A Dissertation for the Degree of Doctor of Philosophy

The Role of Nucleotide-binding Oligomerization Domain 2 on the Immunomodulatory Function of Mesenchymal Stem Cells in Mouse Colitis Model

마우스 결장염 모델에서 중간엽 줄기세포의 면역 조절능에 대한 노드 2 수용체의 역할

By

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Program in Zoonotic Animal Diseases,

College of Veterinary Medicine,

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ABSTRACT

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Mesenchymal stem cells (MSCs) are multipotent adult stromal cells that can self-renew and differentiate into various cell types of mesodermal lineage. Moreover, MSCs are recently known to possess regulatory function on immune cells which makes them a promising tool for the treatment of inflammatory and autoimmune diseases. The interaction between MSCs and immune cells through soluble factors and adhesion

molecules has been reported to be crucial for the immunomodulatory effect of MSCs.

Innate immune receptors, such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs), are known to trigger an initial immune responses against microbial infection. Although studies suggest that activation of TLRs modulate the function of mesenchymal stem cells (MSCs), little is known about the role of NLRs on the MSC function. In this study, I investigated whether Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and 2 (NOD2), well-known receptors in NLR family, regulate the functions of human umbilical cord blood-derived MSCs (hUCB-MSCs). TLR2, TLR4, NOD1, NOD2 and receptor interacting protein 2 (RIP2), an adaptor protein of NOD1 and NOD2 were expressed in hUCB-MSCs. Stimulation with each agonist ;Pam3CSK4 for TLR2, Lipopolysaccharide (LPS) for TLR4, L-Ala-gamma-D-Glu-meso-_diamino-pimelic acid (Tri-DAP) for NOD1, and muramyl dipeptide (MDP) for NOD2, led to Interleukin (IL)-8 production in hUCB-MSC, suggesting that the expressed receptors are functional in hUCB-MSC. None of agonists influenced proliferation of hUCB-MSCs. I next examined whether TLR and NLR agonists affect the differentiation of hUCB-MSCs. Pam3CSK4 and Tri-DAP strongly enhanced osteogenic differentiation through the induction of ERK phosphorylation in hUCB-MSCs, and LPS and MDP also slightly augmented osteogenesis. Treatment of U0126 (MEK1/2 inhibitor) restored osteogenic differentiation enhanced by Pam3CSK4. Tri-DAP and MDP slightly inhibited adipogenic differentiation of hUCB-MSCs, but Pam3CSK4 and LPS did not. On chondrogenic differentiation, all TLR and NLR agonists could promote chondrogenesis of hUCB-MSCs (Kim et al., 2010).

Decreased levels or function of NOD2 are associated with Crohn's disease. NOD2 is known to regulate intestinal inflammation, and also is expressed by human umbilical cord blood-derived mesenchymal stem cells, to regulate their differentiation. I investigated whether NOD2 is required for the anti-inflammatory activities of MSCs in mice with colitis. Colitis was induced in mice by administration of dextran sulfate sodium (DSS) or trinitrobenzene sulfonic acid (TNBS). Mice then were given intraperitoneal injections of NOD2-activated hUCB-MSCs, and colon tissues and mesenteric lymph nodes were collected for histologic analyses. Administration of hUCB-MSCs reduced the severity of colitis in mice. The anti-inflammatory effects of hUCB-MSCs were greatly increased by activation of NOD2 by its ligand, MDP. Administration of NOD2-activated hUCB-MSCs increased anti-inflammatory responses in colons of mice, such as production of interleukin IL-10 and infiltration by T regulatory cells, and reduced production of inflammatory cytokines. A bromodeoxyuridine (BrdU) assay was used to determine the ability of hUCB-MSCs to inhibit proliferation of human mononuclear cells (hMNCs) in culture. Proliferation of mononuclear cells was inhibited significantly by co-culture with hUCB-MSCs that had been stimulated with MDP. MDP induced prolonged production of prostaglandin (PG)E_2 in hUCB-MSCs via the NOD2–RIP2 pathway, which suppressed proliferation of mononuclear cells derived from hUCB. PGE_2 produced by hUCB-MSCs in response to MDP increased production of IL-10 and T regulatory cells. In mice, production of PGE_2 by MSCs and subsequent production of IL-10 were required to reduce the severity of colitis (Kim et al., 2013).

Taken together, these findings suggest that (1) NOD1 and NOD2 as well as TLRs are involved in regulating the differentiation of MSCs, and that (2) activation of NOD2 is required for the ability of hUCB-MSCs to reduce the severity of colitis in mice and NOD2 signaling increases the ability of these cells to suppress mononuclear cell proliferation by inducing the production of PGE_2.

Keywords : Mesenchymal stem cell, Innate immune receptor, NOD2, Differentiation, Immunomodulation, Crohn's disease

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LIST OF ABBREVIATION

hMSC	Human mesenchymal stem cell
hUCB-MSC	Human umbilical cord-derived MSC
CFSE	5,6-Carboxy fluorescein succinimidyl ester
CM	Culture Media
COX-2	Cyclooxygenase-2
DSS	Dextran sulfate sodium
FoxP3	Forkhead box P3
IDO-1	Indoleamine-2,3-dioxygenase-1
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MDP	Muramyl dipeptide
MLN	Mesenteric lymph node
MLR	Mixed lymphocyte reaction
MNC	Mononuclear cell

MPO	Myeloperoxidase
NF-κB	Nuclear factor-κB
NLR	Nod-like receptors
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain 2
PBS	Phosphate-buffered saline
PG	Prostaglandin
RIP	Receptor-interacting protein
siRNA	Small interfering RNA
Th	T-helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNBS	Trinitrobenzene sulfonic acid
Treg	Regulatory T cell
UCM	UCB-MSC conditioned medium

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LITERATURE REVIEW

Function of mesenchymal stem cells

Mesenchymal stem cells (MSCs), which can be alternatively defined as multipotent stromal cells, can self-renew and differentiate into various cell types, such as osteocytes, adipocytes, chondrocytes, cardiomyocytes, fibroblasts, and endothelial cells (Friedenstein et al., 1976; Jiang et al., 2002; Pittenger et al., 1999). Moreover, MSCs have been known to interact with cell types of both innate and adaptive immune systems, which results in the suppressive effect on proliferation, differentiation, and activation of immune cells including T cells, B cells, dendritic cells, and natural killer (NK) cells (Asari et al., 2009; Prigione et al., 2009; Ren et al., 2008; Zhang et al., 2009a). This immunomodulatory effect of MSCs on immune cells is exerted by soluble factors such as prostaglandin-E_2 (PGE_2), indoleamine 2,3-dioxygenase-1 (IDO-1), nitric oxide (NO), transforming growth factor (TGF-β1), hepatocyte growth factor (HGF), and interleukin (IL)-10 (Fig. 1) (Beyth et al., 2005; Krampera et al., 2003; Puissant et al., 2005; Ren et al., 2008; Sato et al., 2007; Tse et al., 2003; Yanez et al., 2006). Indeed, a number of studies have reported that the immunomodulatory ability of MSCs can be usefully applied for the treatment of autoimmune and inflammation-related diseases such as graft-versus-host-disease, inflammatory bowel disease, multiple sclerosis, sepsis, collagen-induced arthritis, and type I diabetes (Augello et al., 2007; Gonzalez et al., 2009;

Le Blanc et al., 2004; Lee et al., 2006; Nemeth et al., 2008; Zappia et al., 2005). Moreover, recent studies have demonstrated that MSCs can also ameliorate allergic diseases such as asthma, rhinitis and dermatitis (Goodwin et al., 2011; Jee et al., 2013; Kapoor et al., 2012; Kavanagh and Mahon, 2011; Nemeth et al., 2010; Su et al., 2011; Sun et al., 2012).

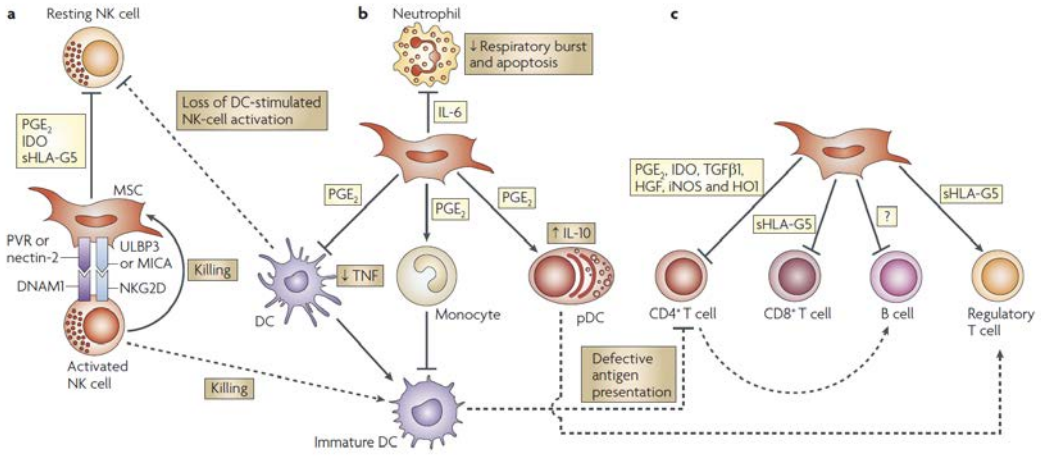

Figure 1. Possible mechanisms of the interactions between MSCs and cells of the innate and adaptive immune systems. (a) Mesenchymal stem cells (MSCs) can inhibit the proliferation and cytotoxicity of resting natural killer (NK) cells and their cytokine production in vitro. These effects are mediated by prostaglandin E_2 (PGE_2), indoleamine 2,3-dioxygenase (IDO) and soluble HLA-G5 (sHLA-G5) released by MSCs. (b) MSCs inhibit the differentiation of monocytes to immature myeloid dendritic cells (DCs), skew mature DCs to an immature DC state, inhibit tumor-necrosis factor (TNF) production by DCs and increase IL-10 production by plasmacytoid DCs (pDCs). MSC-derived PGE2 is involved in all of these effects. (c) Direct inhibition of $CD4^+$ T-cell function depends on the release by MSCs of several soluble molecules, including PGE_2, IDO, transforming growth factor-β1 (TGF-β1), hepatocyte growth factor (HGF), inducible nitric-oxide synthase (iNOS) and haem oxygenase-1 (HO-1). Defective $CD4^+$ T-cell activation impairs helper function for B-cell proliferation and antibody production. (Uccelli et al., 2008).

Innate Immune receptors and their role in MSC function

The mammalian immune system efficiently eliminates infective pathogens through the cooperative interactions of innate and adaptive immunity. The innate immune system recognizes the invading microbial pathogens, eradicates them by phagocytosis and induces inflammatory responses (Akira et al., 2006; Beutler et al., 2007; Hoffmann, 2003; Medzhitov, 2007). The innate immune system senses microbial pathogens using pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), lipoteichoic acid, peptidoglycan (PGN), flagellin and microbial nucleic acid variants (Janeway, 1989). Upon sensing theses PAMPs by PRRs, downstream signaling pathway is activated, promoting an antimicrobial immune response. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) constitute large family of PRRs and initiates immune responses against particular microbes by activating specific signaling pathways (Fig. 2).

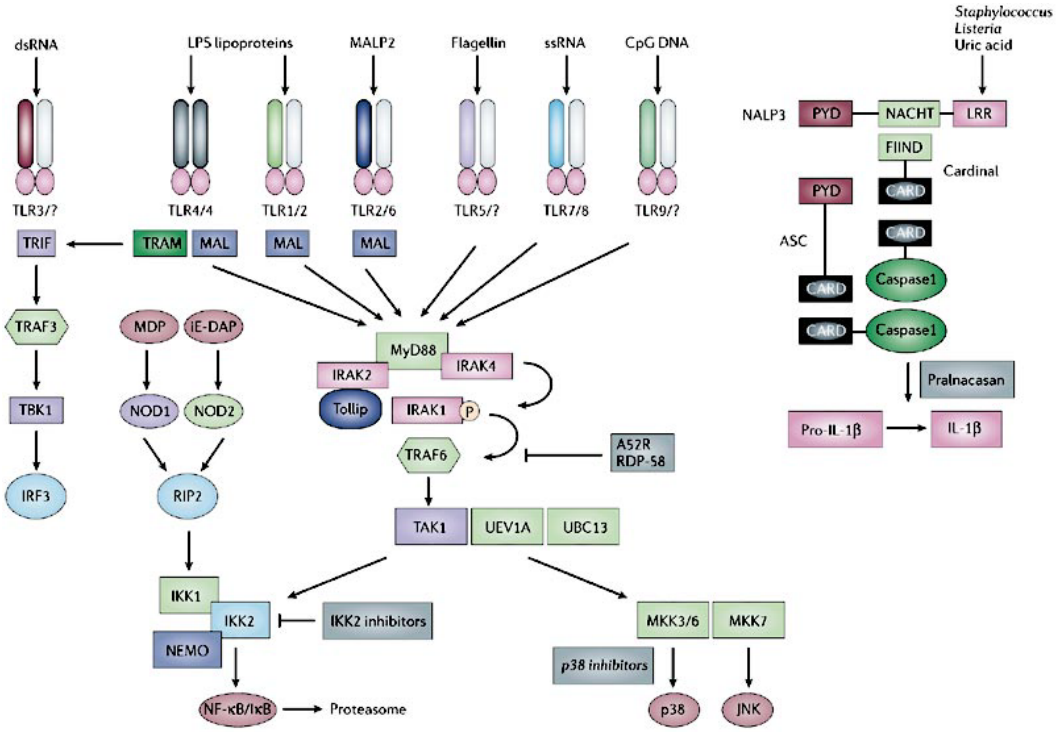

Figure 2. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are key initiators of inflammation during host defense. Acting as dimers, the ten different TLRs display differential recognition of microbial products. NLRs sense microbial products, with NOD1 and NOD2 recognizing the peptidoglycan products, muramyl dipeptide (MDP) and the dipeptide iE-DAP, respectively (O'Neill, 2006).

TLRs are composed of three major domains; extracellular leucine rich repeats (LRRs) in the ectodomain that mediate the recognition of PAMPs, transmembrane domain and intracellular Toll/IL-1R (TIR) domain, which initiates downstream signaling pathways. TLRs are mainly expressed on immune cells, such as macrophages, dendritic cells and B cells. Up to now, 13 members have been identified in mammalian TLR family (Akira et al., 2006; Oldenburg et al., 2012). Certain TLRs (TLR 1, 2, 4, 5 and 6) are expressed on the surface of cells, whereas others (TLR 3, 7, 8 and 9) are detected in the cytosol of cells. TLRs sense microbial components such as triacyl lipopeptides (TLR1/TLR2), dsRNA (TLR3), lipopolysaccharide (TLR4), flagellin (TLR5), ssRNA (TLR7/8), and CpG-DNA (TLR9) to subsequently trigger inflammatory process (Akira et al., 2006). Moreover, TLR2 and TLR4 also serve as sensors for danger signals recognizing endogenous molecules including heat shock proteins, fibrinogen, fibronectin, heparin sulphate, fatty acids, hyaluronic acid, high mobility group box 1 (HMGB1) and β-defensin 2. Most of these endogenous ligands are produced by inflammatory or necrotic cells (Miyake, 2007; Wong and Wen, 2008).

NLRs detect PAMPS and endogenous ligands in the cytosol (Fritz et al., 2006; Inohara et al., 2005; Kanneganti et al., 2007; Meylan et al., 2006). So far, more than 23 NLR family members in humans and 34 genes in mice have been identified (Franchi et al., 2009; Ting et al., 2008). NOD1 and NOD2 are well-known members among the large number of NOD-LRR family, which both contain N-terminal caspase recruitment domain (CARD) for the initiation of signaling, intermediate nucleotide-binding oligomerization domain (NOD), and C-terminal leucine-rich repeats (LRRs) domain for ligand sensing

(Figure 3). NOD1 and NOD2 detect peptidoglycan (PGN) derivatives, γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively. Binding of specific bacterial ligands to NOD1 and NOD2 induces oligomerization and lead to the activation of NF-κB and MAPK and subsequent production of inflammatory cytokines through the recruitment of receptor-interacting serine/threonine kinase (RICK/Rip2), an adaptor molecule, to the NODs via CARD-CARD interaction (Girardin et al., 2001; Inohara et al., 2000). Moreover, NOD1 and NOD2 agonists, in combination with TLR agonists, synergistically induce cytokine production and activation of NF-κB and MAPK in immune cells.(Kim et al., 2008; Park et al., 2007b; Tada et al., 2005)

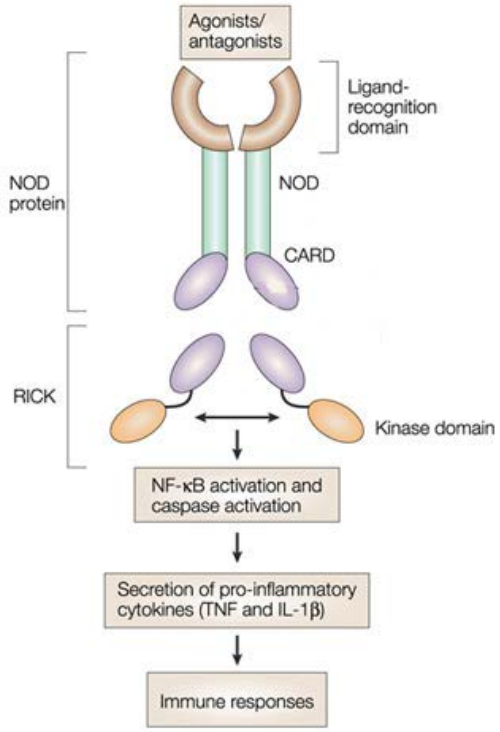

Figure 3. NOD structure and signaling pathway. Signaling pathways mediated by the NOD2 protein-family members, NOD1 and NOD2, are dependent on the RICK to initiate downstream signaling. Rick interacts with NOD1 and NOD2 through CARD-CARD interaction. (Ulevitch, 2004).

Recent studies reported that TLRs and NLRs are expressed in MSCs (Table 1). A recent study showed that hUCB-MSCs expressed TLR1, 3, 5, 9 and 4 (van den Berk et al., 2009). Many studies also determined the functional expression of TLR2 in BM- or adipose tissue-derived MSCs (ASC) (Hwa Cho et al., 2006; Lombardo et al., 2009; Pevsner-Fischer et al., 2007; Tomchuck et al., 2008). There are discrepancies regarding the effect of TLRs on the MSC proliferation. A previous study demonstrated that TLR ligands including PGN, LPS, poly I:C, and flagellin did not affect the proliferation of human ASC (Hwa Cho et al., 2006). Only CpG-ODN, TLR9 agonists, slightly reduced hASC proliferation.(Hwa Cho et al., 2006) This was confirmed by a study of,Lombardo et al. (2009) showing that TLR3 and TLR4 ligands had no effect on the proliferation of hASCs. In contrast, downregulation of MyD88, a common adaptor molecule for TLRs inhibited the proliferation of hASC.(Yu et al., 2008) Additionally, ligands for TLR 3 and 4 enhanced the proliferation of mouse BM-MSCs (Pevsner-Fischer et al., 2007; Wang et al., 2009), suggesting involvement of TLRs on MSC proliferation. Upon MSC differentiation, TLR3 and TLR4 ligands increased osteogenic differentiation of hASCs, but did not affect adipogenic differentiation (Lombardo et al., 2009; Yu et al., 2008). Other TLR ligands (poly I:C, flagellin, CpG-ODN) also did not influence adipogenic differentiation of hASC. (Hwa Cho et al., 2006) Furthermore, regarding immunosuppressive ability of MSCs, Lombardo et al. (2009); (Travassos et al., 2004) showed that TLR activation did not impair the inhibitory effect of hMSCs on the proliferation of peripheral blood mononuclear cells. In contrast, in a study by Platten's group (Opitz et al., 2009), TLR activation enhanced immunosuppressive ability of hMSC

by inducing IDO-1, an enzyme responsible for tryptophan catabolism. In addition, Waterman et al. (2010) observed that TLR4-activated hMSCs secrete pro-inflammatory mediators, while TLR3-activated hMSCs efficiently suppress inflammation. However, although several studies have shown that TLRs regulate the functions of MSCs, little is known about the role of NLRs on MSC functions.

Table 1. Pattern-recognition receptors expressed by MSCs (Le Blanc and Mougiakakos, 2012)**.**

Type of PRR	Source of MSCs	Localization of PRR	Species
Toll-like receptors (TLRs)			
TLR1	Bone marrow, adipose tissue, umbilical cord	Cell surface	Human, mouse
TLR2	Bone marrow, adipose tissue, umbilical cord	Cell surface	Human, mouse
TLR3	Bone marrow, adipose tissue, umbilical cord	Cell surface	Human, mouse
TLR4	Bone marrow, adipose tissue, umbilical cord	Cell surface	Human, mouse
TLR5	Bone marrow, adipose tissue, umbilical cord	Cell surface	Human, mouse
TLR6	Bone marrow, adipose tissue, umbilical cord	Cell surface	Human, mouse
TLR7	Bone marrow	Intracellular	Human, mouse
TLR8	Bone marrow	Intracellular	Human, mouse
TLR9	Bone marrow, adipose tissue, umbilical cord	Intracellular	Human
TLR10	Adipose tissue	Cell surface	Human
NOD-like receptors (NLRs)			
NOD1	Umbilical cord	Intracellular	Human
NOD2	Umbilical cord	Intracellular	Human

Contribution of NOD2 in inflammatory bowel disease

Crohn's disease is a chronic inflammatory disorder which affects the gastrointestinal tract and is one of the inflammatory bowel disease (IBD) (Xavier and Podolsky, 2007). This disease is known to result from an overexuberant immune response to the intestinal commensal microflora. The precise underlying mechanisms for these inappropriate reactions are less-understood. However, the genetic variants of NOD2 are reported to contribute to the development of Crohn's disease (Hugot et al., 2001; Ogura et al., 2001). Three single nucleotide polymorphisms (SNPs) located near or within the LRR region (G908R, R702W, and L1007insC) are associated with increased susceptibility to disease (Figure 4) (Hugot et al., 2001; Ogura et al., 2001).

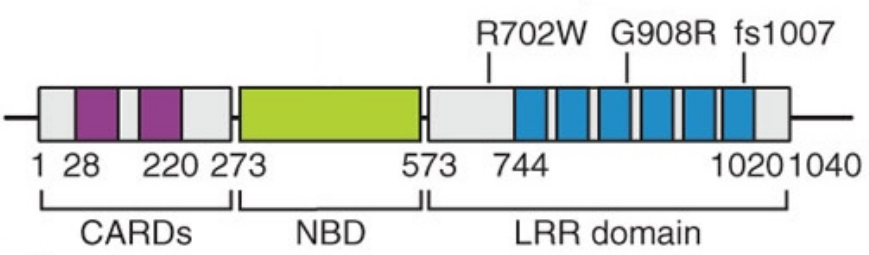

Figure 4. Single nucleotide polymorphisms in NOD2 protein domains (Rivas et al., 2011)**.**

Several studies have shown the role of NOD2 in intestinal inflammation and homeostasis. For example, Noguchi et al. (2009) reported that some of Crohn's disease-associated mutations lead to inhibition of the IL-10 production, a prominent immunosuppressive cytokine (Figure 5). In this study, IL-10 production induced by bacteria was dampened in human monocytes from Crohn's disease patients having frameshift mutation (L1007insC) in NOD2. Another group found that the susceptibility of mice to *Listeria monocytogenes* was increased when NOD2 was deficient due to lower expression of microflora-regulating α-defensin in intestine, which suggests the 'loss of function' by NOD2 mutation (Kobayashi et al., 2005). In contrast, Maeda et al. (2005) showed that macrophages derived from NOD2 mutant mice exhibited the up-regulation of NF-κB and IL-1β production in response to MDP, which suggests the 'gain of function' by NOD2 mutation. Furthermore, enhanced production of inflammatory cytokines such as IL-1β and IL-6 in colons of NOD2 mutant mice resulted in a more susceptibility to DSS-induced colitis. Watanabe et al. (2006) proposed that NOD2 negatively regulate the IL-12 production mediated by peptidoglycan through TLR2 and NOD2 deficiency led to an excessive NF-κB-dependent IL-12 production by antigen presenting cells and an exacerbation in murine antigen-specific colitis. In addition, overexpression of NOD2 in mice led to lower IL-12 production in splenocytes, and these transgenic mice were resistant to peptidoglycan-induced colitis (Yang et al., 2007). These results point to a paramount role of NOD2 in the intestinal homeostasis and their dysfunction is closely associated with IBD (Figure 6). However, further studies are required to define that NOD2 mutation might be loss of function or gain of function.

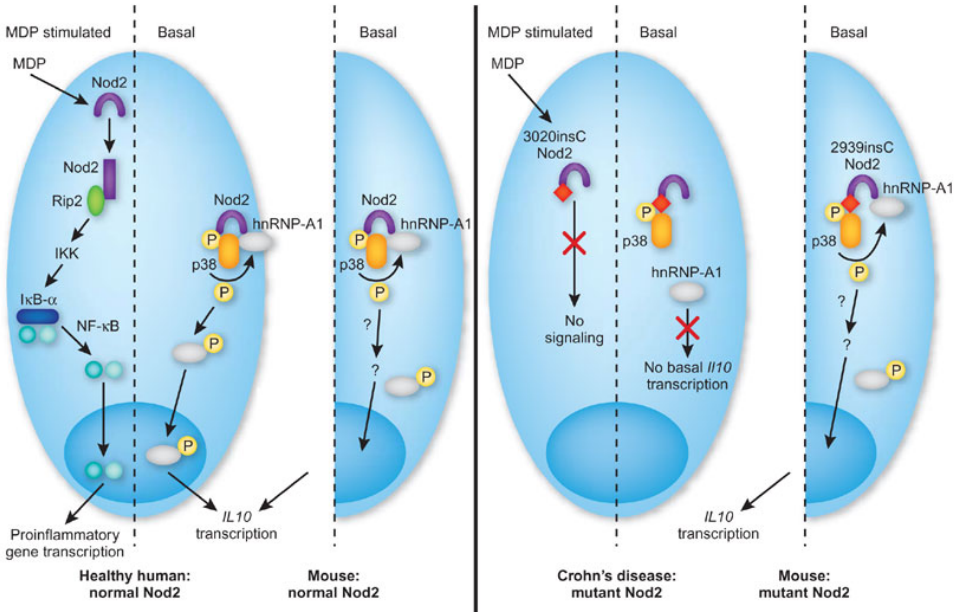

Figure 5. Reduced production of IL-10 in cells expressing Crohn's disease-associated mutations in *Nod2*. Wild-type Nod2 forms a trimolecular complex with active, phosphorylated p38 and the transcription factor hnRNP A1. This association drives IL-10 transcription during the steady-state and after bacterial stimulation (left panel). The Crohn's disease–associated 3020insC mutant Nod2 (right panel) fails to detect MDP and activate the NF-κB pathway, and does not efficiently interact with hnRNP A1; as a result, hnRNP A1 activation and subsequent transcriptional control of the IL-10 promoter is impaired. Reduced IL-10 production is therefore a consequence of this mutation and may contribute to the hyper inflammatory response in the intestine that is characteristic of Crohn's disease. (Philpott and Girardin, 2009).

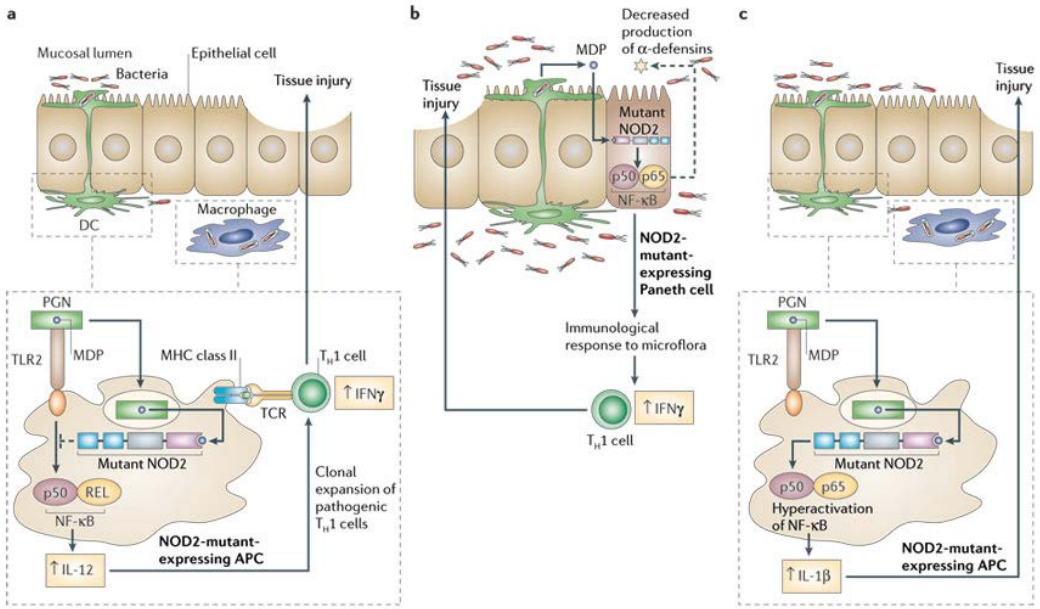

Figure 6. Possible mechanisms of Crohn's disease in patients with mutations in NOD2. (a) In normal mucosa, TLR2-induced activation of NF-κB is negatively regulated by activation of NOD2. By contrast, in mucosa, deficient in NOD2, NF-κB activation is not regulated, and an effector-cell response that supports the inflammation of Crohn's disease ensues. (b) MDP diffuses into Paneth cells and activates NOD2, which then induces secretion of antimicrobial peptides, α-defensins. In normal mucosa, the commensal-bacteria population is down-regulated, and no inflammation occurs. By contrast, in mucosa in which NOD2 is deficient, the lack of α-defensin production leads to bacterial overgrowth that triggers the inflammatory response (c) In APCs with mutations in NOD2, MDP induces excessive IL-1β production. The result is a gain-of-function defect that mediates the inflammation of Crohn's disease (Strober et al., 2006).

MSC therapy for inflammatory bowel disease

Recently, several studies have demonstrated that MSCs derived from various tissues are capable of attenuating inflammatory bowel disease using experimental colitis model (Table 2). Gonzalez et al. (2009) showed that systemic administration of human adipose tissue-derived MSCs (ASCs) or murine ASCs reduced the clinical and histological severity of colitis in mice, improving body weight loss, survival and inflammation in colon. Parekkadan et al. (2011) reported that intravenous transplantation of bone marrow-derived MSCs (BM-MSCs) prevented colitis and increased survival times of colitic mice by increasing the anti-colitic effects of $CD11b^{+}$ cells. Moreover, systemically infused human umbilical cord-derived MSCs (hUC-MSCs) could migrate to inflammatory sites and efficiently alleviate colitis in mice through the regulation of IL-23/IL-17 (Liang et al., 2011). In contrast, a few studies demonstrated that MSCs exacerbate IBD. Nemoto et al. (2013) showed that BM-MSCs stably expressed a higher level of IL-7 and played a crucial role for the development of IBD. Recent studies suggest that resting MSCs do not exert immunoregulatory effect, but obtain their immunosuppressive ability when activated by certain inflammatory cytokines such as interferon-γ (IFN- γ), tumor necrosis factor-α (TNF- α) and interleukin-1β (IL-1β) (Krampera et al., 2006; Ren et al., 2008). For this reason, several groups explored and showed that pretreatment with IFN- γ or IL-1β enhances the therapeutic efficacy of MSC in mice models of colitis (Duijvestein et al., 2011; Fan et al., 2012). In addition, Ko et al. (2010) reported that therapeutic efficacy could be increased when MSCs were coated with antibodies to enhance their delivery to

inflamed colon. These results indicate that MSCs can be a promising tool to treat IBD and additional studies to enhance their therapeutic effect are further required.

Table 2. Study on therapeutic efficacy of MSCs against animal colitis model

Year	Animal	Induction	MSC Source	Route
2008	Rat	4% DSS	Rat BM	IV
2009	Mouse	3mg TNBS	Human, Mouse AD	IP
2010	Mouse	TNBS	Mouse BM	IV, IP
2010	Mouse	TNBS	Human UC	IV
2010	Mouse	5% DSS	Mouse BM	IV
2011	Mouse	$CD4^{+}CD45RB^{hi}$	Mouse BM	IV
2011	Mouse	2.25% DSS 2.75mg TNBS	Human, Mouse BM	IP
2012	Mouse	3% DSS	Human UC	IV
2013	Mouse	3mg TNBS	Human, Mouse AD	IP

CHAPTER I

Implication of NOD1 and NOD2 for the differentiation of multipotent mesenchymal stem cells derived from human umbilical cord blood

1.1 INTRODUCTION

Toll-like receptors (TLRs) are composed of extracellular leucine rich repeats (LRRs) domain that recognizes pathogen-associated molecular patterns (PAMPs) and intracellular Toll/IL-1R (TIR) domain which initiates downstream signaling. TLRs recognize microbial peptides including lipoprotein (TLR2), LPS (TLR4), flagellin (TLR5), dsRNA (TLR3), ssRNA (TLR7/8), and CpG DNA motif (TLR9). Upon sensing specific ligands, TLR-mediated activation of NF-κB and MAPK leads to initiation of inflammatory responses (Akira et al., 2006). Moreover, several endogenous agonists such as heat shock proteins, fibrinogen, fibronectin, heparin sulphate, fatty acids, hyaluronic acid, high mobility group box 1 (HMGB1) and β-defensin 2 are recognized by TLR2 or TLR4 (Wong and Wen, 2008).

Nod-like receptors (NLRs), another PRRs family, are expressed in cytosol and recognize microbial components and danger signals (Chen et al., 2008; Franchi et al., 2009). To now, there are 23 NLR family members identified in humans and 34 genes in mice (Franchi et al., 2009). NLRs are mainly expressed in immune cells, such as dendritic cells, macrophages. However, non-immune cells including epithelial cells and mesothelial cells also express NLRs. As first identified and well-characterzed NLRs, NOD1 and NOD2 are composed of three domains; N-terminal caspase recruitment domain (CARD), intermediate nucleotide-binding oligomerization domain (NOD), and C-terminal leucine-rich repeats (LRRs) domain. NOD1 and NOD2 sense peptidoglycan (PGN) derived peptides; meso-diaminopimelic acid (meso-DAP) for NOD1 and muramyl dipeptide (MDP) for NOD2. Upon stimulation with specific ligands, NOD1 and NOD2 recruit an

adaptor molecule, RICK/Rip2/CARDIAK, through CARD-CARD interaction, which activates NF-κB and MAPK and subsequently induces numerous genes triggering inflammatory process (Girardin et al., 2001; Inohara et al., 2000).

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that can differentiate into not only cells of mesodermal lineage including osteoblasts, adipocytes, chondrocytes, but also cells of other lineages, such as cardiomyocytes, fibroblasts, and endothelial cells (Friedenstein et al., 1976; Jiang et al., 2002; Pittenger et al., 1999). MSCs are expected to be promising candidate tools for the regenerative medicine, due to their differentiation potential. Moreover, MSCs were reported to suppress proliferation, differentiation, maturation and activation of immune cells including T cells, B cells, macrophages, dendritic cells and NK cells (Asari et al., 2009; Prigione et al., 2009; Ren et al., 2008; Zhang et al., 2009a).

The isolation and characterization of MSCs have been described in several species and from different tissues, including bone marrow (BM), peripheral blood, adult fat, skeletal muscle umbilical cord, umbilical cord blood (UCB), Wharton's jelly, amniotic membrane and fluid. Although BM- or adipose tissue-derived MSCs are commonly used for clinical purposes, MSCs from UCB have many advantages because of their immature nature compared with adult cells. Furthermore, hUCB-MSCs do not provide ethical barriers for basic studies and clinical applications as embryonic stem cells do (Gluckman et al., 1997; Grewal et al., 2003).

Recent studies verified the expression of TLRs in MSCs and their role to regulate MSC functions including self-renewal, differentiation, migration, and immunoregulation

(Hwa Cho et al., 2006; Pevsner-Fischer et al., 2007; Tomchuck et al., 2008). Although van den Berk et al. recently demonstrated that TLRs are involved in the regulation of the functions of hUCB-MSCs, most studies have focused on BM- or adipose tissue-derived MSCs (van den Berk et al., 2009). Moreover, the role of NLRs on MSC functions is not well understood yet. This study aimed to investigate the role of NOD1 and NOD2 on the proliferation and differentiation of hUCB-MSCs. I show here that NOD1 and NOD2 as well as TLR2 and TLR4 contribute to the regulation of differentiation in hUCB-MSC.

1.2 MATERIALS AND METHODS

1.2.1 Cell culture

The UCB samples were obtained from the umbilical vein immediately after delivery, with the written informed consent of the mother and approved by the Boramae Hospital Institutional Review Board (IRB) and the Seoul National University IRB (IRB No. 0603/001-002-07C1). The UCB samples were mixed with the Hetasep solution (StemCell Technologies, Vancouver, Canada) at a ratio of 5:1, and then incubated at room temperature to deplete erythrocyte counts. The supernatant was carefully collected and mononuclear cells were obtained using Ficoll (GE healthcare life sciences, Pittsburgh, PA) density-gradient centrifugation at 2,500 rpm for 20 min. The cells were washed twice in PBS. Cells were seeded at a density of 2×10^5 to 2×10^6 cells/cm2 on plates in growth media consisted of D-media (Formula No. 78-5470EF, Gibco BRL) containing EGM-2 SingleQuot and 10% fetal bovine serum (Gibco BRL). After 3 days, non-adherent cells were removed. The adherent cells formed colonies and grew rapidly, exhibiting spindle-shaped morphology.

1.2.2 Flow cytometric analysis

hUCB-MSCs (1×10^6/ml) were stained with FITC- or PE- conjugated antibodies specific for human CD14, CD29, CD31, CD33, CD34, CD44, CD45, CD73, CD90, CD105, CD133, and HLA-DR. Non-specific isotype-matched antibodies served as

controls. All the antibodies were purchased from BD Bioscience (Franklin Lakes, NJ), and flow cytometry analysis was performed on a FACSCaliber using the Cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

1.2.3 RNA extraction and RT-PCR

Total RNA was extracted from hUCB-MSCs by using Easy-spin total RNA extraction kit (Intron Biotechnology, Seongnam, Korea) according to the manufacture's protocol. cDNA was prepared from 1 μg of total RNA by using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo (dT) primers (Invitrogen). The primer sets used are listed in Table 1. The PCR condition consisted of an initial denaturation at 95℃ for 3 min; 30 cycles of 94 ℃ for 30 sec, 60℃ for 30 sec and 72℃ for 1 min; a final extension at 72 ℃ for 10 min. The PCR products were separated on a 1.5% agarose gel, visualized, and photographed using a gel documentation system.

1.2.4 Cytokine production

Briefly, hUCB-MSCs (2×10^4/well) were seeded in MSC medium supplemented with 2% FBS in 96-well plate. Twenty-four hours later, the cells were treated with various doses of Pam3CSK4, LPS, Tri-DAP, and MDP and incubated for additional 24 h. Culture supernatant was collected, centrifuged, filtered through 0.2μm filter and IL-8 concentration was measured using commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol.

1.2.5 hUCB-MSC proliferation

The cells were seeded at 2×10^3/well in 96-well plates in MSC medium supplemented with 2% FBS. Twenty-four hours later, the cells were treated with Pam3CSK4, LPS, Tri-DAP, and MDP at 10 μg/ml concentration and incubated for 4 days. Proliferation was determined by Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) according to manufacturer's instruction.

1.2.6 hUCB-MSC differentiation

Osteogenic differentiation. The cells were incubated in conditioned media containing DMEM low glucose medium, 10% FBS, 0.1 μM dexamethasone, 10 mM beta-glycerophosphate, and 50 μM ascorbate in the absence or presence of TLR and NLR agonists. The cells were grown for 2 weeks, with medium replacement twice a week. Osteogenesis was detected by Alizarin Red staining. Photographs were taken and optical density was measured at 570 nm.

Adipogenic differentiation. The cells were incubated in conditioned media containing DMEM low glucose medium, 10% FBS, 1M dexamethasone, 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.2 mM indomethacin in the absence or presence TLR and NLR agonists. The cells were grown for 3 weeks, with media replacement twice a week. Adiopogenesis was detected by Oil red O staining. Photographs were taken and optical density was measured at 500 nm.

Chondrogenic differentiation. 2×10^5 cells were seeded in 15-mL polypropylene tube and centrifuged to a pellets. The pellets were cultured in 1ml of chondrogenic

medium that contained 10% FBS and 500ng/ml bone morphogenetic protein-2(BMP-2; R&D Systems) for 3 weeks. The chondrogenic differentiation medium was replaced twice a week. The pellets were embedded in paraffin and cut into 3μm sections. For histological evaluation, the sections were stained with toluidine blue following general procedures.

1.2.7 Western Blot

The cells were stimulated with agonists, harvested, and lysed in buffer containing 1% Nonidet-P40 supplemented with complete protease inhibitor 'cocktail' (Roche) and 2 mM dithiothreitol. Lysates were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with primary antibodies such as regular- and phopho-ERK (Cell signaling, Beverly, MA, USA) and GAPDH (Santa Cruz biotechnology, Santa Cruz, CA, USA). After immunoblotting with secondary antibodies, proteins were detected with enhanced chemiluminescence (ECL) reagent (Intron Biotechnology).

1.2.8 Statistical Analysis

The differences in mean values among different groups were tested, and the values were expressed as mean ± SD. All of the statistical calculations were calculated by one-way ANOVA followed by Bonferroni post-hoc test for multi-group comparisons (StatView 5.0; SAS Institute, Cary, NC). Statistical significance is indicated in the figure legends.

Table 1. Names and sequences of the primers for RT-PCR

Gene		Primer sequence
TLR2	F	5’-GATGCCTACTGGGTGGAGAA-3’
	R	5’- CGCAGCTCTCAGATTTACCC-3’
TLR4	F	5’- ACAGAAGCTGGTGGCTGTG-3’
	R	5’- TCTTTAAATGCACCTGGTTGG-3’
NOD1	F	5’- CCACTTCACAGCTGGAGACA-3’
	R	5’- TGAGTGGAAGCAGCATTTTG-3’
NOD2	F	5’- GAATGTTGGGCACCTCAAGT-3’
	R	5’- CAAGGAGCTTAGCCATGGAG-3’
Rip2	F	5’- CCATTGAGATTTCGCATCCT-3’
	R	5’- ATGCGCCACTTTGATAAACC-3’
RPL13A	F	5’- CATCGTGGCTAAACAGGTAC-3’
	R	5’- GCACGACCTTGAGGGCAGCC-3’

1.3 RESULTS

1.3.1 hUCB-MSCs functionally expressed TLR2, TLR4, NOD1, and NOD2

To verify the stem cell phenotypic markers of hUCB-MSCs, hUCB-MSCs were determined for surface marker expression after incubation with fluorescence conjugated specific antibodies. Upon flow cytometric analysis, hUCB-MSCs were found to be negative for CD14, CD31, CD33, CD34, CD45, CD133 and HLA-DR expression but positive for CD29, CD44, CD73, CD90 and CD105 (data not shown). The gene expression of TLR2, TLR4, NOD1, and NOD2 in hUCB-MSCs was examined by RT-PCR. A human monocytic leukemia cell line, THP-1 cells were used as positive control. All receptors tested were expressed in both THP-1 cells and hUCB-MSCs (Fig 1A). TLR4 was expressed strongly in hUCB-MSCs than in THP-1 cells, whereas the gene expression of TLR2, NOD1, and NOD2 was weaker in UCB-MSC (Fig 1A). Rip2, the adaptor protein of NOD1 and NOD2, was also apparently expressed in hUCB-MSCs (Fig 1A). To evaluate the functionality of the receptors, I examined IL-8 production by hUCB-MSCs in response to their specific agonists. Stimulation with Pam3CSK4 (TLR2 agonist), LPS (TLR4), Tri-DAP (NOD1), and MDP (NOD2) led to increased production of IL-8 in hUCB-MSCs in a dose-dependent manner (Fig 1B and C). These findings indicate that NOD1 and NOD2, as well as TLR2 and TLR4, are expressed in hUCB-MSCs and can respond to their specific agonists, implying that all the receptors are functionally expressed.

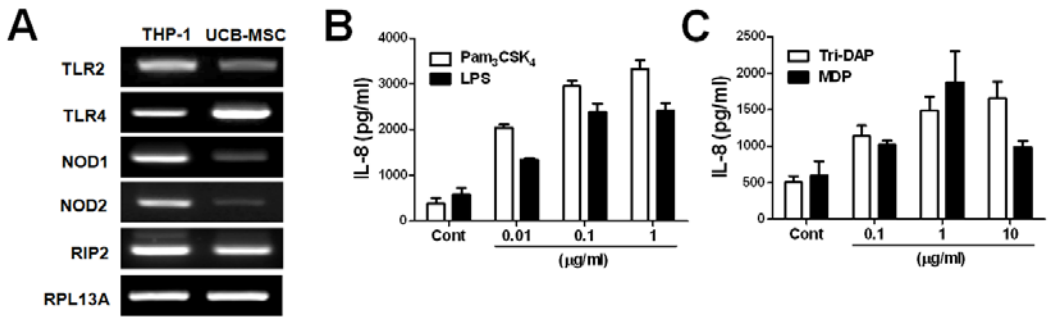

Figure 1. TLRs and NLRs were functionally expressed in hUCB-MSCs.

(A) mRNA expressions of TLR2, TLR4, NOD1, NOD2, and Rip2 were determined by RT-PCR in hUCB-MSCs. **(B-C)** The cells were treated with **(B)** Pam3CSK4, LPS, **(C)** Tri-DAP, and MDP in a dose-dependent manner for 24 h and IL-8 production was determined using a commercial ELISA kit.

1.3.2 hUCB-MSC proliferation was not affected by activation of TLRs and NLRs

TLRs have been found to promote the proliferation of several types of MSC (Pevsner-Fischer et al., 2007; Wang et al., 2009; Yu et al., 2008). To examine whether TLR and NLR activation influence the proliferation of hUCB-MSC, the cells were incubated at the absence or presence of each agonist (Pam3CSK4, LPS, Tri-DAP, and MDP) for 4 days and cell proliferation was determined by CCK-8 analysis. Results showed that none of agonists influenced the proliferation of hUCB-MSC (Fig 2A and B).

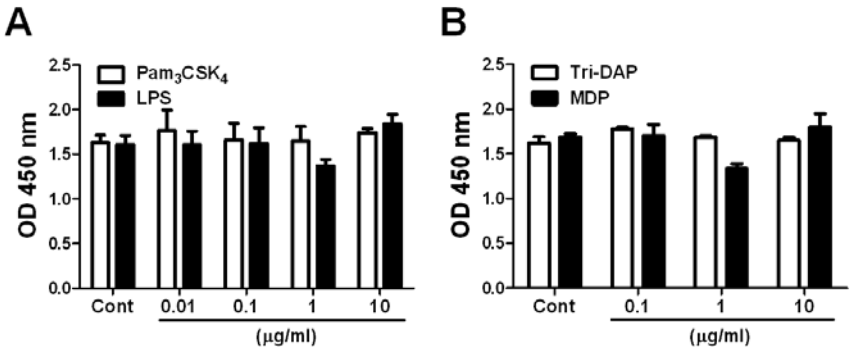

Figure 2. Activation of TLRs and NLRs did not influence the proliferation of hUCB-MSCs.

(A-B) hUCB-MSCs were treated with various doses of **(A)** Pam3CSK4, LPS, **(B)** Tri-DAP, and MDP for 4 days and cell proliferation was determined by CCK-8 kit.

1.3.3 Osteogenic differentiation of hUCB-MSCs was enhanced by activation of TLRs and NLRs

It has been shown that TLRs modulates the differentiation of MSCs (Hwa Cho et al., 2006; Pevsner-Fischer et al., 2007). To determine whether TLRs and NLRs are involved in osteogenic differentiation of hUCB-MSCs, the cells were treated with Pam3CSK4, LPS, Tri-DAP, and MDP and cultured in standard osteogenic medium. During osteogenic differentiation of two different hUCB-MSCs (#618 and #1114), all agonists tested significantly induced higher intensity of the Alizarin red S staining (Fig 3A and B). It has shown that extracellular signal-regulated protein kinases (ERK) activation plays an important role in the osteogenic differentiation of MSCs (Rodriguez et al., 2004). Therefore, I explored whether TLR and NLR agonists lead to ERK activation in hUCB-MSCs. As expected, stimulation by TLR and NLR agonists rapidly induced phosphorylation of ERK in hUCB-MSCs (Fig 3C). To determine whether inhibition of ERK is associated with osteogenic differentiation of hUCB-MSCs, the Pam3CSK4-stimulated cells were treated with U0126 as an MEK1/2 inhibitor. In Alizarin Red S staining, treatment of U0126 restored osteogenic differentiation of hUCB-MSCs enhanced by Pam3CSK4 (Fig3 D and E). These results indicated that both TLR and NLR signaling may promote osteogenic differentiation of hUCB-MSCs through ERK-dependent pathway.

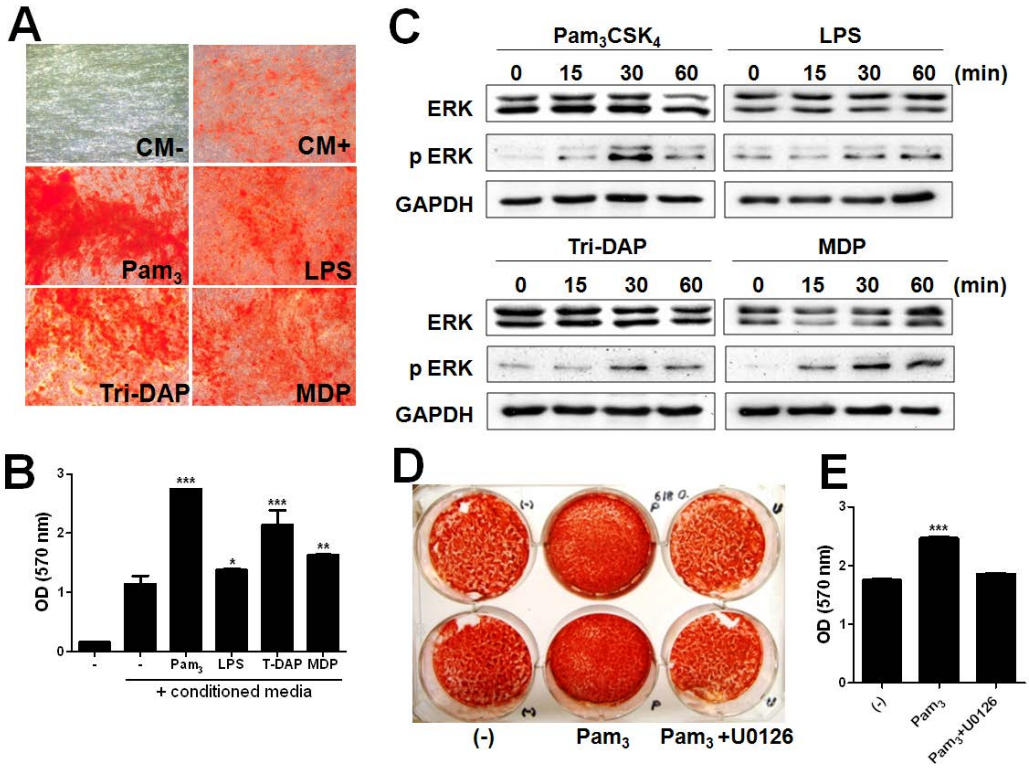

Figure 3. Stimulation with TLR and NLR agonists promoted osteogenic differentiation of hUCB-MSCs through phosphorylation of ERK1/2.

Figure 3. Stimulation with TLR and NLR agonists promoted osteogenic differentiation of hUCB-MSCs through phosphorylation of ERK1/2.

(A-D) hUCB-MSCs were grown in conditioned media at the absence or presence of Pam3CSK4, LPS, Tri-DAP, and MDP (10 μg/ml) for 2 weeks, and culture media was replaced twice per week. **(A)** Osteogenesis was determined by Alizarin Red S at 2 weeks after treatment and **(B)** optical density was determined using ELISA at 570 nm. **(C)** hUCB-MSCs were treated with Pam3CSK4, LPS, Tri-DAP, and MDP for 15, 30, and 60 min and ERK phosphorylation was determined by Western Blot analysis with an anti-phospho-ERK antibody. **(D-E)** hUCB-MSCs were co-treated with Pam3CSK4 and U0126 for 2 weeks and determined by Alizarin Red staining and quantified using ELISA at 570 nm . * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

1.3.4 Adipogenic differentiation of hUCB-MSCs was inhibited by activation of NOD1 and NOD2

To determine the effects of TLR and NLR agonists on adipogenic differentiation of hUCB-MSCs, the cells were incubated at the absence or presence of each agonist for 3 weeks. As shown in Fig 4A-C, stimulation with Tri-DAP and MDP significantly inhibited adipogenic differentiation at 3 weeks after treatment, whereas Pam3CSK4 and LPS did not influence on adipogenic differentiation of hUCB-MSCs (Fig 4A-C). This phenomenon was confirmed in another line of hUCB-MSCs (#1114) (Fig 4D and E). These findings suggest that NOD1 and NOD2 signaling may be involved in adipogenic differentiation of hUCB-MSCs.

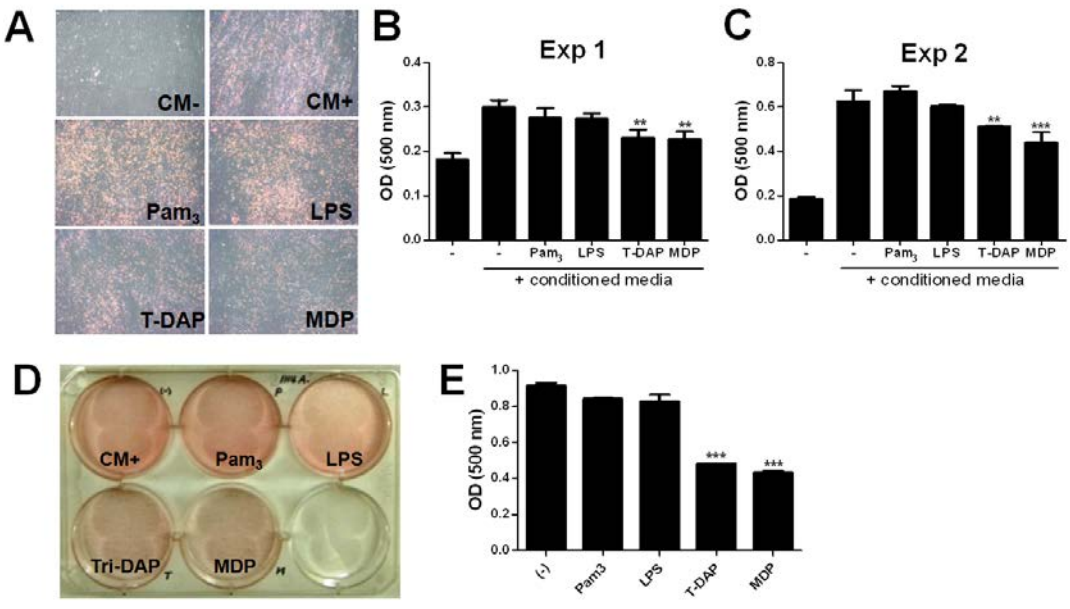

Figure 4. Tri-DAP and MDP inhibited adipogenic differentiation of hUCB-MSCs.

Figure 4. Tri-DAP and MDP inhibited adipogenic differentiation of hUCB-MSCs.

(A-D) hUCB-MSCs were grown in conditioned media at the absence or presence of Pam3CSK4, LPS, Tri-DAP, and MDP (10 μg/ml) for 3 weeks, and culture media was replaced twice per week. **(A)** Adipogenesis was determined by Oil Red O staining and **(B-C)** level of intercellular lipid was determined by measuring absorbance at 500 nm at 3 weeks after treatment. **(D-E)** hUCB-MSCs (#1114) from another umbilical cord blood were grown in conditioned media at the absence or presence of each ligands for 3 weeks, and **(D)** adipogenesis was determined and**(E)** quantified by measuring absorbance at 500 nm. ** $P<0.01$, *** $P<0.001$.

1.3.5 Chondrogenic differentiation of hUCB-MSCs was promoted by activation of TLRs and NLRs

To determine whether TLR and NLRs are involved in chondrogenic differentiation of hUCB-MSCs, hUCB-MSCs were maintained in BMP-2 supplemented chondrogenic medium at the absence or presence of each agonists. All TLR and NLR agonists used increased the diameter of pellets (Fig 5A). All the pellets were positive to toluidine blue staining (Fig 5B). These data suggest that both TLR and NLR signaling may be involved in chondrogenesis of hUCB-MSCs.

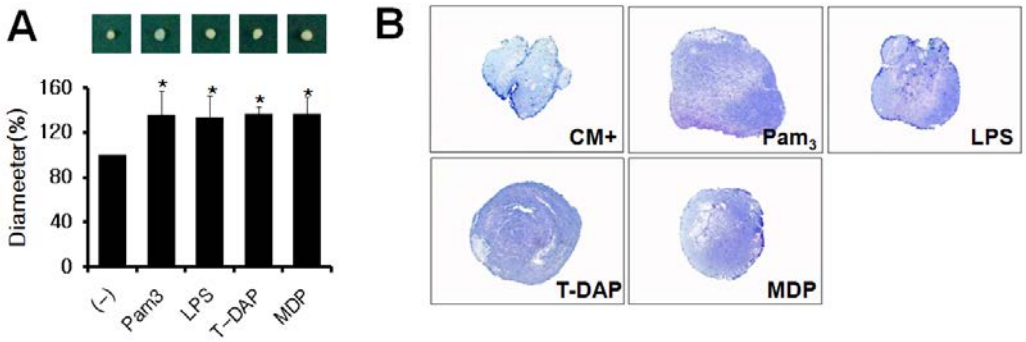

Figure 5. Activation of TLRs and NLRs promoted chondrogenic differentiation of hUCB-MSCs.

(A-B) hUCB-MSCs were prepared as pellets and they were cultured in chondrogenic medium supplemented with 500 ng/ml BMP-2 for 3 weeks. Then, **(A)** the volume of pellets was measured and **(B)** stained with toluidine blue. * $P<0.05$.

1.4 DISCUSSION

NLRs have been known to recognize intracellular microbial components such as PGN derivatives (by NOD1 and NOD2) and flagellin (by NLRC4/IPAF) (Chen et al., 2008). Similar to TLR signaling, NOD1 and NOD2 activate NF-κB and MAPK to trigger inflammatory process. Moreover, NOD1 and NOD2 agonists, in combination with TLR agonists, synergistically induce cytokine production and activation of NF-κB and MAPK in immune cells (Kim et al., 2008; Park et al., 2007b; Tada et al., 2005). These results indicate that NLR signaling might be closely associated with TLR-mediated events. Since TLRs regulate the functions of a various MSCs, in this study, I explored the role of NLRs, particularly NOD1 and NOD2, on hUCB-MSCs function. Additionally, because most studies showed the role of TLRs in BM- or adipose tissue-derived MSCs, I also confirmed the effect of TLR2 and TLR4 on UCB-MSC functions along with NOD1 and NOD2.

A previous study showed that hUCB-MSCs expressed low levels of TLR1, 3, 5, 9 and high level of TLR4 (van den Berk et al., 2009). In my study, both TLR2 and TLR4 were expressed in hUCB-MSCs, and TLR4 expression was much stronger than TLR2. This discrepancy might result from difference of PCR condition used or MSCs origin. Several studies also showed functional expression of TLR2 in BM- or adipose tissue-derived MSCs (Hwa Cho et al., 2006; Lombardo et al., 2009; Pevsner-Fischer et al., 2007; Tomchuck et al., 2008). In addition, in this study, Pam3CSK4 and LPS stimulation led to IL-8 production in hUCB-MSC, implying that hUCB-MSCs functionally express TLR2 and TLR4.

It has been reported that NOD1 is ubiquitously expressed in various cell types, whereas NOD2 is mainly expressed in immune cells (Inohara et al., 2005). In this study, hUCB-MSCs apparently expressed the genes of NOD1 and NOD2, although expression levels were relatively weaker than those of TLR2 and TLR4. In addition, mRNA expression of Rip2/RICK, an adaptor molecule of NOD1 and NOD2, was also determined. Stimulation with Tri-DAP and MDP, NOD1 and NOD2 ligands, led to IL-8 production, which indicates the functional expression of NOD1 and NOD2 in hUCB-MSCs.

The effect of TLRs on the MSC proliferation is controversial. A recent study demonstrated that TLR ligands such as PGN, LPS, poly I:C, and flagellin did not affect the proliferation of human adipose tissue-derived MSCs (hASC) (Hwa Cho et al., 2006). Only CpG-ODN, TLR9 agonists, slightly inhibited hASC proliferation.(Hwa Cho et al., 2006) This phenomenon was confirmed by a study of Lombardo et al. (Lombardo et al., 2009), showing that TLR3 and TLR4 agonists had no influence on the proliferation of hASCs. By contrast, siRNA-mediated inhibition of MyD88, a common adaptor molecule for TLRs except TLR3, suppressed the proliferation of hASC (Yu et al., 2008). Furthermore, TLR agonists such as Pam3Cys and LPS augmented the proliferation of murine BM-MSCs (Pevsner-Fischer et al., 2007; Wang et al., 2009), implying the modulatory role of TLRs on MSC proliferation. Taken together, these results indicate that the effect of TLRs on MSC proliferation might be cell-type specific. In my study, the agonists of NOD1 and NOD2 as well as TLR2 and TLR4 did not influence hUCB-MSCs proliferation. It is necessary to clarify the effect of NOD1 and NOD2 on the proliferation

of MSCs from different tissues.

TLRs are well known to be affect MSC differentiation. In particular, TLRs seem to be involved in osteogenic differentiation rather than adipogenic differentiation of MSCs. TLR3 and TLR4 agonists significantly enhanced osteogenic differentiation of hASCs, but did not affect adipogenic differentiation (Lombardo et al., 2009; Yu et al., 2008). Other ligands for TLR (poly I:C, flagellin, CpG-ODN) also did not influence adipogenic differentiation of hASC (Hwa Cho et al., 2006). Interestingly, PGN significantly suppressed adipogenic differentiation of hASCs (Hwa Cho et al., 2006; Yu et al., 2008). In these studies, PGN was used as TLR2 agonist. However, a previous study by Travassos et al. (Travassos et al., 2004) reported that highly purified PGN was not detected by TLR2. They revealed that cell wall contaminants such as lipoteichoic acid (LTA) or lipoproteins present in PGN are responsible for TLR2-dependent cell activation (Travassos et al., 2004). In my study, Tri-DAP and MDP significantly down-regulated adipogenic differentiation of hUCB-MSCs at 2 and 4 weeks after stimulation, whereas Pam3CSK4 and LPS did not, suggesting that NOD1 and NOD2 are involved in adipogenic differentiation of hUCB-MSCs. It is well known that NOD1 and NOD2 recognize PGN derivatives, meso-DAP and MDP, respectively. Therefore, it is likely that NOD1 and NOD2 are involved in PGN-mediated inhibition of adipogenic differentiation of hASCs. The role of TLR2 on adipogenic differentiation of MSC and the type of receptors originally mediating inhibitory effect of PGN should be clarified. Moreover, several TLR agonists enhance osteogenic differentiation of MSCs (Hwa Cho et al., 2006; Lombardo et al., 2009; Yu et al., 2008). My results showed that all TLR and NLR

agonists promoted osteogenic differentiation of hUCB-MSCs, implying that NLRs as well as TLRs might be involved in osteogenic differentiation of MSCs. ERK phosphorylation is known to be correlated with the osteogenic differentiation of MSCs (Jaiswal et al., 2000; Rodriguez et al., 2004). As TLR and NLR agonists strongly induced osteogenic differentiation of hUCB-MSCs, I examined phosphorylation of ERK in response to agonists. LPS and MDP induced phosphorylation of ERK within 1 h in mouse macrophages (Kim et al., 2008; Park et al., 2007a). In this study, Pam3CSK4 and MDP induced ERK phosphorylation in hUCB-MSCs from 15 min after stimulation and the level or phosphorylation reached to peak at 30 min post-stimulation. LPS and Tri-DAP induced phosphorylation of ERK from 30 min after stimulation. ERK was strongly phosphorylated in the hUCB-MSCs treated with Pam3CSK4, whereas LPS induced only slight activation of ERK, which correlated with intensity of Alizarin Red S staining. In addition, U0126, MEK1/2 inhibitor, inhibited osteogenic differentiation induced by Pam3CSK4. Taken together, one can envision that ERK signaling is critical for osteogenic differentiation of hUCB-MSCs induced by TLR and NLR stimulation. In my knowledge, there is no report about the effect of TLRs on chondrogenic differentiation of MSCs, except the study by Pevsner-Fischer et al (Pevsner-Fischer et al., 2007). They revealed that TLR2 stimulation with Pam3Cys inhibited the differentiation of MSCs into osteogenic, adipogenic, or chondrogenic lineages. However, in this study, all used agonists including Pam3CSK4 enhanced the chondrogenic differentiation potential of hUCB-MSCs. The reason for this discrepancy remains to be elucidated.

In conclusion, the present study revealed novel information that NOD1 and NOD2 as well as TLRs are functionally expressed and are involved in regulating the differentiation of hUCB-MSCs. These findings are expected to provide better understanding of the biological function of MSCs. Further study using in vivo model is needed to clarify physiological role of NOD1 and NOD2 on MSC functions.

CHAPTER II

Human Umbilical Cord Blood Mesenchymal Stem Cells Reduce Colitis in Mice by Activating NOD2 Signaling to COX2

2.1 INTRODUCTION

Nucleotide-binding oligomerization domain 2 (NOD2) is a member of the cytosolic Nod-like receptor family. NOD2 senses muramyl dipeptide (MDP), a small molecule derived from the peptidoglycan of bacterial cell wall, and activates the serine-threonine kinase RICK (RIP-like interacting CLARP kinase) [also known as receptor-interacting protein 2 (RIP2)] (Chen et al., 2009; Inohara et al., 2003). The activation of RICK results in MAPK activation and ubiquitinylation of NF-κB essential Modulator (NEMO) and translocation of nuclear NF-κB subunit into the nucleus (Abbott et al., 2004; Inohara et al., 2000). Finally, nuclear-translocated NF-κB leads to the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, which are key molecules in host innate immune responses.

Several genetic variants of NOD2 are associated with the development of Crohn's disease (Hugot et al., 2001; Ogura et al., 2001). Single nucleotide polymorphisms of NOD2 are genetic risk factors for susceptibility to Crohn's disease (Hugot et al., 2001; Ogura et al., 2001). Several groups have studied the role of NOD2 in intestinal inflammation. Watanabe et al. demonstrated that Nod2 deficiency led to enhanced IL-12 production by antigen presenting cells in response to peptidoglycan and that this deficiency exacerbated antigen-specific colitis in mice (Watanabe et al., 2006). Furthermore, Nod2 transgenic mice overexpressing Nod2 were resistant to peptidoglycan-induced colitis (Yang et al., 2007). In addition, Nod2 deficiency impaired the recruitment of inflammatory monocytes and intestinal clearance of pathogenic E. coli resulting in exacerbated colitis (Kim et al., 2011). These findings indicate that NOD2 may

regulate intestinal inflammation through various mechanisms and that mutation or absence of NOD2 could be a crucial factor for the development of colitis.

My previous study revealed that NOD2 is functionally expressed in hUCB-MSCs and regulate the differentiation of hUCB-MSCs (Kim et al., 2010). Although some Toll-like receptors (TLRs) are known to enhance the immunosuppressive activity of MSCs (Opitz et al., 2009), the role of NOD2 in the immunomodulation of MSCs has not been investigated. I report here that NOD2 activation enhances the protective effect of hUCB-MSCs against both DSS- and TNBS-induced colitis in mice by producing PGE_2 via the NOD2-RIP2 pathway.

2.2 **MATERIALS AND METHODS**

2.2.1 Cell culture

The UCB samples were obtained from the umbilical vein immediately after delivery, with the informed consent of the mother approved by the Boramae Hospital Institutional Review Board (IRB) and the Seoul National University IRB (IRB No. 0603/001-002-10C4). The UCB samples were mixed with the Hetasep solution (StemCell Technologies, Vancouver, Canada) at a ratio of 5:1, and then incubated at room temperature to deplete erythrocyte counts. The supernatant was carefully collected and mononuclear cells were obtained using Ficoll density-gradient centrifugation at 2,500 rpm for 20 min. The cells were washed twice in PBS. For human mononuclear cell (hMNC) culture, cells were cultured in RPMI media with 10% fetal bovine serum. For hUCB-MSC culture, cells were seeded at a density of 2×10^5 to 2×10^6 cells/cm2 on plates in growth media consisted of D-media (Formula No. 78-5470EF, Gibco BRL, Grand Island, NY) containing EGM-2 SingleQuot and 10% fetal bovine serum (Gibco BRL). After 3 days, non-adherent cells were removed. The adherent cells formed colonies and grew rapidly, exhibiting spindle-shaped morphology.

The cells were used for experiments after verifying the stem cell characteristics by observing the differentiation, proliferation and immunological phenotypes of hUCB-MSCs as was previously determined (Seo et al., 2011). For this study, MSCs derived from umbilical cord blood of five different individuals were used, designated as #618, 620, 1180, 1267 and U8.

2.2.2 Colitis induction

C57BL/6J mice (male, 8-10wk old) were obtained from Jackson Laboratory (Bar Harbor, ME) and BALB/c mice (male, 8-10wk old) from SLC (Hamamatsu, Japan). Mice were group housed under specific pathogenic-free conditions in the animal facility of the Seoul National University. All experiments were approved and followed by the regulations of the Institute of Laboratory Animals Resources (SNU-100125-8, SNU-111223-1 and SNU-130130-2 Seoul National University, Korea).

Colitis was induced in mice by the addition of 3% (w/v) dextran sulfate sodium (DSS, MP Biochemicals, Solon, OH) in drinking water for 7 days. hUCB-MSCs were exposed to MDP for 24 h before administration and washed with PBS to remove residual MDP. hUCB-MSCs resuspended in PBS (2×10^6 cells/200 μl) were injected intraperitoneally into mice 1 day after administration of DSS. Body weight and survival rate were monitored over 14 days, and on day 7, colitis severity was measured by evaluating the disease activity index through the scoring of weight loss (0~4), stool consistency (0~4), bleeding (0~4), coat roughness (0~4), mouse activity (0~2), and bedding contamination by stool and blood (0~2). At the peak of disease (on day 10), the mice were sacrificed, colon length and diameter of mesenteric lymph nodes (MLNs) were measured. Histopathological evaluation was performed. Trinitrobenzene sulfonic acid (TNBS, Sigma, St.Louise, MO) colitis was induced by the intrarectal administration of TNBS (3mg) in 40% ethanol into BALB/c mice after presensitization on the skin. Six hours after intrarectal TNBS infusion, hUCB-MSCs were i.p. injected. Mice were monitored for survival rate and body weight loss, and sacrificed on day 5 for colon length

measurement and histopathological evaluation (Fig 1).

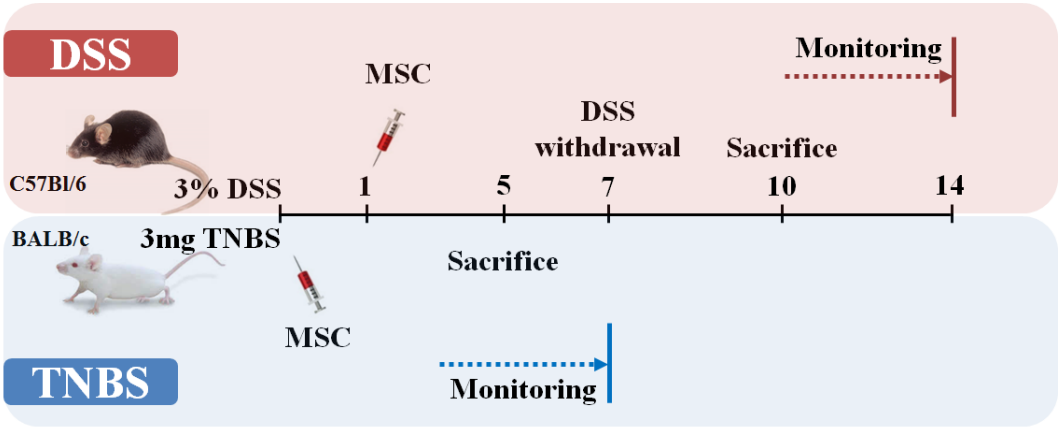

Figure 1. Time Table for colitis induction and hUCB-MSC transplantation

2.2.3 Histopatholigical evaluation

Colon samples from colitic mice were collected, fixed in 10% formalin, subjected to consecutive steps of alcohol-xylene changes, and embedded in paraffin. Sections of 5 μm thickness were prepared and stained with Hematoxylin and Eosin (H&E). Leukocyte infiltration and intestinal damages were graded blindly.

2.2.4 Cytokine production

In vivo Protein lysates were extracted from colonic segments (50 mg tissue/mL) in 50 mM Tris-HCl, pH 7.4, with 0.5 mM dithiothreitol and 10 μg/ml of proteinase inhibitor cocktail (Sigma). Protein extracts were centrifuged at 30,000g for 20 minutes and stored at -80°C. IL-10, IL-6, and IFN-γ concentration was measured using commercial ELISA kit (eBioscience, San Diego, CA) according to manufacturer's

protocol. For detection of PGE2 from mouse serum and colon, serum or protein lysates were purified by multiple affinity removal kit (Agilent technologies, Santa Clara, CA) before measured using ELISA kit (R&D Systems, Minneapolis, MN).

In vitro Briefly, cells were treated with ligands for 24 h and PGE2 production was determined from culture supernatant using commercial ELISA kit (R&D Systems). For IL-10 measurement, hUCB-MSCs were treated with ligands for 24 h. After 5 times washing, fresh RPMI 1640 (Gibco BRL) was added. After 5 days, media was harvested (UCB-MSC Conditioned Medium, UCM). MNCs prepared as described above were cultured with ConA in UCM. After 5 days, culture supernatant was collected, and the IL-10 concentration was measured using an ELISA kit (R&D Systems). Additionally, PGE_2 concentration in UCM was measured.

2.2.5 Myelopoeroxidase assay

Neutrophil infiltration in the colon was determined by measuring myeloperoxidase (MPO) activity. Colon segments were homogenized at 50mg/mL in phosphate buffer (50mM, pH6.0) with 0.5% hexadecyltrimethylammonium bromide. Samples were centrifuged at 30,000g for 15min at 4°C after 3 times of freezing and thawing. The supernatants were diluted 1/30 with 50mM phosphate buffer (pH6.0) containing 0.167mg/mL o-dianisine (Sigma) and 0.0005% H2O2. Changes in absorbance between 1 and 3 minutes at 450nm were measured with spectrophotometer. MPO activity was calculated as units (U) per gram of wet tissues. 1 U MPO activity represents the amount of enzyme required to degrade 1 μM peroxide/min/mL at 24°C.

2.2.6 Immunohistochemistry

Paraffin-embedded sections of colon samples were stained with specific primary antibodies against CD4, CD11b and Foxp3 followed by 2 h incubation with Alexa 488-labeled secondary antibody (1:1,000; Molecular Probes, Eugene, OR). The nuclei were stained with Hoechst 33258 (1:1,000; Sigma). The images were captured with a confocal microscope.

2.2.7 Western Blot

The cells or colon segments were harvested, and lysed in a buffer containing 1% Nonidet-P40 supplemented with a complete protease inhibitor 'cocktail' (Roche) and 2 mM dithiothreitol. Lysates were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with primary antibodies such as NOD2 (Cayman, Ann Arbor, MI), Rip2 (Alexis, Plymouth Meeting, PA), indoleamine-2,3-dioxygenase (IDO-1, Millipore, Billerica, MA), COX-2, GAPDH (Santa Cruz biotechnology, Santa Cruz, CA), Foxp3 and iNOS (Abcam, Cambridge, MA). After immunoblotting with secondary antibodies, proteins were detected with enhanced chemiluminescence (ECL) reagent (Intron Biotechnology, Korea).

2.2.8 Cell tracking

To track the injected cells, hUCB-MSCs were labeled with 10 μM carboxy fluorescein diacetate succinimidyl ester (CFSE: Molecular Probes, Carlsbad, CA) according to manufacturer’s protocol. CFSE-labled cells were i.p. injected. At 1, 3, 5, 7

and 10 days after injection, 10 μm frozen colon sections were cut and examined for green fluorescence with a confocal microscope. For analysis by flow cytometry, cells were isolated from colon segments, MLNs and spleen, followed by digestion with type IV collagenase (0.5 mg/mL) and deoxyribonuclease I (0.5 mg/mL) for 30 minutes at 37°C. After filtration with strainer, cells were analyzed for fluorescence on a FACS Caliber (Becton Dickinson, Franklin Lakes, NJ).

2.2.9 Mixed Lymphocyte Reaction

hUCB-MSCs were treated with 25 μg/ml of mitomycin C at 37°C for 1 h. After five washes, the cells were seeded in 96-well plates at 1×10^4/well. Six hours later, the cells were treated with each agonist and incubated for 24 h. hMNCs prepared as described above were treated with (Concanavalin A) ConA in RPMI media for 1 h and subsequently added to each well of hUCB-MSCs cultured at 1×10^5/well. After 5 days of a mixed leukocyte reaction (MLR), MNC proliferation was determined by Cell Proliferation ELISA, BrdU kit (Roche). For MLR with hUCB-MSCs conditioned medium (UCM), hUCB-MSCs (3×10^5/well) were seeded in 6-well plates, and 24 hours after seeding, MSCs were treated with each agonist. After another 24 hr, MSCs were washed 5 times and fresh RPMI 1640 (Gibco BRL) was added. After 5 days, the media was harvested and MLR as described earlier was then performed in this media. MLRs with hUCB-MSCs and Jurkat (human T cell lymphoblast-like cell line) or mouse splenocytes were performed in the same way.

2.2.10 Nitric Oxide detection

hUCB-MSCs were treated with Pam_3CSK_4, LPS(1 μg/ml) and Tri-DAP, MDP (10 μg/ml) for 24 h and RAW 264.7 cells were treated with 100 ng/ml LPS as a positive control. NO was measured from culture supernatant using a Griess reagent (Sigma) according to the manufacturer's instruction.

2.2.11 RNA interference

Transfection of small interfering RNA(siRNA) into the cells was conducted when they had reached 60% confluence. The siRNAs of NOD2 (siNOD2, J-011388-07), RIPK2 (siRIPK2, M-003602-02) PTGS2 (siCOX-2, L-004557-00) and non-targeting control (siControl #1, D-001810-01) were purchased from Dharmacon (Chicago, IL). Experiments were conducted using DharmaFECT1 (Dharmacon) as a transfection agent and siRNA at a concentration of 100 nmol/L. After 48 h, the medium was changed and the cells were treated with or without each agonist.

2.2.12 Flow cytometric analyses

For analysis of human regulatory T cell population, hUCB-MNCs cultured in UCM were incubated with FITC/anti-CD4 and PerCP/anti-CD25 antibodies. After extensive washing, cells were fixed and permeabilized with human Foxp3 buffer set (BD Biosciences, San Jose, CA) and incubated with PE/anti-Foxp3 antibody. Non-specific isotype-matched antibodies served as controls. All the antibodies were purchased from BD Biosciences. For analysis of mouse regulatory T cell infiltration in colon, cells

isolated from digested colons were incubated with APC/anti-CD4 and PE/anti-CD25. After washing, cells were fixed and permeabilized with mouse FoxP3 Fixation/Permeabilization Concentrate and Diluent (eBioscience) and incubated with FITC/anti-FoxP3 antibodies (eBioscience). All the flow cytometry analyses were performed on a FACS Caliber using the Cell Quest software (BD Biosciences).

2.2.13 Statistical analyses

Mean values among different groups were expressed as mean ± SD. All of the statistical comparisons were made by one-way ANOVA followed by Bonferroni post-hoc test for multi-group comparisons using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Statistical significance is indicated in the figure legends. Statistical significance designated as asterisks is indicated in the Figure legends.

2.3 RESULTS

2.3.1 MDP enhances the protective effect of hUCB-MSCs against DSS-induced colitis in mice

I first explored whether the systemic administration of hUCB-MSCs rescues mice from DSS-induced colitis and whether NOD2 activation enhances the protective effect of hUCB-MSCs against colitis. Intraperitoneal injection of hUCB-MSCs ameliorated the loss of body-weight and decreased the mortality of mice compared with PBS- or fibroblast injections (Fig 2A-B). Significantly, treatment with MDP-stimulated hUCB-MSCs (MDP-MSCs) restored the body-weight of mice with DSS-induced colitis to 90% of that of the control mice without colitis and rescued 100% of the mice from colitis-induced lethality (Fig 2A-B). On day 7, the disease activity index was slightly decreased by treatment with hUCB-MSCs. In contrast, the administration of MDP-MSCs resulted in a significant improvement of the disease activity index (Fig 2C). On day 10, the mice were sacrificed and the length and histopathology of the colon were evaluated. Gross findings revealed a reduction in colon length in mice treated with PBS, however the colon length was moderately restored by treatment with hUCB-MSCs and further improved by treatment with MDP-MSCs (Fig 2D). When NOD2 was down-regulated by siRNA, MDP-MSCs did not improve the loss of body-weight, survival rate, disease activity index, and colon length of mice with experimental colitis (Fig 2A-D).

Upon histological examination, destruction of the entire epithelium, severe submucosal edema, and scattered infiltration of inflammatory cells in the lamina propria and submucosa were observed in the colon of DSS-treated mice (Fig 2E). In hUCB-MSC-treated mice, mucosal destruction and edema in the submucosa were reduced when compared with PBS-treated mice (Fig 2E). Importantly, the administration of MDP-MSCs greatly inhibited the histological damage in the colon and led to a significant decrease in the histological score (Fig 2E). As expected, the administration of NOD2 siRNA-treated hUCB-MSCs neither prevented histological damage nor decreased the histological score (Fig 2E). MDP-MSCs were found to efficiently prevent the enlargement of mesenteric lymph nodes (MLNs) induced by DSS (Fig 2F).

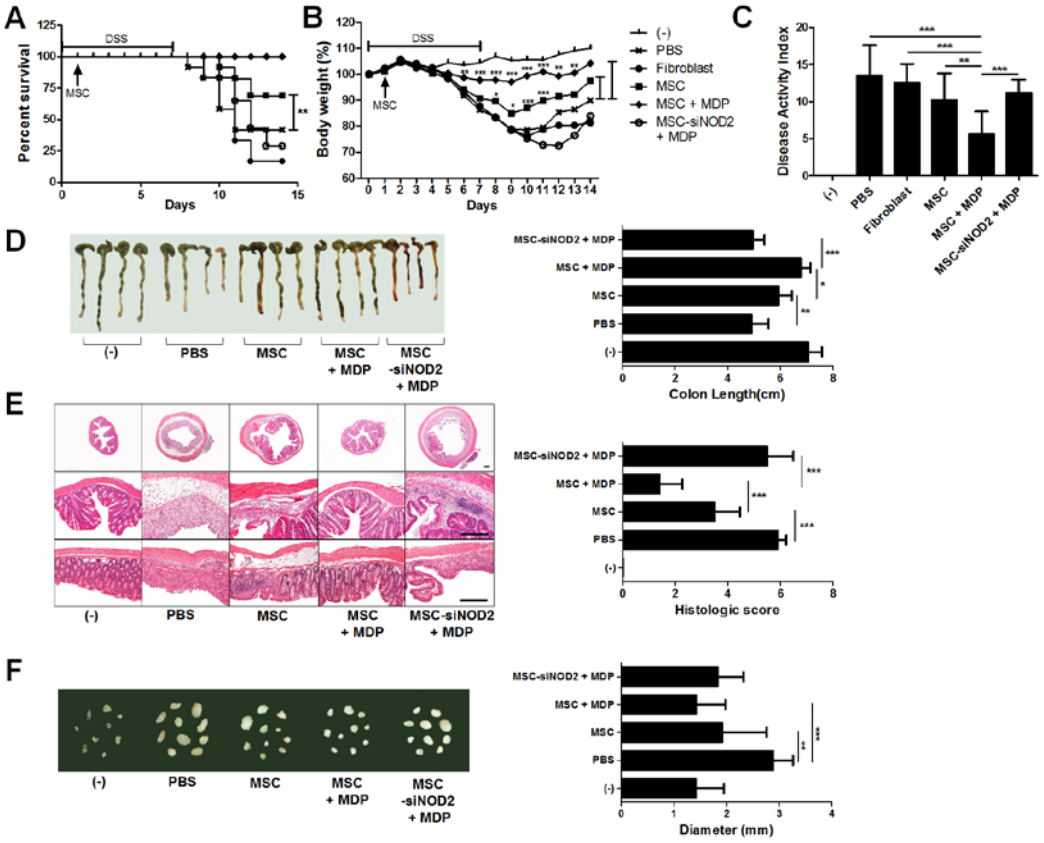

Figure 2. Administration with NOD2-activated hUCB-MSCs enhances the protective effects against DSS-induced colitis in mice.

Figure 2. Administration with NOD2-activated hUCB-MSCs enhances the protective effects against DSS-induced colitis in mice.

(A-C) Clinical progression in DSS-induced colitic mice was monitored. mice#; naive = 10-14, PBS = 12-20, Fibroblast = 6-10, MSC = 12-20, MSC + MDP = 12-20, MSC-siNOD2 + MDP = 12-29 (A) Mantel Cox analysis of survival rate, **(B)** Percentage of body weight loss, **(C)** Disease activity index for colitis severity, **(D-E)** On day 10, animals were sacrificed for further evaluation. **(D)** Colon length measurement. **(E)** Histopathological analysis of colon, Bar = 500 μm, Six mice / group were used. **(F)** Enlargement of mesenteric lymph nodes was evaluated. Five mice / group were used.

* $P<0.05$, ** $P<0.01$, *** $P<0.001$. Results are shown as mean ± SD.

On the other hand, pretreatment of hUCB-MSCs with LPS, a ligand for TLR4, did not decrease lethality or, body-weight loss in colitic mice and did not ameliorate the reduction in colon length or the histological damage as observed with MDP-MSCs (Fig 3A-D). These findings indicate that MDP improves the protective effect of hUCB-MSCs against cellular inflammation in the gut via NOD2-dependent pathway.

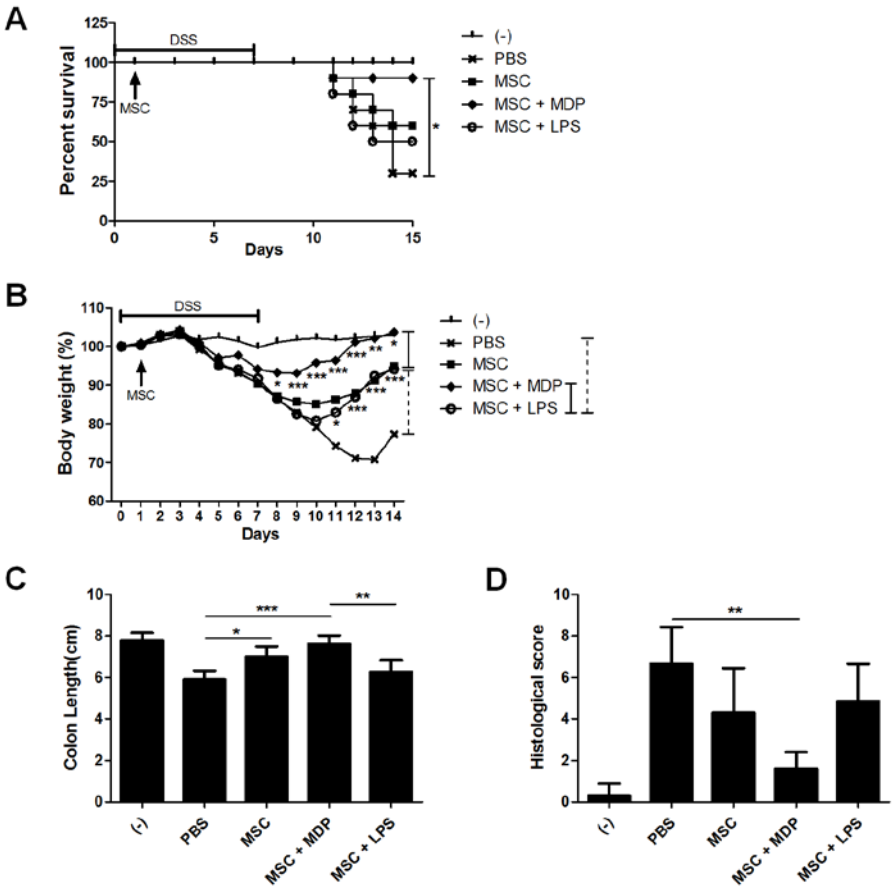

Figure 3. LPS pretreatment did not enhance the protective effects of hUCB-MSCs.

Figure 3. LPS pretreatment did not enhance the protective effects of hUCB-MSCs.

(A–D) Colitis was induced by the addition of 3% DSS in drinking water for 7 days. Mice were injected intraperitoneally with LPS or MDP pretreated hUCB-MSCs (2×10^6 cells) 24 hours after DSS addition. Disease severity was evaluated by gross and histologic analyses. **(A)** Survival rate analysis. **(B)** Body weight loss. Ten mice per group were used. **(C)** Measurement of reduction in colon length. **(D)** Histologic scoring of colon. Four to 5 mice per group were used. *P < .05, **P < .01, ***P < .001. Results are shown as mean ± SD.

2.3.2 MDP enhances the anti-inflammatory activity of hUCB-MSCs in the colon of mice

I next investigated the effect of hUCB-MSCs and MDP-MSCs on the production of pro-inflammatory cytokines associated with DSS-induced colitis. IL-6, IFN-γ, and TNF-α production was markedly increased in the colon tissue of DSS-treated mice, whereas IL-10 production was slightly induced (Fig 4A). Treatment with hUCB-MSCs reduced IL-6, IFN-γ, and TNF-α production in the colon of DSS-treated mice (Fig 4A). MDP stimulation enhanced the ability of hUCB-MSCs to suppress IL-6, IFN-γ, and TNF-α production in the colon of DSS-treated mice, which was abolished by down-regulation of NOD2 with targeting siRNA (Fig 4A). In addition, hUCB-MSCs treatment significantly increased colonic IL-10 production, which was further augmented by MDP stimulation. Similarly, siRNA-induced knockdown of NOD2 reduced the ability of MDP-MSCs to enhance the production of IL-10 in the colon (Fig 4A).

The infiltration of inflammatory cells in the colon of DSS-treated mice was next examined by measuring myeloperoxidase (MPO) activity, which is correlated with the presence of neutrophils. MPO activity and the infiltration of $CD4^+$ and $CD11b^+$ cells were significantly increased in the colon of DSS-treated mice (Fig 4B-D). The administration of hUCB-MSCs reduced the MPO activity and the infiltration of $CD4^+$ and $CD11b^+$ cells in the colon of DSS-treated mice (Fig 4B-D). Similar to the above results, MDP-MSCs further inhibited the MPO activity and the colonic infiltration of $CD4^+$ and $CD11b^+$ cells, a process which was inhibited by transfection with NOD2 siRNA (Fig 4B-D). To

determine whether hUCB-MSCs affect the Treg population in the colon of mice, the colonic infiltration of $CD4^+$ $CD25^+$ $FoxP3^+$ cells was examined by flow cytometry. The administration of hUCB-MSCs led to increased localization of regulatory T cells in the colon (Fig 4E). Moreover, the colonic infiltration of $Foxp3^+$ cells was further increased by MDP-stimulated MSCs, which was suppressed by siRNA transfection targeting NOD2 (Fig 4E). The number of regulatory T cells was confirmed by analysis of $FoxP3^+$ cell infiltration and FoxP3 expression in colonic tissue (Fig 5A-C). These findings indicate that hUCB-MSCs induce anti-inflammatory responses and suppress pro-inflammatory responses in the colon, and these anti-inflammatory activities are enhanced by MDP stimulation in a NOD2-dependent manner.

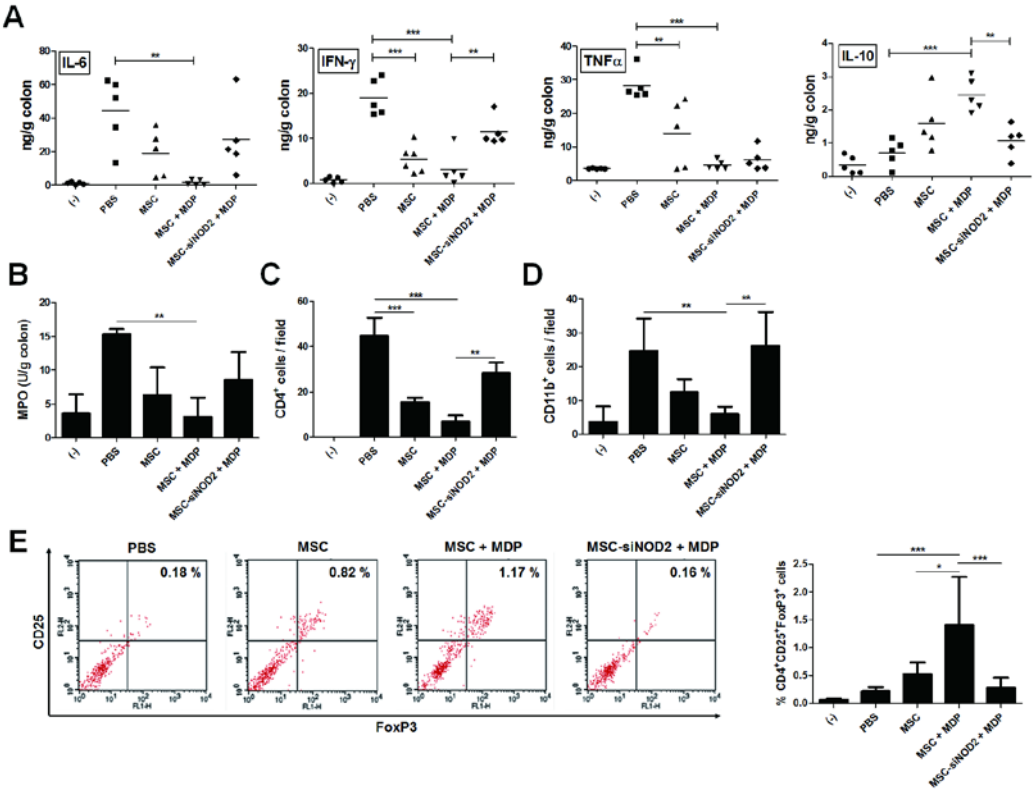

Figure 4. NOD2-activated hUCB-MSCs reduce colonic inflammation in mice.

(A) DSS-induced colitic mice were i.p. injected with hUCB-MSCs and IL-6, IFN-γ, TNF-α and IL-10 levels in colon were determined on day 5 **(B)** Neutrophil infiltration was determined by colonic MPO activity assay. **(C-D)** Inflammatory T lymphocytes and phagocytes infiltration was measured by counting cells per microscopic field on colon sections. **(C)** $CD4^+$ cell counts. **(D)** $CD11b^+$ cell counts. **(E)** Colonic infiltration of $CD4^+CD25^+FoxP3^+$ cells was determined by flow cytometry. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Three to six mice/group were used. Results are shown as mean ± SD.

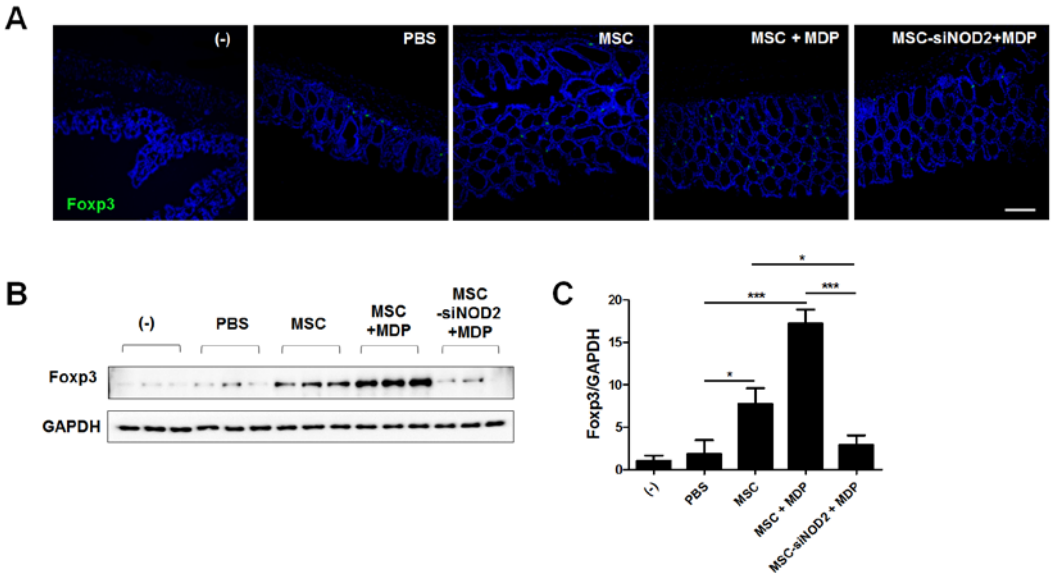

Figure 5. NOD2-activated hUCB-MSCs increased colonic infiltration of Foxp3$^+$ cells. **(A)** Frozen colon sections were immunostained for Foxp3 (Green). Bar, 100 μm. **(B)** Protein levels of Foxp3 in colon segment lysates were detected. **(C)** Quantification of FoxP3 protein levels. *P < .05, ***P < .001. Results are shown as mean ± SD.

Given that the immunosuppressive ability of hUCB-MSCs is significantly increased by NOD2 activation, a cell tracking assay was performed to investigate whether this enhancement is correlated with the migration of hUCB-MSCs to inflammatory sites. To track infused hUCB-MSCs, CFSE-labeled cells were injected into naïve and colitic mice. On days 1 and 3, hUCB-MSCs were detected in the inflamed colon (Fig 6A). However, MDP stimulation did not enhance the trafficking of hUCB-MSCs into the inflamed colon (Fig 6B). To better explore the bio-distribution of hUCB-MSCs, infused CFSE-labeled cells in the inflamed colons were detected by flow cytometry. MDP stimulation did not have any influence on the trafficking of hUCB-MSCs (Fig 6C). Interestingly, hUCB-MSCs were not recruited by the non-inflamed colon compared with the inflamed colons (Fig 6C). The administered cells in the mLNs and spleen of the recipient mice were also detected (Fig 6D).

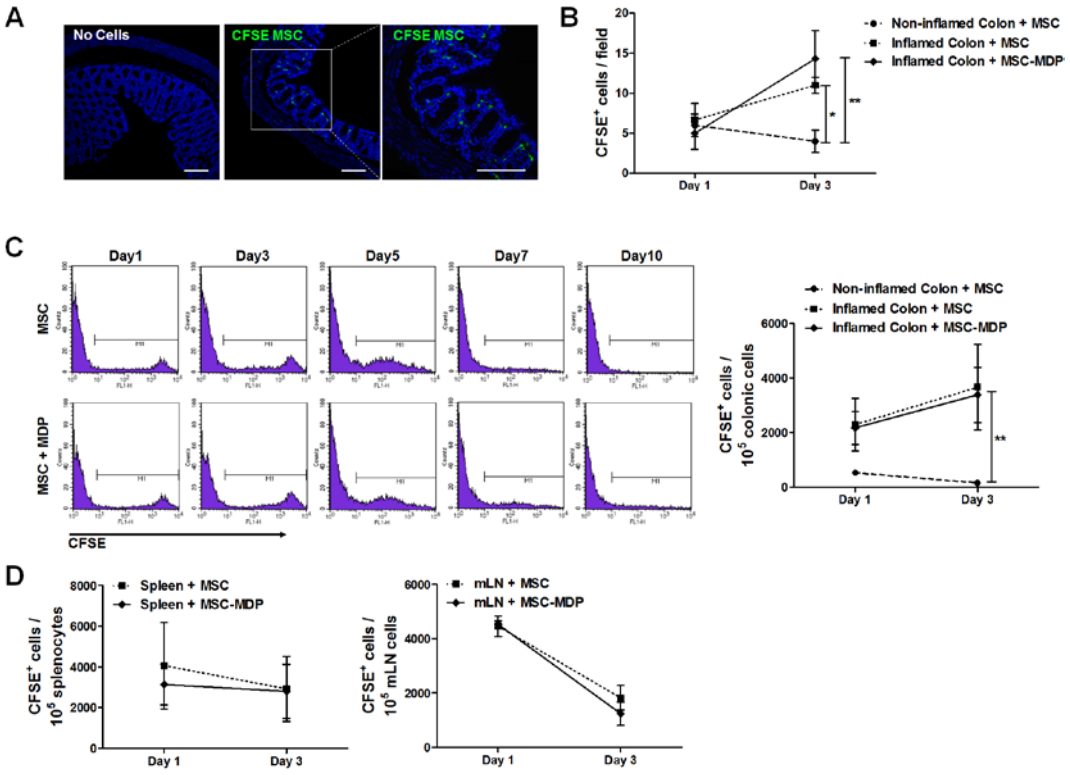

Figure 6. NOD2 activation did not modulate the migratory ability of hUCB-MSCs.

Figure 6. NOD2 activation did not modulate the migratory ability of hUCB-MSCs. **(A and B)** CFSE-labeled hUCB-MSCs were injected intraperitoneally into DSS-induced colitic mice and at 1, 3, and 7 days after injection, the colon sections were examined for green fluorescent cells with a confocal microscope. **(A)** CFSE-labeled cells in frozen colon sections were detected with fluorescent microscopy. Bar, 100 μm. **(B)** The number of green fluorescent cells per microscopic field was counted. Three mice per group were used. **(C and D)** CFSE-labeled cells were injected intraperitoneally into colitic mice. At different time points, colons, MLNs, and spleens were digested for single-cell suspensions and CFSE-positive cells were determined by flow cytometry. **(C)** CFSE-positive cells in single-cell suspensions isolated from inflamed colons were detected on days 1, 3, 5, 7, and 10. The number of CFSE-positive cells in 1×10^5 colonic cells were determined by flow cytometry. **(D)** CFSE-positive cells in single-cell suspensions isolated from spleens and mesenteric lymph nodes were detected on days 1 and 3. Three to 4 mice per group were used. Results are shown as mean ± SD.

2.3.3 NOD2 activation enhanced the protective effect of hUCB-MSCs against TNBS-induced colitis in mice, whereas NOD2 deficiency caused a loss of this effect

I further examined the effect of hUCB-MSCs on the TNBS-induced colitic mice. Infusion of hUCB-MSCs increased the survival rate and decreased the loss of body weight (Fig 7A). MDP-MSCs further improved survival and ameliorated the loss of body weight (Fig 7A). Additionally, shortening of the colon length was significantly prevented by the administration of either hUCB-MSCs or MDP-MSCs (Fig 7B). Histological damage was also ameliorated by the injection of hUCB-MSCs and, was further ameliorated by MDP-MSCs (Fig 7C). These therapeutic effects of MDP-MSCs were abolished when NOD2 was down-regulated (Fig 7A-C).

Moreover, NOD2 deficiency in hUCB-MSCs resulted in a loss of their protective activity against TNBS-induced colitis, as siRNA-induced NOD2 down-regulation in hUCB-MSCs decreased the survival rate and increased the body-weight loss in TNBS-treated mice (Fig 7D). To investigate the generation of immune tolerance in colitic mice by hUCB-MSCs, I assayed whether colitic mice treated initially with hUCB-MSCs or MDP-MSCs could resist a second dose of TNBS without additional treatment with cells. Interestingly, whereas all mice rapidly died after exposure to the second dose of TNBS, the initial inoculation of hUCB-MSCs protected mice from disease recurrence (Fig 7E). Infusions with MDP-MSCs led to the amelioration of body-weight loss and mortality to a greater extent (Fig 7E). These results support the model that NOD2 stimulation plays a crucial role in enhancing the immunomodulatory ability of hUCB-

MSCs, more importantly, these cells cannot maintain their immunomodulatory ability without NOD2.

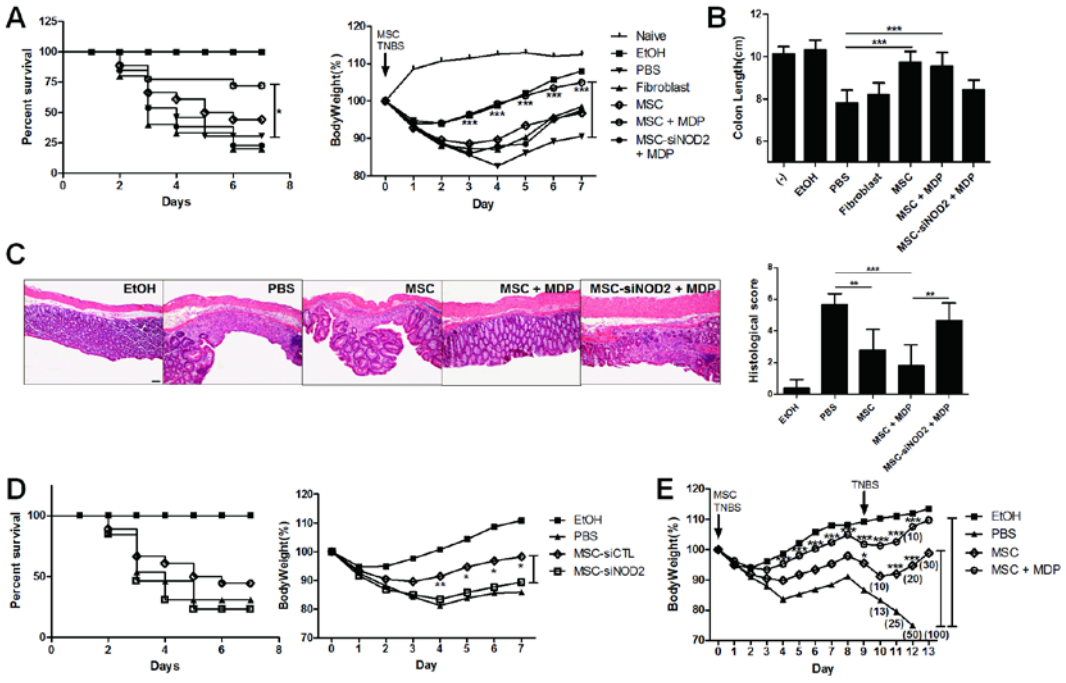

Figure 7. NOD2 is crucial for the protective ability of hUCB-MSCs against TNBS-induced colitis.

Figure 7. NOD2 is crucial for the protective ability of hUCB-MSCs against TNBS-induced colitis.

(A-C) Gross and histological observations in TNBS-induced colitic mice were performed. mice#; naive = 7, EtOH = 8, PBS = 13, Fibroblast = 15, MSC = 18, MSC + MDP = 18, MSC-siNOD2 + MDP = 13. **(A)** Survival rate and body weight loss. **(B)** Measurement of colon length. **(C)** Histopathological evaluation of colon sections, Five mice/group were used, Bar = 500 µm **(D)** NOD2 deficient hUCB-MSCs without MDP stimulation were i.p. injected into colitic mice, Percentage of survival rate and body weight loss were measured, mice#; EtOH = 8, PBS = 13, MSC-siCTL = 18, MSC-siNOD2 = 13. **(E)** Nine days after colitis induction and hUCB-MSCs administration, second dose of TNBS was inoculated, body weight and survival rate were analyzed. Mice #; EtOH = 9, PBS = 8, MSC = 10, MSC + MDP = 10. Numbers in parentheses represent % dead. Results are shown as mean ± SD.

2.3.4 Characterization of hUCB-derived mononuclear cells for mixed lymphocyte reactions

To evaluate whether hUCB-MSCs possess general immunosuppressive property, mononuclear cells from hUCB were isolated and examined for spontaneous and specific antigen-induced T-cell proliferation. hUCB-derived mononuclear cells (hUCB-MNCs) were determined for subpopulation composition including $CD3^+$, $CD4^+$ and $CD8^+$ cells by flow cytometry (Fig 8A). hUCB-MNCs could be efficiently expanded upon stimulation with concanavalin A (ConA), phytohemagglutinin (PHA), anti-CD3 and irradiated MNCs from different UCB as a stimulator (Fig 8B). To verify whether mononuclear cell proliferation is affected by hUCB-MSCs, the UCB-MNCs were co-cultured with the hUCB-MSCs. The proliferation of hUCB-MNCs stimulated with the mitogens was inhibited when they are cultured with hUCB-MSCs (Fig 8C). Moreover, hUCB-MNCs migrated into proximity with the MSC layers (Fig 8D). In addition, hUCB-MNCs co-cultured with hUCB-MSCs expressed lower level of annexin V, implying that the inhibitory effect of hUCB-MSCs on MNC proliferation is not exerted by the induction of apoptosis (Fig 8E). These data suggest that hUBC-MNCs were successfully isolated and characterized and that the hUCB-MSCs exert suppressive effects on MNC proliferation.

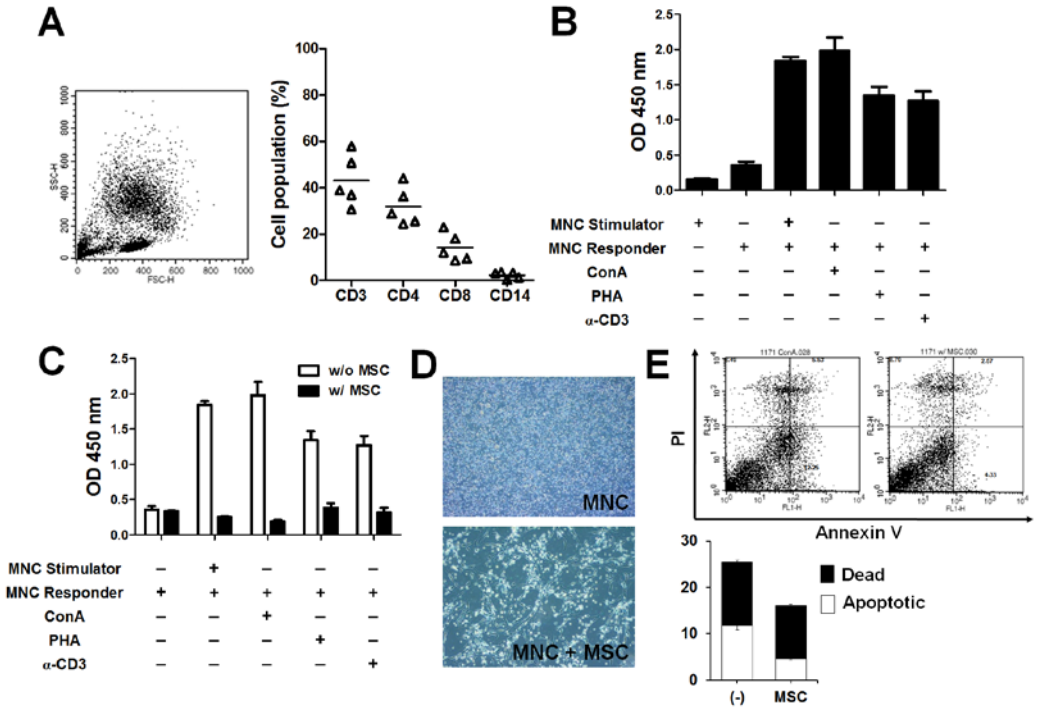

Figure 8. hUCB-MSCs exerted immunosuppressive effects *in vitro*.

(A) Dot plot image for hUCB-MNCs (left panel) MNCs were incubated with FITC-labeled anti-CD3, anti-CD4 and anti-CD8 and analyzed by flow cytometry. **(B)** hUCB-MNCs were stimulated with ConA, PHA, anti-CD3 and irradiated MNCs from different donor as a stimulator and MNC proliferation was determined by BrdU incorporation assay. **(C)** hUCB-MNCs in the absence or presence of MSCs were stimulated and MNCs proliferation was determined. **(D)** Morphological aspects of hUCB-MNCs were observed in the absence (upper panel) and presence (lower panel) of MSCs. **(E)** ConA-stimulated MNCs were cultured with MSCs, and annexin V binding assay was performed using annexin V-FITC apoptosis detection kit after 3 days of culture.

2.3.5 MDP, but not Pam_3CSK_4, LPS, or Tri-DAP, enhanced the inhibitory activity of hUCB-MSCs against the mitogen-induced proliferation of hMNCs and splenocytes

My previous study showed that hUCB-MSCs functionally expressed TLR2, TLR4, NOD1, and NOD2 (Kim et al., 2010). NOD2 expression on protein level was further confirmed by western blotting and immunofluorescent staining (Fig 9).

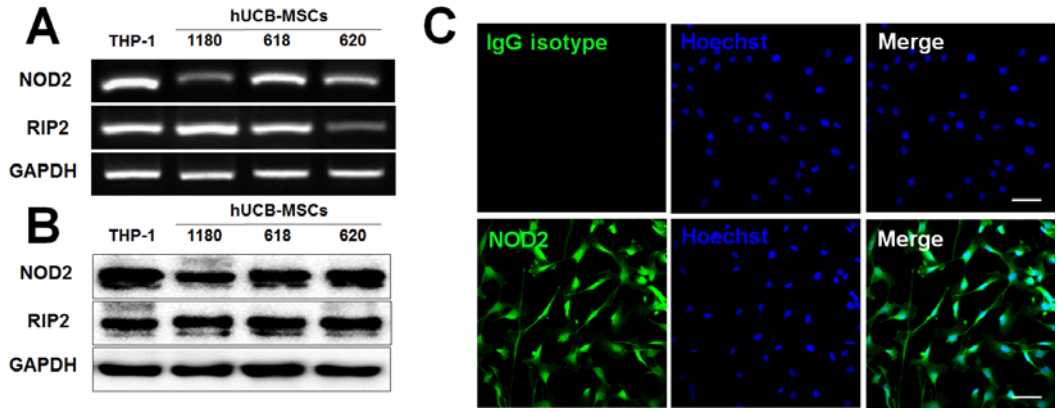

Figure 9. NOD2 expression in hUCB-MSCs. (A-C) mRNA and protein expression of NOD2 and Rip2 in hUCB-MSCs were examined by **(A)** RT-PCR, **(B)** western blotting and **(C)** immunocytochemistry. A human monocytic leukemia cell line, THP-1 cells were used as positive control.

The mixed leukocyte reaction (MLR) assay was performed to explore whether the TLR and NOD1/NOD2 agonists affected the inhibitory effect of hUCB-MSCs on the proliferation of human mononuclear cells (hMNCs). Under conditions of cell-cell contact, hUCB-MSCs markedly inhibited the proliferation of mitogen-induced hMNCs (Fig 10A). However, stimulation with TLR agonists (Pam_3CSK_4 and LPS) and NOD1/NOD2 agonists (Tri-DAP and MDP) did not alter the inhibitory effect of hUCB-MSCs on hMNC proliferation (Fig 10A). Since soluble factors can also mediate the immunosuppressive activity of MSCs(Aggarwal and Pittenger, 2005; Beyth et al., 2005; Di Nicola et al., 2002; Ren et al., 2008), I next examined whether soluble factors produced by hUCB-MSCs could influence hMNCs proliferation. To prepare culture media (CM), hUCB-MSCs were incubated with the indicated TLR and NOD1/NOD2 agonists for 24 h, washed, and incubated with fresh media. After an additional 5 days of incubation, the CM of control and agonist-treated UCB-MSCs (#618) were prepared, and hMNCs were cultured in the presence of the CM. The proliferation of hMNCs was slightly inhibited in the presence of CM from unstimulated hUCB-MSCs (UCM) (Fig 10B). Remarkably, hMNCs proliferation was further suppressed in the presence of CM from hUCB-MSCs stimulated with MDP (MDP-UCM), but not with other agonists (Fig 10B). The same results were obtained with CM from another preparation of hUCB-MSCs (#620) (Fig 10C). In addition, the proliferation of human Jurkat cells and xenogeneic mouse splenocytes was also suppressed in the presence of UCM and this suppression was augmented by MDP-UCM (Fig 107D-E). These findings suggest that soluble factors selectively induced by NOD2 stimulation augment the immunosuppressive property of hUCB-MSCs.

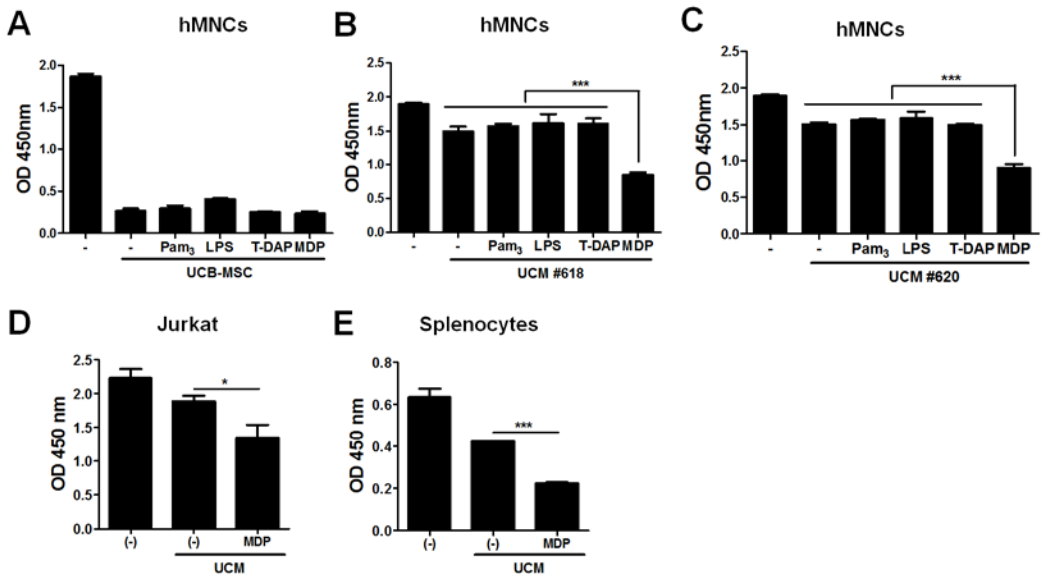

Figure 10. MDP enhances the immunosuppressive effect of hUCB-MSCs.

(A) After treatment with ligands, hUCB-MSCs were co-cultured with hUCB-MNCs and MNC proliferation was determined by the BrdU kit. **(B-C)** hUCB-MNCs were cultured with the culture media of hUCB-MSCs (UCM). hUCB-MNC proliferation was determined by the BrdU kit. UCM#618 and #620; UCM from #618 and #620 hUCB-MSCs **(D)** Human Jurkat cells and **(E)** mouse splenocytes were cultured in the presence of UCM and their proliferation was determined. * $P<0.05$, *** $P<0.001$. Results show one representative experiment out of at least three. Results are shown as mean ± SD.

2.3.6 PGE_2 produced by hUCB-MSCs in response to MDP was responsible for hMNCs suppression

Soluble factors such as indoleamine 2,3 dioxygenase-1 (IDO-1), nitric oxide (NO), and prostaglandin E_2 (PGE_2) are potential candidates that may modulate the immunosuppressive activity of MSCs (Aggarwal and Pittenger, 2005; Beyth et al., 2005; Di Nicola et al., 2002; Ren et al., 2008). ELISA assay was conducted to assess whether TLR and NOD1/NOD2 agonists induce the production of such soluble factors in hUCB-MSCs. Western blot analysis revealed that none of the agonists could induce the expression of IDO-1 in UCB-MSCs (Fig 11A). In addition, although LPS induced NO production in mouse macrophages, none of the agonists enhanced NO production or iNOS expression in hUCB-MSCs (Fig 11B). These findings suggest that IDO-1 and NO are not the factors responsible for the MDP-induced immunosuppression of hUCB-MSCs.

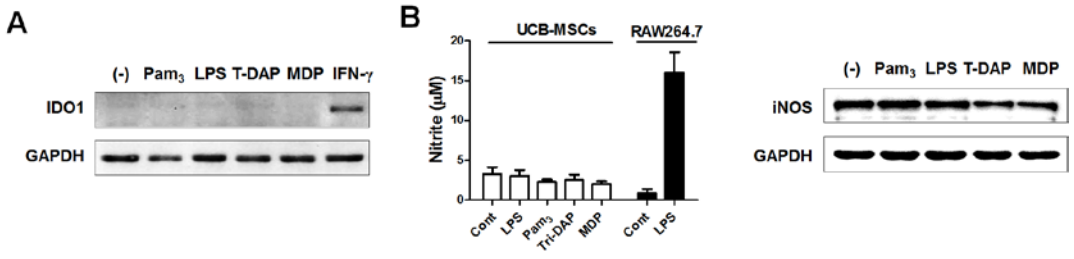

Figure 11. Failure of induction in IDO-1 activation and NO production by Toll-like receptor (TLR) and NOD-like receptor (NLR) ligands stimulation.

(A–C) hUCB-MSCs were treated with Pam3CSK4, LPS, Tri-DAP, and MDP for 24 hours. **(A)** The protein level of IDO-1 was determined using Western blot analysis. **(B)** The supernatant of hUCB-MSCs treated with TLR and NLR ligands were collected and NO production was examined by the Griess reaction. LPS-treated RAW264.7 cells were used as positive control. **(C)** Protein expression level of inducible NO synthase (iNOS) was detected by immunoblotting. Results show 1 representative experiment of 3.

PGE_2 is a key soluble factor through which human umbilical cord-MSCs suppress the proliferation of human monocytes and T cells (Chen et al., 2010; Cutler et al., 2010). I found that LPS induced a slight increase in PGE_2 production in hUCB-MSCs, whereas Pam_3CSK_4 and Tri-DAP did not (Fig 12A). In contrast, MDP induced a robust production of PGE_2 in hUCB-MSCs after 24 h of stimulation (Fig 12A). In addition, incubation with MDP led to an increase in the protein expression of COX-2, a key enzyme in PGE_2 production (Fig 12B). When measured in CM, PGE_2 levels were much higher in MDP-UCM than in UCM (Fig 12C), suggesting that NOD2 activation can lead to the prolonged secretion of PGE_2 in hUCB-MSCs.

To determine the effect of PGE_2 on mitogen-induced monocytes proliferation, ConA-treated hMNCs and mouse splenocytes were cultured in the presence of various doses of PGE_2. The proliferation of hMNCs and mouse splenocytes was significantly inhibited by PGE_2 in a dose-dependent manner (Fig 12D-E). Moreover, the inhibitory effect of MDP-UCM on hMNCs proliferation was abolished by indomethacin, a pan COX inhibitor (Fig 12F). These findings suggest that PGE_2 is a critical factor involved in mediating the immunosuppressive activity of hUCB-MSCs which is enhanced by MDP.

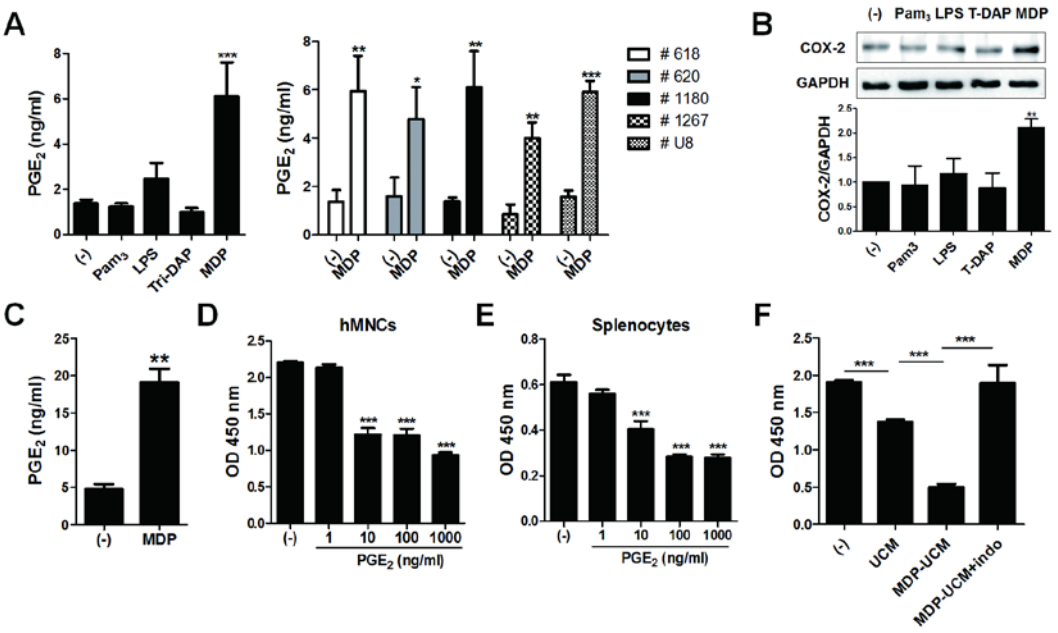

Figure 12. MDP-induced PGE_2 is responsible for anti-inflammatory activity of hUCB-MSCs *in vitro* and *in vivo*.

(A-B) hUCB-MSCs were treated with each indicated ligand. **(A)** PGE_2 concentration was measured from culture supernatant by ELISA. **(B)** Cellular COX-2 expression was determined by Western Blot analysis. **(C)** PGE_2 concentration in UCM was detected. **(D)** hMNCs and **(E)** mouse splenocytes were cultured at the presence of various doses of PGE2 and their proliferation was determined by the BrdU kit. **(F)** hUCB-MSCs were treated with MDP alone or MDP + indomethacin and UCM was collected. hUCB-MNCs were cultured in the presence of each UCM and MNC proliferation was determined. * P<0.05, ** P<0.01, *** P<0.001. Results show one representative experiment out of at least three. Results are shown as mean ± SD.

2.3.7 NOD2 and RIP2 were essential for COX-2 expression and the production of PGE_2 by MDP-stimulated hUCB-MSCs

NOD2 and RIP2 are critical for MDP-induced immune responses (Park et al., 2007a). Therefore, I investigated whether NOD2 and RIP2 were also required for COX-2 expression and the production of PGE_2 by hUCB-MSCs in response to MDP. The protein expression of NOD2 and RIP2 in UCB-MSCs was significantly inhibited by siRNA transfection. Importantly, the down-regulation of NOD2 and RIP2 expression by siRNA inhibited MDP-induced COX-2 expression and the production of PGE_2 in the UCM (Fig 13A-B). Furthermore, down-regulation of NOD2 and RIP2 in hUCB-MSCs suppressed the inhibitory effect of the MDP-UCM on hMNCs proliferation (Fig 13E).

To investigate whether the basal expression and/or activation of NOD2 in hUCB-MSCs play a role in the production of PGE_2, COX-2 expression and subsequent PGE_2 secretion was evaluated after NOD2 down-regulation by siRNA. Interestingly, NOD2 inhibition significantly impaired the basal expression of COX-2 in hUCB-MSCs (Fig.13C). In addition, NOD2 deficiency caused decreased secretion of PGE2 from hUCB-MSCs during both short-term (24 h) and prolonged (5 day) incubation (Fig 13D). Moreover, the immunosuppressive effect of hUCB-MSCs against hMNC proliferation was diminished by the down-regulation of NOD2 (Fig 13F).

Taken together, my results indicate that MDP can increase PGE_2 production in hUCB-MSCs via the NOD2-RIP2 pathway, which enhances the immunosuppressive

properties of hUCB-MSCs. Additionally, the presence of NOD2 is essential for the basal synthesis and secretion of PGE_2.

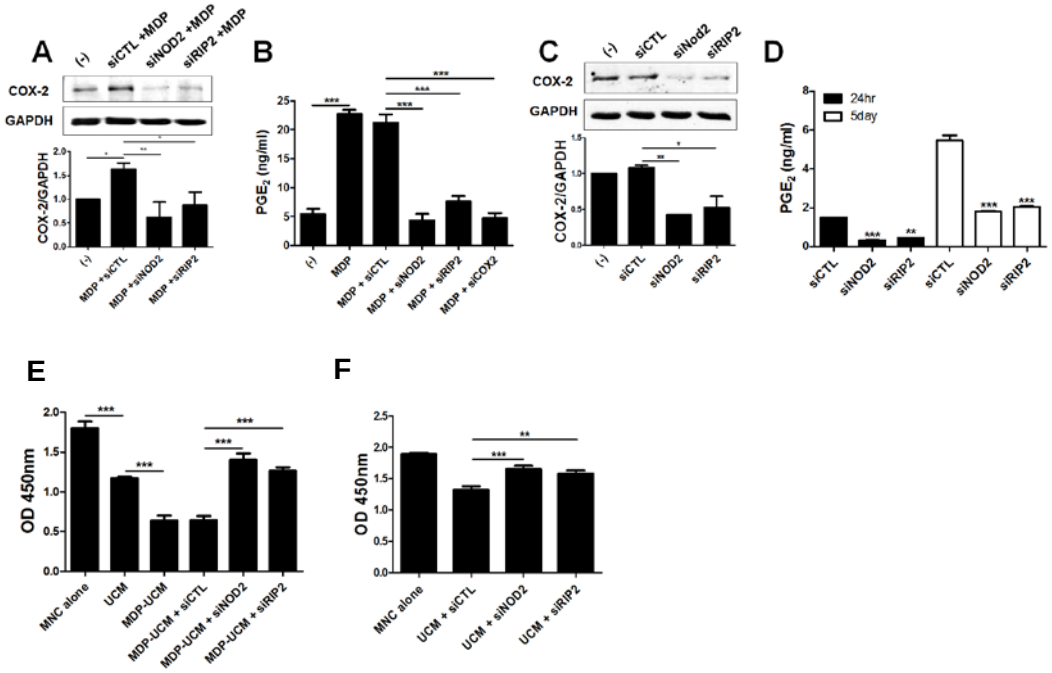

Figure 13. MDP enhances immunosuppressive effect of hUCB-MSCs through NOD2-RIP2 dependent pathway.

Figure 13. MDP enhances immunosuppressive effect of hUCB-MSCs through NOD2-RIP2 dependent pathway.

(A) hUCB-MSCs were transfected with siRNAs, and then were treated with MDP. Protein levels of COX-2 were examined by Western Blot analysis. **(B)** UCM was harvested from cells treated with MDP after siRNA transfection. PGE_2 concentration was measured in UCM by ELISA. **(C)** siRNA transfected hUCB-MSCs without MDP stimulation were determined for COX-2 expression on protein level. **(D)** hUCB-MSCs were treated with siRNA without MDP stimulation, and PGE_2 secretion was detected from culture supernatant using ELISA kit. Additionally, PGE_2 concentration in UCM was measured. **(E)** hUCB-MNCs treated with Concanavalin A (ConA) were cultured for 5 days in the presence of UCM harvested from siRNA transfected hUCB-MSCs. hUCB-MNC proliferation was determined by the bromodeoxyuridine (BrdU) kit. **(F)** MLR using NOD2 and RIP2 siRNA-transfected UCM was performed and hMNC proliferation was determined. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Results show 1 representative experiment of 2 or 3. Results are shown as mean ± SD.

2.3.8 IL-10 production and the regulatory T cell population were increased in hMNCs by UCM pre-stimulated with MDP

A previous study revealed that PGE_2 produced by bone marrow stromal cells is important for IL-10 production by host macrophages (Nemeth et al., 2009). Because MDP stimulation led to PGE_2 production in hUCB-MSCs, I examined whether IL-10 production by hMNCs is increased in the presence of MDP-UCM. hUCB-MSCs alone did not produce IL-10 in the presence or absence of MDP stimulation (data not shown). Although hMNCs produced small amount of IL-10, its production was upregulated in the presence of UCM (Fig 14A). In addition, IL-10 production by hMNCs was further increased by MDP-UCM (Fig 14A). When NOD2 and RIP2 were down-regulated in UCB-MSCs, the ability of MDP-UCM to enhance IL-10 production by hMNCs was suppressed (Fig 14A). In addition, COX-2 down-regulation also inhibited the ability of MDP-UCM to enhance IL-10 production by hMNCs (Fig 14A).

The effect of UCM on the differentiation of hMNCs into regulatory T cells (Treg) was further examined. The Treg population in hMNCs was increased in the presence of UCM and further increased by MDP-UCM (Fig 14B). Similarly, NOD2, RIP2 or COX-2 inhibition suppressed the ability of MDP-UCM to enhance the Treg population (Fig 14B). These findings indicate that MDP-induced PGE_2 in CM is critical to enhance IL-10 production by hMNCs and the induction of hMNC differentiation into Tregs.

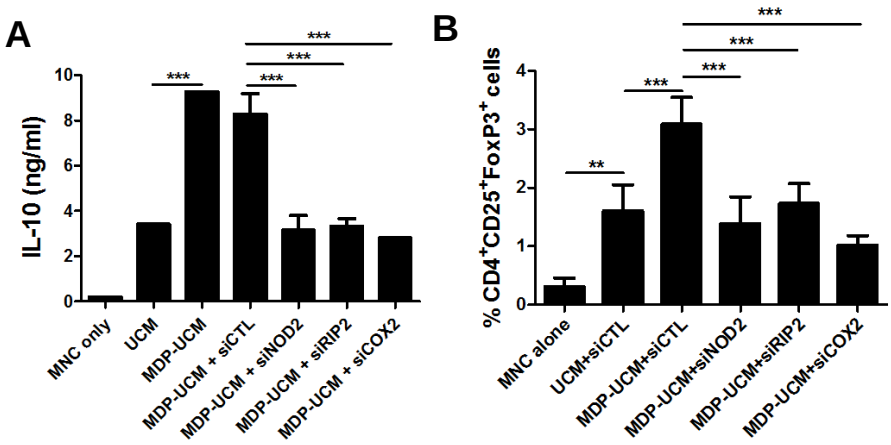

Figure 14. MDP enhances anti-inflammatory effect of hUCB-MSCs via NOD2-RIP2 dependent pathway.

(A) hUCB-MNCs were cultured in UCM and IL-10 production was measured in the culture supernatant. **(B)** hUCB-MNCs cultured in UCM were analyzed for regulatory T cell population by Flow Cytometry. Results are one representative experiment out of two or three or cumulative of three independent experiments. ** P<0.01, *** P<0.001. Results are shown as mean ± SD

2.3.9 MDP-mediated robust PGE_2 production from hUCB-MSCs played a crucial role and subsequent IL-10 induction played a partial role in the protective effects against colitis in vivo

To explore the physiological role of PGE_2 in the immunosuppressive activity of NOD2-activated hUCB-MSCs, COX-2-inhibited cells were administered to colitic mice. Significantly, COX-2 inhibition abolished the ability of MDP-MSCs to suppress lethality and disease activity in DSS-induced colitic mice (Fig 15A). In the same manner, COX-2 inhibition led to a loss of the ability of MDP-MSCs to prevent mortality and body-weight loss associated with TNBS treatment (Fig 15B).

I further examined PGE_2 level in the serum or colon of mice and showed that PGE_2 production is elevated by transplanted MSCs. On day 3 and 5, PGE_2 production in both the serum and colon of DSS-induced colitic mice was significantly elevated by MSCs transplantation and further increased by MDP stimulation (Fig 15C). As expected, the administration of NOD2- or COX-2-inhibited MDP-MSCs did not cause an increase in PGE_2 production compared with PBS-treated group (Fig 15C). Additionally, on day 5, the COX-2 expression intensity of MSCs detected in the mouse colon was higher when stimulated with MDP (Fig 16).

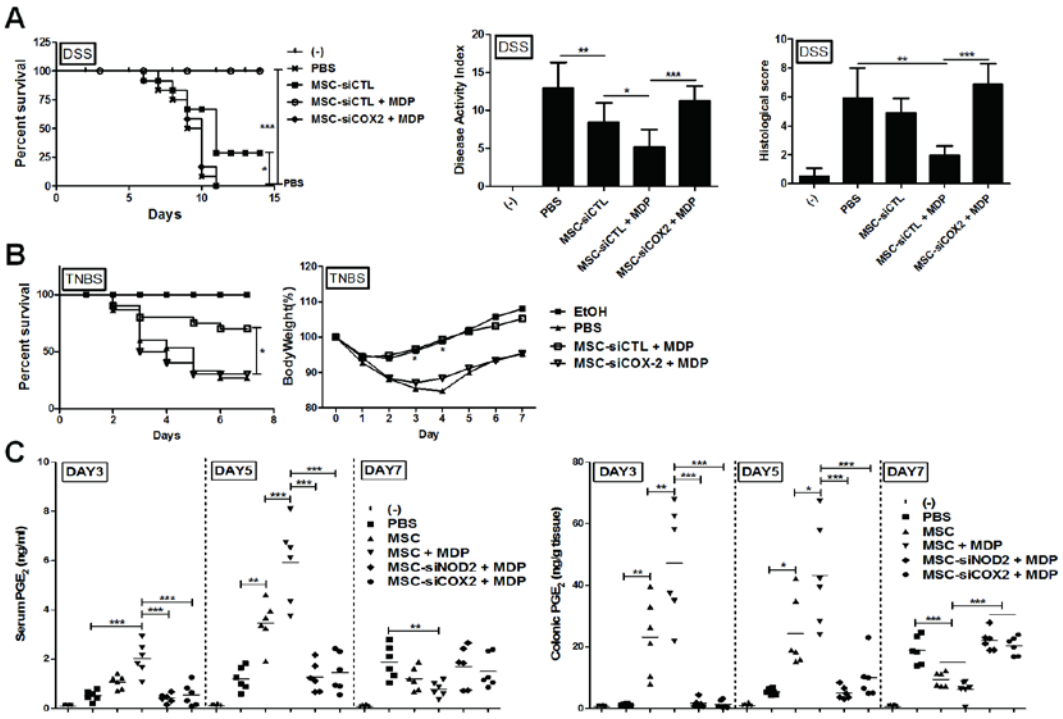

Figure 15. NOD2-mediated PGE_2 production is crucial for the attenuation of colitis.

(A) siRNA for COX-2 was transfected into hUCB-MSCs. Cells were stimulated with MDP and i.p. injected into DSS-induced colitic mice. Survival rate, disease activity index and histopathological score were analyzed. mice#; naive = 10, PBS = 12, MSC-siCTL = 12, MSC-siCOX2 + MDP = 12, MSC-siCTL + MDP = 10, **(B)** COX-2 inhibited hUCB-MSCs were administered into TNBS-induced colitic mice and disease progress was monitored. mice#; EtOH = 8, PBS = 15, MSC-siCTL + MDP = 20, MSC-siCOX2 + MDP = 10 **(C)** PGE2 concentration was measured in both serum and colon of hUCB-MSCs transplanted colitic mice at day 3, 5 and 7. * P<0.05, ** P<0.01, *** P<0.001. Results are shown as mean ± SD

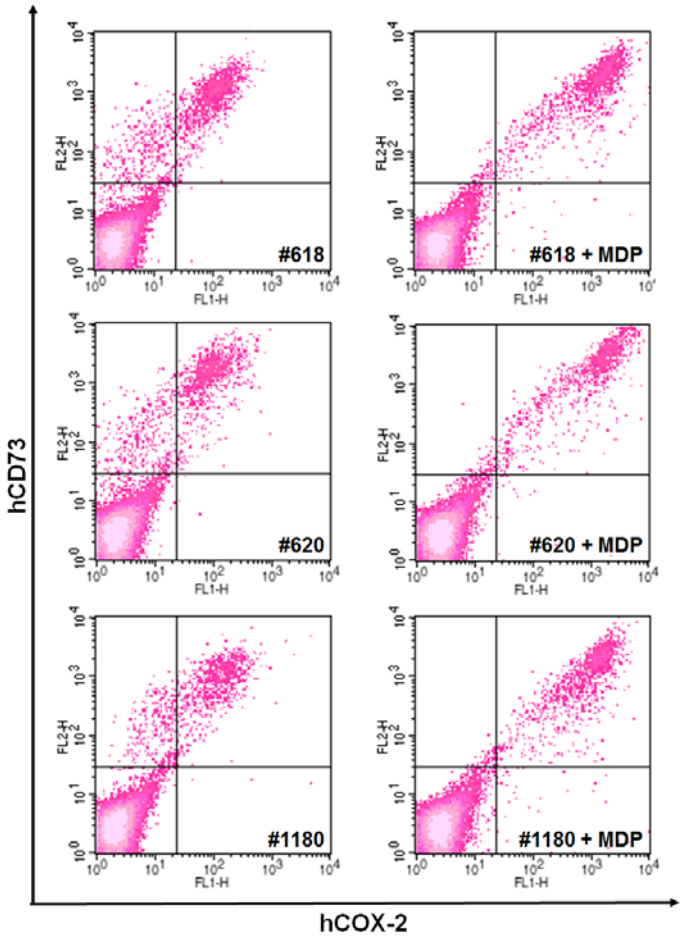

Figure 16. Intracellular COX-2 expression level of hUCB-MSCs detected in mice colon was increased by MDP stimulation.

hUCB-MSCs and MDP-MSCS were injected intraperitoneally into colitic mice. On day 3, colons were digested for single-cell suspensions and incubated with human CD73, permeabilized, and then incubated with human COX-2 antibodies. Intensity of human COX-2 expression among human CD73-positive cells was determined by flow cytometry.

To demonstrate that IL-10 induction by MSCs injection is correlated with PGE_2 production from MSCs, COX-2-down-regulated MDP-MSCs were administered to DSS-induced colitic mice. COX-2 inhibition remarkably decreased the induction of IL-10 production by MDP-MSCs (Fig 17). An IL-10 neutralizing antibody was inoculated daily to investigate whether IL-10 induced by transplantation of MDP-MSCs has any effect on colitis severity. Surprisingly, although IL-10 neutralization impaired the protective effect of MDP-MSCs, it did not abolish the therapeutic effect completely (Fig 18). These results indicate that IL-10 plays a partial role in the attenuation of colitis. Taken together, my findings confirm the physiological evidence regarding the significance of PGE_2 production by transplanted MSCs and the subsequent IL-10 induction in recipient mice during colitis progression.

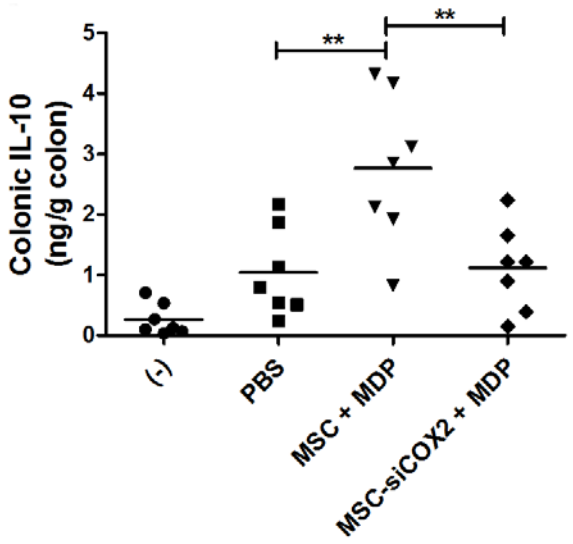

Figure 17. COX-2 inhibition decreased colonic IL-10 level by MDP-MSC transplantation.

DSS-induced colitic mice were injected intraperitoneally with MDP-MSCs or COX-2–inhibited MDP-MSCs and mouse IL-10 levels in colon were determined on day 5 by enzyme-linked immunosorbent assay. **P <0.01.

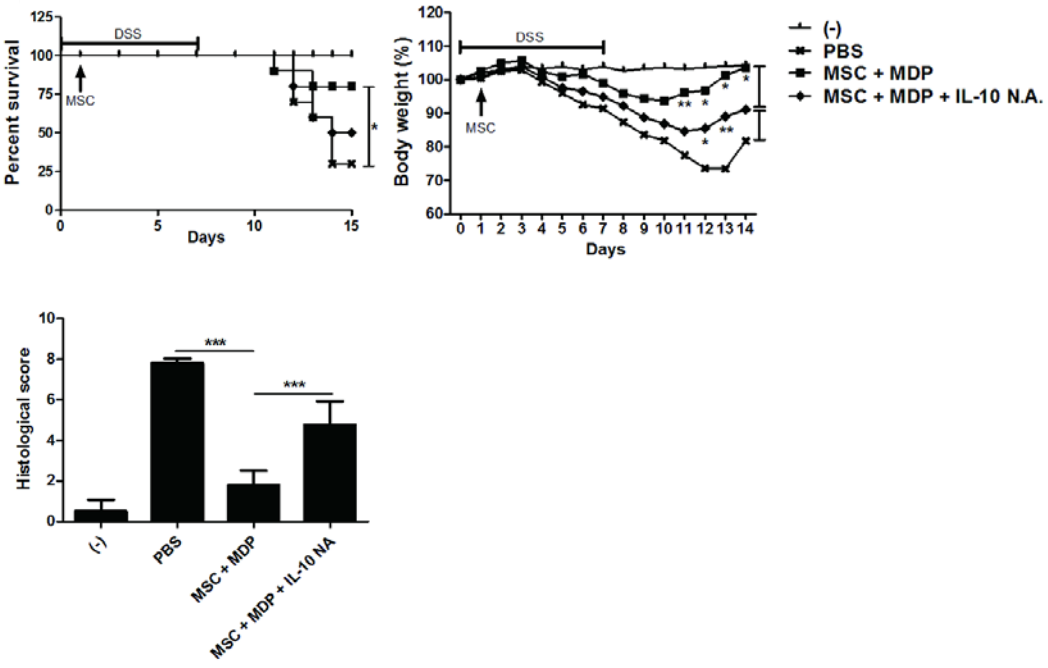

Figure 18. PGE_2-mediated IL-10 production contributes to the attenuation of colitis.

IL-10 neutralizing antibody was i.p. injected daily through day 1 to day 5 into hUCB-MSCs-administered colitic mice. Survival rate, body-weight loss and histological score were evaluated. mice#; naive = 10, PBS = 10, MSC + MDP = 10, MSC + MDP + IL-10 neutralizing antibody (N.A.) = 10. * P<0.05, ** P<0.01, *** P<0.001. Results are shown as mean ± SD

2.4 DISCUSSION

Recently, a previous study showed that NOD1 and NOD2 are functionally expressed in UCB-MSCs and regulate their differentiation (Kim et al., 2010). Because several NOD2 genetic variants are associated with susceptibility to Crohn's disease, I sought to determine the role of NOD2 in the protective effect of hUCB-MSCs against experimental colitis and the mechanism underlying the immunosuppressive property of UCB-MSCs.

The therapeutic effect of MSCs on experimental colitis is characterized by improvement of the survival rate and reduction of disease activity (Gonzalez et al., 2009; Zhang et al., 2009b). In this study, the systemic application of hUCB-MSCs improved these parameters, which is in accordance with the effect of different types of MSCs on experimental colitis (Gonzalez et al., 2009; Zhang et al., 2009b). The main finding of this study is that NOD2 stimulation selectively enhances the protective effect of hUCB-MSCs against experimental colitis. MDP-stimulated hUCB-MSCs abrogated the weight loss and histological severity and protected mice from the lethality associated with DSS- or TNBS-induced colitis. These findings led us to examine whether NOD2 activation affects the inhibitory effect of hUCB-MSCs on mitogen-induced monocytes proliferation. Previous studies showed that cell-cell contact is partly required for the immunosuppressive activity of MSCs under *in vitro* conditions (Ren et al., 2008). In this study, the proliferation of hMNCs was significantly inhibited by hUCB-MSCs under cell-cell contact. Under these conditions, the stimulation of hUCB-MSCs with TLR and NOD1/NOD2 agonists did not affect the inhibitory effect of hUCB-MSCs on the

proliferation of hMNCs. Therefore, the effect of soluble factors on the inhibition of cell proliferation was assessed. The proliferation of hMNCs was inhibited by approximately 20% in the presence of the CM of hUCB-MSCs. Remarkably, stimulation with MDP, but not other agonists, enhanced the inhibitory effect of CM on the proliferation of hMNCs and xenogenic mouse splenocytes. These findings indicate that soluble factors secreted by hUCB-MSCs in response to MDP may play a critical role in mediating immunosuppression.

It is well known that soluble factors mediate T cell suppression by MSC (Aggarwal and Pittenger, 2005; Beyth et al., 2005; Di Nicola et al., 2002; Ren et al., 2008). NO was found to mediate the immunosuppressive properties of MSCs (Ren et al., 2008). I investigated whether TLR and NOD1/NOD2 agonists induce NO production in hUCB-MSCs. These results revealed that none of the agonists elicited NO production, suggesting that NO may not be a crucial factor for the immunosuppressive effect of hUCB-MSCs.

The activity of IDO, an enzyme participating in the conversion of tryptophan into kynurenine, plays a critical role in the suppression of MNCs proliferation by MSCs (Stagg, 2007). Opitz *et al*. reported that TLR activation enhances the immunosuppressive activity of BM-MSCs by inducing IDO-1 (Opitz et al., 2009). In the present study, although IFN- γ up-regulated the protein expression of IDO-1 in hUCB-MSCs, the same effect was not observed with any of the agonists. These findings suggest that the induction of IDO-1 expression by TLR and NOD1/NOD2 agonists may depend on the

source of MSCs and, at least in hUCB-MSCs, IDO-1 has no role in the enhancement of hUCB-MSCs immunosuppressive activity by MDP.

PGE_2 is a soluble factor that mediates most of the immunosuppressive effects that AD-MSCs and BM-MSCs exert on dendritic cell maturation and activated T cell proliferation (Yañez et al., 2010). Moreover, a recent study by Chen *et al*. revealed that PGE_2 is critical for the immunosuppressive activity of human umbilical cord-derived MSCs (UC-MSCs) (Chen et al., 2010). This study demonstrated that the inhibition of PGE_2 synthesis almost completely inhibited the immunosuppressive effects of UC-MSCs (Chen et al., 2010). Additionally, stimulation with MDP but not other agonists led to PGE_2 production and COX-2 protein expression in hUCB-MSCs. I show here that PGE_2 induced by MDP is a crucial soluble factor responsible for the immunosuppressive properties of hUCB-MSCs, which are mediated by the NOD2-RIP2 signaling pathway. More interestingly, I found that the expression of NOD2 in hUCB-MSCs is indispensable for the production of PGE_2.

LPS and TNF-α induce PGE_2 production in bone marrow stromal cells, which reprograms macrophages to increase their IL-10 production (Nemeth et al., 2009). In the present study, even though MDP did not directly induce IL-10 production by hUCB-MSCs, the administration of hUCB-MSCs induced IL-10 production in colitic mice, and this production was further increased by MDP stimulation via the activation of NOD2 signaling to COX-2. I further provided evidences that IL-10 induced by MDP-MSCs infusion presents partial rescue in the protective effect against colitis, suggesting the existence of IL-10-independent mechanisms. PGE_2 has been shown to directly inhibit the

activation and expansion of T cells through the regulation of IL-2 production and IL-2 responsiveness (Kolenko et al., 1999; Walker et al., 1983). Moreover, PGE_2 has been reported to regulate the balance between different types of T-helper (Th) cell-mediated inflammation (Snijdewint et al., 1993). The main finding of this study is that PGE_2 shifts the balance from Th1 responses to Th2 responses. Besides, it is well known that activated Th1 cells are crucial for the progression of both Crohn's disease and experimental colitis (Bouma and Strober, 2003). With these findings, one can envision that MSCs-derived PGE_2 might exert protective effect against colitis by directly suppressing T cells or attenuating Th1 responses independently of IL-10. In addition to its direct inhibitory effects on T cells, PGE_2 is known to promote the development of Tregs (Baratelli et al., 2005). And MSCs treatment increased Treg population with suppressive function on T cell-mediated inflammation in vitro and in vivo (Gonzalez et al., 2009). My study also reveals that activation of NOD2 enhances the induction of Tregs by hUCB-MSCs in a PGE_2-dependent fashion. Taken together, these results indicate that NOD2 activation induces PGE_2 production by hUCB-MSCs, which leads to an increase in IL-10 production and Treg population, and concerted action of PGE_2 with subsequent suppressive factors are required for complete attenuation of colitis by hUCB-MSCs (Fig 15). My results suggest that the use of hUCB-MSCs can be a new therapeutic alternative as a cell-based therapy of inflammatory bowel disease.

GENERAL CONCLUSION

Pattern recognition receptors (PRRs) are known to trigger an innate immune response against microbial infection. MSCs were found to express TLRs. Although recent studies showed that activation of TLRs modulate the MSC functions including proliferation, differentiation, migration, and immunomodulation, little is known about the role of NLRs on the MSC function. Therefore, the first study suggested that NLRs deserve more attention in the studies regarding stem cell function-related complications. In the first study, I investigated whether NOD1 and NOD2 regulate the functions of hUCB-MSCs. In hUCB-MSCs, TLR2, TLR4, NOD1 and NOD2 were functionally expressed. However, none of TLR and NLR ligands influenced the proliferation of hUCB-MSCs. On differentiation, TLR and NLR ligands could promote both osteogenesis and chondrogenesis of hUCB-MSCs. More interestingly, only NOD1 and NOD2 activation slightly inhibited the adipogenic differentiation of hUCB-MSCs. My findings suggest that TLRs and NLRs differently modulate the hUCB-MSC differentiations (Fig 1).

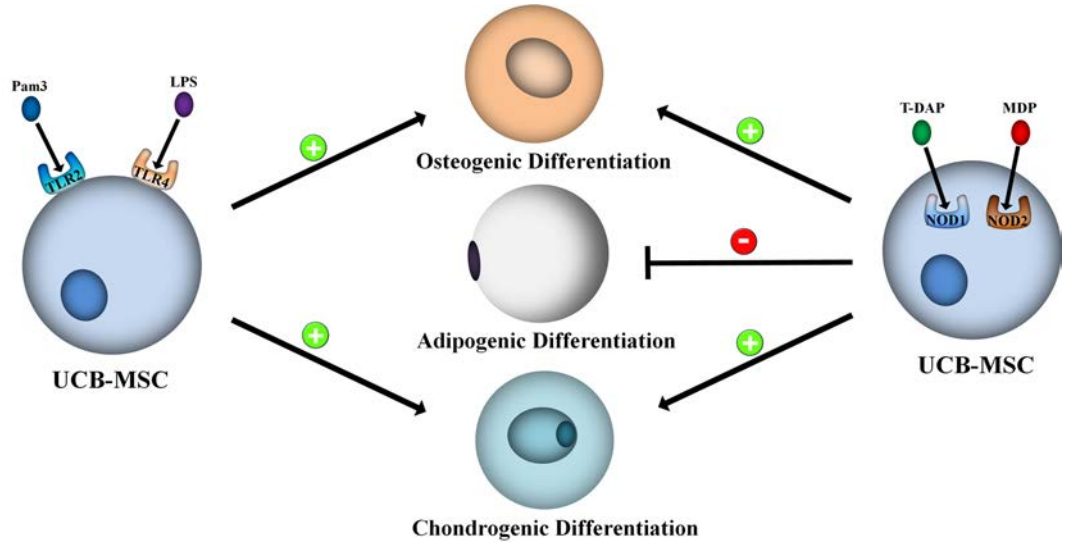

Figure 1. Schematic diagram showing the role of PRRs in differentiation of hUCB-MSCs. TLR2, TLR4. NOD1 and NOD2 are functionally expressed in hUCB-MSCs and involved in the regulation of MSC differentiation into cell types of mesodermal lineage.

Nucleotide-binding oligomerization domain 2 (NOD2) is known to play the role in intestinal inflammation and homeostasis. And genetic variants of NOD2 have been reported to be associated with the development of Crohn's disease. Although studies suggest that NOD2 on host intestine modulate the inflammation, little is known about the role of NOD2 on the transplanted cell function. In the second study, I investigated the roles of NOD2 in hUCB-MSCs and underlying mechanisms for the regulation of immune responses in inflammatory bowel diseases using experimental mouse colitis model. In the first study, I showed that NOD2 is functionally expressed in hUCB-MSCs. Interestingly, in my unpublished data, I found that the expression of NOD2 in human bone marrow MSCs was undetectable on mRNA level and were weak in protein level. Therefore, I sought to investigate the role of NOD2 in the immune modulation of hUCB-MSCs. Here, I showed the evidence that NOD2 activation by MDP, the bacterial peptidoglycan derivative, augmented the protective effect of hUCB-MSCs against both DSS- and TNBS- induced mouse colitis. More interestingly, the present study revealed novel information that MDP can enhance the immunosuppressive ability of hUCB-MSCs by increasing PGE_2 secretion through a NOD2-RIP2-dependent pathway and subsequently, elevated PGE_2 induces well-known anti-inflammatory cytokine, IL-10 production form mononuclear cells and regulatory T cell differentiation (Fig 2).

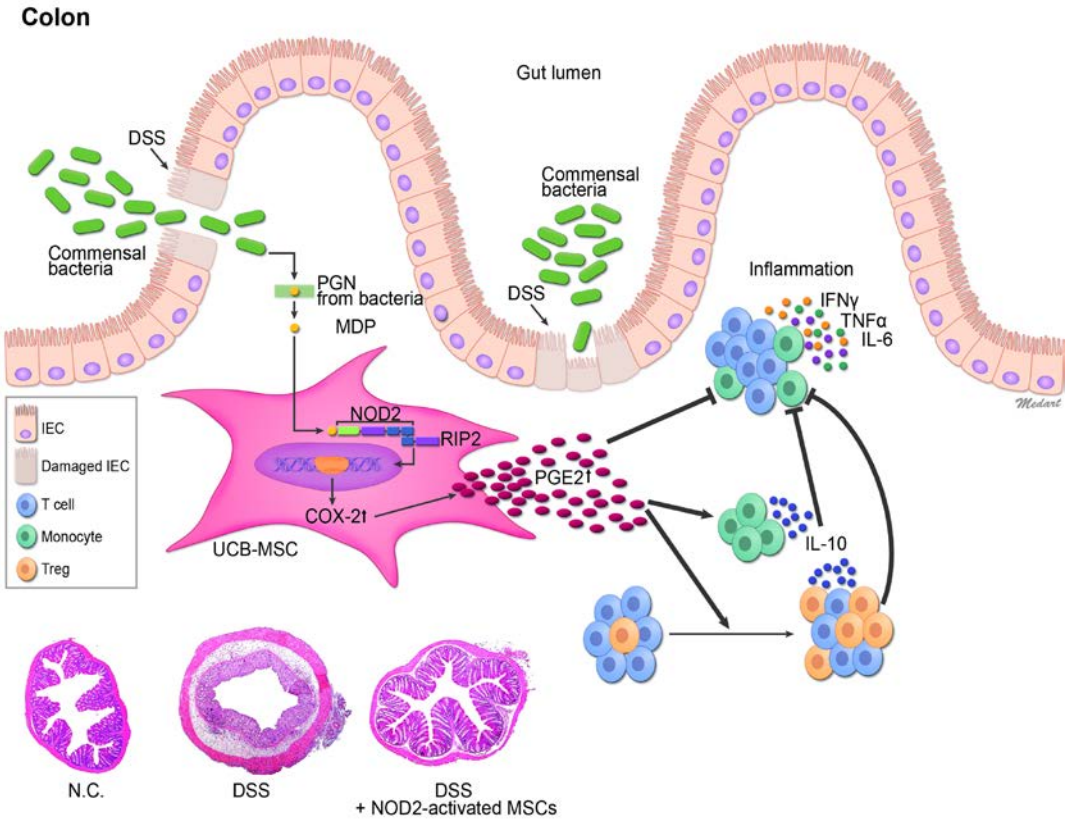

Figure 2. Schematic diagram of proposed mechanism for NOD2-mediated immunomodulatory ability of hUCB-MSCs in colitis.

Muramyl dipeptide (MDP), a small molecule derived from the peptidoglycan of bacterial cell wall, invades into submucosa through disrupted epithelium and activates NOD2-RIP2 signaling pathway in migrated hUCB-MSCs to produce PGE_2 through the up-regulation of COX-2. PGE_2 production leads to an increase in IL-10 production and Treg differentiation and concerted actions of PGE_2 with its subsequent suppressive factors reduce the inflammation in colon. IEC, intestinal epithelial cell

These findings could provide novel insights into both fields of stem cell transplantation and NOD2 function in IBD. In several studies regarding stem cell transplantation, no study so far has been able to show that bacterial components affect the therapeutic efficacy of MSCs. However, in this study, the therapeutic efficacy of hUCB-MSCs against experimental colitis was significantly improved by NOD2 activation with MPD, a PGN derivative of bacterial cell wall. This result supports the possibility that MSCs could be activated upon sensing the components of commensal bacteria and that MSC-microbiota interaction might be required for sufficient activation of MSC immunoregulatory function. More importantly, I have proven that NOD2 deficiency results in the down-regulation of PGE_2 production and loss of immunosuppressive effect, implying that PRR in MSCs could have ligand-independent functions. This finding further suggests that determination of single nucleotide polymorphisms in NOD2 should be performed before the administration of MSCs, since NOD2 mutation might lead to loss of therapeutic function. Further studies with PRR mutation or deficiency in MSCs could reveal additional mechanisms regarding the interaction between microbiome and MSC, and the consequence of PRR mutation in MSC functions.

My findings also stress a key point for the controversial role of NOD2 mutation associated with Crohn's disease; whether this mutation might be a loss of function or gain of function, according to the studies reported so far. NOD2 is the first gene, firmly identified as Crohn's disease-associated gene (Hugot et al., 2001; Ogura et al., 2001). More than 60 genetic variants in NOD2 have been reported, among them, three major variants, G908R, R702W, and L1007insC, are known to be intimately associated with

Crohn's disease. Particularly, the susceptibility to Crohn's disease is increased to approximately 30 times when individuals carry homozygous frameshift mutation, L1007insC (Seiderer et al., 2006). It is generally accepted that polymorphism-mediated NOD2 dysfunction is a predisposing factor for increased susceptibility to Crohn's disease, since NOD2 plays crucial role for intestinal homeostasis by linking innate immune responses with the adaptive immune tolerance to commensal microflora. However, mechanisms by which NOD2 defect contributes to the development of Crohn's disease have been controversial. Several hypotheses were proposed for variant NOD2 proteins and they can be divided into those supporting that NOD2 mutation leads to defect in normal function (loss of function), and those advocating that variant NOD2 proteins result in activation of inflammatory responses (gain of function). The first hypothesis conjectures that NOD2 contributes to the epithelial defense against enteric bacteria by regulating the secretion of α-defensins from Paneth cells, intestinal secretary cells. And alterations in defense mechanism by mutant NOD2 lead to the change in the composition of the microflora and overgrowth of pathogenic bacteria, suggesting that Crohn's disease-associated NOD2 mutations might result from a functional loss (Ahmad et al., 2002; Hisamatsu et al., 2003; Kobayashi et al., 2005; Wehkamp et al., 2004). The second hypothesis suggests that NOD2 negatively regulates TLR signaling pathway and that mutant from of NOD2 is responsible for dysregulation of TLR signaling and subsequent over-production of IL-12, an important cytokine for type I helper T cell-mediated pathogenesis of Crohn's disease, implying that NOD2 mutant exerts a loss of its regulatory function (Bouma and Strober, 2003; Mannon et al., 2004; Watanabe et al.,

2004). In the third hypothesis, NOD2 variants are proposed to trigger IL-1β processing, through the abnormal activation of IL-1β converting enzyme, thereby identifying the NOD2 mutation as a gain of function (Hogquist et al., 1991; Maeda et al., 2005; McAlindon et al., 1998; Siegmund et al., 2001; Yoo et al., 2002). In addition to IL-1β triggering, another hypothesis advocates that Crohn's disease-associated mutant NOD2 protein might activate its adaptor protein, RIP2, more strongly than normal protein (Hollenbach et al., 2005). All of these hypotheses are non-mutually exclusive and may be effective in combination. In this study, NOD2 inhibition in hUCB-MSCs resulted in the down-regulation of PGE_2 production, followed by complete loss of immunomodulatory effect. Although siRNA-mediated inhibition of NOD2 is not equivalent to Crohn's disease-associated NOD2 polymorphisms, my finding suggests that NOD2 variants might lead to a loss of function phenotype, thereby proposing a new hypothesis for mutant NOD2 function. The intestinal stem cells are supported by their niche that consists of mesenchymal and epithelial cells (Medema and Vermeulen, 2011). Theses mesenchymal cells as an endogenous intestinal niche are proposed to contribute to intestinal stem cell homeostasis (Farin et al., 2012). Since PGE_2 is known to play crucial role in intestinal homeostasis and inflammation (Berg et al., 2002; Chinen et al., 2011), one can envision that intestinal MSCs might contribute to gut homeostasis via PGE_2 regulation in response to bacterial components such as MDP and that NOD2 defects in MSCs could be one of predisposing factors for the development of Crohn's disease. However, an identification of the mesenchymal niche is yet incomplete. Furthermore, a recent study reports that unidentified mesenchymal cells migrate to sites of intestinal inflammation and contribute

to the restoration of homeostasis through the secretion of certain factors (Powell et al., 2011). In this respect, it is apparent that future work will require the precise identification of intestine-residing mesenchymal cells and their functions in intestinal homeostasis and inflammation.

I anticipate that these findings could provide a better understanding of the immune-related characteristics of MSCs and provide a basis for development of highly efficient cell therapy to treat several intractable diseases.

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국문 초록

마우스 결장염 모델에서 중간엽 줄기세포의 면역 조절능에 대한 노드2 수용체의 역할

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중간엽 줄기세포는 분열을 통해 자신과 동일한 세포를 만들어낼 수 있는 자가재생능과 다른 종류의 세포로 바뀔 수 있는 분화능을 가지고 있을

뿐만 아니라, 최근 들어 중간엽 줄기세포는 여러 가지 면역세포의 증식, 성숙 및 활성화를 억제할 수 있는 면역조절능 역시 가지고 있는 것으로 알려져 있다. 이러한 줄기세포의 면역조절능이 보고된 이후로 자가면역질환, 이식거부반응 등의 다양한 면역관련 질환의 치료에 줄기세포를 치료제로서 사용하기 위해 많은 연구 및 임상시험이 이루어지고 있다.

톨 (Toll) 유사 수용체 및 노드 (NOD) 유사 수용체는 선천 면역 수용체로서 미생물에 의한 감염에 대항하여 초기 면역 반응을 촉진한다. 톨 유사 수용체가 중간엽 줄기세포의 기능을 조절할 수 있다는 연구 보고가 있지만 노드 유사 수용체가 세포 기능에 미치는 영향에 대한 연구는 거의 보고된 바가 없다. 본 연구에서는 사람 제대혈 유래 중간엽 줄기세포의 노드 1 또는 노드 2 수용체 발현여부를 조사하고, 줄기세포가 발현하는 수용체가 세포 기능에 어떠한 영향을 미치는지 확인해 보았다. 사람 제대혈 유래 중간엽 줄기세포는 톨 유사 수용체 2 와 톨 유사 수용체 4 뿐만아니라 노드 1 및 노드 2 수용체를 발현하였으며 각 수용체에 해당하는 리간드로 수용체를 자극하였을 때 인터루킨-8 이 생성됨을 확인하였다. 어떤 리간드도 제대혈 유래 중간엽 줄기세포의 자가재생능에는 영향을 주지 않았다. 반면에 모든 리간드가 줄기 세포의 골분화를 향상시켰으며 이는 ERK 의 인산화 유도를 통해 일어나는 현상임을 확인하였다. 노드 1 및 노드 2 수용체를 활성화 시키면 제대혈 유래 중간엽 줄기세포의 지방분화가 미약하게 억제되었으나,

톨 유사 수용체의 활성화는 아무런 영향을 주지 않았다. 연골분화에 있어서는 모든 수용체의 활성화가 분화를 촉진하였다.

노드 2 의 결핍 또는 변이는 크론병과 관련이 있으며 또한 장내 염증을 조절하는 것으로 알려져 있다. 첫 번째 연구를 통해 사람 제대혈 유래 중간엽 줄기세포가 노드 2 를 발현하며 노드 2 가 세포 분화능에 영향을 미친다는 것을 확인하였다. 본 연구에서는 줄기세포의 노드 2 가 면역조절능에서도 역할을 하는지 확인하고자 하였으며 이를 위해 마우스 크론병 유사 모델을 이용하였다. 마우스에 화학물질을 이용하여 궤양성 결장염을 유발한 뒤 정상 줄기세포와 노드 2 를 해당 리간드인 muramyl dipeptide (MDP)로 활성화시킨 줄기세포를 복강투여 하였다. 줄기세포의 주입은 결장염의 증상을 완화시켰으며 노드 2 활성화는 이 효과를 증진시켰다. 또한 결장 내의 염증성 싸이토카인의 분비를 완화시켰으며 항염증성 싸이토카인의 분비 및 억제성 T 림프구의 생성을 증가시켰다. 노드 2 를 활성화시킨 줄기 세포는 효율적으로 단핵세포의 비특이적 증식을 억제하였으며 이러한 효과는 MDP 에 반응하여 줄기세포가 분비하는 프로스타글란딘 E_2 에 의한 것임을 확인하였다. 노드 2 활성화 줄기세포 유래의 프로스타글란딘 E_2 는 인터루킨-10 과 억제성 T 림프구의 생성을 증가시켰으며 마우스의 결장염 증상을 완화시켰다.

이 결과는 (1) 톨 유사 수용체뿐만 아니라 노드 유사 수용체가 사람 제대혈 유래 줄기세포에 기능적으로 발현되며 세포의 분화에 영향을 미친다는 것과 (2) 특히, 줄기세포의 면역조절능을 이용하여 마우스 결장염 모델을 치료하기 위해 노드 2 수용체가 필요하며 해당 수용체의 활성화는 프로스타글란딘 E_2 의 분비를 통한 면역조절능의 증진을 유도할 수 있음을 의미한다.

주요어 : 중간엽줄기세포, 선천면역수용체, 노드 2, 분화, 면역조절, 크론병

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