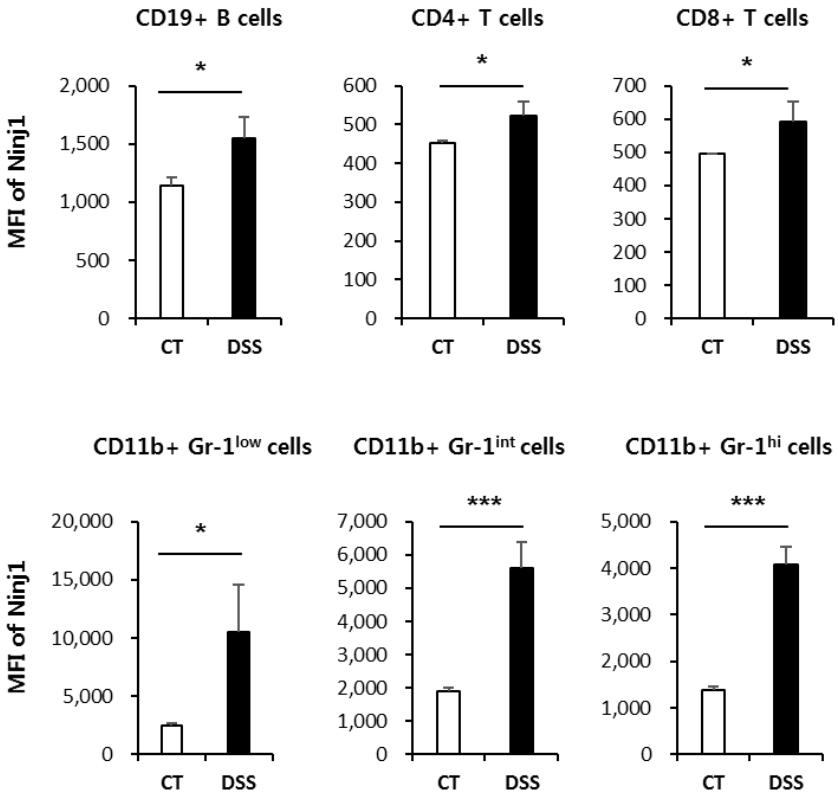


Figure 19. Ninjurin1 expression on lymphocytes and myeloid cells in spleen of mice.

Single cell suspensions were isolated from the spleen of 1.5% DSS-treated mice or control mice, and stained with surface markers. Shown are the representative FACS histograms for Ninjurin1 expression with mean fluorescence intensities (MFIs). * $p < 0.05$, *** $p < 0.005$ compared to the CT group.

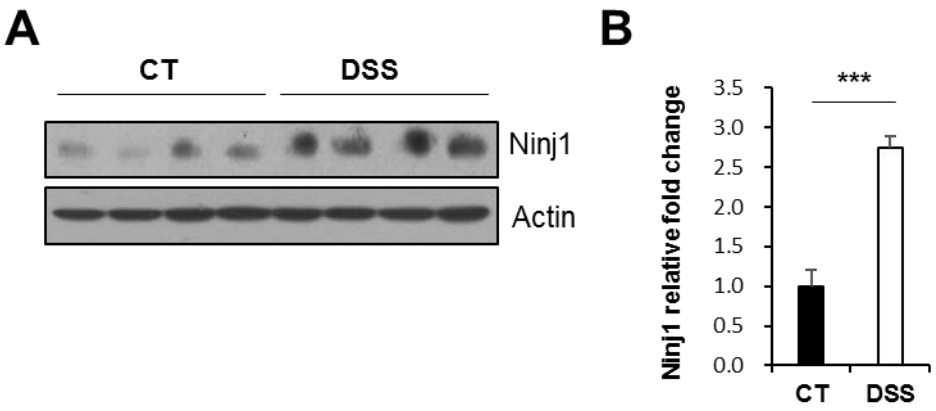


Figure 20. Ninjurin1 expression in peritoneal macrophages.

Ninjurin1 and actin protein expressions were detected in peritoneal macrophages extracted from untreated and 2.5% DSS-treated WT mice; n = 4 per group. Densitometry represents relative protein levels of Ninjurin1. Similar results were observed in three independent experiments. Values expressed are mean ± SD. *** $p < 0.005$, Student's t-test. CT, control.

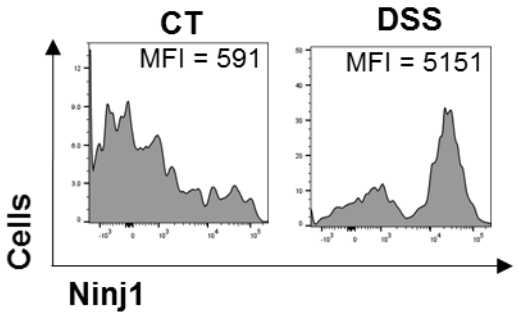


Figure 21. Induction of Ninjurin1 in macrophages of DSS-treated mice was analyzed by flow cytometry.

Shown are the representative FACS histograms for Ninjurin1 expression within macrophage gate (7-AAD^{-}, CD45^{+}, CD11b^{hi}, F4/80^{+}) with mean fluorescence intensities (MFIs).

6. Intestinal barrier is not affected by Ninjurin1

Epithelial barrier destruction is also associated with IBD. Tight junction proteins are located in the apical region of the intestinal epithelium, and form selectively permeable barriers. Increased permeability and loss of tight junction-related proteins are prerequisites for the development of colitis (Chelakkot et al., 2018). When mimicking the intestinal barrier in vitro, the Caco2 cell line is the most popular model because of its ability to spontaneously differentiate into an enterocyte-like phenotype. I evaluated Ninjurin1 expression in Caco2 cells and detected a relatively low expression level (Figure 22A). This result was parallel to my immunofluorescence data which reveals that Ninjurin1 expression is mainly detected in non-epithelial cells (Figure 11). I subsequently overexpressed Ninjurin1 in Caco2 cells with Ninjurin1 expressing adenovirus and subjected the cells to immunofluorescence staining with the anti-Zo-1 antibody. Regular distribution of Zo-1 protein in a Caco2 monolayer was unaffected by the overexpression of Ninjurin1 (Figure 22B). DSS was applied to Caco2 monolayers to mimic intestinal barrier dysfunction; this treatment disrupted the Zo-1 distribution in the Caco2 monolayer, with no significant changes in Zo-1 distribution upon Ad-Ninjurin1 treatment (Figure 22B). Overexpression of Ninjurin1 in intestinal epithelial cells did not alter the intestinal barrier function.

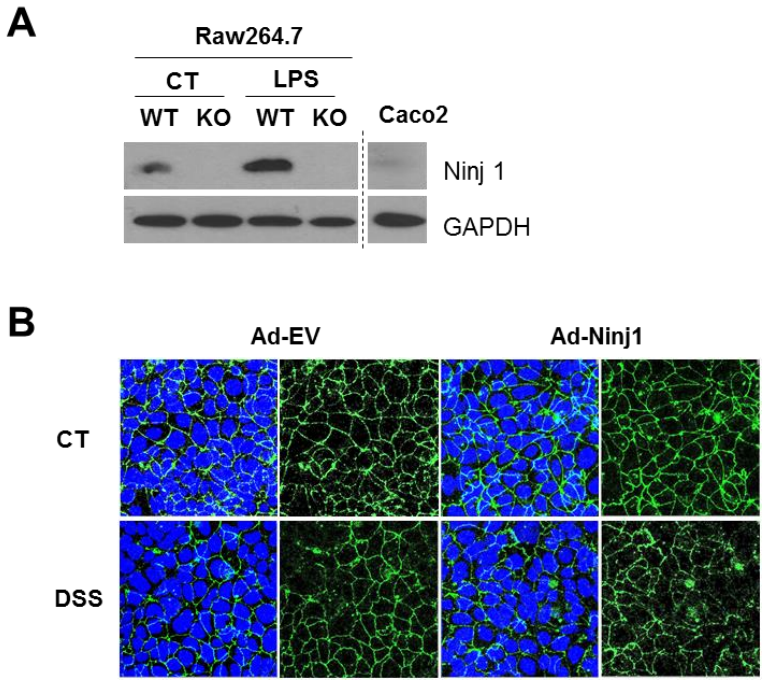


Figure 22. Ninjurin1 function in intestinal epithelial cells.

(A) Ninjurin1 expression in Raw 264.7 and Caco2 cells was detected by Western blotting. (B) The effect of Ninjurin1 on the structure of tight junction complexes *in vitro*. Caco2 cell monolayers were infected with Ad-EV (adenovirus-empty vector) or Ad-Ninjurin1, and subsequently incubated with 5% DSS. Cell monolayers were stained for ZO-1 and images were collected by confocal microscopy. ZO-1 (green), nuclei (DAPI, blue), and CT, control.

7. Migration capacity of macrophages is not influenced by Ninjurin1 expression during colitis development

During the development of intestinal inflammation, macrophage infiltration is vital for pathogenesis. Interestingly, I did not observe decreased prevalence of myeloid cells in the spleen tissues and lamina propria of Ninjurin1-deficient mice (Figure 23). To confirm this result, I analyzed $CD11b^+$ $F4/80^+$ macrophages extracted from lamina propria by FACS analysis. DSS treatment resulted in marked infiltration of macrophages into the colonic tissues of both Ninjurin1-deficient and WT mice (Figure 24A, B). Using immunofluorescent staining, I further confirmed that there were no changes in the number of $F4/80^+$ macrophages in the colon tissues from DSS-treated WT and Ninjurin1 KO mice (Figure 24C). To investigate Ninjurin1 expression in the colon during colitis, the tissues were subjected to immunohistochemical analysis. I observed Ninjurin1 staining in a subpopulation of cells that are also positive for the macrophage marker F4/80 (Figure 25). These findings indicate that Ninjurin1 expressed on macrophages does not modulate the migration capacity of macrophages.

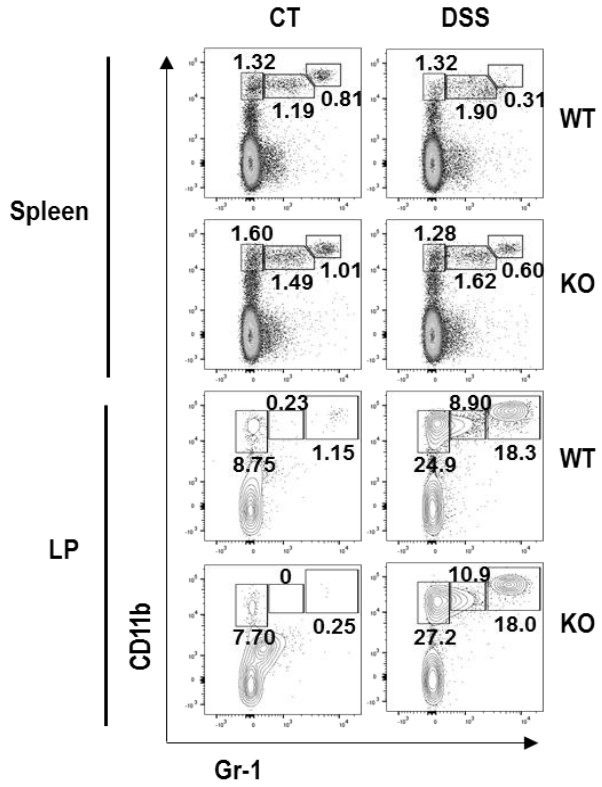


Figure 23. Infiltrating myeloid cells of DSS-treated mice were analyzed by flow cytometry.

Single cell suspensions were isolated from the spleen and lamina propria (LP) of 1.5% DSS-treated mice or control mice, and stained with surface markers. The representative FACS profiles are shown within viable leukocyte gate (7-AAD$^-$ CD45$^+$) with expression of CD11b and Gr-1.

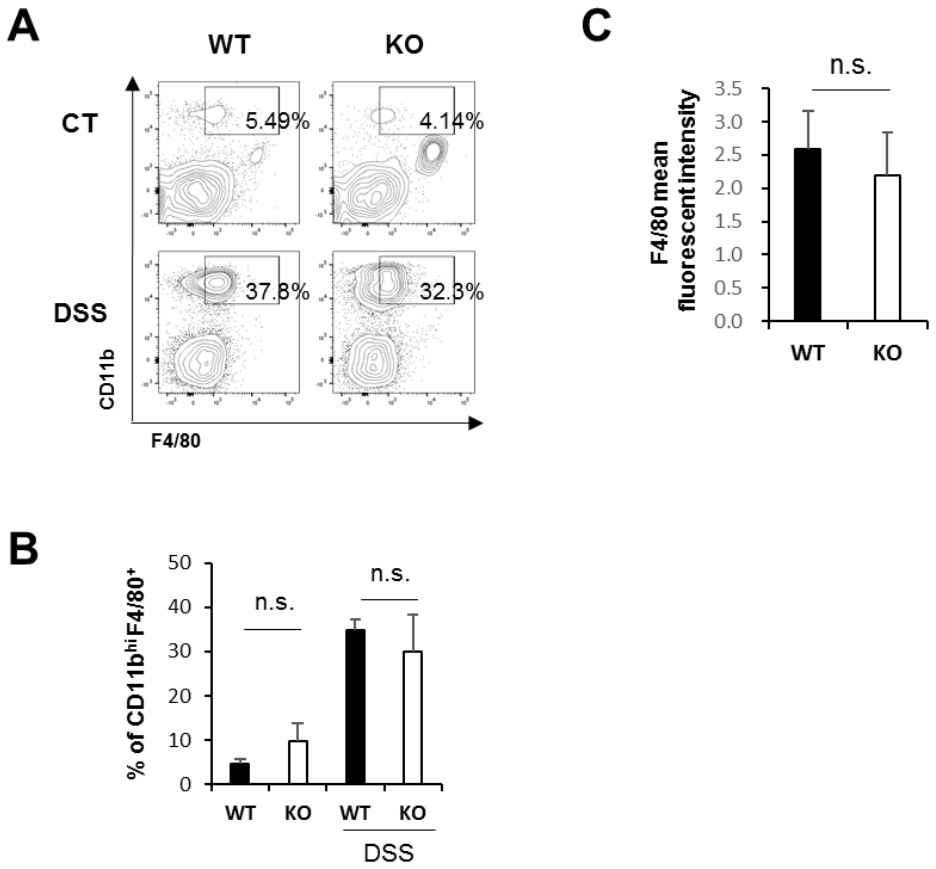


Figure 24. Infiltrating macrophages of DSS-treated mice were analyzed by flow cytometry and immunofluorescent staining.

(A) Single cell suspensions were isolated from the lamina propria of 1.5% DSS-treated mice or control mice, and stained with surface markers. The representative FACS profiles are shown within viable leukocyte gate (7-AAD$^-$, CD45$^+$) with frequencies of CD11b^{hi} F4/80$^+$ macrophages. (B) Cumulative data of CD11b^{hi} F4/80$^+$ macrophages; n=3 per group. Data represent mean ± SEM., Student's t-test. CT, control. (C) Immunofluorescence staining of F4/80 in colon tissue sections from mice.

The quantification of F4/80 immunofluorescence is presented as the mean fluorescence intensity; n=3 per group. Values are mean ± SD. Student's t-test. Not significant (n.s.).

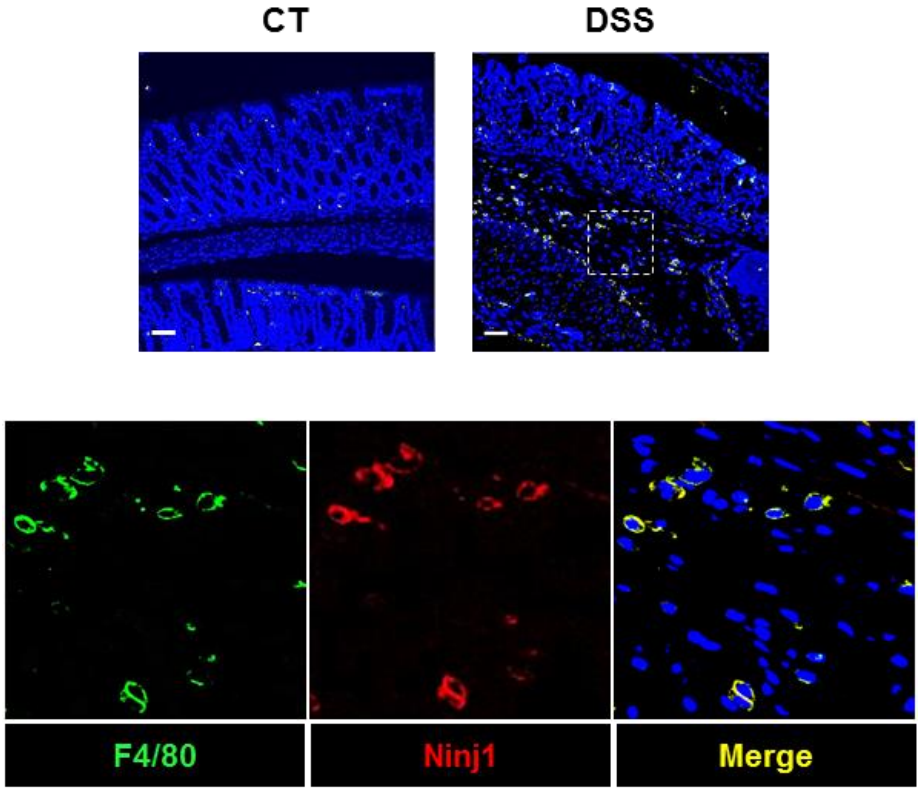


Figure 25. Confocal microscopy image of colon section from untreated (CT) and 1.5% DSS-treated WT mouse.

Sections were stained with antibodies against Ninjurin1 (red), F4/80 (green), and nuclei (DAPI, blue). Scale bars, 50 μm. Boxed area in upper panel is magnified in lower panel.

8. Ninjurin1 in macrophages enhances production of cytokines modulating colon inflammation

To elucidate the role of Ninjurin1 in macrophages and in inflammatory stimuli, I compared gene expressions in peritoneal macrophages from WT and Ninjurin1 KO mice. Several studies suggest that peritoneal macrophages are implicated in the development of colitis, even though these cells are not present at the inflammatory site (Eissa et al., 2017; Wang et al., 2013). Here, I determined that pro-inflammatory cytokines and chemokines such as IL1β, IL6, and CCL2 are upregulated in peritoneal macrophages from WT mice compared to the levels in peritoneal macrophages from Ninjurin1 KO mice (Figure 26A). Using qRT-PCR, I validated differences in the gene expression of pro-inflammatory cytokines in peritoneal macrophages (Figure 26B). In addition, I investigated the secretion of 40 different cytokines and chemokines in conditioned culture media of Raw264.7 cells. Initially, the Ninjurin1 gene was knocked out by using the CRISPR-Cas9 system, which was confirmed by western blot analysis (Figure 27A). In response to LPS, Ninjurin1 KO Raw264.7 cells secreted a lesser amount of pro-inflammatory cytokines than that secreted by WT cells (Figure 27B, C). This result was consistent with that in a previous study demonstrating that induced expression of pro-inflammatory cytokines with LPS was diminished when Ninjurin1 function was blocked with a Ninjurin1-blocking peptide (Jennewein et al., 2015). I also found that Ninjurin1 KO Raw 264.7 cells stimulated with LPS show decreased mRNA expression of IL1β and CCL2, whereas LPS-treated WT cells exhibit significantly increased IL1β and CCL2 levels (Figure 28A, B).

Moreover, overexpression of Ninjurin1 in THP-1 cells increased mRNA expressions of IL1β and IL6 (Figure 29). Furthermore, examining the gene expression of a cytokine in colons of DSS-treated mice revealed a decrease in mRNA expressions of IL1β and IL6 in colons of DSS-treated Ninjurin1 KO mice compared to DSS-treated WT mice (Figure 30A). In peritoneal macrophages from DSS-treated Ninjurin1KO mice, diminished IL1β expression was obtained, as compared to peritoneal macrophages from DSS-treated WT mice (Figure 30B). To confirm the above results, colon explant culture was performed, and the amounts of IL1β and IL6 was determined by ELISA. The amounts of IL1β and IL6 expressed in the colon explant culture media from WT mice was significantly higher than Ninjurin1 KO explants (Figure 31). Collectively, these results indicate that Ninjurin1 expression contributes to the activation of macrophages.

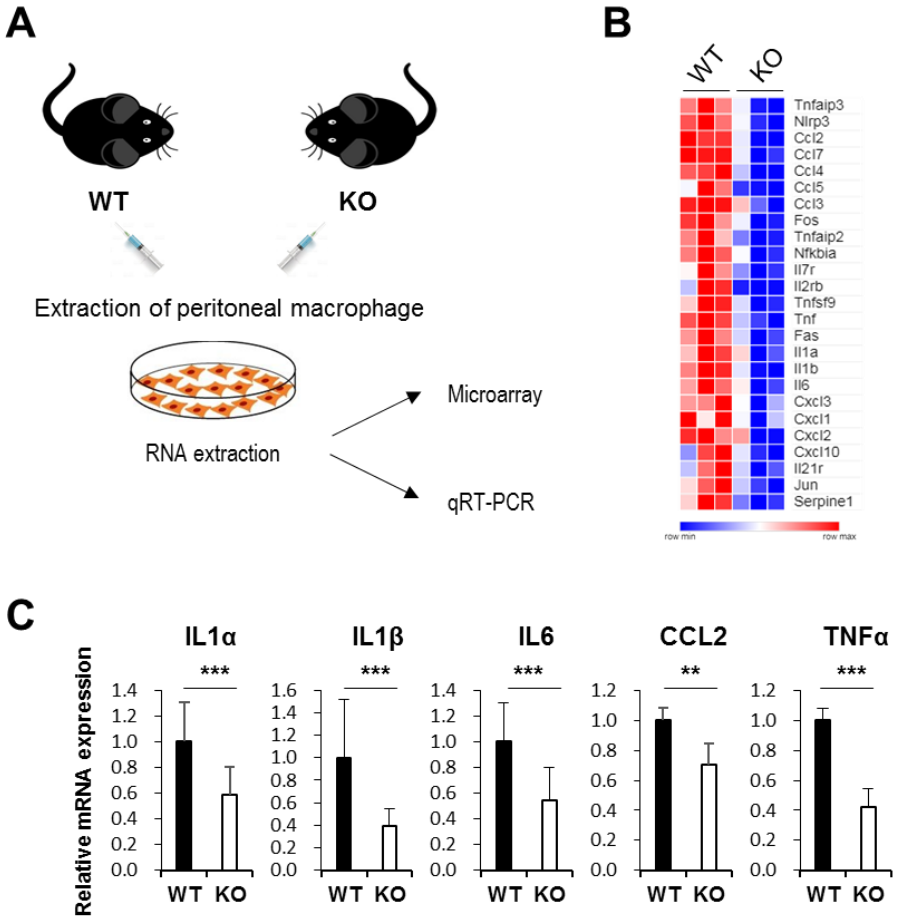


Figure 26. Differences in mRNA expression profiles of macrophages from WT and Ninjurin1 KO mice.

(A) A schematic diagram shows experiment using peritoneal macrophages. (B)Heat map of inflammation-related gene expression in peritoneal macrophages from WT and Ninjurin1 KO mice based on microarray analysis; n = 3 per group. (C) qRT-PCR was performed to confirm the genes of interest identified by microarray analysis. Data are presented as mean ± SEM. ** $p < 0.01$, *** $p < 0.005$, Student's t-test.

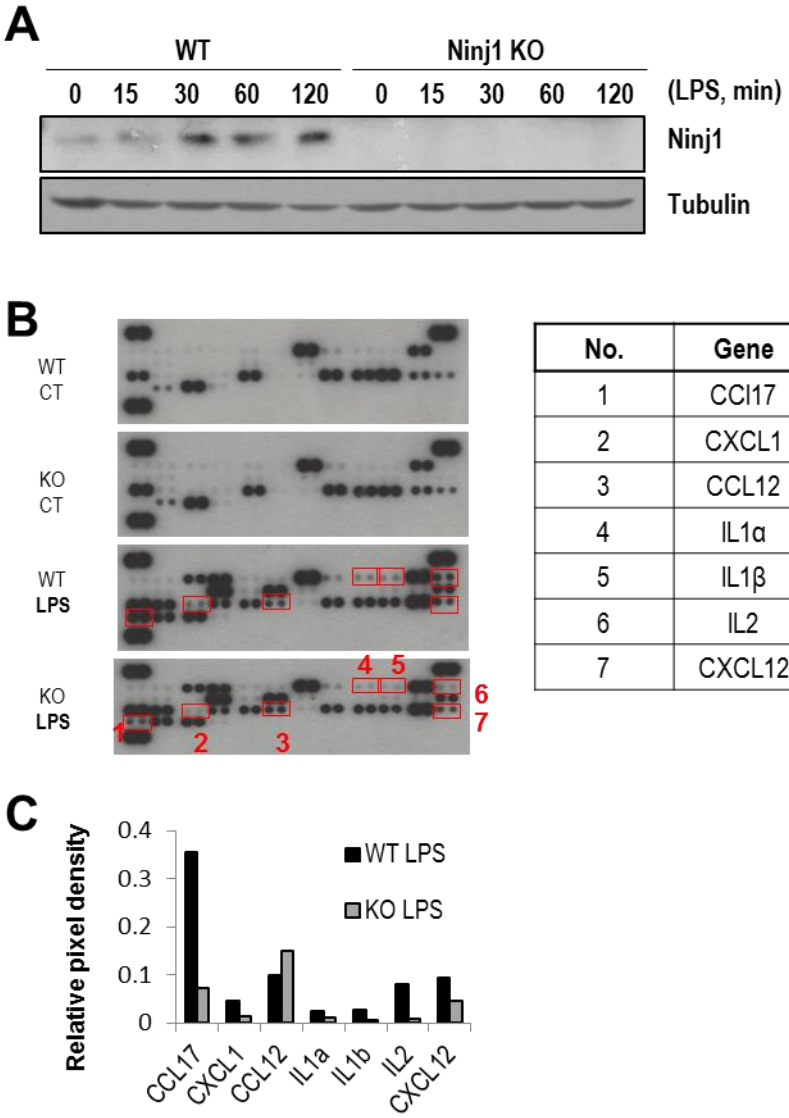


Figure 27. Analysis of cytokines and chemokines secreted by WT and Ninjurin1 KO macrophages.

(A) Raw264.7 WT and Ninjurin1 KO cells were treated with 0.5 μg/ml of LPS for the indicated duration. Western blot analysis was performed to detect the expression

of Ninjurin1 and tubulin. (B) Supernatants were collected from Raw264.7 WT and Ninjurin1 KO cells in the presence and absence of 0.5 μg/mL of LPS for 12 h. Cytokines and chemokines in the supernatants were analyzed by using a mouse cytokine array panel. (C) Densitometry represents relative levels of each spot on the array.

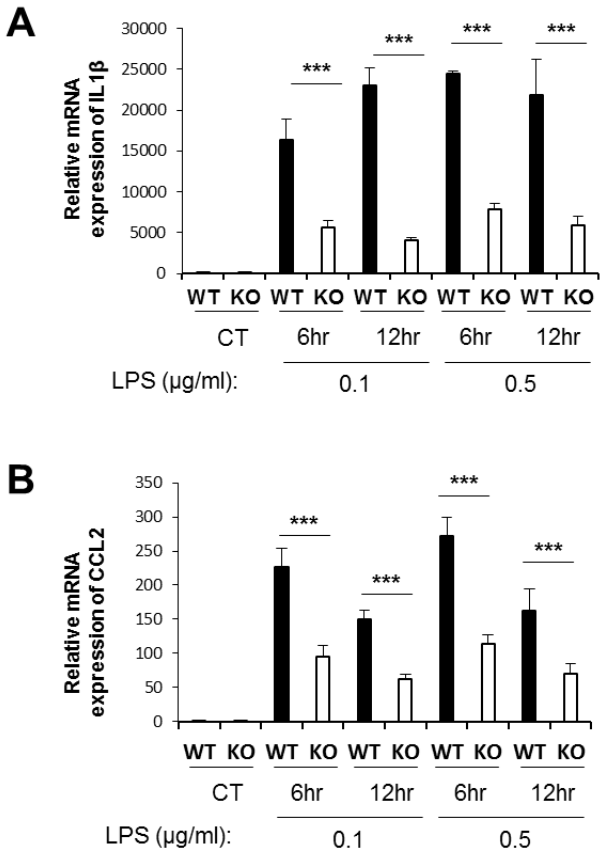


Figure 28. Ninjurin1 in Raw264.7 cells regulate the inflammatory response.

WT and Ninjurin1 KO Raw264.7 cells were treated with 0.1 or 0.5 μg/mL of LPS for the the indicated duration. The mRNA expressions of IL1β and CCL2 were detected and normalized by GAPDH. Data are presented as mean ± SEM. *** $p < 0.005$, Student's t-test. CT, control.

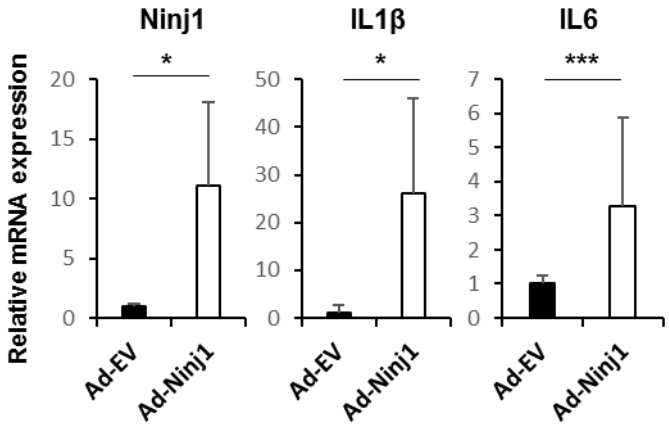


Figure 29. Overexpression of Ninjurin1 increases mRNA expression of IL1β and IL6.

THP-1 cells were infected with Ad-EV (adenovirus-empty vector) or Ad-Ninjurin1. The mRNA expressions of Ninjurin1, IL1β, and IL6 were detected by qRT-PCR and normalized by GAPDH. Data are presented as mean ± SEM. * $p < 0.05$, *** $p < 0.005$, Student's t-test.

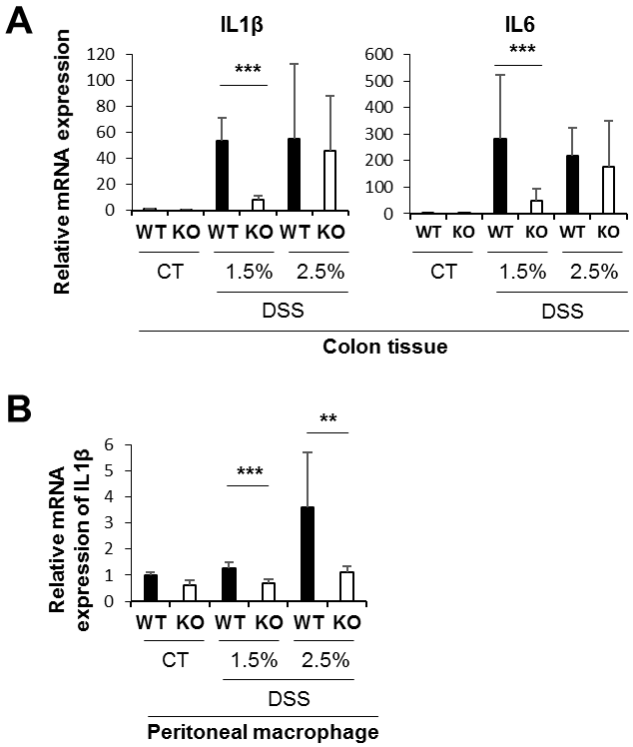


Figure 30. Loss of Ninjurin1 from macrophages reduces mRNA expression of IL1β and IL6.

(A) Relative mRNA expressions of IL1β and IL6 were analyzed by performing qRT-PCR using colon tissues from untreated and DSS-treated WT and Ninjurin1 KO mice; $n = 3$ per group. The expression values are normalized to GAPDH, and the plots are presented as mean ± SEM. *** $p < 0.005$, Student's t-test. Similar results were observed in three independent experiments. (B) Relative mRNA expression of IL1β was analyzed by performing qRT-PCR using peritoneal macrophages from untreated and DSS-treated WT and Ninjurin1 KO mice; $n = 3$ per group. Data are presented as mean ± SEM. ** $p < 0.01$, *** $p < 0.005$, Student's t-test. CT, control.

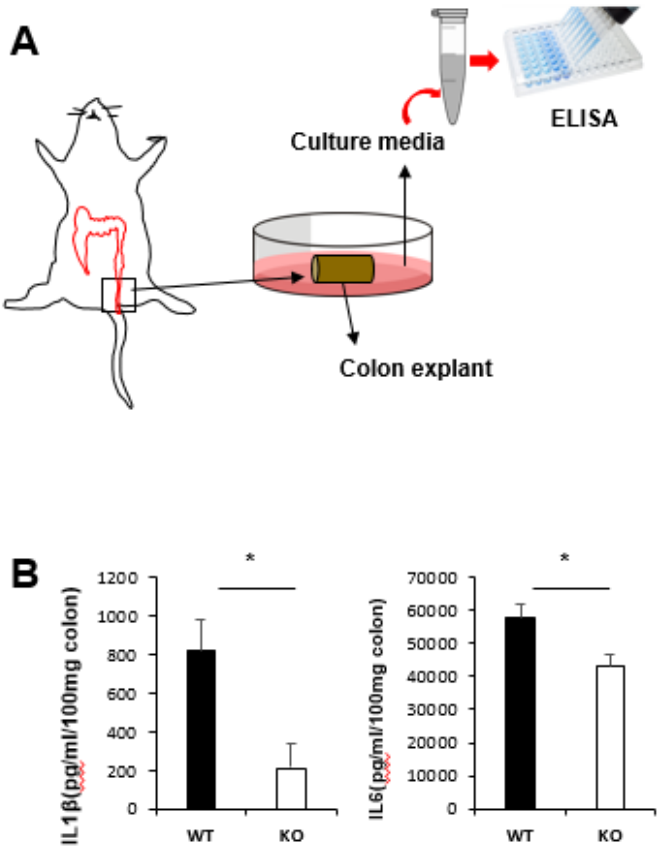


Figure 31. Detection of IL1β and IL6 secreted from colon explant.

(A) Schematic diagram of colon explant experiment. (B) WT and Ninjurin1 KO mice were treated with 1.5% DSS for 8 days. Mice were sacrificed on day 8 after initiating DSS treatment. Colonic tissue explants were harvested, and secreted IL1β and IL6 levels were assessed by ELISA; $n = 4$ per group. Data are presented as mean ± SEM. * $p < 0.05$, Student's t-test. Similar results were observed in three independent experiments.

9. Ninjurin1 inhibition in macrophages reduces production of cytokines

I examined whether inhibition of Ninjurin1 affects the intestinal inflammation. To inhibit function of Ninjurin1, I treated Ninjurin1 antibodies or blocking peptides in BMDMs. As shown in Figure 32A, Ninjurin1 antibodies did not prevent mRNA expression of IL1β compared to LPS only treated BMDMs. However, Ninjurin1 peptides reduced mRNA expressions of pro-inflammatory cytokines compare to LPS treatment alone (Figure 32B). These in vitro data suggest that Ninjurin1 blocking peptides may show a similar effect during development of experimental colitis in mice. Thus, WT mice were treated with the blocking peptide, $Ninjurin1_{26\text{-}37}$, to inhibit Ninjurin1. The mRNA expressions of IL1β and IL6 were dramatically reduced by inhibiting Ninjurin1 with the blocking peptide during the development of colitis (Figure 33A). The amounts of IL1β in colon explant cultures from $Ninjurin1_{26\text{-}37}$-treated mice with DSS was also lower than that obtained from mice treated with DSS alone (Figure 33B left). Although the difference between the two groups was not significant, there was a trend toward decreased IL6 expression in $Ninjurin1_{26\text{-}37}$-treated mice with DSS compared to mice treated with DSS alone (Figure 33B right). These results imply that Ninjurin1 inhibition decreases the intestinal inflammatory response.

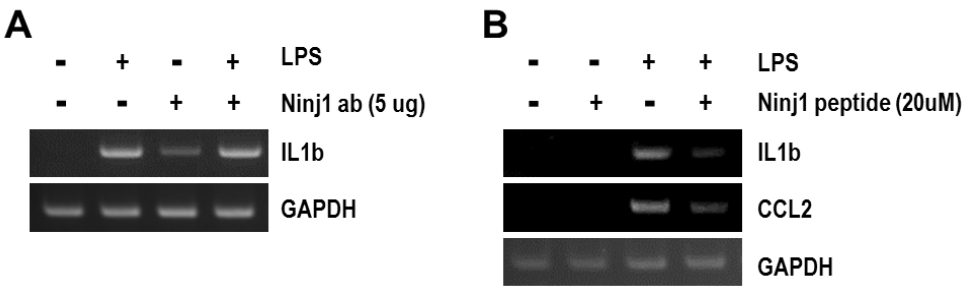


Figure 32. Ninjurin1 inhibition with Ninjurin1 antibodies or blocking peptides.

(A) BMDMs were incubated with 5 μg of Ninjurin1 antibodies for 4 h and 0.1 μg/ml of LPS for 3 h. The mRNA expression of IL1β was detected by RT-PCR and normalized by GAPDH. (C) BMDMs were treated with 20 μM of Ninjurin1 peptides for 3 h 30 min and 0.1 μg/ml of LPS for 3 h. The mRNA expressions of IL1β and CCL2 were detected by RT-PCR and normalized by GAPDH.

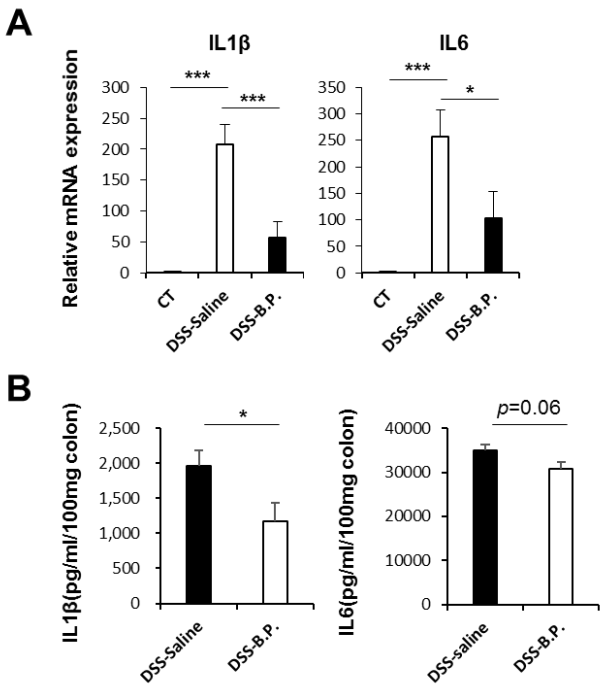


Figure 33. IL1β and IL6 production from colon tissues with Ninjurin1 blocking peptides.

(A) WT mice were intravenously injected with 0.9% NaCl (saline) as control, or Ninjurin1 blocking peptide (B.P.), and treated with 1.5% DSS for 8 days. Mice were sacrificed on day 8 after initiating DSS treatment. IL1β and IL6 mRNA expressions in colon tissue were analyzed by qRT-PCR; $n = 4$, untreated; $n = 5$, DSS + saline; $n = 6$, DSS + Ninjurin1 blocking peptide. Data are presented as mean ± SEM. *** $p < 0.005$, Student's t-test. (B) Colonic tissue explants were harvested, and levels of secreted IL1β and IL6 were assessed by ELISA; $n = 5$, DSS + saline; $n = 6$, DSS + Ninjurin1 blocking peptide. Data are presented as mean ± SEM. * $p < 0.05$, Student's t-test. CT, control.

10. Ninjurin1 deficiency in myeloid cells decreases susceptibility to experimental colitis

To confirm that Ninjurin1 in cells of myeloid lineage has an important function in colon inflammation, I generated mice with a myeloid cell-specific Ninjurin1 deficiency and studied the pathogenesis of colitis (Figure 34A, B). Ninjurin1 cWT (conditional wild-type: Ninjurin1$^{fl/fl}$) mice were crossed with Lyz-Cre mice to generate Ninjurin1 conditional KO mice (Ninjurin1$^{fl/fl}$; Lyz-Cre+ mice, designated as Ninjurin1 cKO). Western blot analysis validated Ninjurin1 expression in peritoneal macrophages and BMDM of the cWT and Ninjurin1 cKO mice (Figure 35A). I also examined intestinal inflammation in cWT and Ninjurin1 cKO mice; the cWT mice exhibited significant body weight loss, compared with Ninjurin1 cKO mice (Figure 35B). The cWT mice also show marked colon shortening (Figure 36A, B). Relative mRNA expressions of IL1β, IL6, and CCL2 were significantly lower in the colon of DSS-treated Ninjurin1 cKO mice than cWT mice (Figure 37). The amounts of IL1β and IL6 expressed in the colon explant culture media from cWT mice were higher than that obtained from Ninjurin1 cKO mice, which is consistent with results of mRNA expression analysis (Figure 38). These results indicate that absence of Ninjurin1 in myeloid cells is sufficient to alleviate experimental colitis.

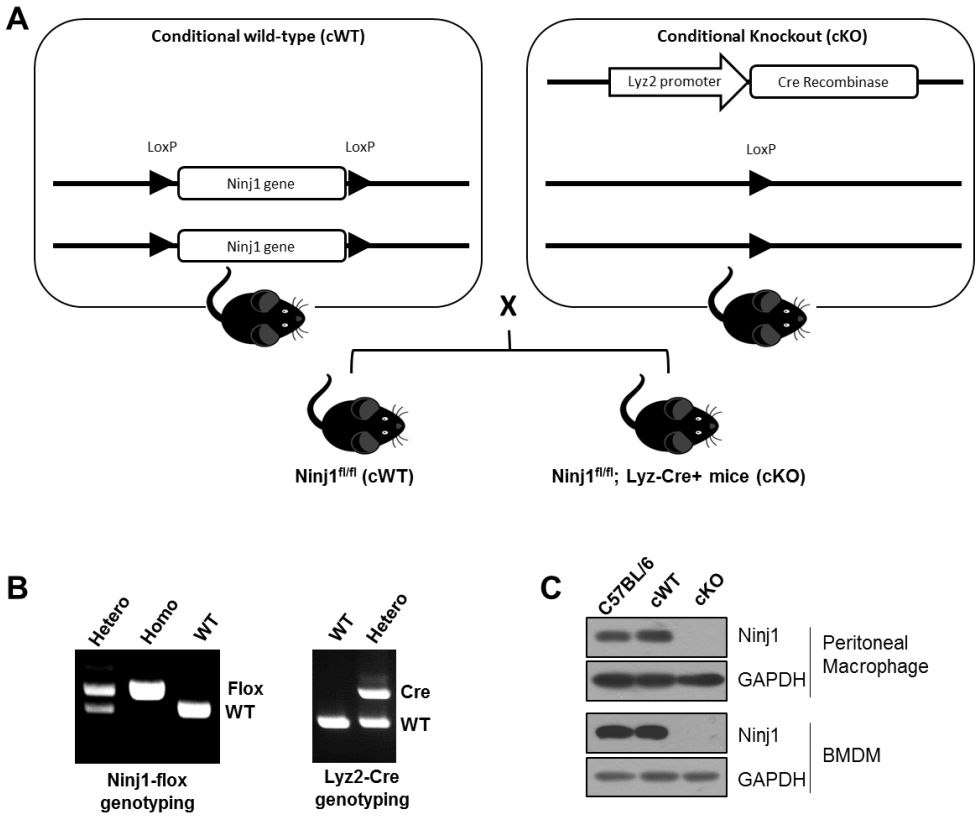


Figure 34. Generation of myeloid cell specific Ninjurin1 deficient mice.

(A) Gene structure of Ninjurin1$^{fl/fl}$ (cWT) and Ninjurin1$^{fl/fl}$; Lyz-Cre+ mice (Ninjurin1 cKO). (B) Mouse genotype was verified using PCR of genomic tail DNA.(C) Peritoneal macrophages and bone marrow-derived macrophages were isolated from cWT (conditional wild-type: Ninjurin1$^{fl/fl}$) and Ninjurin1 cKO (conditional KO: Ninjurin1$^{fl/fl}$; Lyz-Cre+); Ninjurin1 was detected by immunoblotting, using GAPDH as the internal control.

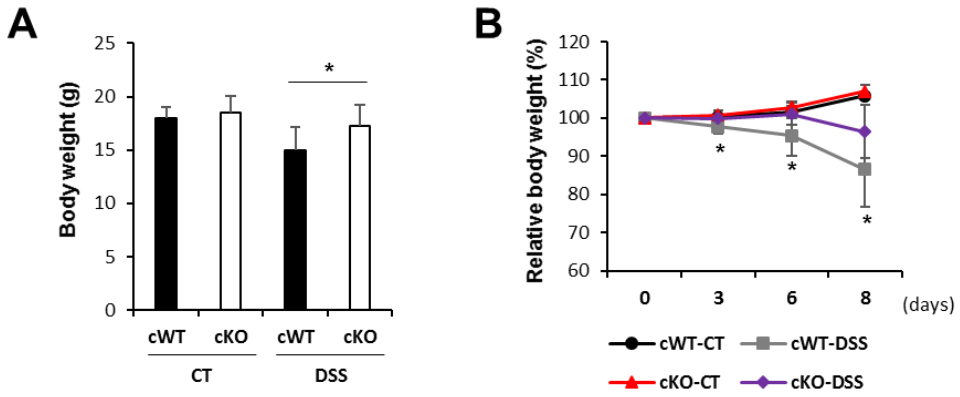


Figure 35. Ninjurin1 deficiency on macrophages reduces colonic inflammation during DSS treatment.

(A) cWT and Ninjurin1 cKO were administered 1.5% DSS for 8 days; n = 5, normal group; n = 8, DSS group. Body weight is presented on day 8 after initiation of treatment (B) Body weight loss is presented as a percentage of initial weight (mean ± SD). Student's t-test comparing 1.5% DSS treated cWT mice and 1.5% DSS treated Ninjurin1 cKO mice, * $p < 0.05$. Similar results were observed in four independent experiments.

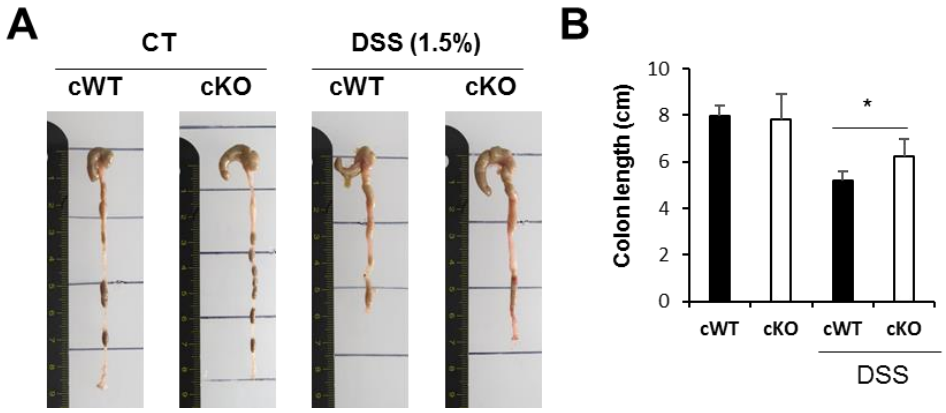


Figure 36. Colon length of DSS treated cWT and Ninjurin1 cKO mice.

(A) Representative images of colons from cWT and cKO mice. (B) Colon lengths were quantified. Data are presented as mean ± SD. * $p < 0.05$, Student's t-test.

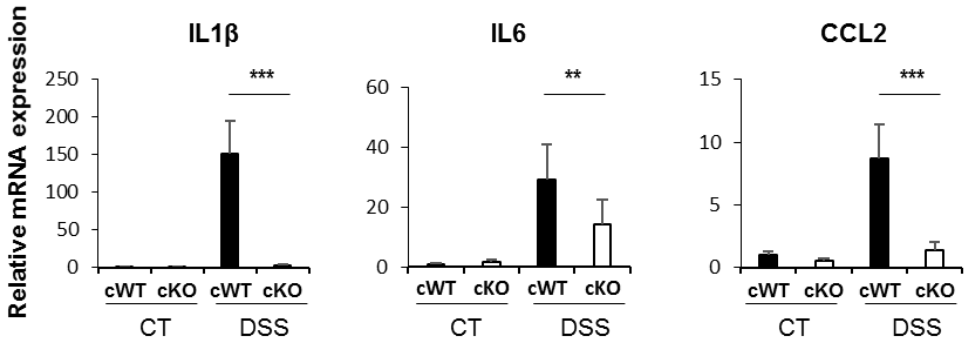


Figure 37. Relative mRNA expressions of IL1β, IL6, and CCL2 extracted from colons of cWT and cKO mice.

Data are presented as mean ± SEM. ** $p < 0.01$, *** $p < 0.005$, Student's t-test.

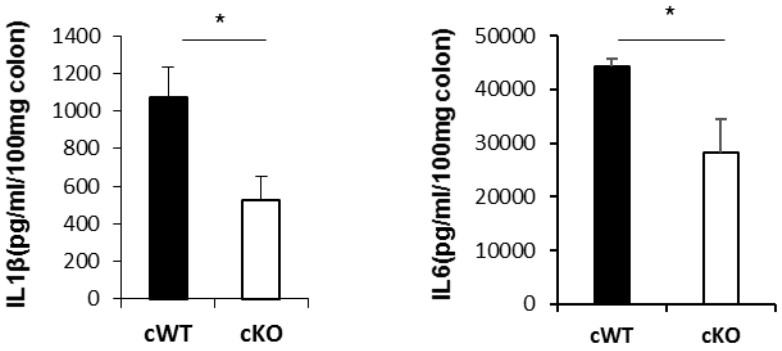
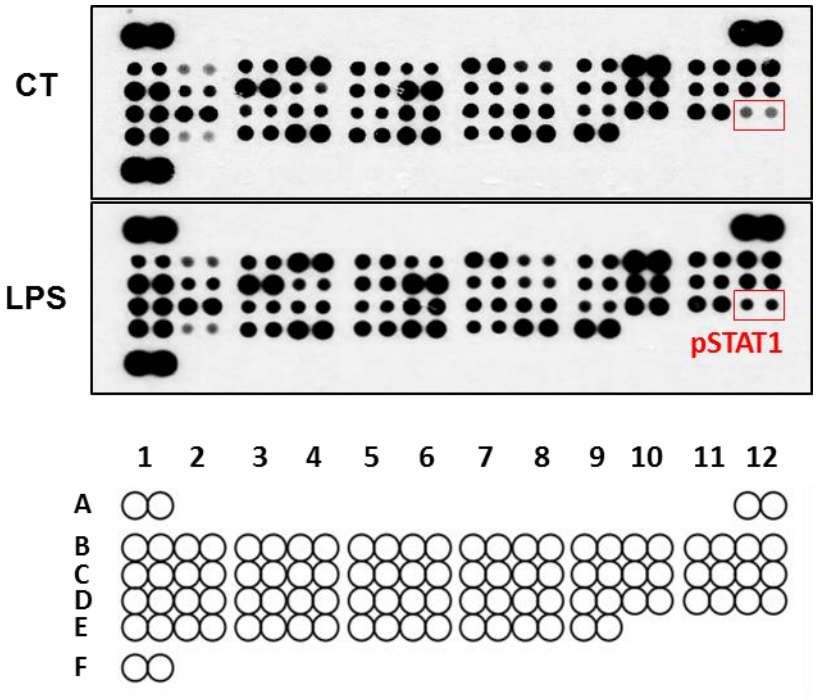


Figure 38. Detection of IL1β and IL6 secreted from colon explant from cWT and cKO mice.

Colonic tissue explants were harvested, and IL1β and IL6 secretions were assessed by ELISA; n = 4 per group. Similar results were observed in two independent experiments. Data are presented as mean ± SEM. * $p < 0.05$, Student's t-test. CT, control.

11. STAT1 is activated in macrophages under inflammatory conditions.

To evaluate the expression of inflammatory markers under inflammatory conditions, I used NF-κB pathway array. THP-1 cells were incubated in the absence or presence of LPS and the lysates were evaluated on the array. Of the several inflammatory markers, signal transducer and activator of transcription 1 (STAT1) activation was enhanced most dramatically in the presence of LPS compared to untreated sample (Figure 39). STAT1 is one of the transcription factors, which are activated as a result of binding to cytokine receptors. Phosphorylation of STAT1 at Ser-727 and Tyr-701 are required for DNA binding and transcription activity. A previous paper studied that activation of TLR2 and TLR4 results in phosphorylation of STAT1 in macrophages (Rhee et al., 2003). When I treated LPS in THP-1 cells for 2 h, phosphorylation of STAT1 was increased (Figure 39). Interestingly, another well-known inflammation related proteins such as p65 showed less dramatic changes than STAT1 activation (Figure 39).



Coordinate	Target	Coordinate	Target	Coordinate	Target	Coordinate	Target
A1, A12, F1	Reference Spots						
B1	ASC	C1	IKKγ	D1	NFκB1	E1	STAT2
B2	BCL10	C2	IL-1	D2	NFκB2	E2	STAT2 (pY689)
B3	CARD6	C3	IL-17	D3	NGF R	E3	STING
B4	CD40	C4	IL-18	D4	p53	E4	TLR2
B5	cIAP1	C5	IRAK1	D5	p53 (pS46)	E5	TNF R1
B6	cIAP2	C6	IRF5	D6	RelA	E6	TNF R2
B7	FADD	C7	IRF8	D7	RelA (pS529)	E7	TRAF2
B8	Fas	C8	JNK1/2	D8	c-Rel	E8	TRAIL R1
B9	IκBα	C9	JNK2	D9	SHARPIN	E9	TRAIL R2
B10	IκBε	C10	LTBR	D10	SOCS6		
B11	IKK1	C11	Metadherin	D11	STAT1 p91		
B12	IKK2	C12	MYD88	D12	STAT1 (pY701)		

Figure 39. NF-κB-related protein regulation in LPS-treated THP-1 cells.

THP-1 cells were incubated with 0.1 μg/ml of LPS for 2 h. Cell lysates were analyzed by human NF- κB pathway array.

12. Ninjurin1 modulates PKCδ/θ activation

In LPS-TLR signaling pathway, STAT1 has been known to be phosphorylated by PKC. Pharmaceutical inhibition of PKCδ/θ led to dephosphorylation of STAT1, which shows the evidence that PKCδ/θ is an upstream kinase of STAT1 (Wallerstedt et al., 2010). Western blot analysis was performed to identify the molecular pathway by which Ninjurin1 modulates PKCδ/θ activation. PKCδ/θ in colons of DSS-treated WT mice show increased activation levels, compared to DSS-treated KO mice (Figure 40). To verify this result, I administered LPS to WT and Ninjurin1 KO Raw 264.7 to mimic macrophage activation during development of colitis. In WT cells, LPS stimulation significantly increased the activation of PKCδ/θ (Figure 41). However, LPS-treated Ninjurin1 KO cells showed no difference in the activation of PKCδ/θ compared to WT cells (Figure 41). Moreover, LPS-administered WT and Ninjurin1 KO Raw264.7 cells were treated simultaneously with rottlerin (rott), a PKC δ/θ inhibitor, to examine the IL1β mRNA expression levels. As expected, LPS-treated WT Raw264.7 cells show markedly increased mRNA expression of IL1β compared to LPS-treated Ninjurin1 KO Raw264.7 cells (Figure 42). Treatment with rott decreases the mRNA expression of IL1β in LPS-treated WT Raw264.7 cells, as compared to LPS treatment alone (Figure 42). However, in Ninjurin1 KO cells, almost no change was observed with combined treatment of rott and LPS, as compared to cells treated with LPS alone (Figure 42). To determine whether PKCδ/θ can regulate Ninjurin1 expression in a feedback fashion, I transfected WT Raw264.7 cells with siRNA against PKCδ; expression of Ninjurin1was unchanged in PKCδ-

deficient cells (Figure 43A). I verified this result with WT Raw264.7 cells in the presence of the PKCδ inhibitor. Inhibition of PKCδ by using rott did not change Ninjurin1 expression (Figure 43B). Taken together, these results indicate that Ninjurin1 regulates PKCδ/θ activation, leading to the production of cytokines.

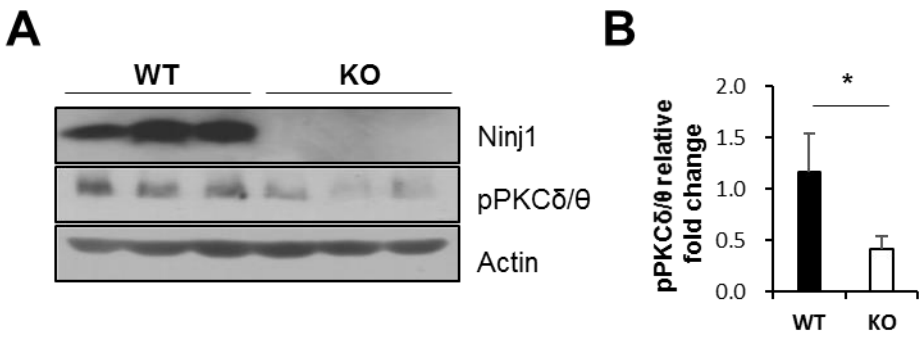


Figure 40. The PKCδ/θ activation is regulated by Ninjurin1 in colon tissues.

(A) Immunoblot analysis of Ninjurin1, pPKCδ/θ, and Actin performed on colonic tissues extracted from 1.5% DSS-treated WT and Ninjurin1 KO mice; $n = 3$ per group. (B) Densitometry represents relative protein levels of pPKCδ/θ. Values presented are mean ± SD. * $p < 0.05$, Student's t-test.

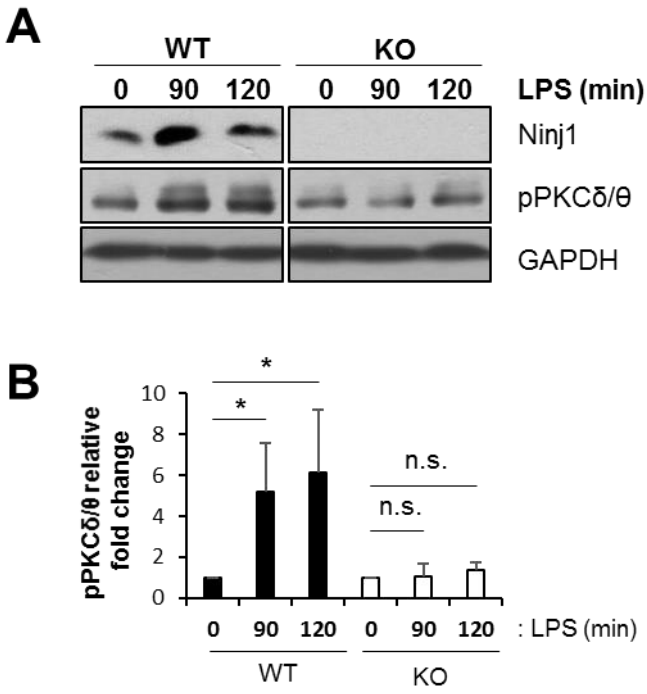


Figure 41. The PKCδ/θ activation is regulated by Ninjurin1 in macrophages.

(A) WT and Ninjurin1 KO Raw264.7 cells were treated with 0.5 μg/mL of LPS for 2 h. Western blot analysis was performed to detect the expression of Ninjurin1, pPKCδ/θ, and GAPDH. (B) Densitometry represents relative protein levels of pPKCδ/θ. n.s., not significant. Values presented are mean ± SD. * $p < 0.05$, Student's t-test. Similar results were observed in three independent experiments.

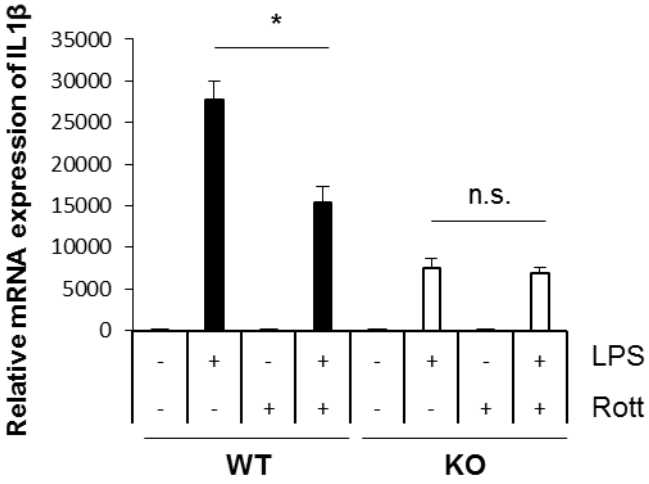


Figure 42. Inhibition of PKC δ/θ reduces IL1β production.

WT and Ninjurin1 KO Raw264.7 cells were treated with 5 μg/mL of rott (rottlerin) and LPS (0.5 μg/mL) for 6 h. qRT-PCR was performed to detect mRNA expressions of IL1β. Data are presented as mean ± SEM. * $p < 0.05$, Student's t-test. Similar results were observed in three independent experiments.

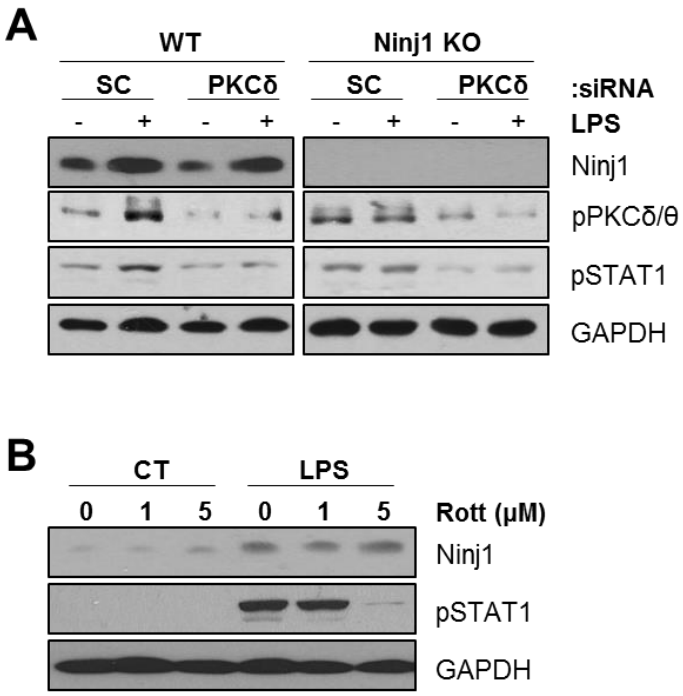


Figure 43. PKC δ/θ is downstream of Ninjurin1.

(A) Scrambled siRNA (SC) and PKCδ siRNA were applied to WT and Ninjurin1 KO Raw264.7 cells, followed by treatment with 0.1 μg/mL of LPS for 2 h. Ninjurin1, pPKCδ/θ, pSTAT1, and GAPDH protein levels were detected by immunoblotting. (B) WT and Ninjurin1 KO Raw264.7 cells were incubated with 1 or 5 μg/mL of rottlerin (rott) for 6 h, followed by treatment with 0.5 μg/mL of LPS for 2 h.

13. Ninjurin1 does not regulate M2 polarization

Macrophages are polarized into two types: M1 and M2. M1 macrophages have pro-inflammatory phenotypes, called classically activated macrophages. M2 macrophages are known to have anti-inflammatory properties such as tissue repair and cell proliferation, called alternatively activated macrophages (Italiani and Boraschi, 2014). Cytokines like IL1β and IL6, which I discussed above, are pro-inflammatory cytokines that are related to M1 macrophages. According to previous papers, signal transducer and activator of transcription 6 (STAT6) has an essential role in macrophage M2 polarization (Gong et al., 2017; Szanto et al., 2010). Activation of STAT6 modulates transcriptional activation of M2-specific genes such as arginase 1 (Arg-1), chitinase-like protein 3 (Ym-1), and resistin-like α (Fizz-1) (Goenka and Kaplan, 2011). To study that Ninjurin1 modulates M2 polarization, I incubated BMDMs from WT and Ninjurin1 KO mice with IL4. STAT6 activation was not changed in WT and Ninjurin1 KO macrophages (Figure 44A). In parallel to this result, I could not detect any changes in the mRNA expression of anti-inflammatory cytokines in IL4-treated BMDM from WT and Ninjurin1 KO mice (Figure 44B).

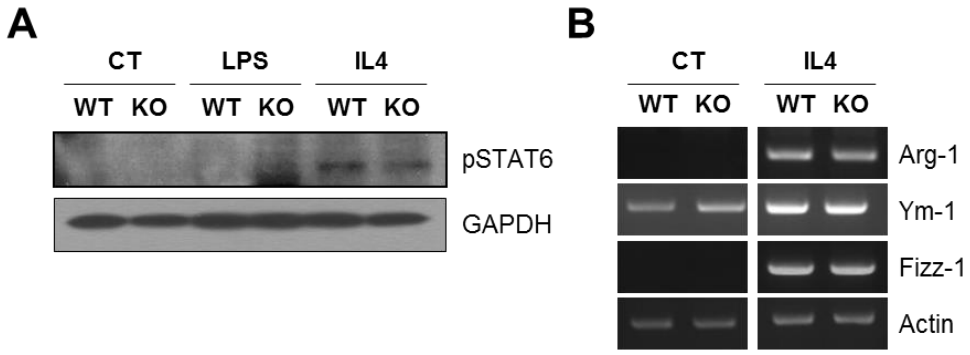


Figure 44. M2 macrophage polarization in the presence or absence of Ninjurin1.

Bone marrow-derived macrophages (BMDM) were extracted from WT and Ninjurin1 KO mice incubated with 10 ng/mL of IL4 for 12 h or 0.5 μg/ml of LPS for 2 h. CT, control. (A) pSTAT6 and GAPDH protein levels were detected by immunoblotting. (B) Anti-inflammatory cytokines were detected by RT-PCR.

IV. Discussion

The results of this study illustrate that Ninjurin1 in macrophages is a regulatory factor involved in the development of colitis. Using myeloid cell-specific Ninjurin1-deficient mice as well as conventional Ninjurin1 KO mice, I show that the loss of Ninjurin1 attenuates intestinal inflammation. These findings clearly demonstrate that a deficiency of Ninjurin1 in macrophages decreases intestinal inflammation, resulting in reduced pathogenesis.

In recent years, several papers have reported that Ninjurin1 expression is induced under inflammatory conditions (Ahn et al., 2014b; Cho et al., 2013; Ifergan et al., 2011; Jennewein et al., 2015). In accordance with these previous results, I identified that protein expression of Ninjurin1 is induced in inflamed colon tissues of WT mice. I presumed that the increased expression of Ninjurin1 in inflamed colon homogenates is the result of induction of its expression on macrophages. Although macrophage infiltration is shown to be a critical step in the development of colitis, there were no changes in the numbers of macrophages extracted from colons of DSS-treated WT and Ninjurin1 KO mice. Ninjurin1-deficient and Ninjurin1-expressing macrophages have similar capacities to migrate to the site of a lesion, thereby suggesting that the difference in colitis incidences seen in WT and Ninjurin1 KO mice are not determined by differences in macrophage infiltration, but rather by activation of macrophages. These results conflict a previous report dealing with the role of Ninjurin1 in colon cancer. During colon cancer development, overexpression of Ninjurin1 suppresses

the migration of macrophages, resulting in the alleviation of cancer development (Woo et al., 2016). Macrophages in tumor tissue directly facilitate the enhancement of growth and mobility of tumor cells. As a tumor progresses, cancer cells train macrophages to adopt new characteristics, allowing them to contribute to migration, angiogenesis, intravasation, and extravasation (Condeelis and Pollard, 2006; Duff et al., 2007). Thus, tumor-associated macrophages (TAM) function in differently than macrophages of normal tissue. Moreover, I analyzed previously published mRNA profiles and determined that Ninjurin1 expression is not induced in colon cancer tissues of mice, as compared to mice with normal colons (GSE31106) (Tang et al., 2012). In this paper, I present the different roles of Ninjurin1 in macrophages under intestinal inflammatory conditions as compared with TAM during colon cancer development.

Similar to the previous paper that analyzed the role of Ninjurin1 in colon cancer, several reports have demonstrated that Ninjurin1 mediates migration of cells in inflammatory conditions (Ahn et al., 2014a; Ahn et al., 2014b; Ifergan et al., 2011; Jang et al., 2016; Jennewein et al., 2015; Yang et al., 2017). Thus, it was surprising that during colitis development, Ninjurin1 did not alter the migration capacity of macrophages, which was seen only in lung fibrosis. Upon bleomycin treatment, Ninjurin1 KO mice ameliorated lung fibrosis; however, the number of infiltrating macrophages from both KO and WT mice were not different (Choi et al., 2018). During development of colitis and lung fibrosis, the Ninjurin1 pathway controlled activation of the macrophages, resulting in the triggering an inflammatory response.

The interaction of LPS and macrophage is important in the pathogenesis of DSS-induced colitis model (Ingalls et al., 1999). LPS-activated macrophages in colon tissues trigger the onset of mucosal edema and diarrhea (Dang et al., 2015). LPS is a well-known activator of TLR4 on macrophages, and for full activation of inflammatory signals, PKCδ/θ is considered an important factor in the TLR4-mediated pathway (Bhatt et al., 2010; Kontny et al., 2000). In the current study, the combined treatment with PKCδ/θ inhibitor and LPS resulted in diminished cytokine secretion, as compared to LPS-alone treatment group (Figure 42). Therefore, I assume that Ninjurin1 regulates TLR4-PKCδ/θ activation under inflammatory conditions.

I also present evidence that my results from the murine colitis model are relevant to human IBD patients. By analyzing published data, I detected a correlation between Ninjurin1 expression and IBD (Figure 8A). Furthermore, the transcriptional profile in peripheral blood mononuclear cells (PBMC) revealed that mRNA expression of Ninjurin1 is increased in PBMC of IBD patients, as compared to levels obtained in PBMC of healthy subjects (Figure 45) (Burczynski et al., 2006). Macrophage is one of the cell types present in PBMCs, and myeloid cells in human PBMC are known to present the highest levels of Ninjurin1 expression among the leukocytes (Ifergan et al., 2011). Thus, I deduce that there is a positive correlation between Ninjurin1 and IBD.

Over-active immune response is one of the main etiologies that cause IBD (Zenlea and Peppercorn, 2014). Immunosuppressive agents have been considered as the therapeutic focus. Infliximab, adalimumab, and natalizymab are beneficial in the

treatment of CD and UC (Colombel et al., 2009; Targan et al., 2007; van Dullemen et al., 1995). According to a previous report, treatment of Ninjurin1 blocking peptide diminishes systemic inflammation in septic mice (Jennewein et al., 2015). In the current study, I observed that the attenuated gut inflammation in mice treated with Ninjurin1 blocking peptide compared to untreated mice during DSS treatment, which exhibits its potential as an immunosuppressive therapy.

In conclusion, my results demonstrate that Ninjurin1 in macrophages contributes to intestinal inflammation in experimental colitis. Therefore, I propose that targeting Ninjurin1 may be a promising therapeutic approach to decreasing the pathogenesis of colitis.

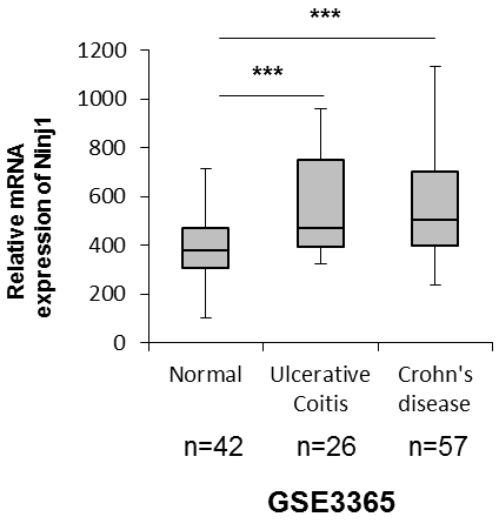


Figure 45. Analysis of Ninjurin1 expression in peripheral blood mononuclear cells from UC and CD patients.

The gene expression data (GSE3365) were obtained from GEO database. *** $p <$ 0.01, Welch's T test.

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국문 초록

마우스 대장염에서 대식세포 Ninjurin1의 기능 연구

Nerve injury-induced protein 1 (Ninjurin1)은 손상된 신경 주위 슈반세포에서의 발현이 처음으로 알려진 세포막 단백질이다. Ninjurin1은 염증반응에서 대식세포의 이동과 부착에 관여하여 염증반응을 조절한다고 알려져 있다. 또한 Ninjurin1은 대식세포, 단핵구, 그리고 호중구와 같은 혈액 유래 면역세포에서 강력하게 발현된다고 보고되었다. 현재까지 많은 연구가 대식세포에서 대해 이루어져 있지만 생리학적 현상에 대한 보고는 제한적이며 자세한 염증조절기전이 알려지지 않아 염증성 질환에서의 Ninjurin1의 작용 기전을 밝히는 연구가 필요할 것으로 생각되었다.

염증성 대장질환은 위장관의 만성 염증 상태를 나타내는 용어로서 크론병과 궤양성 장염으로 구분되며 이들 모두 염증의 발생과 완화가 반복되는 것이 특징이다. 대장염 발생 시, 여러 면역 세포들 중 선천 면역을 담당하는 대식세포의 활성화가 주된 역할을 하는 것으로 알려져 있다. 본 연구에서는 Ninjurin1의 염증 조절 기전을 대장염 발생과정에서 확인하고자 하였다.

공개된 유전체 빅데이터인 Gene Expression Omnibus에서 Ninjurin1과 대장염의 상관관계를 분석한 결과 대장염 환자들의 대장 조직 샘플 내 Ninjurin1 발현 양이 정상 대장 조직에 비하여 유의적으로 증가함을 확인

하였다. 또한 대장염 환자들의 림프구 및 단핵구에서 Ninjurin1의 mRNA 발현 양이 유의적으로 증가하는 것을 확인하였다. 따라서 대장염 발생 과정에서 Ninjurin1이 중요한 역할을 할 것으로 생각하였다.

본 연구에서는 대조군과 Ninjurin1 유전자 결손 마우스(Ninjurin1 KO)에 dextran sodium sulfate (DSS)를 8일 동안 처리하여 대장염을 유발하였다. Ninjurin1 KO 마우스에서는 대조군 마우스에 비해 대장염 병변이 미약하였고 대장염 유도에 따라 대식세포에서 Ninjurin1의 발현이 현저하게 증가하는 것이 관찰되었다. 대장염 부위의 대식세포 수를 분석한 결과 Ninjurin1의 발현은 대식세포의 이동성에는 관여를 하지 않는다는 것을 확인하였다.

Microarray를 수행하여 대식세포에서 Ninjurin1이 결손 됨에 따라 대부분의 염증 관련 유전자의 발현이 현저하게 저하되었음을 확인하였다. LPS에 의한 마우스 대식세포주의 염증 활성화 과정에서 Ninjurin1 유전자의 발현을 억제하면 염증성 사이토카인 유전자가 대조군에 비해 적게 증가하였다. 또한 대조군 마우스 대장조직에 비해 Ninjurin1 KO 마우스 대장조직에서 사이토카인 발현이 낮았다.

대장염 발생에서 대식세포의 Ninjurin1이 중요한 역할을 하고 있음을 확인하기 위하여 대식세포 특이적으로 Ninjurin1의 발현을 억제한 마우스에 대장염을 유발하였다. Ninjurin1이 대식세포에서 결손 됨에 따라 대조군에 비해 더 미약한 병변이 관찰되었고, 대장 조직으로부터 분비된 사이토카인을 측정한 실험에서도 대조군에 비해 사이토카인의 분비가 감소되어 있음을 확인하였다.

대식세포에서 Ninjurin1이 염증 신호를 전달하는 기전을 확인한 결과, 대조군에 비해 Ninjurin1 KO 마우스의 대장 조직에서 Protein Kinase C (PKC)

δ/θ의 활성화가 감소되어 있음을 관찰하였다. 마우스 유래 대식세포주에서 LPS를 처리한 실험에도 일관된 결과를 얻었다.

본 실험에서는 위 일련의 실험들을 통하여 다음과 같은 몇 가지 현상 및 기전을 밝혔다. (1) 대장염 유도시 대조군 마우스에 비해 Ninjurin1 KO 마우스에서 대장염 병변이 미약하게 관찰되었다. (2) Ninjurin1이 대식세포 특이적으로 억제된 마우스에서도 대조군 마우스에 비해 병변이 덜 증가하였다. (3) 대식세포에서 Ninjurin1 발현의 억제는 IL1β와 같은 염증성 사이토카인의 분비를 감소시켰다. (4) Ninjurin1은 PKCδ/θ 단백질의 활성화를 조절하여 염증성 사이토카인의 분비를 유도하였다.

따라서 본 연구는 대식세포에서 Ninjurin1이 PKCδ/θ 활성화를 유도하고 염증성 사이토카인의 분비를 증가시켜 결과적으로 대장염 발생을 일으키는 작용 기전을 규명하였으며 이로 인해 대장염 유발의 원인으로서 Ninjurin1의 가능성을 제시한다.

주요어: Nerve injury-induced protein 1 (Ninjurin1), 염증성 대장염, 대식세포, Protein Kinase C δ/θ (PKC δ/θ), 염증성 사이토카인

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