A Dissertation for the Degree of Doctor of Philosophy

Anti-inflammatory Mechanisms of Human Umbilical Cord Blood-derived Mesenchymal Stem Cells on Atopic Dermatitis and Rheumatoid Arthritis

아토피 피부염과 류마티스 관절염에 대한 인간 제대혈 유래 중간엽 줄기세포의 항염증 기전

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ABSTRACT

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Atopic dermatitis (AD) and rheumatoid arthritis (RA) are representative intractable immune-related diseases characterized by complex allergic inflammatory responses and autoimmunity, respectively. Recently, extensive investigations of the pathogenic immune mechanisms in AD and RA have fueled the development of new biologic therapies that

block specific cytokine networks or cellular pathways. Nonetheless, these agents have been reported to have limited efficacy as well as uncertainty in long term safety. Thus, there is a clear need to develop a novel treatment with efficacy and safety. Cell therapies utilizing mesenchymal stem cells (MSCs) have emerged as a promising alternative to replace current therapeutic approaches due to their unique immunomodulatory properties. However, the underlying mechanisms of MSCs on specific disease pathogenesis-related immune cells and the relevant efficacies are not fully elucidated. Therefore, the aims of this study are to investigate the therapeutic potency of hUCB-MSCs on AD and RA, and to provide the better understanding of anti-inflammatory mechanisms of MSCs on disease pathogenesis-related immune cells in allergic inflammatory responses and autoimmunity.

AD is a chronic and relapsing allergic skin disorder characterized by eczematous skin lesions and severe pruritus. Because AD is difficult to cure completely and its recurrence is frequent, no satisfactory therapeutics is currently available. Although few studies have shown the potential use of MSCs for the attenuation of AD, the precise mechanisms of interaction between MSCs and AD pathogenesis-related immune cells are barely verified. In the first study, I investigated the therapeutic efficacy of human umbilical cord blood-derived MSCs (hUCB-MSCs) against murine atopic dermatitis and explored distinct mechanisms that regulate their efficacy. AD was induced in mice by the topical application of *Dermatophagoides farinae*. Naïve or activated-hUCB-MSCs were administered to mice, and the severity of clinical symptoms was determined. The subcutaneous administration of nucleotide-binding oligomerization domain 2 (NOD2)-activated-hUCB-MSCs exhibited prominent protective effects against AD, and suppressed

the infiltration and degranulation of mast cell (MC). A β-hexosaminidase assay was performed to evaluate the effect of hUCB-MSCs on MC degranulation. NOD2-activated MSCs reduced the MC degranulation via NOD2-cyclooxygenase 2 (COX2) signaling. In contrast to bone marrow-derived MSCs (BM-MSCs), hUCB-MSCs exerted a cell-to-cell contact-independent suppressive effect on MC degranulation through the higher production of prostaglandin E_2 (PGE_2). Additionally, transforming growth factor (TGF)-β1 production from hUCB-MSCs in response to IL-4 contributed to the attenuation of MC degranulation by down-regulating FcεRI expression in MCs. In conclusion, the subcutaneous application of NOD2-activated hUCB-MSCs can efficiently ameliorate AD, and MSC-derived PGE_2 and TGF-β1 are required for the inhibition of MC degranulation.

RA is one of the most common chronic autoimmune diseases accompanied by progressive synovitis leading to joint impairment as well as systemic complications. Recent studies have reported that monocytic lineage cells and their derived cytokines play pivotal roles in the pathogenesis of RA. Although several studies have shown the therapeutic potency of MSCs for the attenuation of RA, the underlying mechanism of MSCs on monocyte/macrophage has not been clearly investigated. I thus examined the therapeutic efficacy of hUCB-MSCs against murine collagen-induced arthritis (CIA) and explored integrated mechanisms mainly focused on macrophage. The systemic administration of hUCB-MSCs significantly ameliorated the severity of CIA to a similar extent in anti-TNF-α-treated group. hUCB-MSCs exerted this therapeutic effect against CIA through the regulation of macrophage functions. To verify the regulatory effects, human and murine macrophages were co-cultured with hUCB-MSCs. Activation of COX-2 and tumor

necrosis factor-stimulated gene (TSG)-6 in hUCB-MSCs by TNF-α stimuli polarized naïve macrophage toward M2 type. In addition, hUCB-MSCs down-regulated nucleotide binding domain and leucine-rich repeat pyrin 3 (NLRP3) inflammasome activation via paracrine loop of IL-1β signaling. These immune-balancing effects of hUCB-MSCs were reproducible in co-culture experiments using PBMCs isolated from patients with active RA. These data suggest that can exert simultaneous regulation of multiple cytokine pathways in response to elevated inflammatory cytokines in RA-related immune microenvironment, implying hUCB-MSCs can be an attractive candidate for refractory RA patient.

Taken together, these findings imply that (i) locally infused hUCB-MSCs exert therapeutic effect against AD by suppressing the MC degranulation and NOD2 activation in MSCs can enhance the immunosuppressive effect by increasing the release of PGE_2, and that (ii) systemically delivered hUCB-MSCs can significantly ameliorate RA and they act as a cellular modulator of macrophages through the simultaneous regulation on the production of TNF-α and IL-1β by macrophages in response to disease-related inflammatory milieu. Consequently, this study suggests the possibility that hUCB-MSCs can be a promising strategy for the therapy of allergic disorders and autoimmune diseases.

Keywords: Mesenchymal stem cells, Atopic dermatitis, Mast cell, Rheumatoid arthritis, Macrophage, NLRP3 inflammasome

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LIST OF ABBREVIATIONS

1-MT	1-methyl-tryptophan
AD	Atopic dermatitis
ATP	Adenosine triphosphate
β-hex	Beta-hexosaminidase
BM-MSC	Bone marrow-derived mesenchymal stem cells
Cas-1	Caspase-1
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CFSE	5,6-Carboxy fluorescein succinimidyl ester
CIA	Collagen-induced arthritis
CII	Bovine type II collagen
COX-2	Cyclooxygenase-2
CM	Conditioned medium
DAPI	4',6-Diamidino-2-phenylindole
DC	Dendritic cell
Df	*Dermatophagoides farina*
ELISA	Enzyme-linked immunosorbent assay
FB	Human dermal fibroblast

FcεRI	High affinity IgE receptor
HLA-DR	Human leukocyte antigen-antigen D related
hMSC	Human mesenchymal stem cell
hUCB-MSC	Human umbilical cord blood-derived MSC
IDO-1	Indoleamine-2,3-dioxygenase-1
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
iPSC	Induced pluripotent stem cell
i.v.	Intravenous
L-NAME	*N*-Nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
MC	Mast cell
MDP	Muramyl dipeptide
NLR	Nod-like receptor
NLRP3	Nucleotide binding domain and leucine-rich repeat pyrin 3
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain 2

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PDS	Prednisolone
PGE_2	Prostaglandin E_2
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RIP2	Receptor-interacting protein 2
s.c.	Subcutaneous
STAT6	Signal transducer and activator of transcription 6
siRNA	Small interfering RNA
TGF-β	Transforming growth factor beta
Th	Helper T lymphocyte
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TSG-6	Tumor necrosis factor-stimulated gene/protein 6

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LITERATURE REVIEW

Current Management and Treatment of Atopic Dermatitis

Atopic dermatitis (AD), a typical examination of type I hypersensitivity reaction, is an allergic itchy skin disease accompanied by epidermal barrier dysfunction, recurrent eczematous skin lesions and intense pruritus (Leung and Bieber, 2003). AD has inflicted a considerable socioeconomic burden on the quality of life for patients and their relatives, affecting 3-10% of adults and approximately 20% of children all over the world (Odhiambo et al., 2009; Silverberg and Hanifin, 2013). A number of immunologic abnormalities including an imbalance towards a Th2 response, elevated levels of serum IgE and blood eosinophils, and impaired cell-mediated immunity have generally known to be responsible for the allergic responses in AD (Wallach and Taieb, 2014). Namely, elevated IgE-mediated mast cell degranulation in response to environmental allergens leads to the infiltration of lymphocytes and eosinophils into the cutaneous lesion (Schneider et al., 2013). Although Th2 cells are considered to play an essential role the exacerbation of AD, it has been recently reported that other subsets of helper T cells such as Th1, Th17 and Th22 might be involved in the pathogenesis of AD (Guttman-Yassky et al., 2011; Suarez-Farinas et al., 2013). Furthermore, many patients with AD might develop other symptoms of atopy such as food allergy, allergic rhinitis and asthma. Because of the complicated inflammatory pathogenesis, AD cannot be cured completely at present. Thus, the aims of current clinical

management of AD are simply to improve clinical symptoms and to achieve long-term disease control with multiple approaches (Fig 1), which include restoration of epidermal barrier function with emollients, avoidance of risk factors and application of topical corticosteroids or calcineurin inhibitors for anti-inflammatory therapy. Anti-histamines can be also helpful to improve the itching, especially at night time (Weidinger and Novak, 2016). However, these approaches induce only temporary improvement and have a greater risk of severe side effects with long-term application due to non-specific suppression of immune responses (Ring et al., 2012). More recently, new biologic agents that specifically block target molecules have been developed and applied to treat AD. Particularly, dupilumab, a monoclonal antibody directed against the alpha subunit of IL-4 receptor that blocks both IL-4 and IL-13, represented impressive efficacy in patients with moderate to severe AD (Montes-Torres et al., 2015). Despite the beneficial effect of the biologic drugs, they have been reported to have considerable limitations associated with the safety and efficacy (Borrebaeck and Carlsson, 2001; Chames et al., 2009).

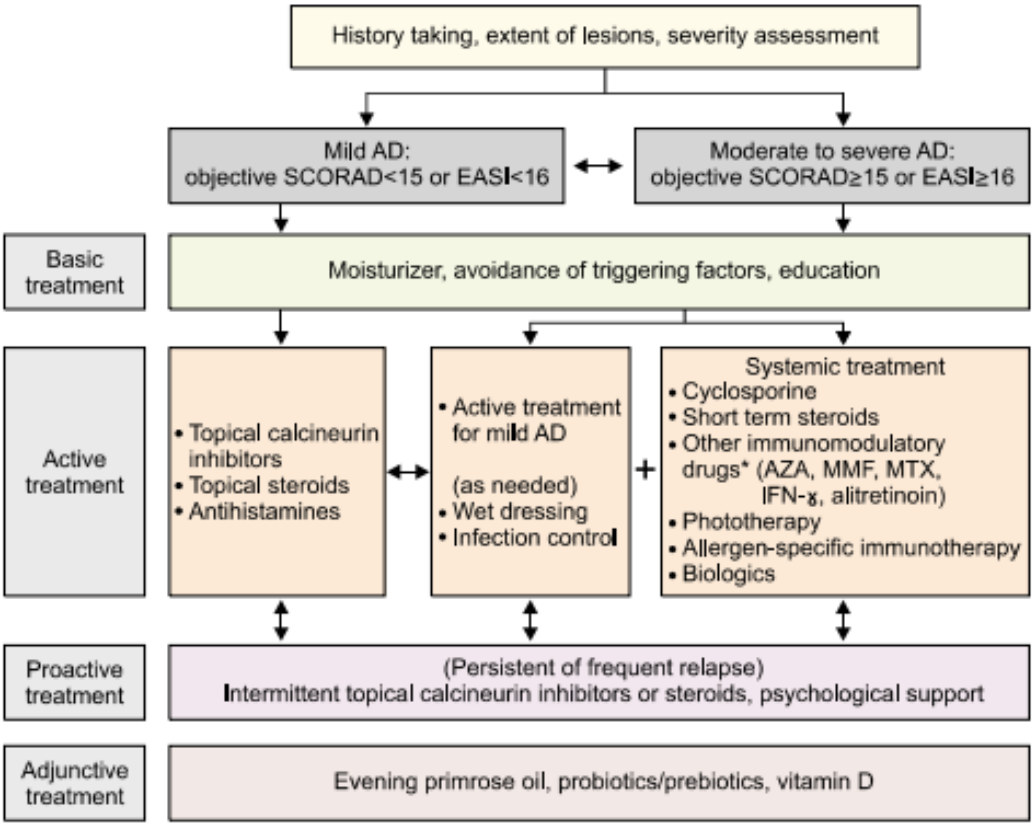


Figure 1. Treatment algorithm for atopic dermatitis

SCORAD = SCORing atopic dermatitis; EASI = eczema area and severity index; AZA = azathioprine; MMF = mycophenolate mofetil; MTX = methotrexate

(Kim et al., 2015b)

Current Therapeutic Approaches for Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common and chronic progressive autoimmune disease that has substantial personal, social and economic costs. It affects primarily on the joint tissues leading to joint destruction as well as disability, but frequently accompanied by extra-articular manifestations and systemic comorbidities such as pulmonary fibrosis, renal damage and heart injury. It thus should be regarded as a systemic syndrome rather than a single disease (McInnes and Schett, 2011). The etiology of RA is unknown, and the prognosis is guarded. The pathogenesis of RA is characterized by the impaired immune system including auto-reactive T cells and auto-antibody producing B cells generates undesirable inflammatory responses. Early diagnosis is important to determine optimal therapeutic approach and success, especially in patients with high disease activities, presence of auto-antibodies and early joint damage (Smolen et al., 2016). Therefore, the primary goal of RA treatment is to maximize long-term quality of life through control of symptoms and prevention of disease exacerbation. Because inflammation is the apex of clinical features and symptoms, attenuation of excessive inflammation is the most important way to achieve these goals (Huizinga and Knevel, 2015). Namely, current treatment approaches for RA include non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, disease-modifying anti-rheumatic drugs (DMARDs) and biologic response modifiers (Koenders and van den Berg, 2015). Previously, treatment for RA was typically stared with NSAIDs and glucocorticoids to relive pain and reduce inflammation, but they offered only temporary improvement or were often associated with serious long-

term side effects due to non-specific suppression of host immune responses. Currently, patients with newly diagnosed RA are treated with more aggressive strategies including DMARDs such as methotrexate (MTX), leflunomide or sulfasalazine to reduce painful symptoms and slow the progression of the disease. Furthermore, advances in understanding of the autoimmune inflammatory mechanisms in RA have fueled the development of new biologic therapeutics that specifically block target molecules, with beneficial outcome. Biologicals, also referred as biological DMARDs, are reserved for patients who do not adequately respond to conventional DMARDs. Currently approved biologicals for RA include anti-TNF-α (infliximab, etanercept, adalimumab and golimumab), anti-IL-6 (tocilizumab), anti-IL-1 (anakinra), anti-B-cell (rituximab) and anti-T-cell co-stimulation (abatacept) (Table 1). Among them, the first and most widely used biologicals is the group of TNF-α inhibitors. Biological DMARDs monotherapy particularly targeting to TNF-α or combination therapy with conventional DMARDs and steroids is the most widely used at present. However, despite the success of these biologicals, many open issues remain.

Table 1. DMARDs and recommendation doses for RA treatment

	Molecule type	Usual dose*	Loading dose	Comments
Conventional synthetic DMARDs				
Methotrexate	Small chemical	25 mg once weekly*	No	Starting dose 10 mg—escalation to 25 mg within 4–8 weeks; folate use important (suggest 10 mg/week or 1 mg/day)
Sulfasalazine	Small chemical	3 g/day*	No	Starting dose 1 g, escalation to 3 g/day within 4–8 weeks
Leflunomide	Small chemical	20 mg/day	Optional	Loading dose associated with more gastrointestinal side-effects
Hydroxychloroquine	Small chemical	400 mg/day	No	For mild arthritis or as combination therapy
Biological DMARDs				
TNF inhibitors				
Adalimumab	Human monoclonal antibody	40 mg every 2 weeks subcutaneously	No	Biosimilars expected
Certolizumab pegol	F(ab') fragment of a humanised monoclonal antibody	200 mg every 2 weeks subcutaneously	Yes	
Etanercept	IgG–Fc-receptor construct (fusion protein)	50 mg/week subcutaneously	No	Biosimilar approved
Golimumab	Human monoclonal antibody	50 mg/month subcutaneously	No	
Infliximab	Chimeric monoclonal antibody	3–10 mg/kg intravenously every 4–8 weeks	Yes	Biosimilars approved
Anti-B-cell				
Rituximab	Chimeric monoclonal antibody	1000 mg intravenously every 6 months	No	Biosimilars expected
Anti-T-cell co-stimulation				
Abatacept	IgG–Fc-receptor construct (fusion protein)	125 mg/week subcutaneously	No	Intravenous dosing available
Anti-IL 6R				
Tocilizumab	Humanised monoclonal antibody	162·6 mg/week subcutaneously		Intravenous dosing available; sarilumab (anti-IL6R [Regeneron, Tarrytown, NY, USA]) and anti-IL6 cytokine antibodies (sirukumab [Janssen, Springhouse, PA, USA]) in development
Targeted synthetic DMARDs				
Janus kinase inhibitors				
Tofacitinib	Small chemical	5 mg twice daily	No	JAK1/2/3 inhibitor; once daily medication in development; baricitinib (Eli Lilly, Indianapolis, IN, USA), a JAK1/2 inhibitor, has completed phase 3 trials

IL6R=interleukin 6 receptor. IL6=interleukin 6. DMARD=disease-modifying antirheumatic drug. TNF=tumour necrosis factor. *Contraindicated or dose reductions needed with renal or hepatic impairment; for adverse events see package inserts.

(Smolen et al., 2016)

Limitations of Current Medications

As described earlier, immunosuppressants such as corticosteroids and NSAIDs have been commonly used for relieving pain and reducing excessive inflammation, but they have a greater risk of long-term side effects involved in gastrointestinal tract and other body systems (Ring et al., 2012). Targeted biologic therapies thus have made major inroads into the treatment of a wide range of inflammatory diseases including AD and RA. However, despite dramatic advances in the development of therapeutic options for AD and RA, there have been still considerable limitations to be overcome. The central obstacles in biological therapies include their limited and non-uniform efficacy and serious adverse effects. Although dupilumab have been reported to be significantly effective against moderate to severe AD, a few clinicians and researchers have found undesirable clinical responses or undulating outcomes (Chames et al., 2009). In case of RA, while promising remissions by TNF-α inhibitors, including infliximab, etanercept and adalimumab, have been reported in the treatment of RA, large numbers of patients still fail to achieve a sufficient clinical response or cannot tolerate these agents. In addition, these medications may carry a potential risk of side effects including serious opportunistic infections or malignancies, and the use of biologicals in pregnancy is still inconclusive (Raja et al., 2012; Verstappen et al., 2011). Therefore, the safety and efficacy of these drugs should be further investigated in long-term follow-up studies. Furthermore, there is a clear unmet need for developing a novel therapeutic strategy with safety and efficacy. To ensure this end, stem cell therapy can be a promising substitute to replace current medications.

Benefits of Stem Cell Therapy

Stem cell therapies have been expected to bring new hope to patients suffering from a wide range of injuries and disorders. To date, many clinical investigations using various types of stem cells such as pluripotent stem cells, neural stem cells and MSCs in regenerative medicine have been conducted in a large number of pathologic conditions and diseases (Trounson and McDonald, 2015). Among them, MSCs can be the most anticipated candidates for stem cell therapy because they have lots of benefits compared to other types of stem cells for various reasons (Fig 2). Briefly, MSCs are easily isolated from adult and fetal tissues and are successfully expanded *in vitro* (Bianco et al., 2008). They also avoid the ethical concern that surrounds embryonic stem cells (ESCs) and the adverse complications such as teratoma formation by induced pluripotent stem cells (iPSCs). In addition, immuno-privileged features that include rare or no expression of MHC class I, very limited expression of MHC class II and lack of co-stimulatory molecules of the B7 family allow them to be advantageous for allogeneic transplantation without potential risks for immunological rejection. Therefore, these outstanding properties make MSCs to become one of the most attractive option for treating autoimmune and immune-related diseases. Up to now, there are nearly 500 registered clinical trials in different phases aimed at exploring the therapeutic effects of MSCs worldwide (Fig 3) (Gao et al., 2016), which led to registration for the clinical use of MSCs as a future drug. Prochymal, an allogeneic BM-MSC, was approved for the use in steroid resistant severe acute pediatric graft-versus-host disease (GvHD) in Canada and New Zealand (Prasad et al., 2011; Ratcliffe et al., 2013).

Moreover, up to six autologous or allogeneic MSC-based drugs have approved, locally and abroad, for the treatment of specific indications such as severe limbal stem cell deficiency in the eye, acute myocardial infarction, cartilage defects in degenerative arthritis and anal fistula in Crohn's disase. Many other applications are now progressing in clinical trials, some with benefits to patients (Trounson and McDonald, 2015).

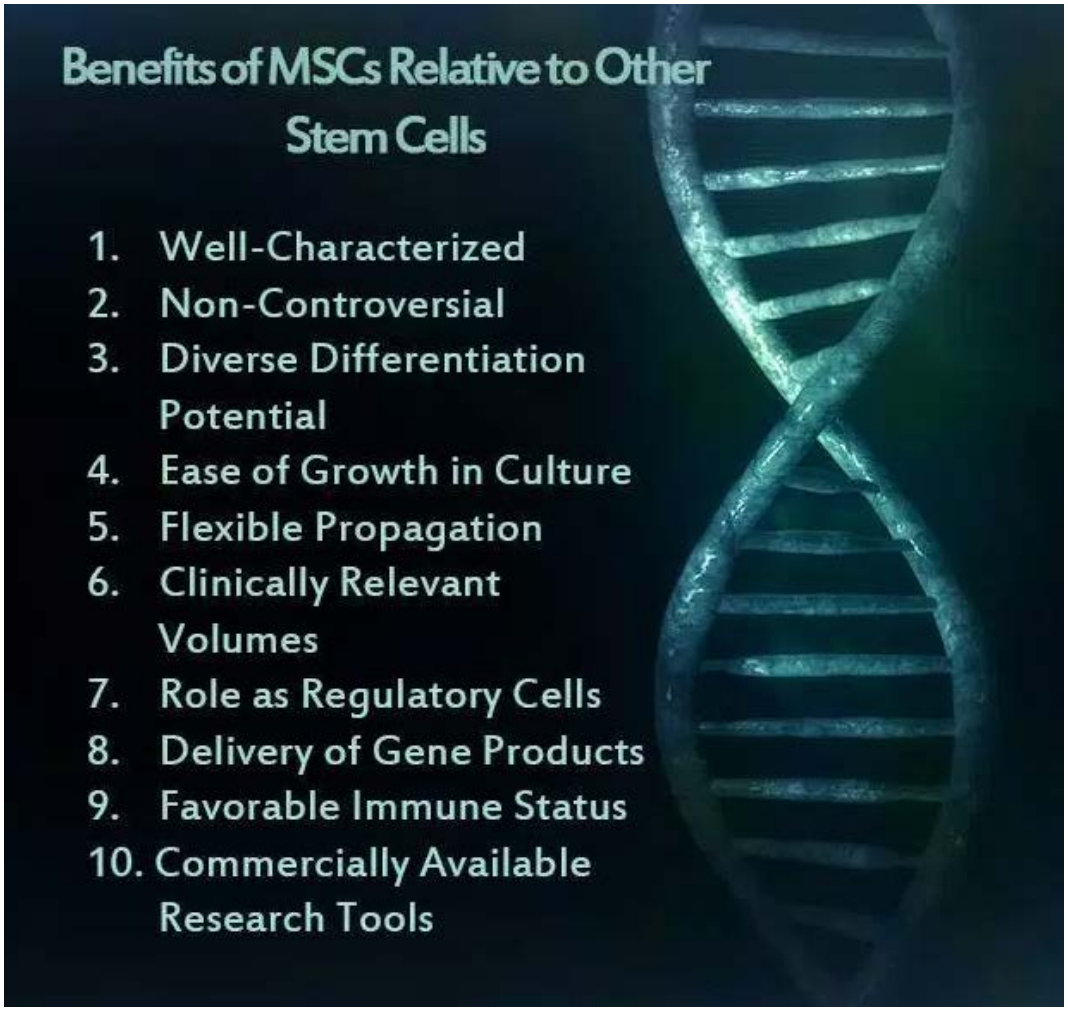
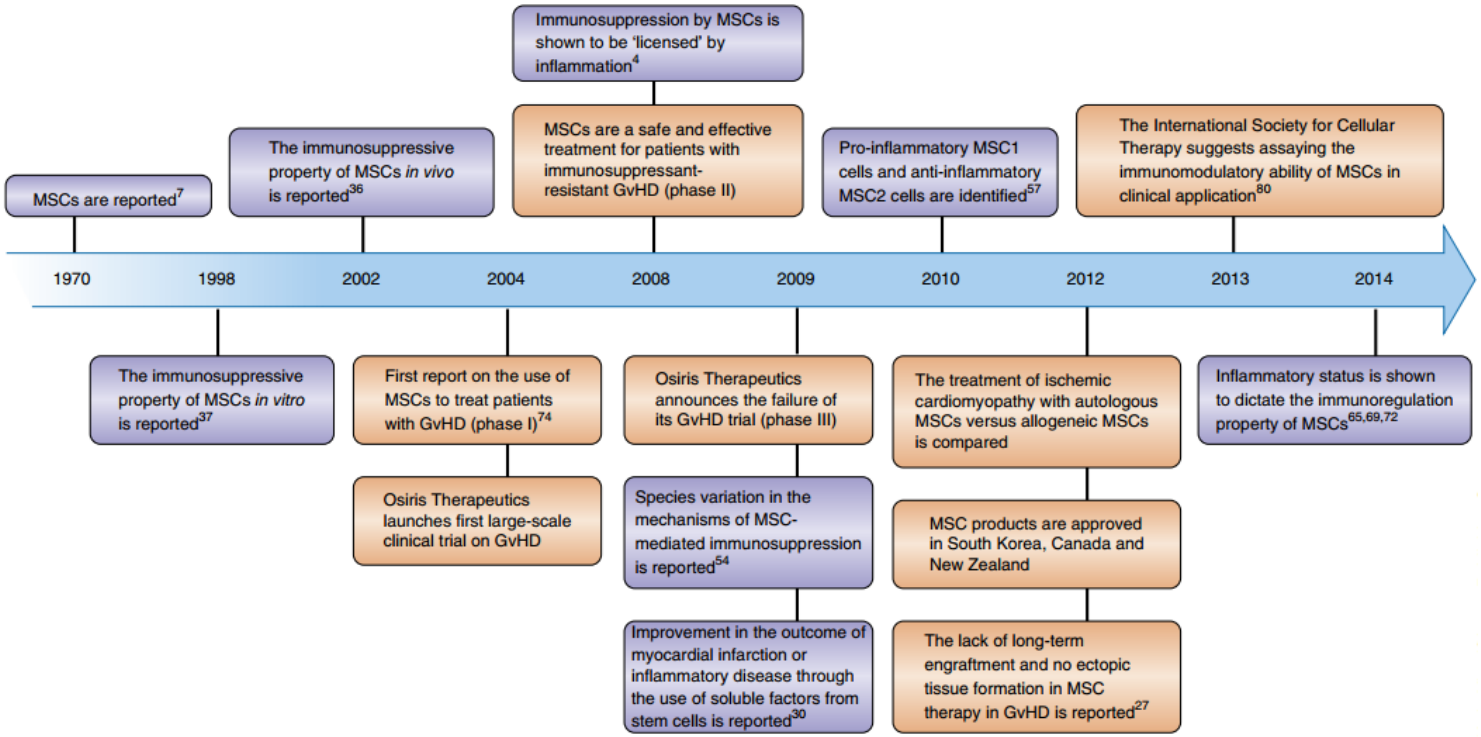


Figure 2. Benefits of MSCs relative to other stem cells.

(Source: Mesenchymal Stem Cells - Advances & Application, BioInfromant, 2015)

Figure 3. Timeline for major events in studies of the immunosuppressive effect of MSCs



Purple boxes summarize findings from preclinical studies; orange boxes show results of clinical applications.

(Wang et al., 2014)

Cell therapies utilizing MSCs are being explored as a novel therapy for intractable immune-relate diseases. Indeed, in the majority of completed pilot clinical trials in various diseases such as myocardial injuries, osteoarthritis, pulmonary diseases and autoimmune disorders, patients with MSC therapy showed successful tolerance and improved clinical features (Sharma et al., 2014). In addition, cell therapies using MSCs have been proven to be definitely safe over the past decade (Connick et al., 2012; Lalu et al., 2012; Le Blanc et al., 2008). Although these results indicate that MSC-based therapy can be a promising candidate for the treatment of immune-related diseases, many obstacles still remain. Major hurdles in stem cell therapy are to identify the exact mechanisms by which injected cells might exert their efficacy against target disease and to optimize process of cell preparation and treatment protocol. As studies with more stronger scientific evidence of likely clinical benefits and demonstrated mechanisms of action evolve from preclinical trials, it might be expected that the conversion to registered stem cell therapeutics will increase with time (Trounson and McDonald, 2015). Moreover, since there is considerable heterogeneity in MSCs, it is necessary to establish of optimal protocol, a careful manipulation of appropriate cell sources and a better mechanistic understanding of disease- and target cell-specific immunomodulation (Gao et al., 2016; Wang et al., 2014). More extensive scientific investigations and further well-designed clinical trials are absolutely necessary to obtain maximal clinical benefits from MSC therapy.

Immunomodulatory Properties of Mesenchymal Stem Cells

MSCs are stromal-derived adult multipotent cells that were originally identified and isolated from bone marrow as precursor cells of osteogenic lineage (Friedenstein et al., 1974). Currently, MSCs can be expanded from various tissues such as umbilical cord blood (UCB), adipose tissue (AT), amniotic fluid, dental pulp, skin and muscle (Campagnoli et al., 2001; Hoogduijn et al., 2014). Moreover, MSCs possess the ability to differentiate into more specialized cell types, including osteoblasts, adipocytes, chondrocytes, cardiomyocytes, endothelial cells, β-pancreatic islet cells and potentially neuronal cells (Caplan, 1991; Kopen et al., 1999; Pittenger et al., 1999). In addition to these stem/progenitor functions, MSCs have also been revealed to have broad and unique immunomodulatory properties and are capable of influencing on proliferation, function, recruitment and fate of both the innate and adaptive immune cells, including T cells, B cells, dendritic cells (DCs) and natural killer (NK) cells (Corcione et al., 2006; Glennie et al., 2005; Spaggiari et al., 2006; Zhang et al., 2009a). Although the underlying mechanisms have yet to be elucidated, various studies have demonstrated that MSC immunomodulation is likely mediated through direct cell-to-cell contact and/or paracrine mechanism by secreting of diverse mediators. Since MSCs typically exhibit low levels of major histocompatibility complex (MHC) class I and rarely express MHC class II or co-stimulatory molecules, cell contact might not be the primary mechanisms of MSC immunomodulation (Chamberlain et al., 2007; Le Blanc et al., 2003). Growing advance suggests that this effect is exerted through the release of soluble mediators such as

prostaglandin E_2 (PGE_2), indoleamine 2, 3-dioxygenase (IDO), transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), nitric oxide (NO) and interleukin-10 (IL-10) (Fig 4) (Bassi et al., 2012; Gao et al., 2016; Ma et al., 2014; Munir and McGettrick, 2015; Ren et al., 2008a). Indeed, MSCs have been employed in the treatment for experimental animal models of autoimmune and inflammation-related disorders such as graft-versus-host-disease (GvHD), inflammatory bowel disease (IBD), multiple sclerosis (MS), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Gonzalez et al., 2009; Guo et al., 2011; Liu et al., 2009; Sun et al., 2009; Zhang et al., 2009c). More recently, several studies have also reported that MSCs can attenuate allergic disease including asthma, atopic dermatitis and rhinitis (Goodwin et al., 2011; Kapoor et al., 2012). Although results have not been consistent altogether, MSCs have been shown to be mainly encouraging in preclinical studies, which rapidly brought them into the clinic.

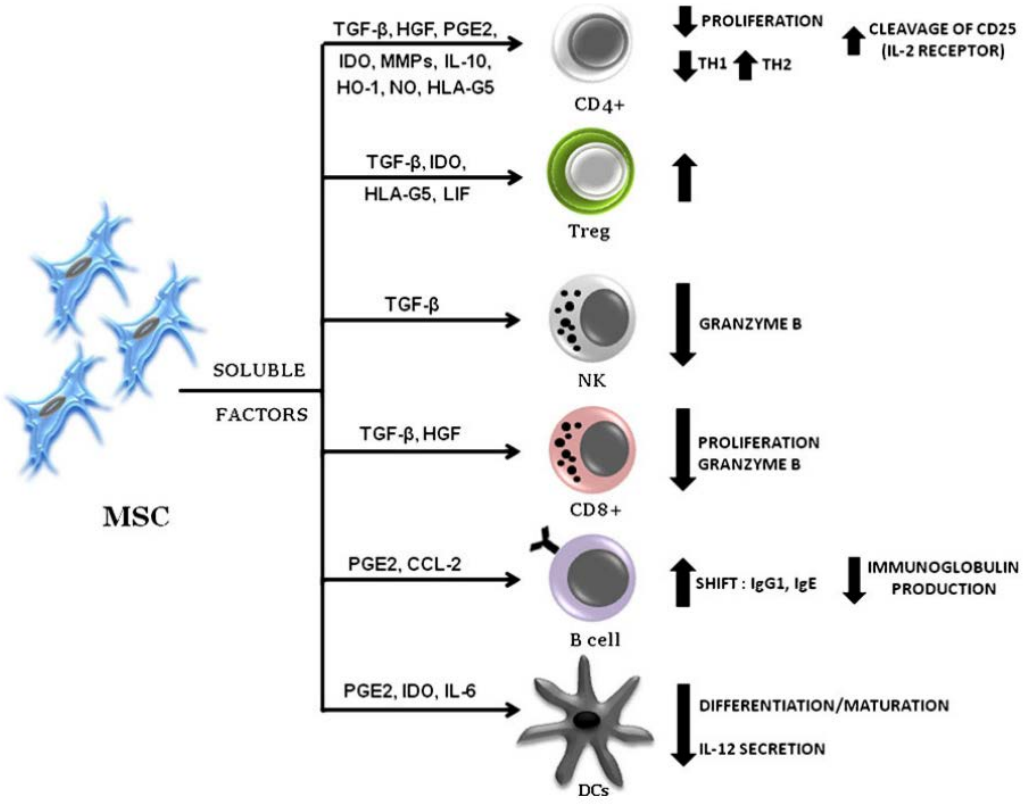


Figure 4. Immune regulatory effects of MSC-secreted soluble factors in several cells of the immune system.

Several MSC-secreted soluble factors have been identified as being responsible for their immune regulation properties in dendritic cells (DCs), and T and B cells and include TGF-β, PGE_2, IDO, HGF, LIF (leukemia inhibitory factor), IL-10, IL-6, NO, HLA-G5 (human leukocyte antigen-5), MMPs (metalloproteinase-9), CCL2 (CC-chemokine ligand 2), CCL5 (chemokine C-C motif ligand 5, RANTES) and HO-1 (Bassi et al., 2012).

Innate Immune Apparatus and Mesenchymal Stem Cells

The immune system refers to the complex response consist of multiple cellular, molecular and tissue components that protect the host from a variety of infectious pathogens and toxic microorganisms. This defense mechanism is typically divided into the greatest dichotomy separated innate immunity from adaptive immunity. Of that, the innate immunity is genetically programmed and rapid acting procedures to detect invading microorganisms. The core components of the innate immune system are mechanical epithelial barrier and cellular players including DCs, NK cells and phagocytic leucocytes. The well-established microbial sensors are so-called pattern recognition receptors (PRRs) of innate immune system, which sense pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), peptidoglycan (PGN) and microbial nucleic acid variants (Hoebe et al., 2004; Iwasaki and Medzhitov, 2010). Upon recognition of PAMPs, PRRs trigger a downstream cascade of chemokines and cytokines contributed to anti-microbial inflammatory responses.

Since the discovery of Toll-like receptors (TLRs) in the mid-1990s, there has been rapid advances in the understanding of innate immune recognition of specific microbial pathogen. In addition, several classes of cytoplasmic PRRs, including Nod-like receptors (NLRs) and RIG-I-like receptors (RIGs), were also identified and these PRRs have been known to play an important role in both innate and adaptive immunity through complicated network of signaling pathway (Fig 5) (Kawai and Akira, 2010; Yoneyama and Fujita, 2009).

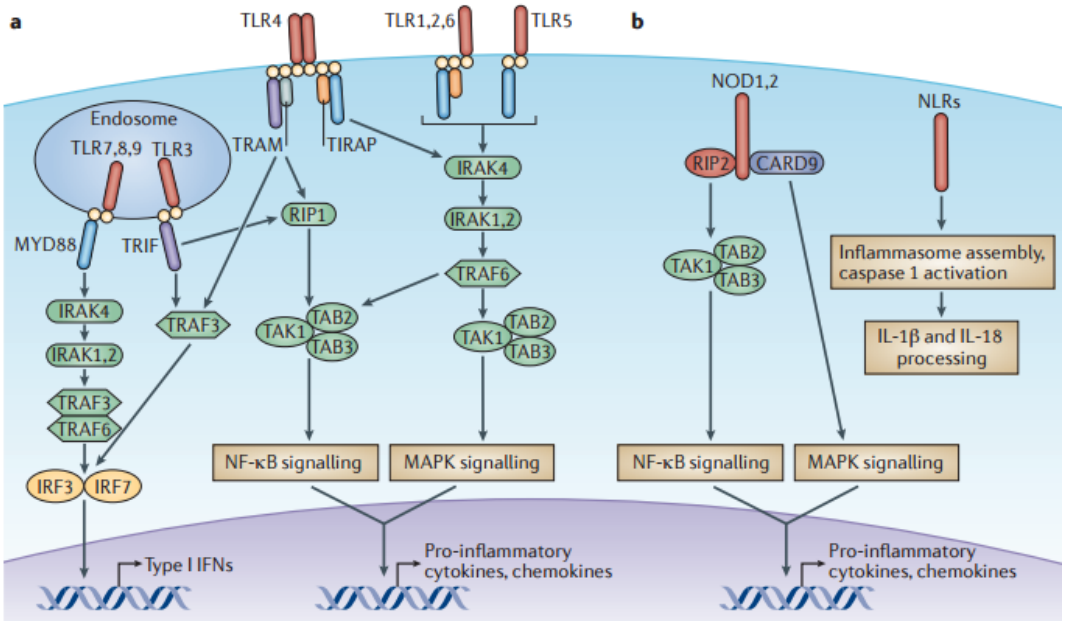


Figure 5. Pattern recognition receptors of the innate immune system and their signaling pathways.

Figure 5. Pattern recognition receptors of the innate immune system and their signaling pathways.

(a) TLRs, which function as dimers (shown for TLR4 only), sense microbial molecular patterns (such as LPS and dsRNA) on the cell surface and in the endosomal compartment and activate signaling pathways through TIR domain-containing adaptor and other downstream signaling mediators. TLR signaling ultimately leads to the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling (pathways not shown in detail), as well as IFN-regulatory factors (IRFs), resulting in the induction of pro-inflammatory cytokines, chemokines and type I interferons (IFNs) and, consequently, the activation of cellular antimicrobial functions. **(b)** NLRs sense components of peptidoglycan in the cytosol and activate receptor-interacting protein 2 (RIP2) and downstream signaling pathways, including the MAPK and NF-κB signaling pathways. This leads to the induction of pro-inflammatory cytokines and chemokines, and the activation of antimicrobial functions. NLRs are activated by diverse microbial signature molecules and other danger signals in the cytosol. After activation, they recruit the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), leading to inflammasome assembly, caspase 1 activation, and the processing and secretion of the cytokines interleukin-1β (IL-1β) and IL-18. CARD9, caspase recruitment domain-containing 9; IRAK, IL-1 receptor-associated kinase; TAK1, TGF-β-activated kinase 1; TRAF, TNF receptor-associated factor (Hancock et al., 2012).

Recently, several studies have reported that TLRs and NLRs are expressed in MSCs, suggesting that they might exert inherent role on early immune responses (Table 2). Since stimulation of these PRRs results in receptor internalization and the activation of NF-κB, MAPK and AKT signaling pathways, PRRs in MSCs act as not only sensor molecules related with infections or injuries but responder to environmental stimuli. Indeed, a few studies reported that stimulation of the PRRs in MSCs by particular microenvironment such as hypoxic condition or specific agonists could enhance immunomodulatory properties of MSCs. Although the TLR participation in MSC immunomodulation remains still controversial, it is generally regarded that TLR3 activation in MSCs supports immunosuppression by up-regulating anti-inflammatory cytokines, whereas activation of TLR4 induces MSCs toward pro-inflammatory phenotype (Opitz et al., 2009; Tomchuck et al., 2008; Waterman et al., 2010). Recently, Kim et al. reported that NOD1 and NOD2 receptors also were functionally expressed in cytosolic regions and participated in regulating the differentiation and immunoregulatory properties of MSCs (Kim et al., 2010b). Further, activation of NOD2 by pretreatment of appropriate ligand, muramyl dipeptide (MDP), resulted in the enhancement of therapeutic efficacy in experimental colitis murine model (Kim et al., 2013). However, in spite of growing evidences have accumulated that PRRs regulate the properties of MSCs, there is an obvious need to understand the precise mechanisms of NLRs on MSC functions.

Table 2. Pattern-recognition receptors expressed by MSCs

Type of PRR	Source of MSCs	Localization	Species
Toll-like receptors (TLRs)			
TLR1	BM / AT / UC (blood)	Cell surface	Human / Mouse
TLR2	BM / AT / UC (blood)	Cell surface	Human / Mouse
TLR3	BM / AT / UC (blood) / DP	Cell surface	Human / Mouse
TLR4	BM / AT / UC (blood) / DP	Cell surface	Human / Mouse
TLR5	BM / AT / UC (blood)	Cell surface	Human / Mouse
TLR6	BM / AT / UC (blood)	Cell surface	Human / Mouse
TLR7	BM	Intracellular	Human / Mouse
TLR8	BM	Intracellular	Human / Mouse
TLR9	BM / AT / UC (blood)	Intracellular	Human
TLR10	AT	Cell surface	Human
NOD-like receptors (NLRs)			
NOD1	UC (blood)	Intracellular	Human
NOD2	UC (blood)	Intracellular	Human

** BM = bone marrow, AT = adipose tissue, UC = umbilical cord, DP = dental pulp

(Le Blanc and Mougiakakos, 2012)

More recently, the novel regulatory apparatus of innate immune responses, called ‘inflammasome’, have newly identified. Inflammasomes are multi-protein complexes that recognize inflammatory stimuli, such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), leading to the activation of caspase-1, which then promotes secretion of pro-inflammatory cytokines, including interleukine-1β and interleukin-18, followed by pyroptosis, a form of cell death induced by microbial pathogen (Franchi et al., 2012; Schroder and Tschopp, 2010; Strowig et al., 2012). Major research interests can be divided into those discovering new types of inflammasome, those investigating the activation mechanism of each inflammasome by microbial peptide or other signals, and those uncovering the role and regulatory mechanism of inflammasomes in diseases. Up to now, six members (NLRP1, NLRP3, NLRP6, NLRC4, NAIP, and AIM2) have been identified in mammalian inflammasomes according to their ligand sensing receptors, and all types, except AIM2, contain intermediate nucleotide-binding oligomerization domain (NOD) (Fig 6) (Boyden and Dietrich, 2006; Mariathasan and Monack, 2007) (Zhao et al., 2011). Each inflammasome is reported to activate downstream signaling upon recognizing a specific microbial component. Interestingly, NLRP3 inflammasome cannot be activated by only one ligand and needs additional signaling, such as ATP for its activation, whereas other types can be activated by recognition of a specific ligand, which makes NLRP3 inflammasome an attractive component to investigate among innate immune sensors (Fig 7). Therefore, further studies on the exact role of inflammasome in specific diseases is highly needed and potentially a novel target in diagnosis and treatment.

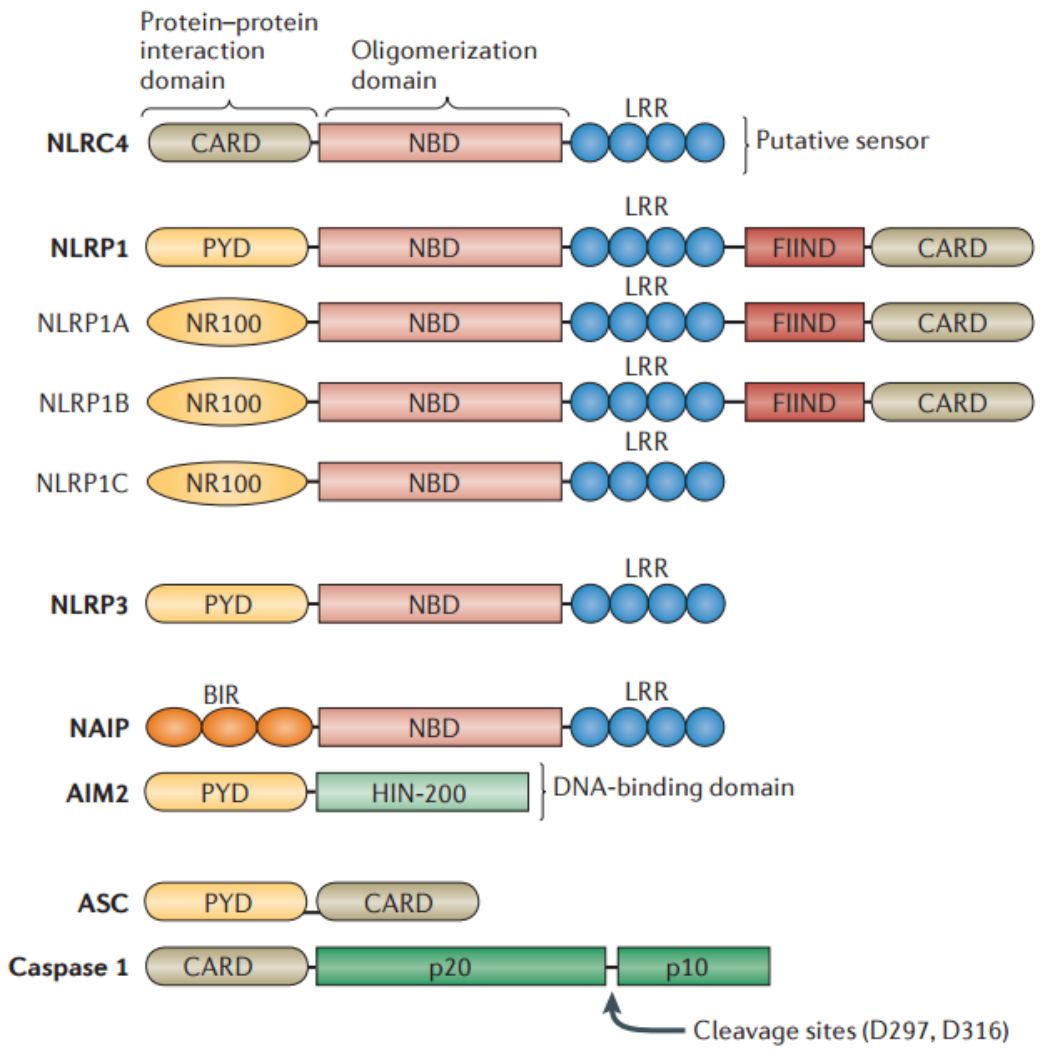


Figure 6. Inflammasome components and domain structure.

Figure 6. Inflammasome components and domain structure.

The activation and formation of inflammasome complexes is mediated through several protein domains. In NOD-like receptors (NLRs), the putative sensory component is formed by the carboxy-terminal leucine-rich repeat (LRR). Oligomerization of NLRs is mediated by the nucleotide-binding domain (NBD). The pyrin domain (PYD) mediates protein–protein interactions between the inflammasome sensor and the adaptor apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) (ASC), which also contains a PYD. The CARD of ASC mediates protein–protein interactions with the CARD of procaspase 1. NOD-, LRR- and CARD-containing 4 (NLRC4) and NOD-, LRR- and PYD-containing 1 (NLRP1) can also directly interact with procaspase 1 through their respective CARDs. NLRP1 contains a unique function-to-find domain (FIIND), which is involved in inflammasome activation through auto-proteolysis. In the murine proteins NLRP1A, NLRP1B and NLRP1C, the amino-terminal PYD is replaced by an NR100 domain (amino-terminal domain of rodent NLRP1 of about 100 amino acids), which has no known homologue in humans. Neuronal apoptosis inhibitory proteins (NAIPs) are a subfamily of the NLRs and contain a bacculovirus inhibitor of apoptosis repeat (BIR) domain. In absent in melanoma 2 (AIM2), the HIN-200 DNA-binding domain is the putative sensory component; the PYD in AIM2 mediates interactions with ASC (Walsh et al., 2014).

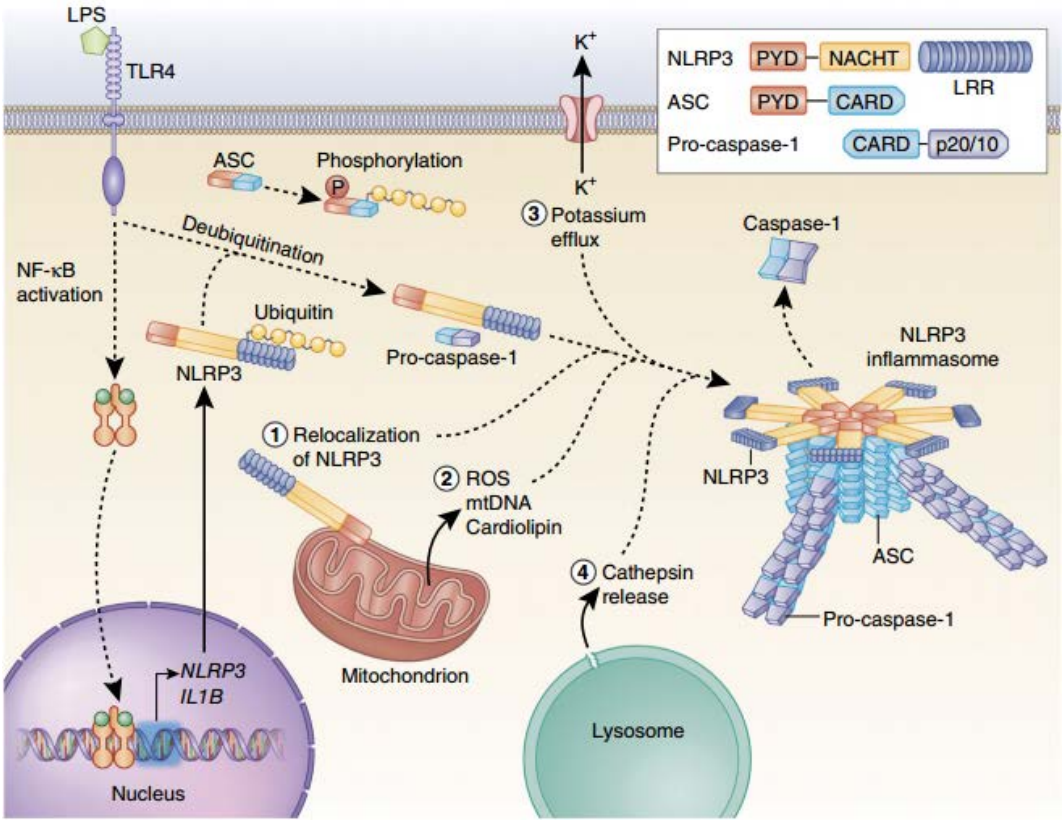


Figure 7. Mechanisms of NLRP3 inflammasome activation

Figure 7. Mechanisms of NLRP3 inflammasome activation.

NLRP3 must be primed before activation. Priming involves two distinct steps. First, an NF-κB–activating stimulus, such as LPS binding to TLR4, induces elevated expression of NLRP3 (as well as IL1β), which leads to increased expression of NLRP3 protein. Additionally, priming immediately licenses NLRP3 by inducing its deubiquitination. The adaptor protein ASC must become linearly ubiquitinated and phosphorylated for inflammasome assembly to occur. After priming, canonical NLRP3 inflammasome activation requires a second, distinct signal to activate NLRP3 and lead to the formation of the NLRP3 inflammasome complex. The most commonly accepted activating stimuli for NLRP3 include relocalization of NLRP3 to the mitochondria, the sensation of mitochondrial factors released into the cytosol (mitochondrial ROS, mitochondrial DNA, or cardiolipin), potassium efflux through ion channels, and cathepsin release following destabilization of lysosomal membranes. Recent studies have determined that activated NLRP3 nucleates ASC into prion-like filaments through PYD-PYD interactions. Pro-caspase-1 filaments subsequently form off of the ASC filaments through CARD-CARD interactions, allowing autoproteolytic activation of pro-caspase-1. Inset shows domain arrangement of the NLRP3 inflammasome components. Pro-caspase-1 and caspase-1 domains are simplified for clarity, the CARD domain is actually removed by cleavage, and two heterodimers form with the p20 and p10 effector domains (p20/10) (Guo et al., 2015).

Inflammasomes have closely associated with various inflammation-related and autoimmune diseases, including neurodegenerative diseases and metabolic syndromes because aberrant inflammasome signaling contributes to pathology in these diseases (Guo et al., 2015; Lamkanfi and Dixit, 2012). Inflammasomes have been known to play either causative or contributing roles, as well as exaggerate the pathophysiology in response to host-derived factors. In particular, dysregulation of NLRP3 inflammasome has been implicated in almost age-related condition, which indicates that age is a risk factors for chronic inflammatory disease like Alzheimer's disease (AD), atherosclerosis, type 2 diabetes and gout. Several recent studies have reported that the NLRP3 inflammasome is triggered by a variety of metabolic byproducts associated with these disease (Doyle et al., 2012; Halle et al., 2008; Masters et al., 2010). Moreover, genetic mutations in components of inflammasome complexes have been associated with a tendency for the development of several immune-related disorders. Notably, three single nucleotide polymorphisms (SNPs) located within the LRR domain are reported to be associated with increased susceptibility to inflammatory bowel disease (IBD) (Hugot et al., 2001; Ogura et al., 2001), and genetic variants at NLRP3 and CARD8 loci are revealed to be closely related to RA susceptibility and responsiveness to anti-TNF-α treatment (Mathews et al., 2014). Therefore, the therapeutic inhibition of inflammasome pathway or prognostic approaches using genetic information has led to create new insight for the treatment and intervention of inflammatory diseases.

Hypersensitivity reaction

The same immune responses that protect host from infection can also impose an undesirable damage not only on a pathogen, but on own cells and tissues. This inappropriate immune response is termed hypersensitivity or allergy. Namely, hypersensitivity is defined as inappropriate and excessive reaction that is launched an exaggerated immune response against innocuous antigens, which can results in significant tissue damage or even death. The first description of a hypersensitivity reaction was reported by two French scientists, Paul Portier and Charles Riche, in the early twenties century. Since that time, a number of immunologists have demonstrated that there are multiple types of hypersensitivity reaction. P.G.H Gell and R.R.A Coombs subsequently proposed a classification scheme in which hypersensitivity reactions are largely divided into four types based on the mechanisms involved and time required for the reaction (Gell and Coombs, 1963). While three of them are mediated by humoral immunity, the fourth type is associated with cell-mediated immunity (Table 3) (Rajan, 2003).

Type I hypersensitivity reactions, also known as immediate or anaphylactic hypersensitivity, encompass the most common allergic responses, including asthma, food allergies and atopic dermatitis. Certain types of antigens, referred to as allergens, induce humoral antibody response, leading to the generation of antibody-secreting plasma cells and memory cells. In particular, the plasma cells increase the secretion of immunoglobulin type E (IgE), followed by binding with high affinity IgE receptor (FcεRI) on the surface of tissue mast cells (MCs) and blood basophils (Abramson and Pecht, 2007). These MCs and

basophils coated by IgE are said to be sensitized. Subsequent encounter with the same allergen cross-links the membrane-bound IgE on sensitized MCs and basophils, triggering degranulation and release of pharmacologically active preformed mediators (e.g. histamine, tryptase, kininogenase and chemotactic factors) and synthesis of new mediators (e.g. leukotriens, prostaglandins and platelet activation factor) (Ariza et al., 2014). These mediators cause vasodilation, smooth muscle spasm and infiltration with eosinophils and type 2 helper T (Th2) cells. The clinical manifestations of type I hypersensitivity reactions can range from localized disorder, such as eczema and hay fever, to life-threatening conditions like systemic anaphylaxis. Healthy individuals mount IgE responses only as a defense against parasitic infection. However, some persons, may have an abnormalities called atopy, are predisposed to generate IgE antibodies and to develop of immediate hypersensitivity reactions against common environmental antigens (Nimmagadda and Evans, 1999). Atopic disorders most commonly affect the nose, skin, eyes and lungs, such as atopic dermatitis, allergic rhinitis, immune-mediated urticaria and some case of asthma.

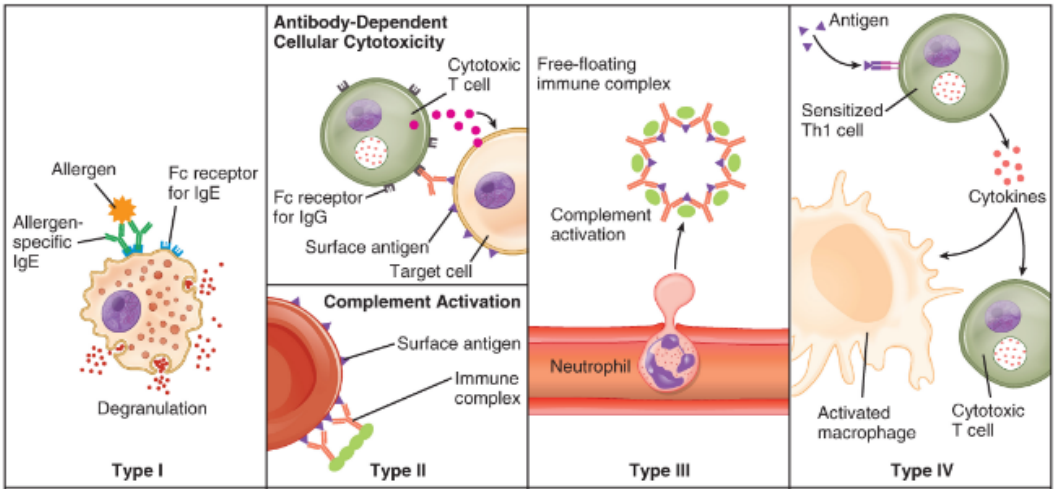
Type II hypersensitivity reaction involve antibody-mediated destruction of cells by immunoglobulins of heavy chain classes other than IgE and complement proteins. When IgG or IgM binds to cell-surface antigens, the antigen-antibody complex can induce cell destructions by antibody-dependent cell-mediated cytotoxicity (ADCC) complement mechanism, or both. Transfusion reactions related with the ABO blood type are a typical example of type II hypersensitivity (Yamamoto, 2004). Type II hypersensitivity reactions include hemolytic diseases, such as erythroblastosis fetalis and drug-induced hemolytic anemia, and anti-glomerular basement membrane diseases like Goodpastrue syndrome.

In type III hypersensitivity reactions, the deposition of soluble antigen-antibody immune complexes can facilitate the activation of complement system or bind to and activate certain type of immune cells, resulting in the recruitment of neutrophils with resultant tissue damage. These complexes generally induce the clearance of antigen by phagocytes, however, in some cases the presence of immune complexes can lead to tissue-damaging type III reaction. Sterile inflammation in various autoimmune diseases including systemic lupus erythematosus, multiple sclerosis and rheumatoid arthritis results from type III hypersensitivity in response to autoantigens (Davidson and Diamond, 2001). Indeed, persistent antibody responses to specific autoantigens are an identifying signature of the disease and complexes are deposited in the flared tissues. Besides, Arthus reaction, one example of a localized type III reaction, has been extensively used as an experimental tool (Hazenbos et al., 1996).

Type IV hypersensitivity, commonly called a Delayed-type hypersensitivity (DTH), is the only category that is dependent on purely cell-mediated branch. The hallmarks of a DTH response are its initiation by T cells, the delay required for the reaction to develop, and recruitment of macrophages as the primary effector cellular component. Generally, Th1 subtypes are primarily activated and clonally expanded during the sensitization phase after the first exposure to antigen. In the effector phase after subsequent exposure of sensitized Th cells to antigen, Th1 cells secrete a variety of cytokines and chemokines that recruit macrophages and other phagocytic cells to the lesion. Other Th cell subset, such as Th17 and Th2, and cytotoxic T cells are also considered to participate in DTH response. The classical example of this response is tuberculin (Montoux) reaction

which is the diagnostic test for finding a tuberculosis infection by intradermally injecting a small amount of purified protein derivative (PPD) (Bansal and Sharma, 2012). If an individual have ever been exposed to the Mycobacterium tuberculosis, the skin will react to the antigens by developing a local induration and erythema at the site within 2 days. Another forms of DTH include contact dermatitis, hypersensitivity pneumonitis and many forms of drug hypersensitivity (Adam et al., 2011).

Table 3. The four types of hypersensitivity responses



IgE-Mediated Hypersensitivity	IgG-Mediated Cytotoxic Hypersensitivity	Immune Complex-Mediated Hypersensitivity	Cell-Mediated Hypersensitivity
IgE is bound to mast cells via its Fc portion. When an allergen binds to these antibodies, crosslinking of IgE induces degranulation.	Cells are destroyed by bound antibody, either by activation of complement or by a cytotoxic T cell with an Fc receptor for the antibody (ADCC)	Antigen–antibody complexes are deposited in tissues, causing activation of complement, which attracts neutrophils to the site	Th1 cells secrete cytokines, which activate macrophages and cytotoxic T cells and can cause macrophage accumulation at the site
Causes localized and systemic anaphylaxis, seasonal allergies including hay fever, food allergies such as those to shellfish and peanuts, hives, and eczema	Red blood cells destroyed by complement and antibody during a transfusion of mismatched blood type or during erythroblastosis fetalis	Most common forms of immune complex disease are seen in glomerulonephritis, rheumatoid arthritis, and systemic lupus erythematosus	Most common forms are contact dermatitis, tuberculin reaction, autoimmune diseases such as diabetes mellitus type I, multiple sclerosis, and rheumatoid arthritis

(Source : Owen et al., Immunology 7th edition, 2013 and http://cnx.org)

CHAPTER I

Concerted Action of PGE_2 and TGF-β1 from Human Umbilical Cord Blood-derived Mesenchymal Stem Cells Alleviates Atopic Dermatitis by Reducing Mast Cell Degranulation

1.1 INTRODUCTION

Mesenchymal stem cell (MSC) is a promising tool for the therapy of immune disorders. However, their efficacy and mechanisms in treating allergic skin disorders are less verified. I sought to investigate the therapeutic efficacy of human umbilical cord blood-derived MSCs (hUCB-MSCs) against murine atopic dermatitis (AD) and to explore distinct mechanisms that regulate their efficacy. AD was induced in mice by the topical application of *Dermatophagoides farinae (Df)*. Naïve or activated-hUCB-MSCs were administered to mice, and clinical severity was determined. The subcutaneous administration of nucleotide-binding oligomerization domain 2 (NOD2)-activated hUCB-MSCs exhibited prominent protective effects against AD, and suppressed the infiltration and degranulation of mast cells (MCs). A β-hexosaminidase assay was performed to evaluate the effect of hUCB-MSCs on MC degranulation. NOD2-activated MSCs reduced the MC degranulation via NOD2-COX2 signaling. In contrast to bone marrow-derived MSCs, hUCB-MSCs exerted a cell-to-cell contact-independent suppressive effect on MC degranulation through the higher production of prostaglandin E_2 (PGE_2). Additionally, TGF-β1 production from hUCB-MSCs in response to IL-4 contributed to the attenuation of MC degranulation by down-regulating high affinity IgE receptor (FcεRI) expression in MCs. In conclusion, the subcutaneous application of NOD2-activated hUCB-MSCs can efficiently ameliorate AD, and MSC-derived PGE_2 and TGF-β1 are required for the inhibition of MC degranulation.

1.2 MATERIALS AND METHODS

1.2.1 Isolation and culture of MSCs

UCB samples were obtained from the umbilical vein immediately after delivery, and the informed consent of the mother was given and approved by the Boramae Hospital Institutional Review Board (IRB) and the Seoul National University IRB (IRB No. 1109/001-006). The UCB samples were mixed with 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. Cells were seeded at a density of 2×10^5 to 2×10^6 cells/cm^2 on plates in growth medium consisting 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 that grew rapidly and, exhibited spindle-shaped morphology.

hBM-MSCs were purchased from Cell Therapy Center in Severance hospital , Yonsei University (Approval No. 4-2008-0643). Cells were cultured in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum. The medium was changed every 3 or 4 days.

1.2.2 Atopic dermatitis induction in NC/Nga mice

NC/Nga mice (male and female, 8wk old) were obtained from SLC (Hamamatsu, Japan) and group housed under specific pathogenic-free conditions in the animal facility of the Seoul National University. All experiments were approved by and followed the regulations of the Institute of Laboratory Animal Resources (SNU-111117-3 & 140320-1, Seoul National University, Korea).

Atopic dermatitis-like symptoms were induced according to the previously described method. Briefly, the upper backs of the mice were shaved with a clipper. Barrier disruption was achieved using 150 μL of 4% sodium dodecyl sulfate treatment on the shaved dorsal skin and both surfaces of each ear 3-4 hours before the topical application of 100 mg of *Df* extract (Biostir Inc., Hiroshima, Japan). *Df* extract was treated twice a week for three weeks. To test the protective effect, hUCB-MSCs (2×10^6 cells / 200 μL PBS) were administered intravenously or subcutaneously on days 2, 9, 16 and 23 (Fig 1.1A). To evaluate the therapeutic effect, hUCB-MSCs were infused subcutaneously on day 21. For MDP-stimulated MSC injection, hUCB-MSCs were exposed to MDP for 24 h before administration and washed with PBS to remove residual MDP. Prednisolone (3 mg/kg po) was administered daily throughout the entire induction period as a therapeutic control. The sum of the individual scores (0, none; 1, mild; 2, moderate; 3, severe) on dryness, excoriation, erythema and edema was utilized as the clinical severity score. After sacrifice on day 30, serum was collected to detect the concentration of total IgE or IgG1 using a commercial ELISA kit (BD Bioscience, San Jose, CA).

1.2.3 Assessment of clinical severity

Pruritus was assessed by automatic counting of the scratching behavior using the MicroAct automatic measuring system (Neuroscience Inc., Tokyo, Japan). Under anesthesia, mice were equipped with a small Teflon-coated magnet that was inserted subcutaneously into the dorsal side of both hind paws on the day before the recording of scratching behaviors. Movement of the mouse legs with implanted magnets induced an electric current in the observation chamber, which was surrounded by a round coil and the movement was recorded by the Microact® software. The number of scratching behaviors was counted for 2 h before sacrifice.

1.2.4 Histopathological evaluation

Skin samples 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) or toluidine blue. Leukocyte and eosinophil infiltration was determined by H&E staining. Mast cell infiltration and degranulation were measured by toluidine blue staining.

1.2.5 Cell tracking

To track the injected cells, hUCB-MSCs were labled with 10 μM of carboxy fluorescein diacetate succinimidyl ester (CFSE: Molecular Probes, Carlsbad, CA) according to the manufacturer's protocol. CFSE-labled cells were i.v. or s.c. injected. At 1, 3 and 7 days after injection, 10 μm of frozen skin sections were cut and examined for green fluorescence using a confocal microscope.

1.2.6 Mast cell culture

Human MCs were derived from umbilical cord blood. Mononuclear cells were isolated from the cord blood and cultured in the presence of 100 ng/mL stem cell factor (SCF), 50 ng/mL IL-6 and 10 ng/mL IL-10 (R&D Systems, Minneapolis, MN), and floating cells were transferred to new plates containing fresh medium twice a week. The cells were used for experiments when they reached more than 95% purity based on the expression of MC tryptase, as determined by flow cytometry (8 weeks).

1.2.7 Mast degranulation

For IgE-mediated MC degranulation, hMCs were primed in the presence of SCF and 10 ng/mL IL-4 (R&D Systems) for 5 days before the stimulation of degranulation. IL-4-primed hMCs were sensitized with 2 μg/mL human myeloma IgE (Millipore, Billerica, MA) for 24 h on the 4th day of priming. Cells that were re-suspended at 1×10^6 cells/mL were challenged with 1 μg/mL anti-IgE (Millipore) (Fig 1.4A). After 2 hours of challenge,

the induction of MC degranulation was stopped on ice and supernatants were harvested after centrifugation and measured for β-hexosaminidase (β-hex) content. MC supernatant (60 μL) was transferred to a 96-well plate and mixed with an equal volume of substrate solution (7.5 mM ρ-nitrophenyl-N-acetyl-β-D-glucosaminide dissolved in 80 mM citric acid, pH 4.5). The mixture was incubated on a shaking incubator for 90 minutes at 37 °C followed by the addition of 120 μL of 0.2 M glycine (pH 10.7). The absorbance at 450 nm was measured using a microplate reader (TECAN, Zürich, Switzerland). Release of β–hex is calculated as the percentage of total β-hex content in unstimulated mast cells, as determined by lysing cells with 0.1 % Triton X-100. For co-culture experiments of MCs with MSCs, MCs were cultured with MSCs at each step of degranulation induction under either cell-to-cell contact or transwell condition.

1.2.8 Western blotting

The cells or skin samples were harvested, and lysed in a buffer containing 1% Nonidet-P40 supplemented with a complete protease inhibitor 'cocktail' (Roche, Basel, Switzerland) and 2 mM of dithiothreitol. Lysates were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the following primary antibodies: COX-2 and GAPDH (Millipore, Billerica, MA), mast cell tryptase, regular- and phospho-STAT6 and FcεRI alpha (Abcam, Cambridge, MA). After immunoblotting with secondary antibodies, proteins were detected with enhanced chemiluminescence (ECL) reagent (Intron Biotechnology, Seongnam, Korea).

1.2.9 Cytokine production

MSCs were transfected with siRNAs followed by MDP treatment for 24 h. MSCs culture medium (CM) was harvested. PGE_2 and TGF-β1 concentrations in CM were determined using a commercial ELISA kit (PGE_2, R&D Systems, Minneapolis, MN; TGF-β1, eBioscience, San Diego, CA).

1.2.10 Flow cytometric analysis

For the characterization and analysis of human MCs and their degranulation by flow cytometry, hMCs were fixed and permeabilized with an intracellular staining buffer set (BD Biosciences, San Jose, CA) and then incubated with anti-mast cell tryptase antibody after Fc receptor (FcR) blocking using FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). hMCs were incubated with Alexa 488-labeled secondary antibody (Molecular Probes, Eugene, OR). All flow cytometry analyses were performed on a FACS Calibur and the Cell Quest software (BD Biosciences).

1.2.11 RNA interference

Transfection of small interfering RNAs (siRNAs) into cells was conducted when the cells reached 60% confluency. siRNAs against NOD2 (siNOD2, J-011388-07), RIPK2 (siRIPK2, M-003602-02) PTGS2 (siCOX-2, L-004557-00), and TGFB1 (siTGF-β1, L-012562-00), as well as a 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 for 72 h.

1.2.12 Statistical analysis

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

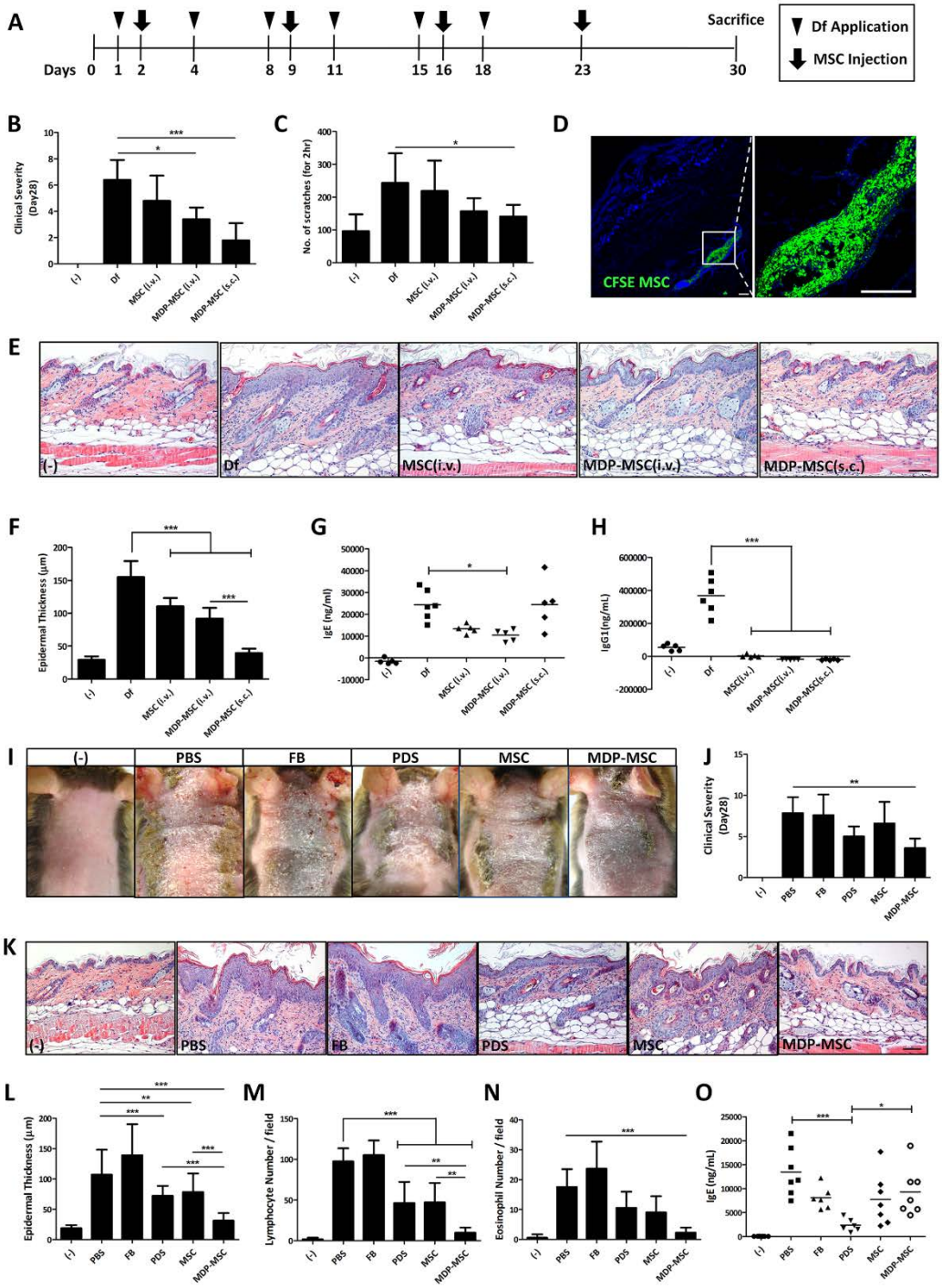
1.3 RESULTS

1.3.1 Subcutaneous administration of MDP-stimulated hUCB-MSCs elicits a robust protective effect against *Df*-induced atopic dermatitis in mice

NOD2 activation by its ligand, muramyl dipeptide (MDP) treatment enhances the protective effect of hUCB-MSCs against experimental colitis via the regulation of COX-2 signaling (Kim et al., 2013). Therefore, I first investigated whether the administration of MSCs or MDP-treated MSCs (MDP-MSCs) could rescue mice from *Df*-induced AD and whether the injection route exerted any difference in the protective effect of MSCs. Intravenous injection of MSCs slightly decreased the clinical severity of AD mice, and this effect was significantly improved by MDP stimulation on MSCs (Fig 1.1B). Interestingly, the protective effect of MDP-MSCs was further enhanced when the cells were infused subcutaneously (Fig 1.1B). Particularly, MDP-MSC treatment significantly restrained scratching behavior in AD mice (Fig 1.1C). CFSE-labeled MSCs were detected in the skin tissue when cells were injected subcutaneously (Fig 1.1D). However, MSCs were hardly detectable when cells were intravenously infused (data not shown). Upon histological evaluation, the dermal inflammation and epidermal hyperplasia observed in AD mice were reduced in the intravenous MSC- and MDP-MSC-injected mice group and was further improved in the subcutaneous MDP-MSC-injected group (Fig 1.1E-F). To explore if MSC

administration could regulate the AD-related serum immunoglobulin (Ig) level, concentrations of serum IgE and IgG1 were assessed. The serum IgE increase in AD mice was significantly attenuated by intravenous UCB-MSC and MDP-MSC injections (Fig 1.1G). However, subcutaneous MDP-MSC infusion did not suppress serum IgE increase (Fig 1.1G). The increase of serum IgG1 was suppressed in all MSC- or MDP-MSC-injected mice (Fig 1.1H).

Then I examined the effects of subcutaneously infused UCB-MSCs and MDP-MSCs compared to the fibroblast (FB) or prednisolone (PDS) treatment. Although injection of FBs could not reduce the clinical severity of AD mice, infusion of MDP-MSCs significantly improved the clinical severity more than daily oral administration of PDS (Fig 1.1I-J). Histological damages including epidermal hyperplasia and infiltration of lymphocytes and eosinophils were significantly attenuated by PDS treatment or MSC injection, and this damage was further ameliorated by MDP-MSC administration (Fig 1.1K-N). Upon serum IgE level analysis, treatment with PDS significantly suppressed the IgE increase (Fig 1.1O). In contrast, serum IgE concentrations of subcutaneous MDP-MSC-infused mice were slightly lower than those of AD mice and significantly higher than those of PDS-treated mice (Fig 1.1O). Taken together, these findings suggest that the subcutaneous administration of MDP-MSCs exhibits a more efficient protective effect against Df-induced AD mice than PDS treatment or intravenous injection MDP-MSCs, and that IgE-independent mechanisms might be involved in this effect.


A
Days 0 1 2 4 8 9 11 15 16 18 23 30
Sacrifice
Df Application
MSC Injection
B
Clinical Severity (Day28)
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No. of scratches (for 2hr)
(-)
Df
MSC (i.v.)
MDP-MSC (i.v.)
MDP-MSC (s.c.)
D
CFSE MSC
E
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Df
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MDP-MSC(s.c.)
F
Epidermal Thickness (μm)
G
IgE (ng/ml)
H
IgG1(ng/mL)
I
(-)
PBS
FB
PDS
MSC
MDP-MSC
J
Clinical Severity (Day28)
K
(-)
PBS
FB
PDS
MSC
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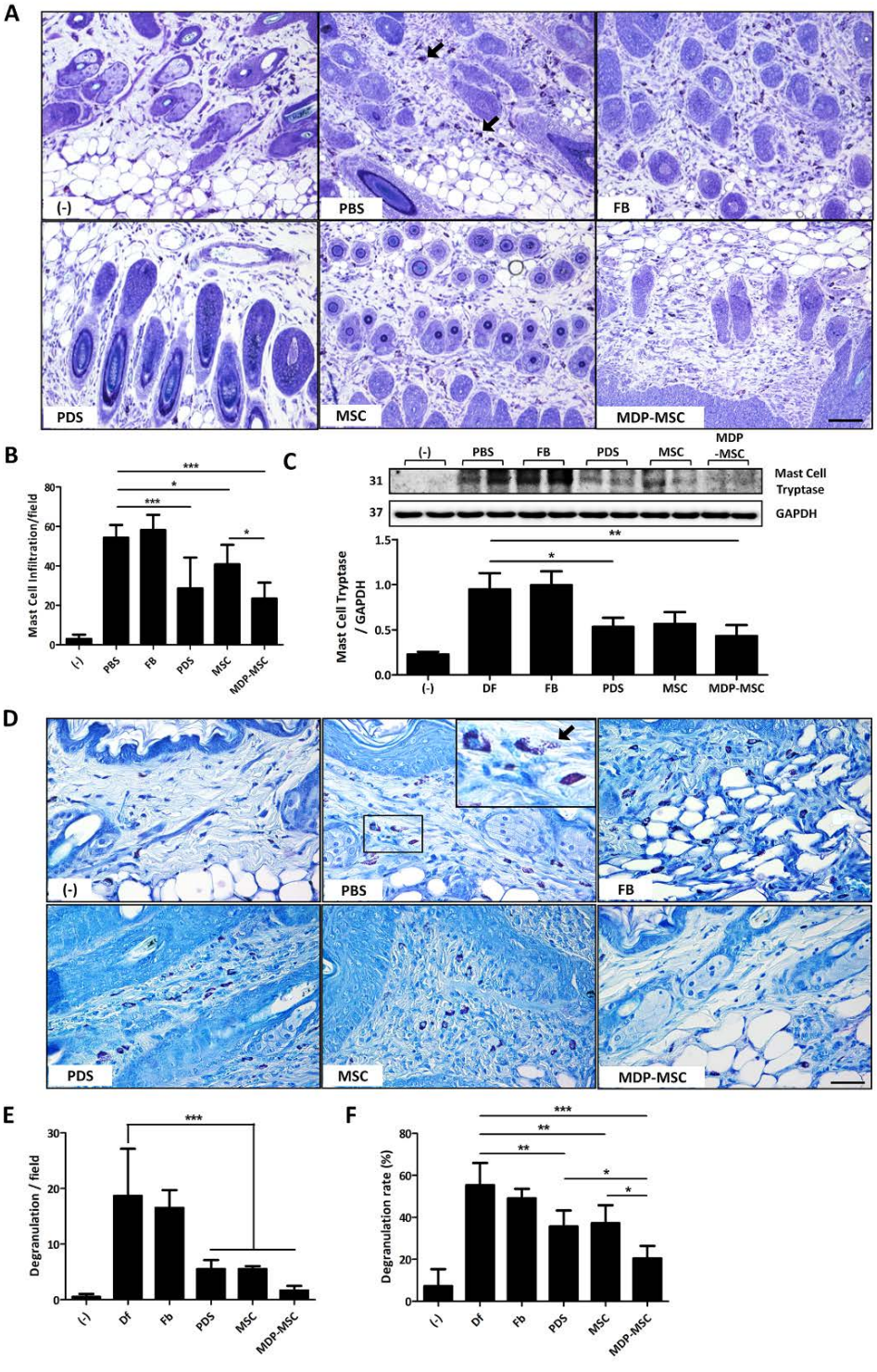
Epidermal Thickness (μm)
M
Lymphocyte Number / field
N
Eosinophil Number / field
O
IgE (ng/mL)

Figure 1.1. Subcutaneous administration of NOD2-activated hUCB-MSCs efficiently prevents the development of mouse atopic dermatitis.

(A-H) Atopic dermatitis was induced by the application of *Dermatophagoides farinae* (*Df*). Mice were intravenously (i.v.) or subcutaneously (s.c.) administered with 2×10^6 hUCB-MSCs. **(A)** Outline for induction of atopic dermatitis and MSC injection. **(B)** Clinical severity was determined. **(C)** The number of scratching behavior was counted. **(D)** CFSE-labeled hUCB-MSCs were subcutaneously injected into AD mice and at 3 days after injection, skin sections were examined for green fluorescent cells with a confocal microscope, Bar = 200 μm. (E-H) On day 30, mice were sacrificed for further evaluation. **(E)** Histological analysis of skin, Bar = 200 μm **(F)** Epidermal thickness was measured. **(G)** IgE and **(H)** IgG1 level in serum were detected. **(I-O)** Atopic dermatitis was induced by Df and mice were subcutaneously injected with 2×10^6 hUCB-MSCs or fibroblasts (FB) weekly. PBS (200 μL) was injected for vehicle control mouse. For therapeutic control group, AD-induced mice were orally administered with prednisolone (PDS) daily. **(I)** MSCs or fibroblasts were subcutaneously administered into AD mice and photographs of skin lesions were taken for gross pathological evaluation. **(J)** Clinical severity was determined. **(K-O)** On day 30, mice were sacrificed for histological and serological evaluation. **(K)** Histological analysis of skin, Bar = 200 μm **(L)** Epidermal thickness was measured. Number of **(M)** lymphocytes and **(N)** eosinophils was counted. **(O)** IgE level in serum was detected. Five to seven mice/group were used. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Results are shown as mean ± SD.

1.3.2 Subcutaneous administration of MDP-MSCs suppresses the infiltration and degranulation of Mast Cells

Given that the local injection of MDP-MSCs might regulate factors other than IgE to protect mice from AD symptoms, I focused on determining the local infiltration and degranulation of mast cells (MCs). MSCs or PDS treatment inhibited the infiltration of MCs (Fig 1.2A-B). The number of infiltrating MCs was confirmed by detecting MC tryptase in AD-induced skin tissue (Fig 1.2C). Interestingly, MSC injection suppressed the number of total degranulated MCs, and the rate of degranulation (Fig 1.2D-F). MDP-MSC administration was found to efficiently reduce the total number and rate of degranulated MCs to a greater extent (Fig 1.2D-F). These results indicate that subcutaneous administration with MDP-MSCs elicits their protective effect against AD symptoms by inhibiting the infiltration and degranulation of locally-acting MCs.


A
(-)
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Mast Cell Infiltration/field
(-)
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(-)
PBS
FB
PDS
MSC
MDP -MSC
31
Mast Cell Tryptase
37
GAPDH
Mast Cell Tryptase / GAPDH
(-)
DF
FB
PDS
MSC
MDP-MSC
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PBS
FB
PDS
MSC
MDP-MSC
E
Degranulation / field
(-)
Df
Fb
PDS
MSC
MDP-MSC
F
Degranulation rate (%)
(-)
Df
Fb
PDS
MSC
MDP-MSC

Figure 1.2. NOD2-activated hUCB-MSCs inhibit both infiltration and degranulation of mast cells.

(A) Paraffin-embedded sections of skin samples from AD mice were stained with toluidine blue and **(B)** the number of mast cells (indicated by arrows in black) was counted, Bar = 200 μm **(C)** Skin samples were lysed and detected for expression of mast cell tryptase. **(D)** Paraffin-embedded sections of skin samples from AD mice were stained with toluidine blue and **(E)** the number of degranulating or degranulated mast cells (indicated by the arrow in black) was counted, Bar = 100 μm **(F)** The rate of degranulating or degranulated mast cells among total mast cells were analyzed. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Results are shown as mean ± SD.

1.3.3 MDP-MSCs exert therapeutic effects against developed atopic dermatitis

I further examined whether MDP-MSCs could reduce already developed AD to prove that MSCs possess the therapeutic ability as well as preventive effect. To this purpose, I performed the single subcutaneous injection of MSCs after the development of AD (Fig 1.3A). Interestingly, MSC injection significantly ameliorated the symptoms of induced AD, analyzed by the evaluation of clinical severity, histopathology and MC degranulation (Fig 1.3B-F). In addition, MDP-MSC infusion further decreased the clinical severity and the epidermal hyperplasia (Fig 1.3B-E). Taken together, these findings suggest that subcutaneously administered MDP-MSCs can not only limit the development of AD, but also alleviate the developed AD.

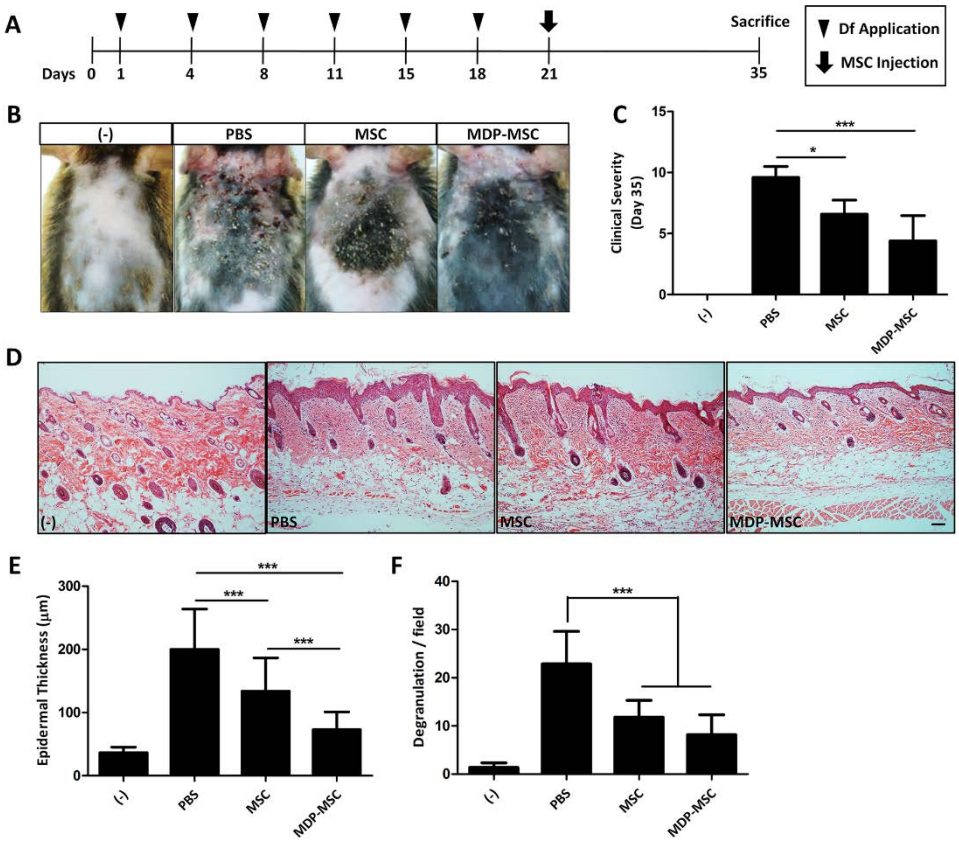

Figure 1.3. NOD2-activated hUCB-MSCs reduce developed murine atopic dermatitis.

(A-C) Atopic dermatitis was induced by the application of *Dermatophagoides farinae* (*Df*). Mice were subcutaneously (s.c.) injected with 2×10^6 hUCB-MSCs. **(A)** Outline for induction of atopic dermatitis and MSC injection. **(B-C)** Clinical severity was evaluated. **(D)** Skin sections were stained for histological analysis, Bar = 200 μm **(E)** Epidermal thickness was measured. **(F)** The number of degranulating or degranulated mast cells was counted. Five mice/group were used. * $P<0.05$, *** $P<0.001$. Results are shown as mean ± SD.

1.3.4 MDP-MSCs prevent the degranulation of MCs through the activation of NOD2 signaling to COX-2 in response to MDP

I further investigated whether MDP-MSCs could suppress the degranulation of MCs in vitro. Human umbilical cord blood-derived MCs were used that composed of more than 95% MC tryptase-positive cells (Fig 1.4A). MSCs or MDP-MSCs were added at either MC sensitization with IgE or MC challenge with anti-IgE and the suppressive effect of MSCs on MC degranulation was determined within the range of 1:100 to 1:1 based on the MSC:MC ratio. MSCs exerted the maximum inhibitory effect on MC degranulation at a ratio of 1:10 (Fig 1.4B-C). Additionally, the point of MSC addition at either MC sensitization or challenge did not have a significant influence on the inhibitory effect of MSCs on MC degranulation (Fig 1.4D). Therefore, I determined the 1:10 cell ratio and added MSCs at the MC challenging point for further experiments. The rate of MC degranulation was significantly reduced by co-culture with MSCs but not with FBs (Fig 1.4E). Remarkably, when MCs were co-cultured with MDP-MSC, this inhibitory effect was enhanced (Fig 1.4E). To verify the underlying mechanisms of this effect exerted by MDP-MSCs, I down-regulated well-known soluble factors produced by MSCs using selective inhibitors or siRNAs. The down-regulation of soluble factors such as indoleamine-2, 3-dioxygenase-1 (IDO-1), nitric oxide (NO) and TGF-β1 did not affect the inhibitory effect of MDP-MSCs on MC degranulation, whereas COX-2-mediated inhibition of PGE2 production significantly abolished this effect (Fig 1.4F). Moreover, the inhibitory effect of MDP-MSCs was impaired when NOD2 or its adaptor protein, RIP2, were down-regulated by specific siRNA transfection (Fig 1.4G). As was shown in previous

study (Kim et al., 2013), MDP stimulation on MSCs significantly enhanced the expression of COX-2 and secretion of PGE_2 by MSCs (Fig 1.4H-I). Furthermore, down-regulation of NOD2, RIP2 and COX-2 expression by siRNA inhibited MDP-induced COX-2 expression and the production of PGE_2 (Fig 1.4H-I). A previous study revealed that multiple PGE_2 receptors (EP receptors) on MCs differentially regulate the response of MCs by PGE_2 stimulation (Feng et al., 2006). Because MDP-mediated PGE_2 production in MDP-MSCs exerted an enhanced inhibitory effect on MC degranulation, I examined whether those multiple EP receptors played different roles using antagonists for each receptor. The inhibitory effect of MDP-MSCs on MC degranulation was diminished by the addition of antagonists for the EP2 and EP4 receptors, whereas antagonists for EP1 and EP3 receptors did not exert any effect (Fig 1.4J). Taken together, these data support the model that MDP-MSCs suppress MC degranulation by producing PGE_2 via NOD2-RIP2-COX2 signaling.

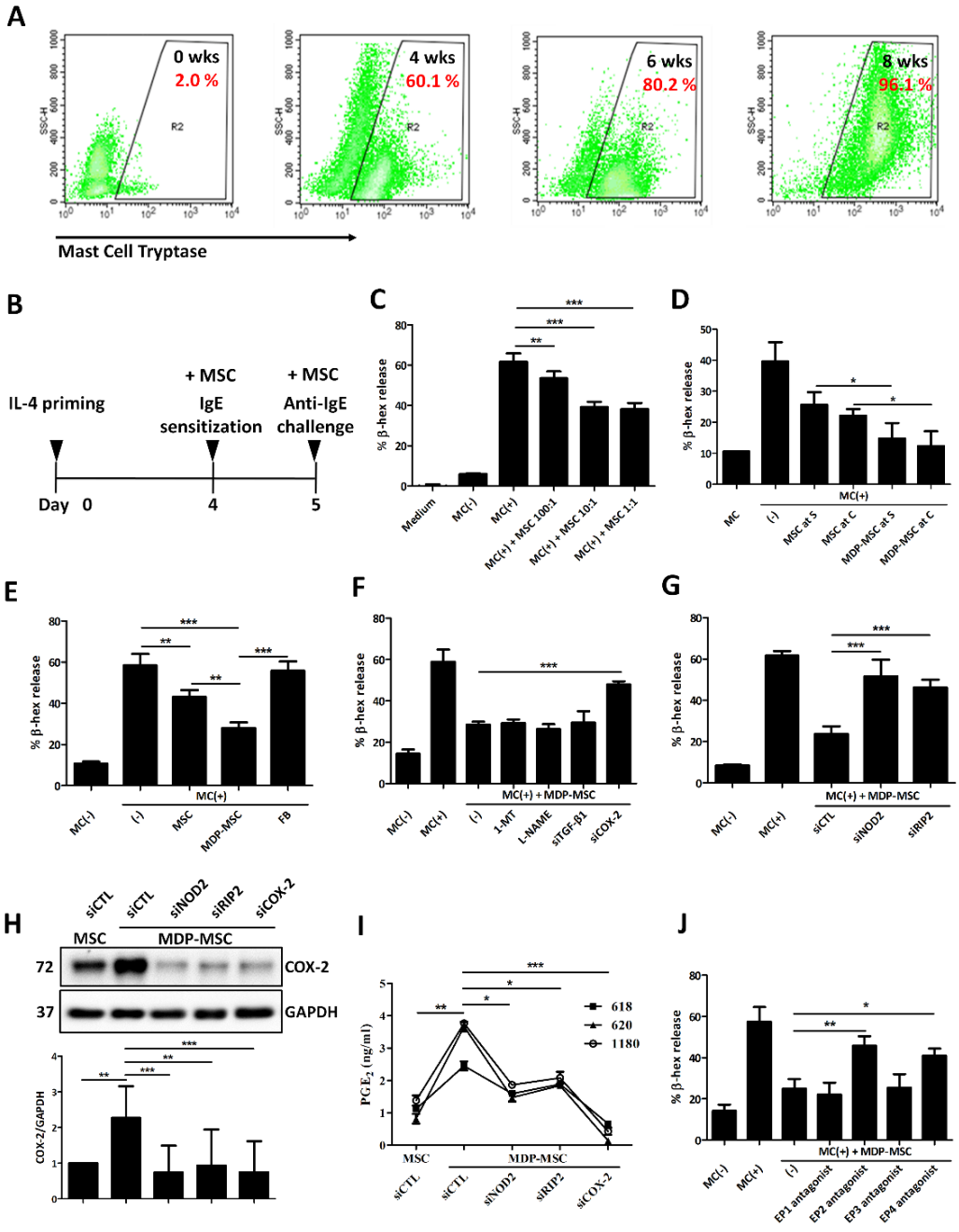

Figure 1.4. NOD2-activated hUCB-MSCs inhibit MC degranulation via NOD2 signaling.

Figure 1.4. NOD2-activated hUCB-MSCs inhibit MC degranulation via NOD2 signaling.

(A) Humans MCs derived from hUCB were analyzed for the expression of MC tryptase by flow cytometry. Purity of MC tryptase-positive cells was determined at 0, 4, 6 and 8 weeks after the induction of MC enrichment. **(B)** Outline for induction of MC degranulation and MSC addition **(C)** Different ratios of MCs and MSCs were co-cultured and β-hexosaminidase (β-hex) release was detected. **(D)** MSCs or MDP-MSCs were added at either MC sensitization with IgE or MC challenge with anti-IgE and the suppressive effect of MSCs on MC degranulation was determined by measuring β-hexosaminidase (β-hex) release. S; sensitization, C; challenge. **(E)** MCs were co-cultured with MSCs, MDP-MSCs or fibroblasts and β-hex release was detected. **(F-G)** MCs were co-cultured with MDP-MSCs after inhibition of **(F)** secretary factors or **(G)** NOD2 and RIP2, and β-hex release was measured. **(H-I)** MSCs were treated with MDP and siRNA for NOD2, RIP2 and COX-2, followed by analysis of **(H)** COX-2 expression and **(I)** PGE_2 production. **(J)** Specific EP receptor-blocked MCs were co-cultured with MDP-MSCs and β-hex release was detected * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Results are 1 representative experiment of 3 or the cumulative of 3 independent experiments. Results are shown as mean ± SD.

1.3.5 UCB-MSCs efficiently inhibit MC degranulation independently of cell-to-cell contact through higher production of PGE_2 than BM-MSCs

Brown et al. reported that the suppression of MC activation by bone marrow-derived MSCs (BM-MSCs) is impaired when MCs are cultured using a transwell system or conditioned medium (CM) from BM-MSCs (Brown et al., 2011). Therefore, I investigated whether the inhibitory effect of UCB-MSCs or MDP-treated UCB-MSCs was maintained when the cell-to-cell contact was absent. Interestingly, UCB-MSCs exerted the inhibitory effect on MC degranulation in co-culture using transwell and MC culture with CM from UCB-MSCs (Fig 1.5A). Furthermore, the suppressive effect was enhanced when MCs were cultured with MDP-MSCs or CM from MDP-MSCs (Fig 1.5A). Down-regulation of NOD2, RIP2 and COX-2 by siRNA reduced the inhibitory effect of MDP-MSCs in transwell co-culture (Fig 1.5B). In contrast, neither BM-MSCs nor MDP-stimulated BM-MSCs suppressed the MC degranulation when co-cultured using the transwell (Fig 1.5C). Because these results suggested that the suppressive effect of UCB-MSCs on MC degranulation in the transwell system was still mediated by NOD2-COX-2 signaling, I measured the basal production of PGE_2 from UCB-MSCs and BM-MSCs. BM-MSCs secreted significantly lower levels of PGE_2 than UCB-MSCs (Fig 1.5D). More interestingly, while UCB-MSCs produced higher levels of PGE_2 in response to MDP, PGE_2 secretion from BM-MSCs was not enhanced by MDP treatment but elevated by LPS treatment (Fig 1.5E-F). However, PGE_2 secretion level of LPS-stimulated BM-MSCs was

still lower than that of un-stimulated UCB-MSCs (Fig 1.5D-E). These findings indicate that UCB-MSCs can maintain their suppressive effect on MC degranulation even when cell-to-cell contact is absent, unlike BM-MSCs, which need cell-to-cell contact to inhibit MC degranulation, and this distinction between two MSCs results from the level of PGE_2 secretion.

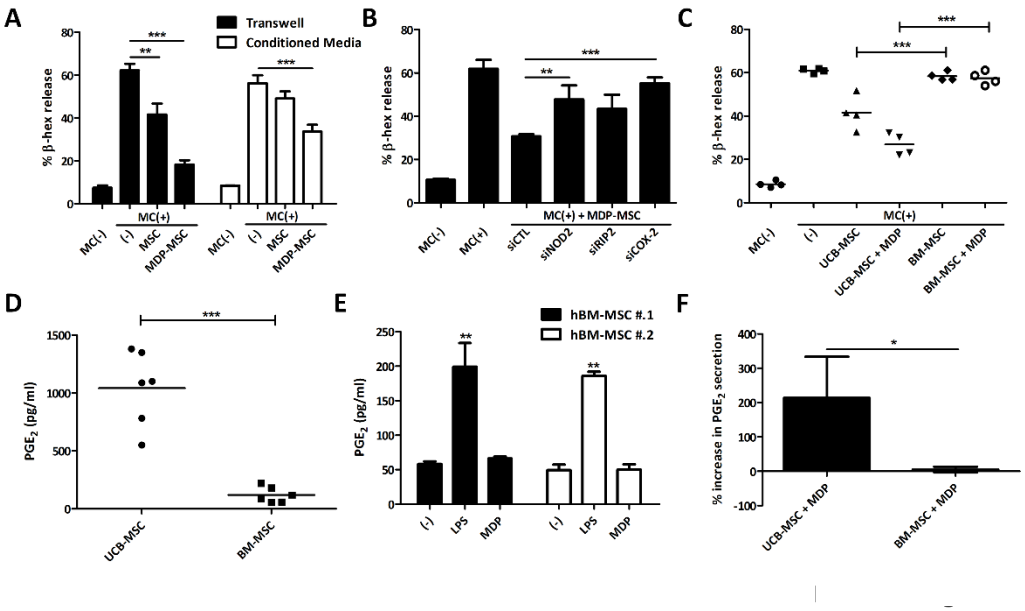

Figure 1.5. UCB-MSCs more effectively suppress MC degranulation than BM-MSCs through the higher production of PGE_2.

Figure 1.5. UCB-MSCs more effectively suppress MC degranulation than BM-MSCs through the higher production of PGE_2.

(A) MCs were cultured with MSCs in transwell system or cultured with MSC-derived conditioned media, and β-hex release was measured. **(B)** NOD2-, RIP2- and COX-2-inhibited MSCs were added to MCs using transwell and β-hex release was measured. **(C)** MCs were co-cultured with UCB-MSCs or BM-MSCs using transwell and β-hex release was detected. **(D-F)** PGE_2 production from MSCs or ligand-treated MSCs was measured. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Results are 1 representative experiment of 3 or the cumulative of 3 independent experiments. Results are shown as mean ± SD.

1.3.6 The suppressive effect of MDP-MSCs on MC degranulation is improved when co-culture is conducted from the beginning of MC priming, and this improvement is mediated by TGF-β1

Since I have found in earlier experiments by me and my colleagues that MDP-MSCs still exerted their inhibitory effect on MC degranulation in a transwell system, I could specifically control the period of co-culture by adding or withdrawing the upper chamber in which MSCs are plated (Fig 1.6A). Therefore, I next explored whether the period of culturing MCs with MDP-MSCs was critical for the suppressive effect. As I showed earlier, the point of MDP-MSC addition at MC sensitization or challenge did not result in any difference on the degranulation-inhibiting effect, whereas MDP-MSC addition at MC priming further improved this effect (Fig 1.6B-C). Moreover, while the down-regulation of COX-2 nearly abolished this effect when MDP-MSCs were added at MC challenge, COX-2 inhibition partially restored the effect when MDP-MSCs were added at MC priming, implying that other soluble factors might be involved in the co-culture that was conducted at MC priming (Fig 1.6D). To find the contributing factors, I inhibited several factors using specific inhibitors or siRNAs. Interestingly, when MDP-MSCs were added at MC priming, down-regulation of TGF- β1 led to a loss in degranulation-inhibiting effect of MDP-MSCs to an extent similar to COX-2 inhibition (Fig 1.6E). Furthermore, the simultaneous inhibition of COX-2 and TGF-β1 abolished the suppressive effect of MDP-MSCs (Fig 1.6F).

To verify the precise mechanism of COX-2 and TGF-β1 in regulating MC

degranulation, either COX-2 or TGF-β1 was down-regulated at a specific period of MC degranulation, from priming to sensitization (α), from sensitization to challenge (β), from challenge to termination of degranulation (γ), by substituting normal MDP-MSCs for siRNA-transfected MDP-MSCs during the intended period. Particularly, COX-2 inhibition led to a loss of the inhibitory effect only when it was inhibited during the γ period, and TGF-β1 inhibition decreased the inhibitory effect during the α period (Fig 1.6G-H). These results prove that co-culture period-specific action of PGE_2 and TGF-β1 from MDP-MSCs is required for the complete prevention of MC degranulation.

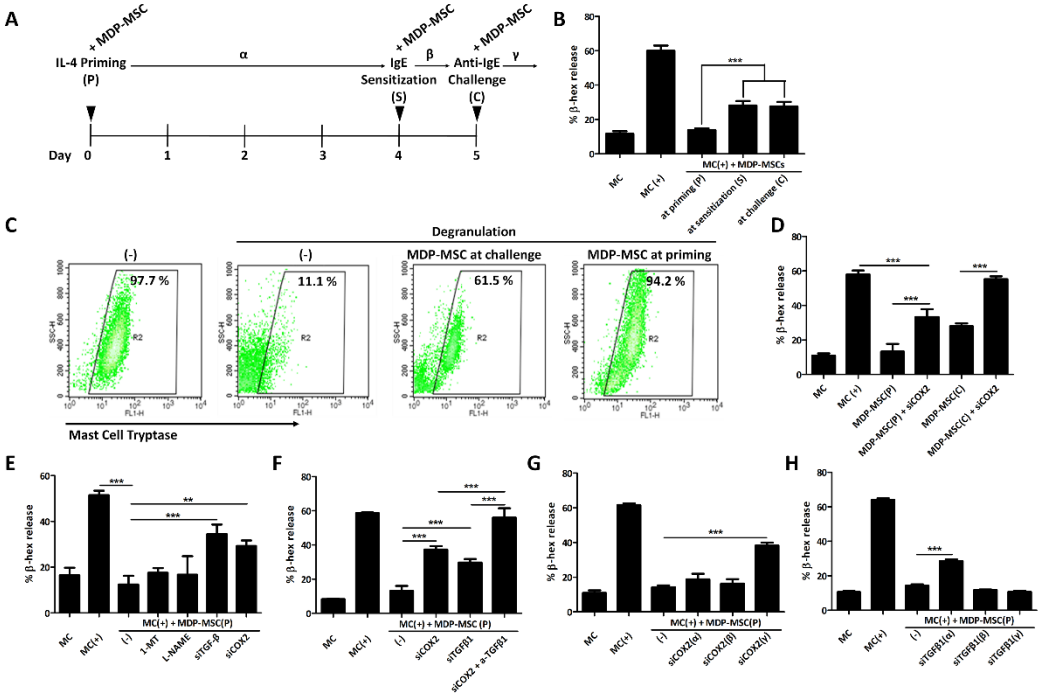

Figure 1.6. The suppressive effect of NOD2-activated hUCB-MSCs is co-culture period-dependent and is mediated by TGF-β1.

Figure 1.6. The suppressive effect of NOD2-activated hUCB-MSCs is co-culture period-dependent and is mediated by TGF-β1.

(A) Outline for induction of MC degranulation and MDP-MSC addition in transwell system. **(B-C)** MDP-MSCs were added to MCs at different time point of degranulation. **(B)** β-hex release was detected and **(C)** MC tryptase-positive cells were measured. **(D)** COX-2-inhibited MDP-MSCs were added to MCs at priming or challenge and β-hex release was detected. **(E-F)** MCs were co-cultured with specific secretary factor-inhibited MDP-MSCs from the beginning of degranulation induction and β-hex release was detected. **(G-H)** COX-2 or TGFβ1 was inhibited in specific period of MC-MSC co-culture and β-hex release was detected. ** $P<0.01$, *** $P<0.001$. Results show 1 representative experiment of at least 3. Results are shown as mean ± SD.

1.3.7 TGF-β1 production from MSCs is stimulated by IL-4 via activation of STAT6 signaling and is responsible for down-regulation of FcεRI expression in MCs

As stimulation through the IL-4 receptor pathway is known to result in increased TGF-β1 production from certain immune cells and BM-MSCs (Elovic et al., 1998; Nemeth et al., 2010), I then assessed whether IL-4, a crucial cytokine for MC priming, could regulate TGF-β1 production from UCB-MSCs. Interestingly, although MDP treatment did not enhance TGF-β1 production from UCB-MSCs, IL-4 treatment up-regulated the secretion of TGF-β1 (Fig 1.7A). The IL-4 receptor has been reported to activate STAT6 signaling (Takeda et al., 1996). Therefore, to investigate if IL-4 treatment enhances TGF-β1 production from UCB-MSCs through the activation of the STAT6 signaling pathway, I determined the phosphorylation of STAT6. As expected, IL-4, but not MDP, rapidly induced the phosphorylation of STAT6 in UCB-MSCs (Fig 1.7B). Gomez et al. reported that TGF-β1 inhibits the FcεRI expression of MCs, a critical component for IgE-mediated MC degranulation (Gomez et al., 2005). To see if TGF-β1 secreted by UCB-MSCs regulates the expression of FcεRI in MCs, I analyzed the expression level of the FcεRI protein after culturing MCs with UCB-MSCs in the presence of IL-4. IL-4 treatment increased the expression of FcεRI α, and interestingly, co-culture with UCB-MSCs significantly inhibited IL-4-mediated up-regulation of FcεRI expression in MCs (Fig 1.7C). Moreover, the inhibitory ability of UCB-MSCs on FcεRI expression was reduced by TGF-β1 inhibition, whereas this ability was not affected by COX-2 inhibition or MDP treatment

in UCB-MSCs (Fig 1.7C and 1.8A). To explore whether basal secretion of TGF-β1 from UCB-MSCs is sufficient to suppress FcεRI expression on MCs, I further determined the expression of FcεRI after culturing IL-4-pretreated MCs with UCB-MSCs in the absence of IL-4. While UCB-MSCs slightly reduced the FcεRI expression in MCs, IL-4-pretreated MSCs significantly suppressed the expression of these receptors (Fig 1.7D and 1.8B). Taken together, these findings indicate that IL-4 stimulation on UCB-MSCs through STAT6 signaling pathway is crucial for the TGF-β1-mediated inhibitory effect on MC degranulation.

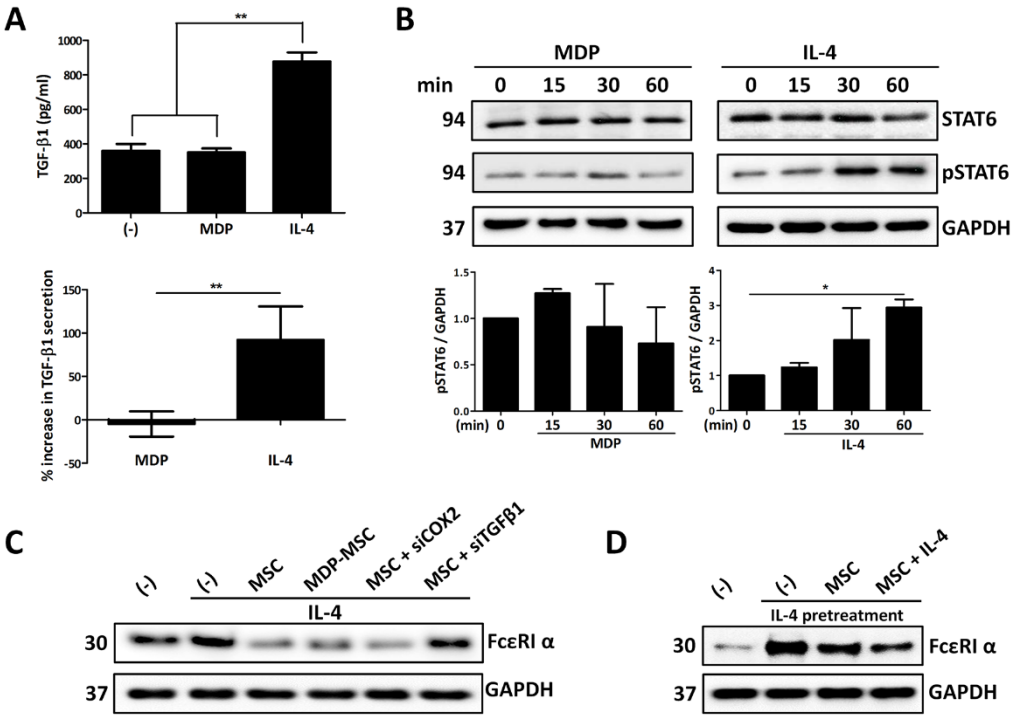

Figure 1.7. hUCB-MSCs produce TGF-β1 upon IL-4 stimulation to regulate the FcεRI expression in MCs.

(A) TGF-β1 production of MSCs in response to MDP or IL-4 was detected. **(B)** STAT6 phosphorylation was detected by immunoblotting. **(C)** FCεRI expression in MCs was determined after co-culture with MSCs in the presence of IL-4. **(D)** IL-4-pretreated MCs were co-cultured with naïve or IL-4-pretreated MSCs and FCεRI expression was detected. * P<0.05, ** P<0.01. Results are 1 representative experiment of 3 or the cumulative of 3 independent experiments. Results are shown as mean ± SD.

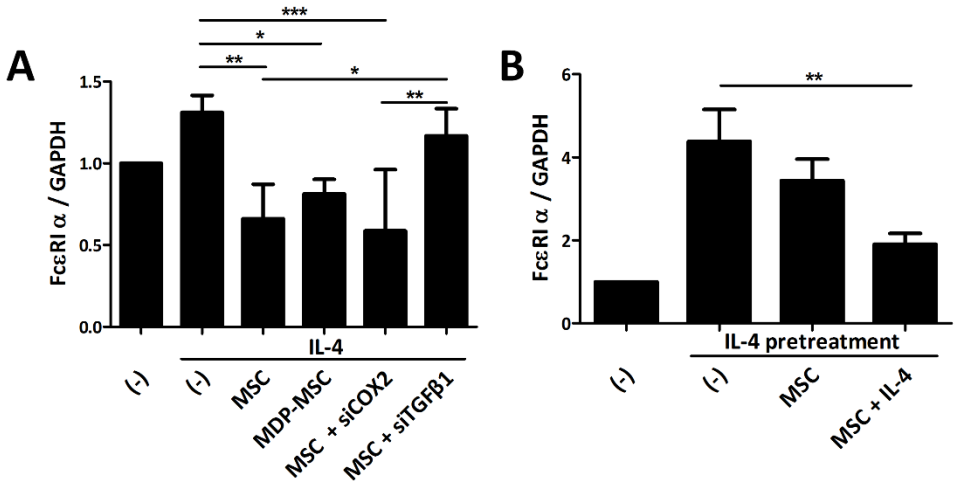

Figure 1.8. Quantification of protein expression in mast cells.

(A-B) Protein expression in MSCs or MCs assessed by immunoblotting was quantified for statistical analysis. **(A)** Quantification of FCεRI expression in MCs (Fig 1.7C). **(B)** Quantification of FCεRI expression in MCs (Fig 1.7D). * P<0.05, ** P<0.01, *** P<0.001. Results are the cumulative of 4 independent experiments. Results are shown as mean ± SD.

1.4 DISCUSSION

Recently, a number of studies have reported that the immunomodulatory ability of MSCs can be usefully applied for the treatment of allergic diseases (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). The majority of these studies assessed the therapeutic efficacy in animal models by determining the systemic inflammatory milieu after intravenous administration of MSCs. None of these studies explored the discrepancies in efficacy that could be exerted by the injection route of cells. Furthermore, because I and my colleagues have recently shown that NOD2 is functionally expressed in hUCB-MSCs and that NOD2 activation by its ligand, MDP, enhances the protective effect of hUCB-MSCs against experimental colitis (Kim et al., 2013; Kim et al., 2010a), I sought to assess the efficacy of intravenously or subcutaneously injected NOD2-activated MSCs (MDP-MSCs) against *Df*-induced AD in mice.

The intravenous application of MDP-MSCs led to higher therapeutic efficacy compared to unstimulated-MSCs administration. More interestingly, subcutaneous application of MDP-MSCs further improved this effect. Particularly, the subcutaneous injection of MDP-MSCs abrogated the histological damages associated with *Df*-induced AD to the extent that was observed in naïve mice. However, the subcutaneous administration of MDP-MSCs could not reduce the increased serum IgE level as the intravenous administration of MSCs or oral administration of PDS did. Since IgE

generation is a general marker for AD (Horan et al., 1992), these results suggest that the subcutaneous injection of MDP-MSCs ameliorate AD by regulating the allergic responses raised after IgE generation.

Itch is one of the severe clinical hallmarks of AD, and continuous scratching caused by itching may be involved in the acceleration of dermatitis symptoms (Boguniewiez, 2005; Proksch et al., 2006). MC degranulation and the release of mediators are known to elicit the itching response in AD (Steinhoff et al., 2003; Tsujii et al., 2009; Ui et al., 2006). Moreover, through the release of cytokines and mediators, mast cells recruit neutrophils, eosinophils and lymphocytes to the site of inflammation (Malaviya and Georges, 2002; Marshall, 2004). In this study, the subcutaneous administration of MDP-MSCs significantly reduced the scratching behavior of AD mice and the infiltration of lymphocytes and eosinophils into skin lesions. I further demonstrated that subcutaneously administered MDP-MSCs efficiently inhibited the infiltration and degranulation of MCs. These findings led me to focus on the interactions between MDP-MSCs and MC-mediated allergic responses.

It is well known that MSCs are capable of suppressing proliferation, maturation, and activation of immune cells through the secretion of soluble factors or cell-to-cell adhesion (Asari et al., 2009; Prigione et al., 2009; Ren et al., 2008b; Zhang et al., 2009b). However, only a few studies have shown the interaction between MSCs and MCs (Brown et al., 2011; Su et al., 2011). The main finding of these studies was that MSCs inhibited MC function via COX2-PGE_2-dependent mechanisms. My previous study showed that NOD2 activation of hUCB-MSCs resulted in the prolonged production of PGE_2. Consistent

with my previous work, in this study, MDP-MSCs suppressed IgE-mediated MC degranulation to a greater extent by secreting PGE_2 via the NOD2-RIP2-COX2 axis.

Human MCs are reported to express multiple EP receptors for PGE_2 that differentially regulate activation responses (Feng et al., 2006). This study revealed that PGE_2 could either induce the activation of MCs via EP3 receptors or suppress the activation via EP2 receptors. This finding was confirmed by the studies of Kay et al. (Kay et al., 2006) and Wang et al. (Wang and Lau, 2006), which showed that PGE_2 inhibited human lung mast cell degranulation by interacting with EP2 receptors and that PGE_2 potentiated the degranulation of human peripheral blood-derived mast cells through EP1/EP3 receptors. In this study, the blocking of EP2 or EP4 receptors on mast cells led to a reduction in the suppressive effect of MDP-MSCs on MC degranulation, suggesting that MSC-derived PGE_2 inhibits MC degranulation possibly by acting on the EP2 and EP4 receptors.

A previous study by Brown et al. (Brown et al., 2011) demonstrated that the suppression of MC activation by BM-MSCs was greatest when the cell-to-cell contact was allowed compared to transwell co-culturing. Moreover, BM-MSC conditioned medium did not exert any inhibitory effect on MC degranulation. However, I showed here that UCB-MSCs consistently exerted their suppressive effect on MC degranulation under both co-culture conditions. Furthermore, even UCB-MSC-derived CM exerted an inhibitory effect on MC degranulation. These findings led me to explore the candidate factors responsible for the discrepancy between BM-MSCs and UCB-MSCs. Interestingly, the basal PGE_2 level of UCB-MSCs was significantly higher than that of BM-MSCs. More interestingly, BM-MSC did not produce a higher level of PGE_2 in response to MDP. Taken together, it is

likely that UCB-MSC-derived PGE_2 is sufficient to exert its degranulation-inhibiting ability even when the cell-to-cell interaction is deficient.

Recently, it has been reported that canine adipose tissue-derived MSCs produce IL-10 and TGF-β1, which effectively ameliorate canine AD (Jee et al., 2013). In my previous study, although the co-culture of hUCB-MSCs with human mononuclear cells resulted in the elevation of IL-10 level in co-culture media, hUCB-MSCs themselves did not secrete sufficient levels of IL-10 to exert any immunoregulatory ability, regardless of their activation by ligands or cytokines (Kim et al., 2013). In this study, the neutralization of IL-10 in co-culture media did not have any influence on the function of MDP-MSCs to regulate MC degranulation (data not shown). Based on these findings, one can envision that hUCB-MSC-derived IL-10 is not associated with the MC-suppressing effect, at least in hUCB-MSCs. TGF-β1 has been known to inhibit mast cell function (Gebhardt et al., 2005; Gomez et al., 2005; Kashyap et al., 2005; Su et al., 2012). In my experimental conditions, the action of TGF-β1 was dependent on the co-culture period. When MDP-MSCs were added to MCs at challenge with anti-IgE, down-regulation of TGF-β1 did not affect the inhibitory ability. However, TGF-β1 was correlated with the additional inhibitory effect, which was exerted independently of COX-2, when MDP-MSCs were cultured with MCs from the beginning of priming with IL-4. Furthermore, TGF-β1 production from hUCB-MSCs was enhanced by IL-4 stimulation via STAT6 phosphorylation, which inhibited FcεRI expression in MCs.

Dendritic cells (DCs), as well as mast cells are known to express the FcεRI (Foster et al., 2003; Maurer et al., 1996). In contrast to mast cells, the regulatory mechanism of

FcεRI signaling in DC function is less understood. Interestingly, recent study reported that DCs contribute to a negative feedback mechanism to regulate allergic inflammation through IgE/FcεRI signaling (Platzer et al., 2014). Although several studies have shown the modulatory effect of MSCs on the maturation and activation of DCs (Chiesa et al., 2011; Zhang et al., 2009b), none have focused on the regulation of FcεRI expression in DCs by MSCs and its functional consequences in allergic inflammation. Therefore, further studies regarding the MSC-mediated alteration of FcεRI expression and its function in DCs could reveal novel mechanisms elucidating the interaction between DCs and MSCs in the developments of allergic inflammation.

In conclusion, the present study revealed novel information suggesting that the subcutaneous administration of hUCB-MSCs could be an efficient therapeutic alternative for AD, and that MDP-stimulated PGE_2 production and IL-4-stimulated TGF-β1 production from hUCB-MSCs are required to inhibit MC degranulation. These findings are expected to provide a better insight for the therapeutic approach to allergic disease using MSC-based cell therapy.

CHAPTER II

Systemic Delivery of Human Umbilical Cord Blood-derived Mesenchymal Stem Cells Ameliorates Rheumatoid Arthritis via Regulation of NLRP3 Inflammasome in Macrophages

2.1 INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease accompanied by progressive synovitis, bone and cartilage destruction leading to joint impairment as well as systemic complications. The pathogenesis of RA is complicated but the orchestrated interaction of abundant pro-inflammatory cytokine pathways and cellular components have been known to play an essential role in RA disease process. Frequently, RA is characterized by undesirable activation of T cells leading to abnormal production of autoantibodies against the portion of normal immunoglobulins, known as rheumatoid factors (RF). Subsequently, macrophages activated by autoantibody produce inflammatory cytokines, which contribute to the intense inflammatory responses leading to tissue damage and clinical manifestations (Firestein, 2003; McInnes and Schett, 2011). Therefore, current therapeutic strategies for the treatment of RA target these cytokines. Because tumor necrosis factor(TNF)-α plays a principal role in the pathogenesis of RA, anti-TNF-α biologic agents (etanercept, infliximab and adalimumab) have brought a dramatic clinical achievement in RA patients (Taylor, 2010). Moreover, blockade of interleukin (IL)-1 (anakinra) and IL-6 (tocilizumab) have been introduced because these cytokines are reported to be involved in the pathogenesis of RA (Koenders and van den Berg, 2015). However, despite widespread use of the targeted therapies, up to 50% of patients with RA still fail to respond adequately and these approaches may carry long-term side effects including serious infections and malignancies (Bongartz et al., 2006; Salliot et al., 2007). Therefore, there are clear unmet demands to develop safe and effective therapeutics without

the potential risk of complications.

Cell-based therapies utilizing mesenchymal stem cells (MSCs) have been spotlighted as a promising tool for the treatment of a wide range of immune-related diseases such as graft-versus-host disease, inflammatory bowel disease, multiple sclerosis and atopic dermatitis and rheumatoid arthritis (Glenn et al., 2014; Introna and Rambaldi, 2015; Kim et al., 2015a; Nagaishi et al., 2015). These therapeutic trials are based on the immunoregulatory capabilities of MSCs. Importantly, several groups have reported the active interaction between MSCs and various types of both innate and adaptive immune cells such as T lymphocytes, B lymphocytes, dendritic cells (DC) and natural killer (NK) cells (Corcione et al., 2006; Di Nicola et al., 2002; Sotiropoulou et al., 2006; Spaggiari and Moretta, 2013). Direct cell-to-cell contact and paracrine action by soluble factors have been reported to be crucial for the immunomodulatory ability of MSCs (English, 2013; Ma et al., 2014). Previous studies conducted by my own group revealed the anti-inflammatory effects of xenogeneic human umbilical cord blood-derived MSCs (hUCB-MSCs) against murine experimental colitis and atopic dermatitis (Kim et al., 2013; Kim et al., 2015a). However, the therapeutic efficacy and the mechanisms of action can be altered by the disease-related immunologic microenvironment or the manipulation of MSCs.

Although several groups have demonstrated the preventive and curative efficacy of MSCs in RA with different mechanisms of action, they have focused profoundly on the regulation of immunocompetent cells, mainly autoreactive T and B lymphocytes (De Bari, 2015; Gonzalez et al., 2009). More recently, accumulating evidence has shown that macrophages are responsible for exacerbation of inflammatory responses and collateral

damage in RA (Kinne et al., 2007). Indeed, macrophage produces core cytokines in RA pathogenesis including TNF-α and IL-1β (McInnes and Schett, 2007), targeted by current biologic medications. However, the underlying mechanisms by which MSCs regulate macrophage activation are relatively less well understood from a perspective of systemic immune homeostasis in response to RA-related inflammatory microenvironment.

Herein, the aim of this study was to investigate the therapeutic efficacy of systemic delivered hUCB-MSCs in murine model of collagen-induced arthritis (CIA). To verify the mechanistic action of their anti-inflammatory effects, I explored the possible mechanisms that hUCB-MSCs might modulate multiple macrophage responses in terms of homeostatic immune-balance.

2.2 MATERIALS AND METHODS

2.2.1 Isolation and culture of hUCB-MSCs

hUCB-MSCs were isolated, cultured *in vitro* and characterized as previously described. Briefly Fresh hUCB samples were obtained from Seoul City Borame Medical Center Cord Blood Bank after full-term delivery with written consent of the mother approved by the Institutional Review Board (IRB) of Borame Medical Center and Seoul National University (IRB No. 0603/001-002-07C1). hUCB-MSCs were prepared and verified as described previously (Seo et al., 2011)

2.2.2 CIA induction and treatment

Rheumatoid arthritis-like symptoms were induced by intradermal immunizations of bovine type II collagen (CII, Chondrex) in DBA1/J mice as previously described (Brand et al., 2007). Briefly, 100 μg of CII was emulsified with an equal volume of complete Freund's adjuvant (CFA, Sigma Aldrich). Mice were primary-immunized with CII emulsion at the basis of the tail, followed by a boosting immunization on day 21 according to the same preparation and method. Clinical severity of CIA was assessed by double-blind monitoring every 2-3 days using the macroscopic scoring system as established previously (Delgado et al., 2001). All experimental procedures were approved by the Institute of Laboratory Animal Resources of Seoul National University (Approval no.; SNU-151203-2) and completed in compliance with the approved guidelines.

To evaluate the curative effect, the treatment was begun after the onset of disease when arthritis score reached 3 or more. Mice with established CIA were injected intraperitoneally (i.p.) daily for 5 days with 10^6 cells of hUCB-MSCs or with 100μg of etanercept as a drug control. Human dermal fibroblasts (FB) were infused as a cell control. Alternatively, other mice were given a single intravenous (i.v.) injection of 10^6 hUCB-MSCs at the same phase of disease progression. After euthanasia on day 49, blood and major organs were collected for cytokine analysis and histopathological evaluation.

2.2.3 Histopathologic evaluation

Formalin-fixed limb samples were decalcified with 10% formic acid, subsequently decalcified limbs were processed and paraffin-embedded according to the standard histologic procedures. Serial sections of 5 μm thickness were prepared and stained with hematoxylin and eosin (H&E) and then were assessed microscopically for the extent of inflammation and joint destruction according to the guidelines previously reported ; 0 = normal synovium, 1 = synovial membrane hypertrophy and cell infiltrates, 2 = pannus and cartilage erosion, 3 = major erosion of cartilage and subchondral bone, and 4 = loss of joint integrity and ankyloses (Nishikawa et al., 2003). Sections were stained with safranin O or toluidine blue for the intensified evaluation of cartilage destruction.

2.2.4 Generation and stimulation of macrophages

hUCB-derived primary macrophages were isolated and differentiated as previously described (Kang et al., 2015). Briefly, mononuclear cells (MNCs) were firstly isolated from hUCB, followed by the separation of $CD14^+$ monocytes using CD14 Microbeads (Milteny Biotech). To generate macrophages, monocytes were incubated for 7 days in six-well cell culture plates at a density of $10^5/cm^2$. Cells were refed every 3 days and the adherent cells were cultured in fresh macrophage induction (Fig 2.1). At day 7, the adherent cells were stimulated with 1 μg/ml lipopolysaccharide (LPS, Invivogen) plus 20 ng/ml interferon gamma (IFN-ɣ, PeproTech) at 37°C for 48 hours for activation of macrophages.

THP-1 cells, human acute monocytic leukemia cell line, were purchased from American Type Culture Collection (ATCC). To differentiate to macrophage-like cells, THP-1 cells were seeded in six-well plates at a density of $10^5/cm^2$ and were treated with 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) for 48 hours, followed by stabilization with fresh RPMI-1640 medium (Gibco) without PMA for additional 5 days (Fig 2.1). At day 5, fully differentiated macrophage-like cells were treated with LPS and IFN-ɣ for 48 hours for classical M1 activation. Alternatively, macrophages were primed with 1 μg/ml of LPS for 4 hours, followed by stimulation with 5 μM of nigericin (Invivogen) for 45 minutes for activation of NLRP3 inflammasome.

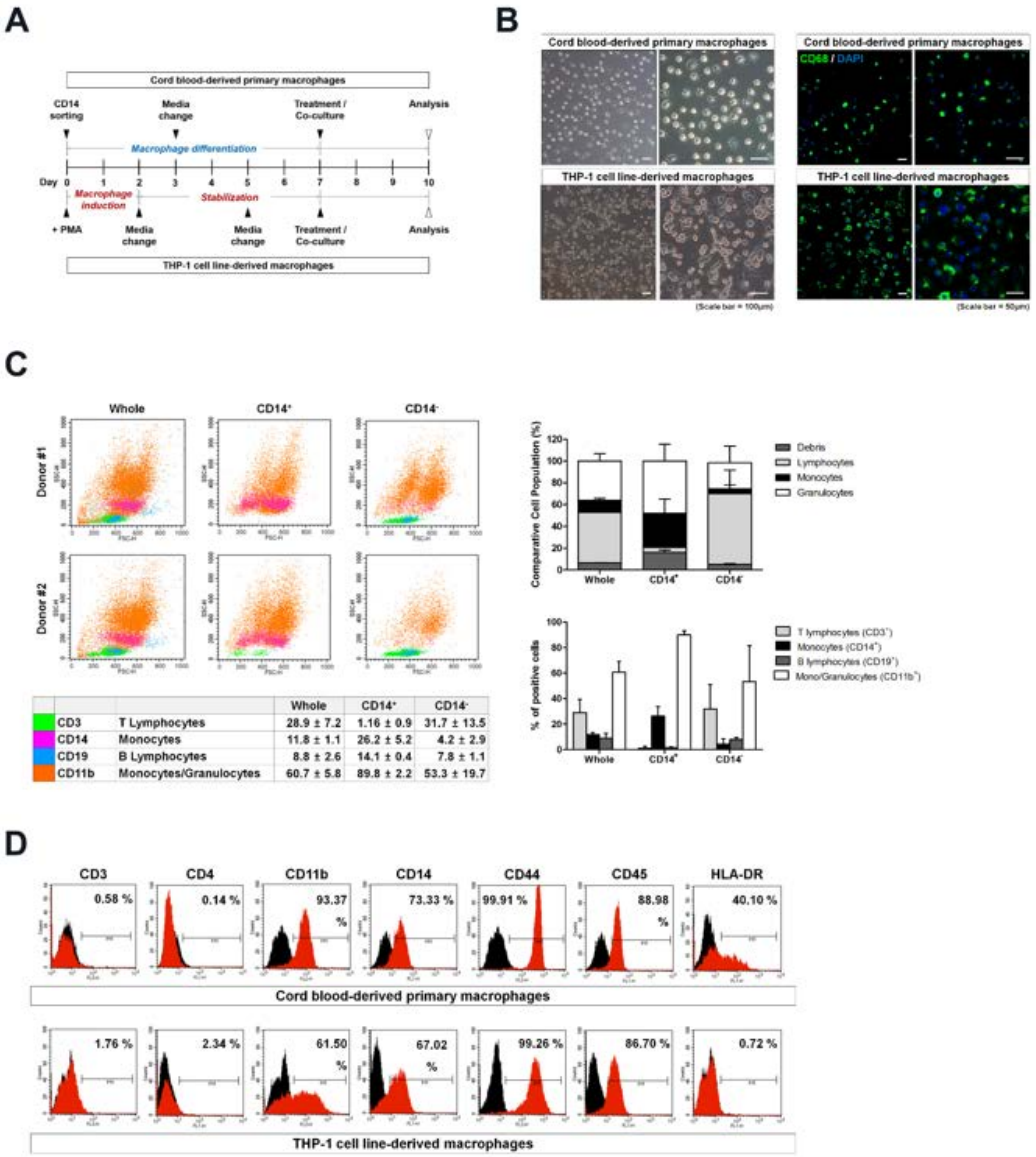

Figure 2.1. Differentiation and characterization of human umbilical cord blood- and THP-1 cell line-derived macrophages

Figure 2.1. Differentiation and characterization of human umbilical cord blood- and THP-1 cell line-derived macrophages

(A) Correlation between human umbilical cord blood- (hUCB-) and THP-1 cell-derived macrophages was established by following protocols. **(B)** Representative phase-contrast images of fully differentiated macrophages, scale bar = 100 μm. Expression level of CD68, a typical lineage marker of the macrophage, was confirmed by immunocytochemistry (ICC), scale bar = 50 μm. **(C)** Identification of various immune cell populations in human umbilical cord blood-derived mononuclear cells and purity of CD14-sorted monocyte/macrophage populations was evaluated by flow cytometry with two different donors. Quantification of comparative immune cell populations including lymphocytes, granulocytes and monocytes was determined by flow cytometry with specific lineage markers. **(D)** Representative surface marker expressions of fully differentiated macrophages from both cord blood and THP-1 was analyzed by flow cytometry. Results are one representative experiment of three or the cumulative of at least three independent experiments. Results are shown as mean ± SD.

2.2.5 Flow cytometric analysis

Flow cytometry was analyzed using a FACS Calibur and the Cell Quest software (BD Biosciences). For characterization of macrophages obtained from hUCB and THP-1, fully differentiated macrophages were harvested and reconstituted with PBS. Macrophages were pretreated with Fc receptor blocking reagent (Milteny Biotech) for 10 min, followed by staining with fluorescent-conjugated antibodies specific for CD3, CD4, CD11b, CD14, CD44, CD73, CD105, CD206 and HLA-DR or respective isotype controls (BD Bioscience) for 1 hour at 4°C in the dark. To assess the M2 polarization, resting macrophages were detached after co-culture with hUCB-MSCs in direct or traswell condition for 48 hours. Subsequently, cells were stained with FITC-conjugated anti-CD14 and with either PE-conjugated anti-CD206 or PE-conjugated anti-CD36 (BD Bioscience).

2.2.6 Cytokine profile

Classically activated M1 macrophages were co-cultured with hUCB-MSCs in direct or transwell for 48 hours and culture supernatant was harvested. Secretion of TNF-α and IL-10 from macrophages was measured using commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D systems). Also, IL-1β and active caspase-1 production in response to NLRP3 stimulation in supernatant was quantified by a commercial ELISA kit (R&D systems).

2.2.7 Patients' samples and *ex vivo* experiments

Peripheral blood and serum samples were obtained from either patients diagnosed with RA or healthy controls. Diagnosis of RA was defined by the 2010 American College of Rheumatology-European League Against Rheumatism (ACR-EULAR) classification criteria and fundamental information and clinical characteristics were shown in Table 4. All the protocols with patient samples were approved by the Institutional Review Board of Borame Medical Center (IRB No. 20131226/16-2013-175-011), and all participants gave written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated and cultured *ex vivo* as previously described (Mathews et al., 2014).

2.2.8 Reagents

Bovine type II collagen (CII) was purchased from Chondrex, Inc. (Redmond, WA) and Freund's adjuvant was purchased from Sigma-Aldrich (St. Louise, MO). Phorbol 12-myristate 13-acetate (PMA) and celecoxib were purchased from Sigma-Aldrich. NLRP3 inflammasome inducers including LPS-EB from E.coli 0111:B4, nigericin and adenosine triphosphate (ATP) were obtained from Invivogen (San Diego, CA). Recombinant human IFN-ɣ, TNF-α and IL-1RA were purchased form PeproTech (Rocky Hill, NJ). Antibodies include anti-CD14-fluorescein isothiocyanate (FITC), anti-CD206-phycoerythrin (PE), anti-CD36-PE and anti-CD86-PE-Cy5.5, all of them were purchase from BD Bioscience (San Jose, CA).

2.2.9 Cell tracking

To trace the migration and distribution of infused cells in vivo, hUCB-MSCs were labeled with 10 μmol/L CFSE (Molecular Probes) according to the manufacturer's recommendation. CFSE-labeled cells were injected intravenously. On 3 days after injection, 10 μm thickness of frozen joint sections were cut and examined for green fluorescence using confocal microscope. To evaluate the hMSC concentration, real-time qPCR was performed with human Alu specific primers in major organs of injected mice. DNAs were isolated from spleen, heart, lung, kidney, liver and joint and concentration of hMSCs in each sample was analyzed according to the method reported previously (Lee et al., 2015).

2.2.10 RNA interference

hUCB-MSCs were transfected with siRNA for TSG-6 (Santa Cruz Biotechnology, Santa Cruz, CA) or control siRNA (Dharmacon) when they had reached 50-60% confluence according to the manufacturer's instructions. Experiments were performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) as a transfection reagent. To confirm successful knockdown of TSG-6 expression, RNA and protein were extracted at 48 hr after the start of transfection and analyzed by RT-PCR and western blotting. At the same time period, transfected hUCB-MSCs were detached and co-cultured with macrophages for another 2 days.

2.2.11 Western blot

hUCB-MSCs were cultured with addition of selective inhibitors for 24 hours and whole cell lysates were harvested with a protein lysate buffer containing 1% Nonidet-P40 supplemented with a complete proteases inhibitor cocktail (Roche, Basel, Switzerland), and 2 mmol/L dithiothreitol. Lysates were loaded to 12% and 15% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transferred to nitrocellulose membranes. Immunoblotted with primary antibodies including COX-2, inducible NO synthase (Abcam, Cambridge, MA), TSG-6 (R&D systems), α-tubulin and glyceraldehyde-3-phosphatate dehydrogenase (Millipore, Billerica, CA). After incubation with secondary antibodies, protein expressions were detected by enhanced chemiluminescence reagent (GE Healthcare Life Science).

2.2.12 Statistical analysis

In vitro data were presented as mean ± standard deviation (SD) from at least three independent experiments. Variables were analyzed using one-way ANOVA followed by the Bonferroni post-hoc test for multi-group comparisons using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA) and p-values less than 0.05 were defined as statistically significant. Spearman's correlation analysis was performed to determine correlations between cytokine levels from the serum and PBMC and the clinical severity of RA patients using SPSS software (IBM). Statistical significance expressed as asterisks is indicated in the figure legends.

Table 4. Fundamental profile of patients with RA (n=15).

	RA patients
Demographics	
Age, years	56.1 (12.3)
Gender, % female	60
Clinical Characteristics	
Rheumatoid factor and/or anti-CCP, % positive	86.7
Disease duration, years	7.0 (11.6)
Patients global assessment of disease activity score, 0-10	4 (2.5)
Routine assessment of patient index data with 3 measurement score	9.1 (5.5)
Erythrocyte sedimentation rate, mm/h	46.3 (33.3)
C-reactive protein, mg/dL	1.7 (1.4)
Prescription history	
Naïve to treatment, %	26.7
Prednisolone (PDS), %	66.7
PDS dose, mg/week	4.8 (1.8)
Methotrexate (MTX), %	60
MTX dose, mg/week	13.9 (1.7)
Combined PDS with MTX, %	53.3

2.3 RESULTS

2.3.1 Systemic delivery of hUCB-MSCs exerts therapeutic effect against mouse CIA model

I and my colleagues previously demonstrated the therapeutic effect of hUCB-MSCs against experimental colitis and atopic dermatitis, and revealed corresponding underlying mechanisms (Kim et al., 2013; Kim et al., 2015a). In this study, I first investigated whether the therapeutic potential of hUCB-MSCs could be reproducible in autoimmune arthritis using CIA model which shares many immunologic, histologic and clinical similarities to RA (Bevaart et al., 2010) and whether the route and the frequency of administration could alter the potency of this effect. Multiple intraperitoneal injection of hUCB-MSCs strongly ameliorated the clinical severity of CIA, as compared with the untreated and FB-injected mice. Interestingly, hUCB-MSCs showed a similar extent of therapeutic effect in comparison with the etanercept, a TNF-α antagonist (Fig 2.2B). Upon histologic evaluation, reduced synovitis and articular destruction were observed in hUCB-MSCs- and etanercept-treated mice (Fig 2.2C). To verify the effect of hUCB-MSCs on the production of inflammatory cytokines closely associated with pathogenesis of CIA, serum level of TNF-α was determined. The concentration of serum TNF-α was increased by CIA induction and its level was remarkably decreased by the application of hUCB-MSCs and etanercept, whereas the infusion of FB did not significantly suppress TNF-α secretion (Fig 2.2D).

Given that several studies have reported that a single intravenous injection of MSCs could sufficiently exhibit the protective and curative effect against CIA (Chen et al., 2013), I further confirmed the efficacy of single injection (Fig 2.3A). As expected, a single intravenous injection of hUCB-MSCs significantly attenuated the symptoms of arthritis (Fig 2.3B). Histologic damages including pannus formation, synovitis and cartilage destruction were markedly alleviated by hUCB-MSC compared to non-treated control (Fig 2.3C). Serum level of TNF-α, IL-1β and IL-6 was generally down-regulated by the infusion of hUCB-MSCs (Fig 2.3D). Among these inflammatory cytokines, IL-6 level was significantly decreased. All mice treated with hUCB-MSCs did not show any side effects and lethality until sacrifice.

Altogether, these findings demonstrate that systemic administration of hUCB-MSCs can exert the significant therapeutic effects against CIA without any noteworthy adverse effects.

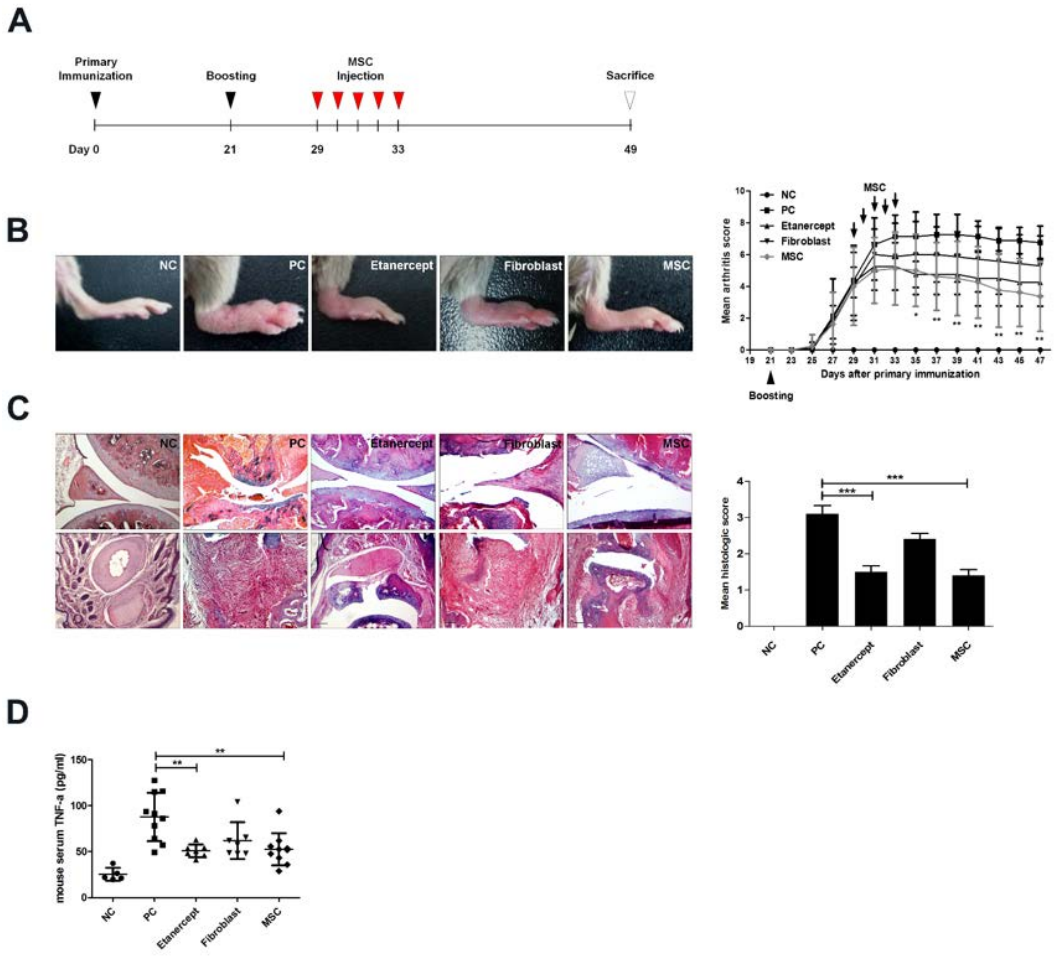

Figure 2.2. Intraperitoneal injection of hUCB-MSCs markedly ameliorates the deterioration of experimental arthritis

Figure 2.2. Intraperitoneal injection of hUCB-MSCs markedly ameliorates the deterioration of experimental arthritis

(A) The schema for collagen-induced arthritis (CIA) induction and treatment of hUCB-MSCs. After the onset of disease, five multiple doses of hUCB-MSCs or etanercetp were administered each day for five days via intraperitoneally (i.p) according to the following procedures. **(B)** Representative gross lesions of hind limb were photographed for clinical assessment. Clinical severity was consistently monitored and arthritis score was calculated until sacrificed. **(C)** All mice were sacrificed on day 49 for histopathological evaluation and paraffin-embedded sections of both patellar and hind phalangeal joints were stained with hematoxylin and eosin (H&E). Representative microscopic images of both joints are shown and histopathological integrity was calculated based on these images, scale bar = 100 μm. **(D)** Serum concentration of tumor necrosis factor α (TNF-α) was measured by enzyme-linked immunosorbent assay (ELISA). Five to ten mice were involved in each group. Results are shown as mean ± SD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

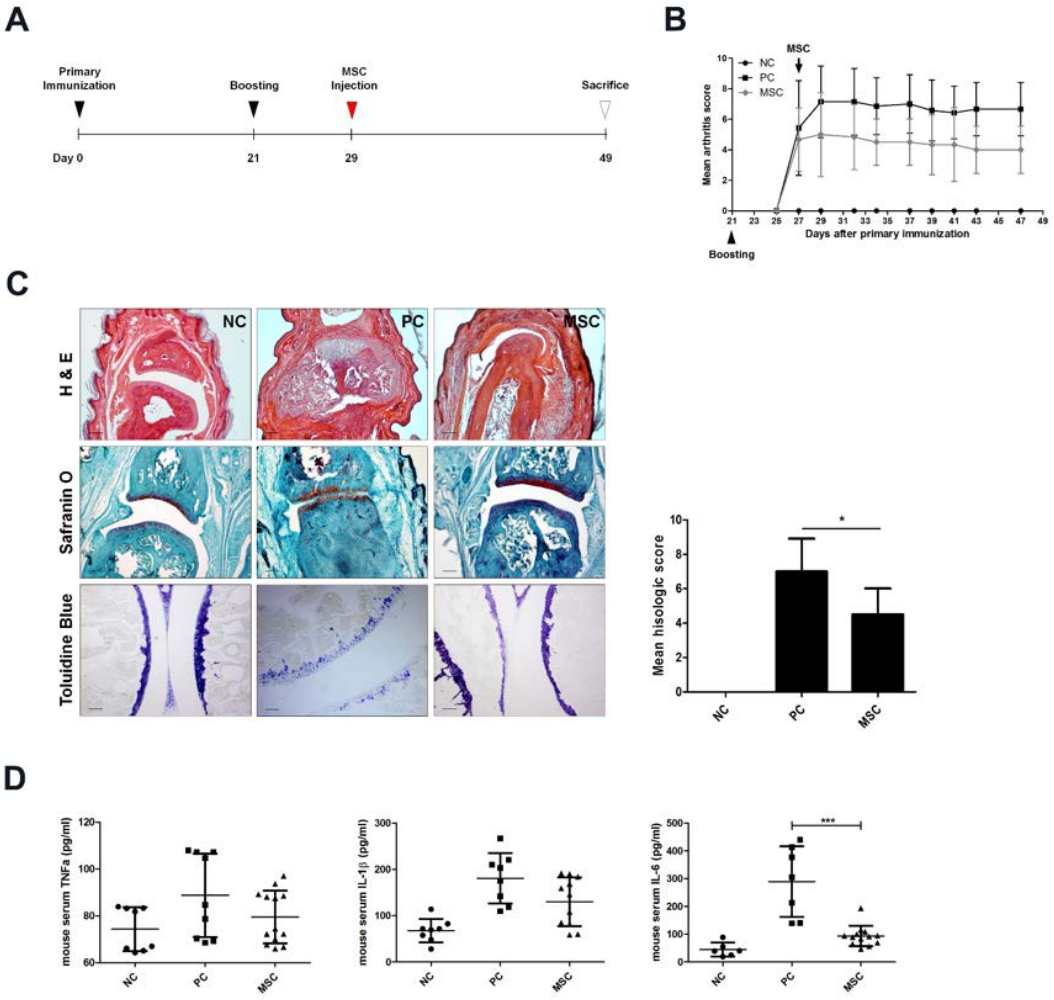

Figure 2.3. A single intravenous administration of hUCB-MSCs sufficiently attenuates established CIA in mice

Figure 2.3. A single intravenous administration of hUCB-MSCs sufficiently attenuates established CIA in mice

(A) Outline for induction of CIA and hUCB-MSCs injection. A single intravenous (i.v) injection of hUCB-MSCs was performed after the onset of arthritis. **(B)** Evaluation of clinical severity was monitored every two or three days and clinical arthritis score was calculated until sacrificed. **(C)** After sacrifice, paraffin-embedded sections of joint tissue were stained with H&E and histologic severity was assessed and calculated. In addition, safranin O and toluidine blue staining were performed for further evaluation of articular and chondral destruction. Representative photomicrographs hind interphalangeal joints stained with each dye are shown, scale bar = 100 μm. **(D)** Serum level of leading pro-inflammatory cytokines including TNF-α, interleukin 1beta (IL-1β) and interleukin 6 (IL-6) was measured by ELISA. Seven to twelve mice per each group were used. Results are shown as mean ± SD. ** $P<0.01$, *** $P<0.001$.

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2.3.2 Systemic infused hUCB-MSCs are mostly distributed in the lung tissue and excreted within two weeks.

The migration and distribution ability of MSCs as well as the paracrine effect plays an important role in suppressing the excessive inflammation. Therefore, I tracked and assessed the systemic injected hUCB-MSCs by real-time qPCR using human Alu gene. The majority of the injected cells were detected in the joint, lung, liver and spleen of mice at 2 hours after administration (Fig 2.4A-B). Consistently, 2 or 3 cases in joint, lung and spleen were detected until a week after cell injection (Fig 2.4A-B). At week 2, hUCB-MSCs were not detected in all analyzed organs of CIA mice (Fig 2.4A-B). All mice administered with hUCB-MSCs did not show any adverse complications and lethality until sacrifice. As a whole, these findings indicate that intravenously injected hUCB-MSCs are safe and successfully distributed in major organs including joint and excreted within a short period of time, suggesting that the ameliorating capability of intravenouly injected hUCB-MSCs might be the consequence of systemic immune-modulation rather than local responses.

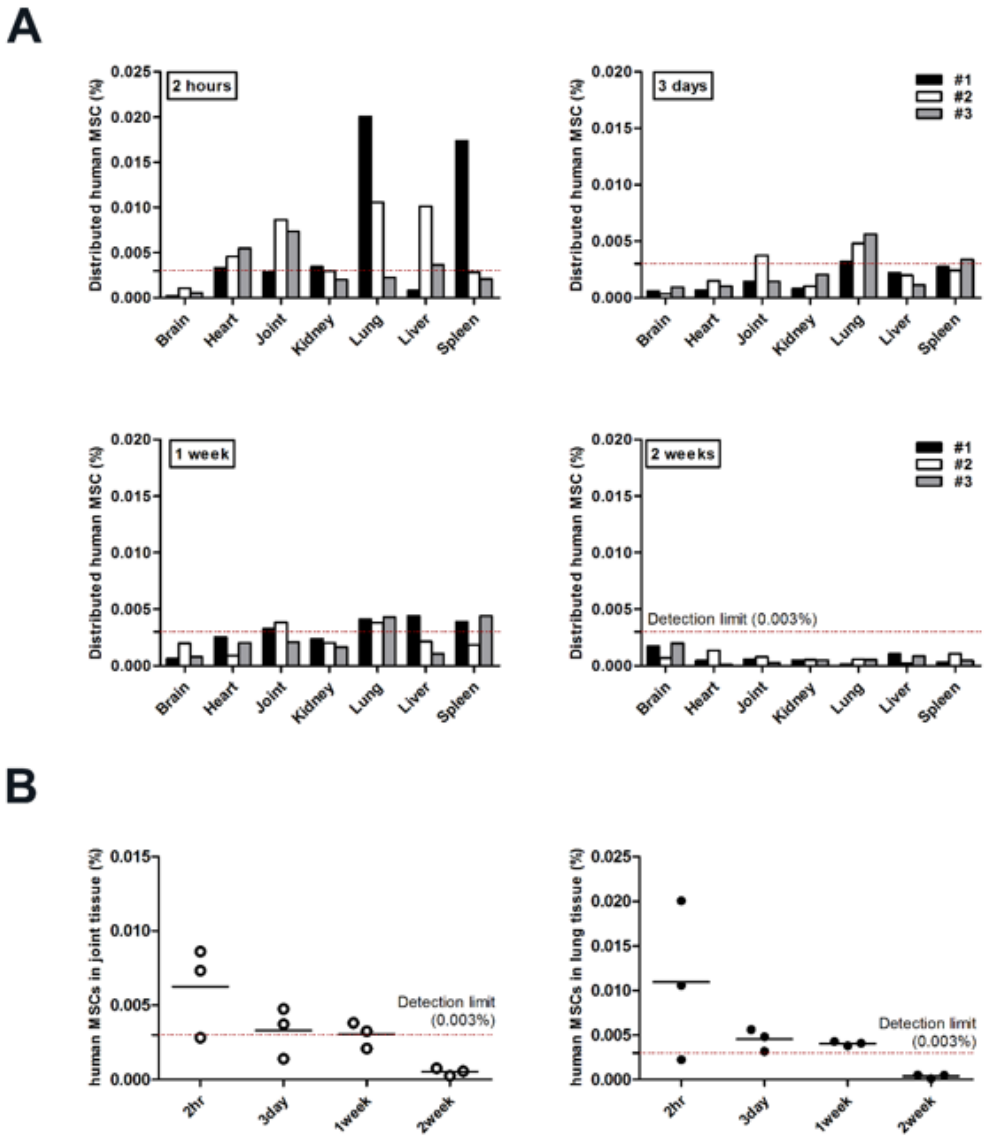

Figure 2.4. Intravenously injected hUCB-MSCs are mostly distributed in the lung and joint and excreted within two weeks.

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(A) Arthritis was induced by the immunization of bovine type II collagen (CII) mixture with complete Freund's adjuvant (CFA). hUCB-MSCs were infused intravenously (i.v), and mice were sacrificed at 2 hours, 3 days, 1 and 2 weeks after cell injection. At the same time, DNAs were isolated from major organs. Concentration of xenogeneic hUCB-MSCs in mice with collagen-induced arthritis (CIA) was evaluated using real-time qPCR with the human-specific ALU gene. **(B)** Distribution alteration of injected cells in lung and joint tissue was analyzed with the course of time. At least 3 mice per group were used and the detection limit was determined on the basis of standard curve (0.003% of human ALU gene).

2.3.3 hUCB-MSCs suppress the activation of M1 type macrophages and induce the generation of M2 type macrophages via TNF-α-mediated activation of COX-2 and TSG-6

Since down-regulated cytokines such as TNF-α and IL-1β are principally derived from macrophages, I sought to investigate whether hUCB-MSCs could modulate the phenotype or the function of macrophages *in vitro* (Bernardo and Fibbe, 2013; Firestein, 2003; Kinne et al., 2007). I thus generated macrophages from both cord blood-derived mononuclear cells and THP-1. The secretion of TNF-α from M1 macrophages was significantly decreased not only when cell-to-cell contact was allowed (Direct) but when transwell was used (Transwell) (Fig 2.5A). Moreover, hUCB-MSCs converted macrophages into anti-inflammatory M2 phenotype. The proportion of cells expressing CD206, the well-established M2 type marker, among $CD14^+$ cells was significantly up-regulated in both direct and transwell group as compared with macrophage control group (Fig 2.6A). Because CD206 is known to be an unstable marker for M2 type determination in THP-1-derived macrophages (Daigneault et al., 2010), M2 polarization of THP-1-derived macrophages was confirmed by measuring the expression of CD36. The proportion of $CD36^+$ M2 macrophages were also increased by hUCB-MSCs similar to the results of $CD206^+$ cells in primary cultured macrophages (Fig 2.5B). These results indicate that hUCB-MSCs could not only inhibit classical M1 activation but also elicit M2 polarization through paracrine fashion.

Considering that a large body of studies has demonstrated that immunomodulation by MSCs is not constitutive but is licensed by inflammatory cytokines (Nemeth et al., 2009; Ren et al., 2008a; Wang et al., 2014), I hypothesized that TNF-α secreted from activated M1 macrophages might influence on regulatory effect of hUCB-MSCs. Therefore, the expression of crucial immunomodulatory factors in hUCB-MSCs was assessed after TNF-α pretreatment. Cyclooxygenase-2 (COX-2) and tumor necrosis factor-stimulated gene 6 (TSG-6) was markedly increased (Fig 2.6B). I thus inhibited these factors in hUCB-MSCs using selective inhibitor or siRNA. Interestingly, the M2 facilitating effect of hUCB-MSCs was almost entirely impaired when COX-2 and TSG-6 were concurrently inhibited, while a single inhibition of each factor displayed partial restoration of M2 polarization (Fig 2.5C). Consistently, concerted contribution of COX-2 and TSG-6 on M2 elicitation was confirmed by determining the secretion of IL-10 (Fig 2.5D).

Taken together, these results suggest that hUCB-MSCs can regulate the macrophage plasticity through the concerted action of COX-2 and TSG-6 signaling enhanced in response to RA-specific inflammatory milieu.

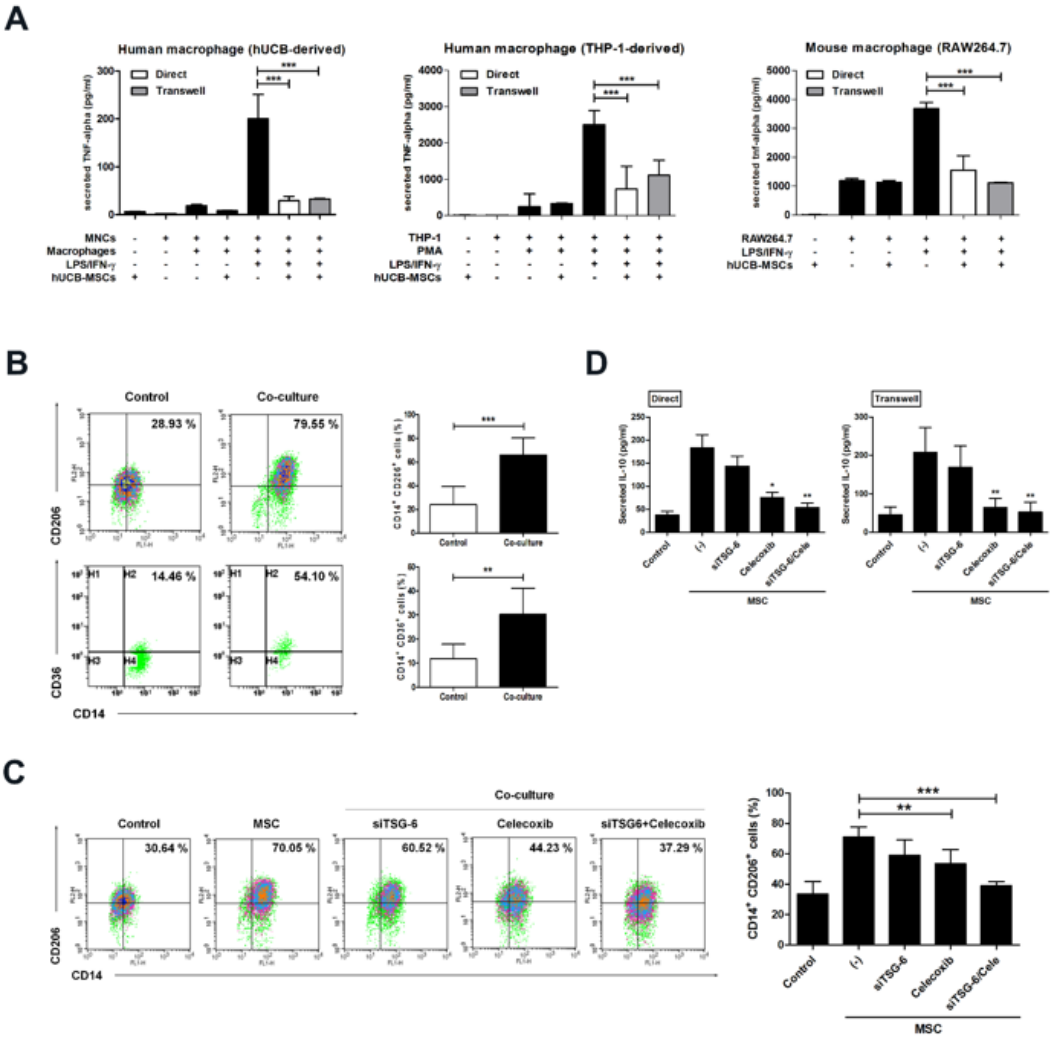


Figure 2.5. hUCB-MSCs suppress activation of M1 macrophages and induce M2 polarization through TNF-α-mediated activation of COX-2 and TSG-6.

Figure 2.5. hUCB-MSCs suppress activation of M1 macrophages and induce M2 polarization through TNF-α-mediated activation of COX-2 and TSG-6.

(A) Human cord blood- and THP-1-derived macrophages or mouse macrophages were activated with LPS and IFN-γ followed by co-culture with hUCB-MSCs in direct or transwell system. The secretion level of TNF-α was measured by ELISA. **(B)** Comparison of M2 specific surface marker expression on macrophages cultured alone (control) and co-cultured with hUCB-MSCs using flow cytometry. **(C)** COX-2 or/and TSG-6-inhibited hUCB-MSCs were added to macrophages and the alterations of CD206 expression was analyzed in the $CD14^+$ fraction for M2 polarization by flow cytometry. **(D)** The secretion of IL-10 in the co-culture supernatants was determined by ELISA. Results are one representative experiment of three or the cumulative of at least three independent experiments. Results are shown as mean ± SD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

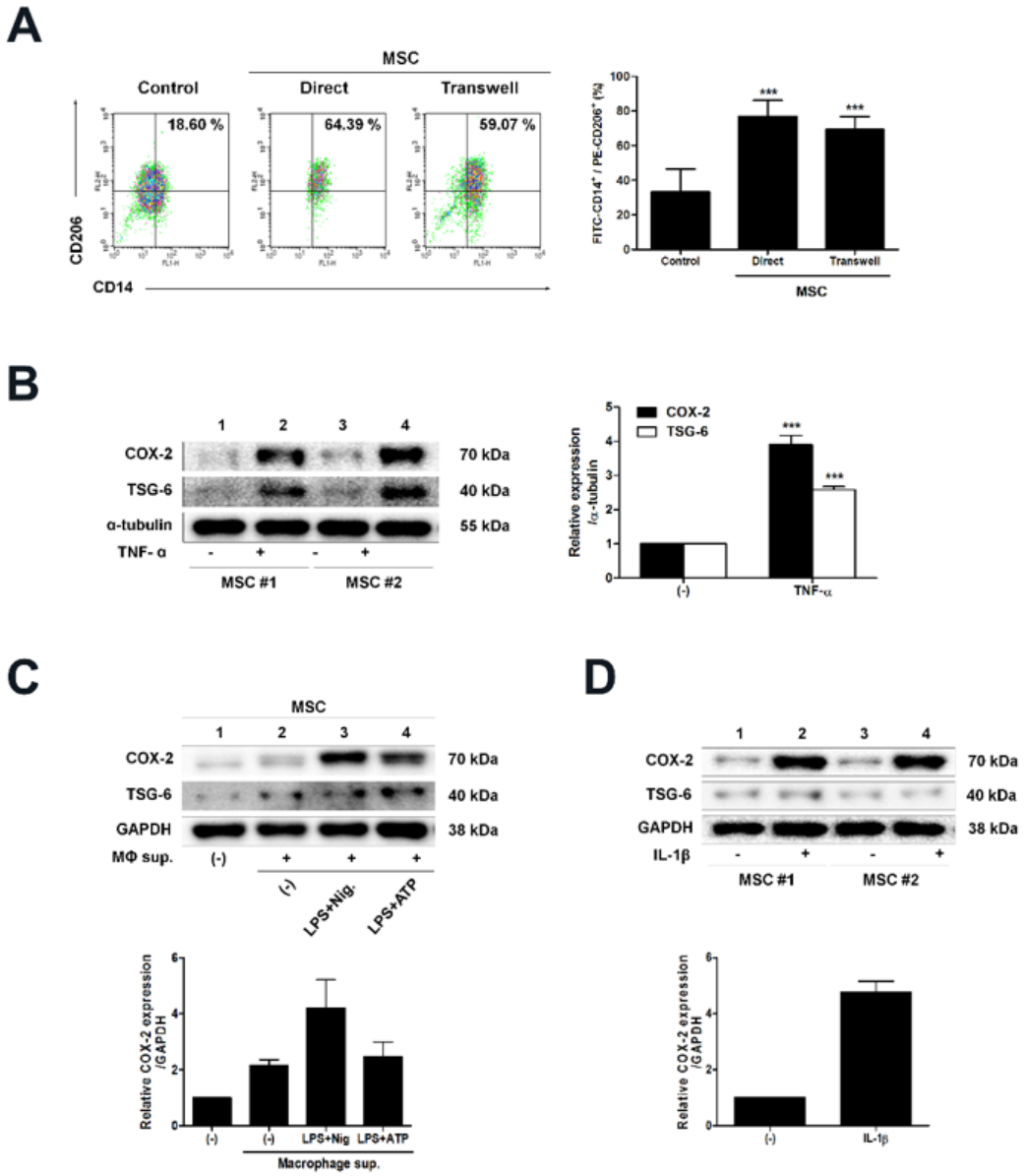


Figure 2.6. Concerted action of COX-2 and TSG-6 signaling enhanced in response to TNF-α and IL-1β contributes to macrophage regulation.

Figure 2.6. Concerted action of COX-2 and TSG-6 signaling enhanced in response to TNF-α and IL-1β contributes to macrophage regulation.

(A) The expression of CD206 among $CD14^+$ macrophages was detected by flow cytometry after co-culture with hUCB-MSCs in direct contact or transwell condition. **(B)** hUCB-MSCs obtained from two different donors were cultured for 2 days with or without recombinant human TNF-α and the protein expression level of pivotal factors, COX-2 and TSG-6, was determined and quantified by western blotting. **(C-D)** Alteration of expression level of these factors after pre-incubation with conditioned media from macrophages **(C)** or pre-treatment with IL-1β **(D)**. Results are one representative experiment of three or the cumulative of at least three independent experiments. Results are shown as mean ± SD. *** $P<0.001$.

2.3.4 hUCB-MSCs suppress the activation of NLRP3 inflammasome in macrophages via IL-1β feedback loop

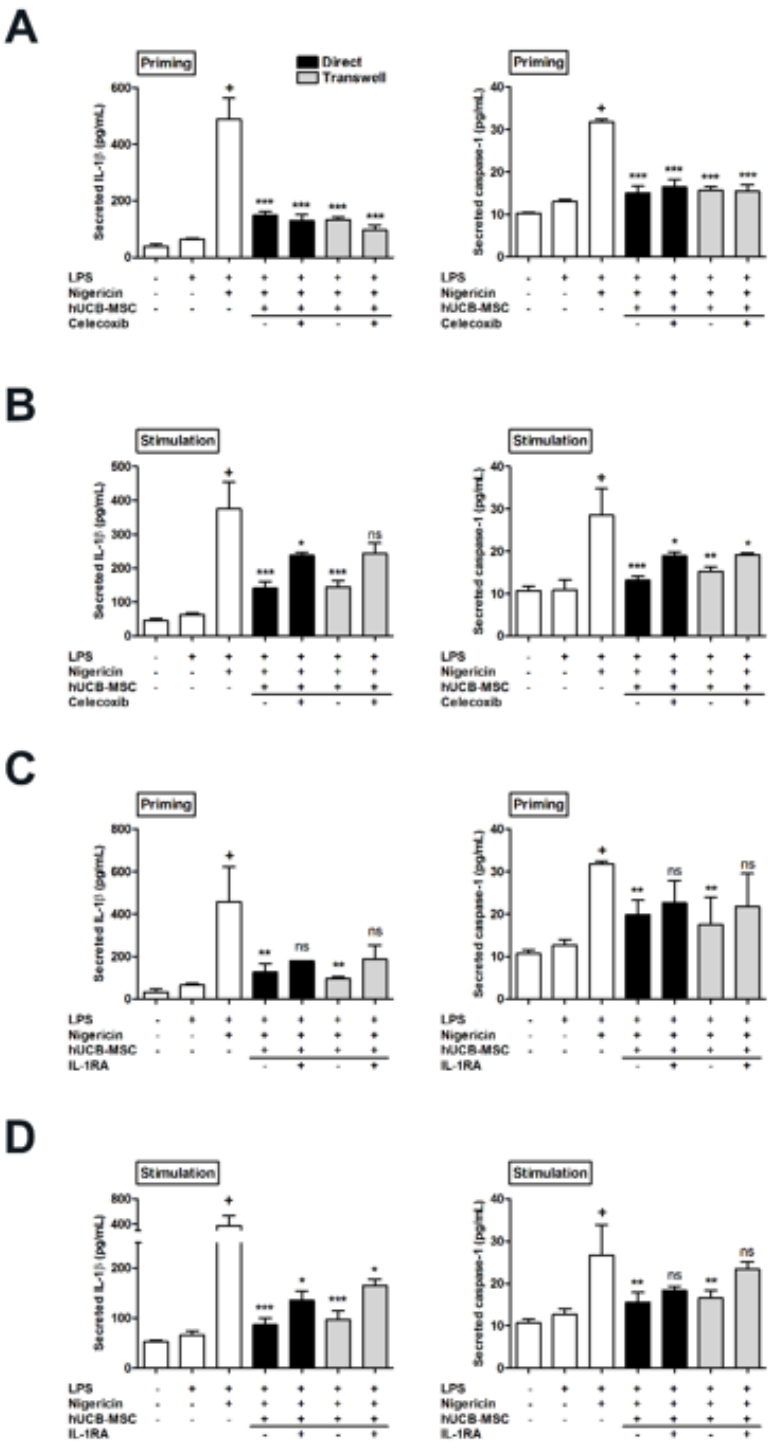
Along with TNF-α, IL-1β has been to play an essential role in RA pathogenesis (Firestein, 2003; Schett et al., 2016). Recently, there have been increasing evidence for the involvement of the NLRP3 inflammasome-mediated IL-1β secretion in RA (Mathews et al., 2014) and only few groups revealed that MSCs could suppress the activation of NLRP3 inflammasomes in macrophages (Oh et al., 2014; Zhu et al., 2016). I thus investigated whether hUCB-MSCs could regulate NLRP3 inflammasome. hUCB-MSCs were added to macrophages at either step of priming with LPS or stimulation with nigericin. Adenosine triphosphate (ATP), the other well-established NLRP3 activator, was also tested but nigericin exhibited much higher efficiency than ATP (data not shown). The concentration of IL-1β and caspase-1 in supernatants was significantly decreased by hUCB-MSCs in both co-culture conditions regardless of the time point of hUCB-MSC addition (Fig 2.7).

Based on these findings, I hypothesized that the suppressive effect of hUCB-MSCs on NLRP3 inflammasome activation might be provoked by specific signals from macrophages. I thus assessed the expression profile of crucial factors in hUCB-MSCs following incubation with or without the conditioned medium (CM) from macrophages after NLRP3 inflammasome activation. Interestingly, the CM of NLRP3 inflammasome-activated macrophages significantly up-regulated COX-2 expression in hUCB-MSC as compared to the CM from resting macrophages (Fig 2.6C). Furthermore, enhancement of COX-2 expression was also observed when hUCB-MSCs were treated with recombinant human IL-1β (Fig 2.6D), suggesting that NLRP3 inflammasome-mediated IL-1β might contribute

to the suppressive effect of hUCB-MSCs through the feedback mechanism.

To further verify that IL-1β-driven COX-2 signaling could be responsible for the suppressive effect on NLRP3 inflammasome activation, hUCB-MSCs were pretreated with celecoxib, a selective inhibitor for COX-2. The blockade of COX-2 partially rescued the secretion of IL-1β and caspase-1 only when hUCB-MSCs were added at stimulation phase (Fig 2.7B). By contrast, inhibition of COX-2 did not result in any alterations when hUCB-MSCs were added at the beginning of inflammasome activation with LPS priming (Fig 2.7A). In addition, I next inhibited IL-1β signaling in hUCB-MSCs with the IL-1 receptor antagonist to confirm the stimulatory effect of IL-1β from activated macrophages on hUCB-MSC funtion. As shown in Figure 2.7B and D, the blockade of IL-1β signaling led to the partial loss of this effect regardless of the co-culture period

Taken together, these results indicate that hUCB-MSCs could suppress NLRP3 inflammasome in macrophage through IL-1β feedback loop, suggesting that the therapeutic effect of hUCB-MSCs in RA might be the consequence of regulation of multiple macrophage functions by targeting various cytokines simultaneously


A
Priming
Direct
Transwell
Secreted IL-1β (pg/mL)
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Celecoxib
B
Stimulation
Secreted IL-1β (pg/mL)
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Stimulation
Secreted caspase-1 (pg/mL)
LPS
Nigericin
hUCB-MSC
IL-1RA

Figure 2.7. hUCB-MSCs negatively regulate NLRP3 inflammasome-mediated IL-1β and caspase-1 production in macrophages through IL-1β feedback loop. **(A-D)** THP-1-derived macrophages were primed with LPS, followed by stimulation with nigericin to activate NLRP3 inflammasome. hUCB-MSCs were added to macrophages at either LPS priming phase or at nigericin stimulating phase in direct or transwell condition. **(A-B)** At 20 hours later, the concentration of IL-1β and mature caspase-1 in culture supernatant was measured by ELISA after with or without pretreatment of selective COX-2 inhibitors to hUCB-MSCs. **(C-D)** Quantification of IL-1β and active caspase-1 in supernatants was determined after with or without IL-1 receptor blocking at each step. All the experiments are repeated at least 3 times. Results are shown as mean ± SD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns = not significant.

2.3.5 Immunologic profiling of serum and PBMC from RA patients

As hUCB-MSCs exerted the regulatory effects on macrophages *in vitro*, I then investigated whether these effects could be reproducible in *ex vivo* cells from patients with active RA. To explore the systemic inflammatory status of RA, I prepared serum and isolated PBMCs from patients and heathy donors. Subsequently, I analyzed baseline concentration of target cytokines. Although variations were observed among patients, the secretion level of TNF-α, IL-1β, IL-6 and even IL-10 was generally up-regulated in serum as well as in cell culture supernatants (Fig 2.8A). Furthermore, PBMCs from RA patients were composed of a high proportion of both $CD14^+$ $CD86^+$ M1 and $CD14^+$ $CD206^+$ M2 macrophages when compared to healthy controls (Fig 2.8B). These results demonstrate that diverse subsets of activated macrophages and numerous cytokines released from these cells are elevated in peripheral blood of patients, suggesting that macrophages might be intimately involved in systemic inflammation of RA.

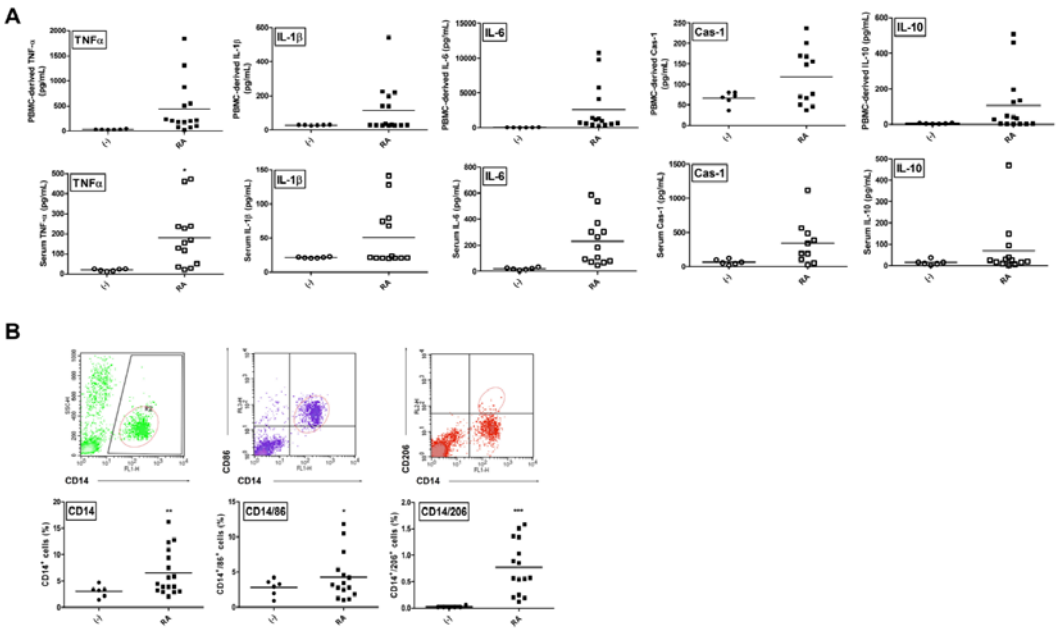

Figure 2.8. Baseline concentration of inflammatory cytokines and proportion of active immune cells are generally up-regulated in RA patients.

(A) Baseline concentration of major pro- and anti-inflammatory cytokines in serum and in culture supernatants of *ex vivo* PBMCs was measured by ELISA. **(B)** Representative dot plot of monocyte/macrophages and M1/M2 macrophage subsets. Total 15 samples from patient with active RA were used. Results are shown as mean ± SD. * P<0.05, ** P<0.01, *** P<0.001.

2.3.6 hUCB-MSCs consistently modulate immune cell functions beneficial for the amelioration of RA disease

To verify the possibility of hUCB-MSCs for clinical application, I next confirmed whether the regulatory effect of hUCB-MSCs might stand up to disease-related immune cells using *ex vivo* PBMCs from RA patients. hUCB-MSCs were added to PBMCs and co-cultured for two days with the ratio of 1:10 and 1:100 based on MSC:PBMC. Consistently with previous *in vitro* results, the secretion of TNF-α, IL-1β and caspase-1 was significantly reduced by co-culture with two different donor-derived hUCB-MSCs, and the release of IL-10 markedly up-regulated by hUCB-MSCs in a few patients (Fig 2.9A). Moreover, to explore the alteration of macrophage plasticity, hUCB-MSCs were co-cultured with PBMCs for two days, followed by the determination of cell surface markers using flow cytometry. Surprisingly, the population of $CD14^+$ $CD206^+$ M2 macrophage was significantly increased by hUCB-MSCs in all of 5 patients examined (Fig 2.9B). Altogether, these results suggested that hUCB-MSCs can effectively regulate circulating macrophages in active RA patients through the preferential induction into M2 phenotype as well as the inhibition of NLRP3 inflammasome-mediated IL-1β secretion, implying that these immune-balancing effects enable hUCB-MSCs to be a promising therapeutic option for RA treatment.

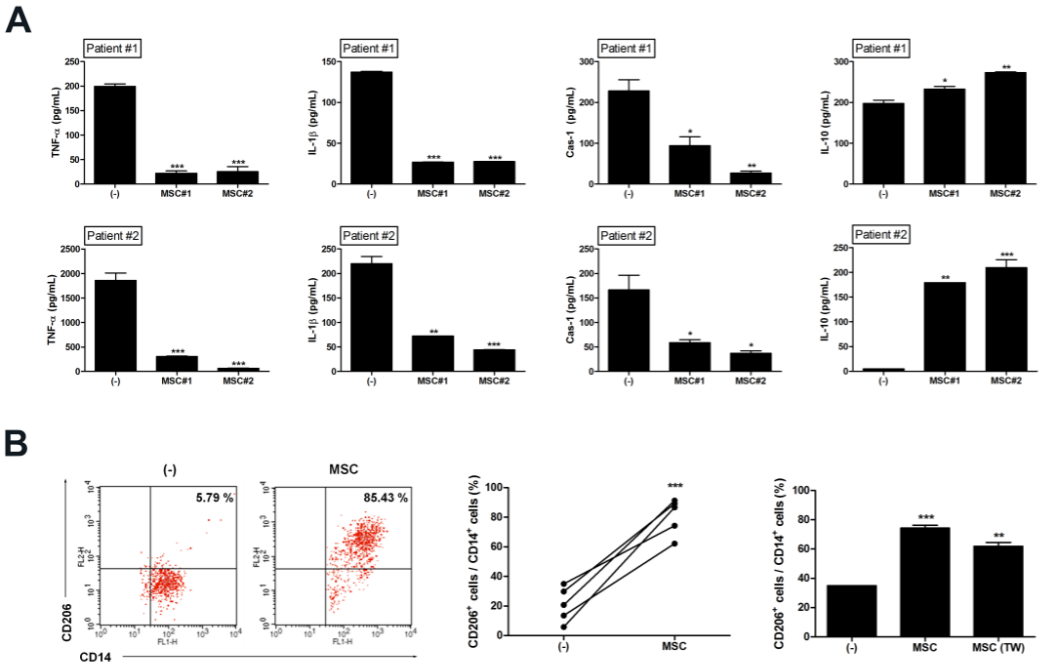

Figure 2.9. hUCB-MSCs effectively regulate patient-derived PBMCs toward anti-inflammatory phenotype.

(A) The secretion of TNF-α, IL-1β, caspase-1 and IL-10 in supernatants of co-cultured PBMC obatained from individual RA patients with hUCB-MSC was measured using ELISA as compared to PBMC alone. **(B)** After co-culture with hUCB-MSCs in direct or transwell, proportion of $CD14^+$ $CD206^+$ M2 population in PBMCs isolated from RA patients or healthy controls was determined by flow cytometry. At least four freshy peripheral blood were involved in patient and control group. Results are shown as mean ± SD. * P<0.05, ** P<0.01, *** P<0.001.

2.4 DISCUSSION

Up to date, a large body of studies has focused on demonstrating the therapeutic effects of MSCs in rheumatic diseases including RA (De Bari, 2015; MacDonald et al., 2011). MSCs have been applied to animal models of RA and result in inconsistent protective and curative effects, varying from remarkable attenuation to no effects (Gonzalez et al., 2009; Liu et al., 2010). These conflicting results might be attributed to several variables including origin of MSCs, number of injected cells and route of administration. Previously, I and my colleagues revealed the anti-inflammatory effect of hUCB-MSCs on experimental colitis and atopic dermatitis (Kim et al., 2013; Kim et al., 2015a). I thus sought to investigate the therapeutic potential of hUCB-MSCs against CIA. In this study, I verify that systemic administration of xenogeneic hUCB-MSCs can significantly ameliorate CIA, probably through the reestablishment of homeostatic inflammatory milieu. Interestingly, local intra-articular delivery of hUCB-MSCs showed little efficacy compared to systemic administration (data not shown), implying that systemic immune constitution rather than reduction of local inflammation might be required for efficient therapy. Intravenously injected hUCB-MSCs successfully migrated to the joint lesion although the majority of cells were trapped in the lung tissue. Subsequently, the injected cells were almost entirely excreted within two weeks after administration. Thereby, in spite of the short period of cell distribution, it seems that

systemically infused hUCB-MSCs can sufficiently suppress local and systemic inflammatory cascades.

MSCs isolated from BM or adipose tissue have been reported to exert therapeutic potential in preclinical model of RA by inhibiting Th1 and Th17 responses and inducing regulatory T cell responses (Chen et al., 2013; MacDonald et al., 2011). Recently, however, accumulating evidence has shown that macrophages play an essential role in the development of chronic inflammatory responses with direct tissue injuries (Kinne et al., 2007; Mantovani et al., 2013). So far, the mechanisms of MSC action on macrophages are not fully elucidated. Therefore, I aimed to demonstrate the effects of MSCs on macrophages due to tight regulation of macrophages can be an attractive strategy for the treatment of RA. MSCs have already been shown to induce the preferential conversion of macrophage toward M2 phenotype (Choi et al., 2011; Nemeth et al., 2009; Zhang et al., 2010). In this study, hUCB-MSCs significantly accelerated M2 polarization through soluble factors in accordance with those previous reports. Recently, inflammasomes have been newly identified as a novel IL-1β generating inflammatory apparatus mainly located in myeloid lineage cells including macrophages. Importantly, it has been reported that genetic mutations in NLRP3 protein are associated with susceptibility and severity of RA (Mathews et al., 2014). Furthermore, a previous study by Choulaki et al. reported that the basal level of NLRP3 and NLRP3-mediated IL-1β are increased in RA patients compared to healthy individuals, and that the activity of NLRP3 inflammasome in response to several ligands of toll-like receptors was also enhanced in patients (Choulaki et al., 2015). Recent study by Oh et al. reported that hBM-MSCs negatively regulate the NLRP3 inflammasome

in macrophages by decreasing mitochondrial reactive oxygen species (ROS) (Oh et al., 2014). However, the underlying mechanisms by which MSCs modulate inflammasome are largely unknown. I show here that hUCB-MSCs can remarkably suppress NLRP3 inflammasome-mediated IL-1β in macrophages, which might be mediated by the enhanced COX-2 signaling in response to IL-1β. Previously known as a pivotal mediator in MSC immunomodulation, COX-2 signaling might be contribute to the regulation of NLRP3 inflammasome. Further extensive investigation are absolutely required to verify core factors involved in the regulation of NLRP3 inflammasome.

The therapeutic strategies for RA have undergone impressive advances over the past two decades (Selmi et al., 2014). Recently, targeted therapies that block specific cytokine networks or cellular pathways have been developed, leading to a beneficial results in the treatment of RA (Koenders and van den Berg, 2015). Particularly among them, TNF-α inhibitors including etanercept, infliximab and adalimumab were reported to be strikingly effective against active RA (Koenders and van den Berg, 2015). However, their efficacy is so far limited and they have uncertainty about long-term safety (Bongartz et al., 2006; Salliot et al., 2007; Scott and Kingsley, 2006). I revealed here that hUCB-MSCs can remarkably suppress NLRP3 inflammasome-mediated IL-1β as well as TNF-α. While the current biologic therapies target a single cytokine, hUCB-MSCs can act as a simultaneous regulator of macrophage-derived multiple cytokines and contribute to the re-establishment of systemic immune-balance. Therefore, cell therapy using hUCB-MSCs can be successfully applied to non-responders who do not respond or cannot tolerate current anti-cytokine therapies.

Moreover, these simultaneous immunomodulatory effect of hUCB-MSCs was consistent with RA-related immune cells obtained from RA patients. The secretion of TNF-α, IL-1β and IL-6 from RA-related immune cells was generally down-regulated by hUCB-MSCs. Furthermore, hUCB-MSCs efficiently polarized patient macrophages toward M2 subset to a greater extent observed in the experiments using primary cultured or THP-1 derived macrophages. Taken together, these findings suggest that the therapeutic potential of hUCB-MSCs through the concurrent modulation on macrophages can be applied for actual patients with RA.

In conclusion, the current study revealed that hUCB-MSCs could act as a cellular modulators of macrophages through the simultaneous regulation on the production of TNF-α and IL-1β, and that the systemic administration of hUCB-MSCs could be an attractive therapeutic alternative for RA.

GENERAL CONCLUSION

MSCs have become the major stem cell types for cell therapy due to their attractive potential for multiple differentiation and immunomodulatory properties. Indeed, they have been used in preclinical models and clinical trials of various disorders. Growing evidence suggests that MSCs possess greater prophylactic or therapeutic potential in immune-related diseases. Although MSCs has been shown to be definitely safe and generally well-tolerated, several clinical trials yielded ambiguous results on the effect of MSCs. These obscure results might be attributed to sources and numbers of MSCs, timing and frequency of injection and insufficient standardization of MSC isolation and preparation. Therefore, it is need to be overcome these obstacles before their widespread application in the clinic.

In this study, I demonstrated the therapeutic efficacy of hUCB-MSCs in two different immune-related disease, specifically focused on Th2 dominant allergic inflammatory responses and Th1/Th17 dominant autoimmunity. Although the pathogenesis is quite different between those diseases, hUCB-MSCs showed significant therapeutic efficacy in common with different mechanisms of action. Therefore, this study may provide the evidence and the references in the field of stem cell therapy.

In the first study, I revealed that the subcutaneous local application of hUCB-MSCs exerted more efficient therapeutic effect against atopic dermatitis mouse model compared with intravenous administration. Moreover, NOD2 activation in hUCB-MSCs further enhanced the protective effect including the regulation of mast cell function. NOD2-mediated PGE_2 production and IL-4-mediated TGF-β1 production from hUCB-MSCs played crucial role to inhibit mast cell degranulation (Fig 8). Therefore, this study provides novel insight into the field of cell therapy for allergic disease including atopic dermatitis by developing highly effective MSCs and elucidating its interaction with mast cells.

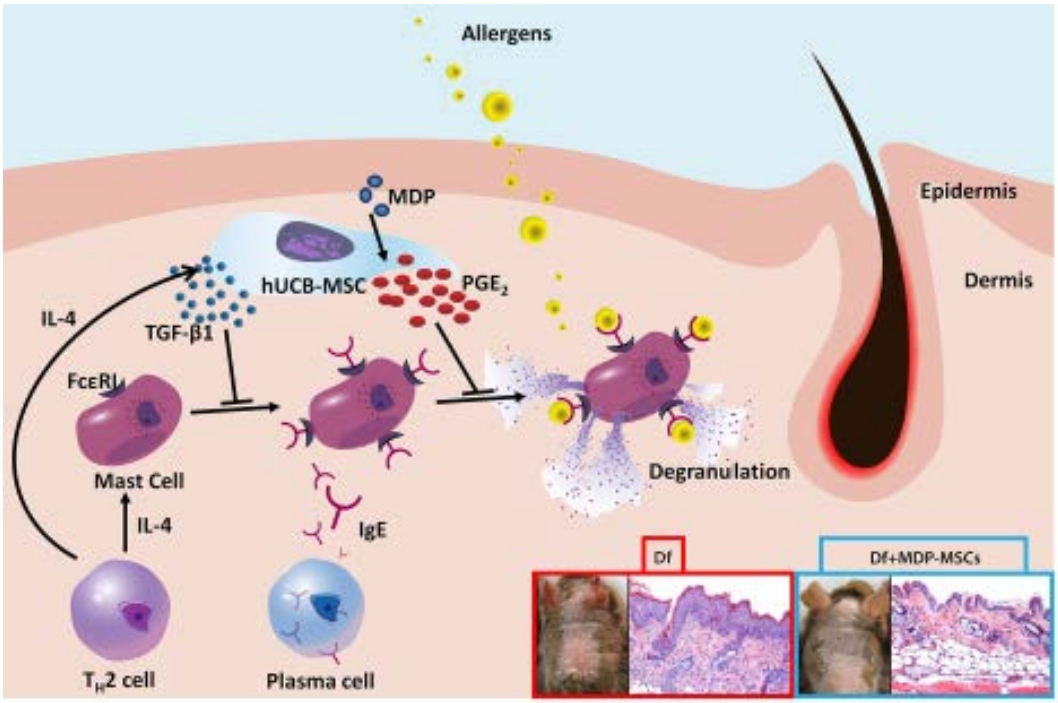

Figure 8. Schematic diagram of proposed mechanisms for NOD2-mediated therapeutic effect of hUCB-MSCs against atopic dermatitis through the regulation of mast cell degranulation.

In the second study, I aimed to investigate the therapeutic potential of hUCB-MSCs against rheumatoid arthritis. The intraperitoneal and intravenous systemic delivery of hUCB-MSCs exhibited significant beneficial effect on established collagen-induced arthritic mice compared with etanercept-injected group. Particularly, hUCB-MSCs efficiently modulate of macrophage functions by targeting multiple inflammatory cytokines simultaneously, and activation of COX-2 signaling in hUCB-MSCs in response to RA disease-specific dominant TNF-α and IL-1β principally contributed to induce M2 polarization and to down-regulate NLRP3 inflammasome activation. Therefore, this study suggest the possibility of hUCB-MSCs as a cell therapeutics for refractory RA patients and provide a better understanding of RA-disease specific mechanisms between MSCs and macrophages (Fig 9).

Consequently, I anticipate that these findings could provide a better understanding of the distinct immunomodulatory mechanisms of MSCs on disease-specific immune cells and bring new insights for the treatment of numerous intractable immune-related diseases, especially for refractory patients.

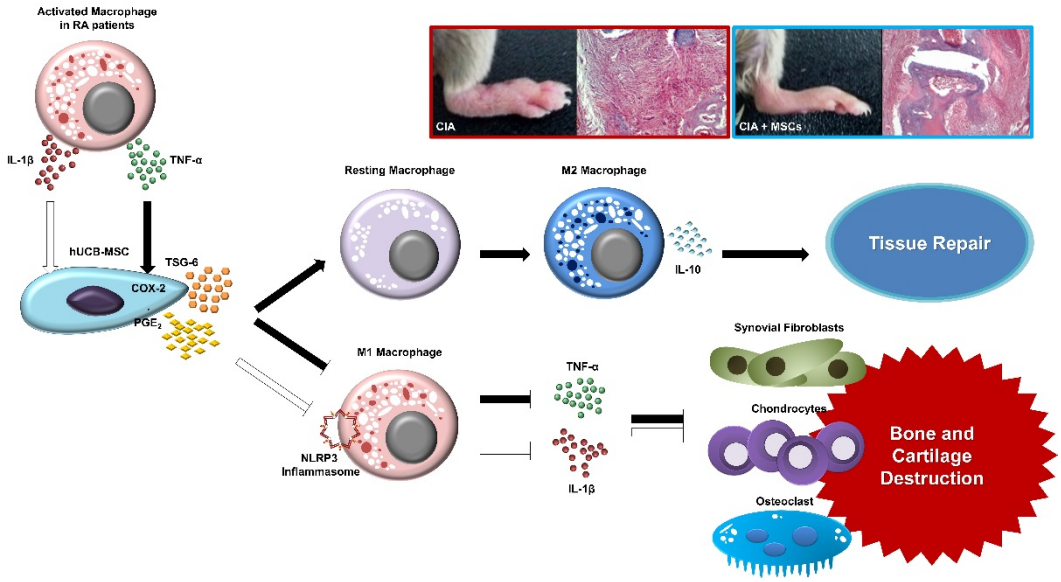

Figure 9. Schematic illustration of suggested mechanisms for therapeutic effect of hUCB-MSCs against rheumatoid arthritis through the simultaneous modulation of macrophage functions.

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국문 초록

아토피 피부염과 류마티스 관절염에 대한 인간 제대혈 유래 중간엽 줄기세포의 항염증 기전

서울대학교 대학원

수의학과

수의병인생물학 및 예방수의학 전공

신 태 훈

(지도교수: 강경선)

아토피 피부염과 류마티스 관절염은 대표적인 난치성 면역질환으로 각각 복잡한 알러지 반응과 자가면역반응을 특징으로 한다. 최근, 아토피 피

부염과 류마티스 관절염의 발병 관련 주요 면역 기전에 대한 광범위한 연구는 특정 사이토카인이나 세포 경로를 타겟으로 하는 새로운 생물학적 제제의 개발을 가속화시켰다. 하지만, 이러한 제제들은 효과가 제한적일 뿐 아니라 장기 투여에 따른 안전성이 아직 검증되지 않았다. 따라서 안전성과 효능을 겸비한 새로운 치료제의 개발이 요구된다. 중간엽 줄기세포는 특유의 면역조절능으로 인해 다양한 염증질환에서 현재의 치료제를 대신할 수 있는 새로운 해법이 될 수 있을 것으로 기대되고 있다. 그러나 질환 특이적 면역세포를 조절하는 줄기세포의 근원적 작용기전이나 그에 따른 실질적 효과에 대해서는 아직 더 자세한 연구가 필요하다. 따라서, 본 연구에서는 아토피 피부염과 류마티스 관절염에 대한 인간 제대혈 유래 중간엽 줄기세포의 치료효과를 확인함과 동시에 알러지성 면역질환과 자가면역질환 특이적 면역세포를 조절하는 중간엽 줄기세포의 항염증 기작을 분석하고자 하였다.

아토피 피부염은 습진성 피부 병변과 심각한 소양증을 동반하는 만성 알러지성 피부질환이다. 아토피성 피부염은 완치가 어렵고 빈번히 재발하기 때문에 만족할 만 한 치료제가 없는 실정이다. 몇몇의 연구에서 중간엽 줄기세포 적용에 따른 아토피 피부염 완화 효과를 제시하였으나, 아토피 피부염 관련 면역세포와 중간엽 줄기세포 사이의 명확한 상호작용기전은 아직 제대로 밝혀지지 않았다. 따라서 첫 번째 연구를 통해 인간 제대혈 유래 중간엽 줄기세포의 아토피 피부염 마우스 모델에 대한 치료 효과를 입증하고, 그 조절 기작을 심도 있게 분석하고자 하였다. 집진드기 추출물(*Dermatophagoides*

farinae)의 반복 도포를 통해 아토피 피부염을 유발한 마우스 모델에 제대혈 유래 중간엽 줄기세포 투여 시 현격한 피부염 예방 및 치료 효과를 나타냄을 확인하였다. 특히 NOD2를 미리 자극시킨 활성화 줄기세포의 경우 그 효과가 더욱 증강되었고, 특징적으로 과립세포의 병변부로의 침윤과 탈과립을 저해하는 것으로 나타났다. β-hexosaminidase 분석 결과, 활성화 줄기세포는 NOD2-COX-2 신호체계를 통하여 과립세포의 탈과립 과정을 저해하는 것으로 나타났다. 가장 연구가 많이 이루어진 골수 유래 줄기세포와 달리 제대혈 유래 줄기세포는 직접적 세포접촉과는 무관하게 높은 PGE_2 분비 기작을 통해 탈과립을 억제하였다. 또한 제대혈 유래 중간엽 줄기세포는 IL-4 에 반응하여 TGF-β1을 분비하고, 이것이 과립세포의 IgE 수용체 발현 저하를 유발함으로써 과립세포 탈과립을 완화하였다. 결론적으로, 제대혈 유래 중간엽 줄기세포의 피내 투여는 PGE_2와 TGF-β1의 복합작용기전에 의해 아토피 피부염을 효과적으로 개선하였고, NOD2 활성화는 치료효율을 더욱 증강시킴을 확인하였다.

류마티스 관절염은 성 진행성의 관절활막염에 따른 관절 손상뿐만 아니라 전신 합병증까지 수반하는 가장 흔한 자가면역질환의 하나이다. 최근 단구성 계열의 염증세포와 그들이 분비하는 싸이토카인이 류마티스 관절염의 발병 기전에 중요하다고 밝혀졌다. 많은 연구들이 류마티스 관절염에 대한 중간엽 줄기세포의 치료 효과를 보여주었으나, 단핵구나 대식세포와 같은 질환 특이적 면역세포를 조절하는 줄기세포의 기전에 대해서는 명확히 알려지지 않았

다. 그래서 두 번째 연구에서는 콜라겐 유도 관절염 마우스 관절염 모델에 대한 인간 제대혈 유래 중간엽 줄기세포의 치료효과를 확인하고, 동시에 대식세포를 중심으로 한 항염증 기전을 밝히고자 하였다. 전신적 경로로 투여한 제대혈 유래 중간엽 줄기세포는 현재 류마티스 관절염 환자에 주요하게 사용되고 있는 TNF-알파 저해제인 etanercept와 유사한 정도의 현저한 관절염 치료효과를 나타내었다. 제대혈 유래 중간엽 줄기세포는 대식세포 기능조절을 통하여 이러한 CIA 완화효과를 나타내었다. 조절 효과의 검증을 위하여 사람과 마우스 유래 대식세포를 줄기세포와 공배양하였다. TNF-α에 의해 줄기세포에서 활성화된 COX-2와 TSG-6 신호가 대식세포를 항염증성 M2 유형으로 극성화시킬 뿐 아니라, IL-1β의 피드백 작용에 반응해 중간엽 줄기세포는 NLRP3 inflammasome의 활성을 감소시켰다. 이러한 면역균형유지작용은 실제 류마티스 관절염 환자에서 분리한 질병 특이적 면역세포들과의 공배양 시에도 동일하게 유지되었다. 이러한 결과는 중간엽 줄기세포가 류마티스 관절염 질병 특이적 염증미세환경에서 우세하게 존재하는 염증성 사이토카인에 반응하여 다수의 사이토카인 체계를 동시에 조절할 수 있기 때문에, 인간 제대혈 유래 중간엽 줄기세포는 난치성 류마티스 관절염 환자 치료에 촉망 받는 대체제가 될 수 있음을 시사한다.

이 연구 결과들은 (1) 과립세포의 탈과립 억제를 통한 알러지성 질환에 대한 인간 제대혈 유래 중간엽 줄기세포의 치료효과를 밝히고, NOD2 활성화라는 줄기세포의 기능증강 방법을 제시함과 동시에, (2) 인간 제대혈 유래

중간엽 줄기세포의 전신 투여는 류마티스 관절염에 현저한 개선 효과가 있으며, 염증 미세환경에 반응해 TNF-α와 IL-1β의 생성을 동시에 조절하는 대식세포의 조절자로 작용할 수 있음을 밝혔다. 결과적으로, 본 연구는 알러지 질환과 자가면역질환에 대한 전도유망한 치료전략으로서의 제대혈 유래 중간엽 줄기세포의 적용 가능성에 대한 근거를 제시한다.

주요어: 중간엽 줄기세포, 아토피 피부염, 비만세포, 류마티스 관절염, 대식세포, NLRP3 인플라마솜

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