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Doctoral Dissertation

間葉幹細胞上類鐸受體 2 配體增強間葉幹細胞於氣喘模式小鼠之療效

Toll-Like Receptor 2 Ligation Enhances Therapeutic Effects of Mesenchymal Stem Cells on Murine Model of Asthmatic Inflammation

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Toll-like Receptor 2 Ligation Enhances Therapeutic Effects of Mesenchymal Stem Cells on Murine Model of Asthmatic Inflammation

本論文係俞惠潔君（D00421001）在國立臺灣大學臨床醫學所完成之博士學位論文，於民國 107 年 4 月 12 日承下列考試委員審查通過及口試及格，特此證明

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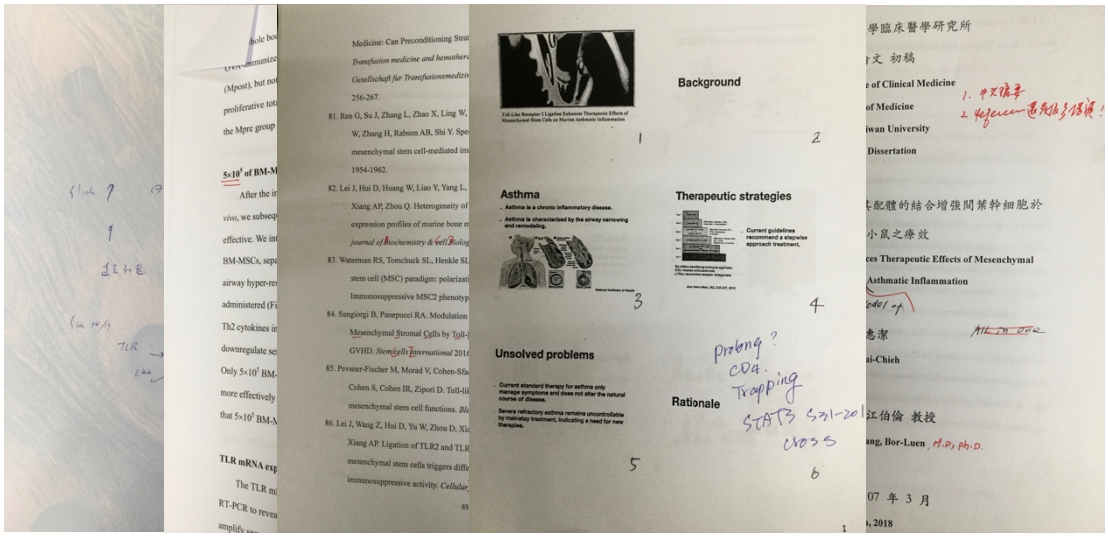
在完成博士學位的路上，儘管碰撞，儘管孤獨，儘管像是一個被放逐到荒島掙扎求生的歷程，因為有了師長與同仁的協助，有了家人與朋友的陪伴，我終於有了享受在荒島上恣意舞蹈的勇氣。



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摘要

現行的治療方法無法達成治癒氣喘的目標，因此發展新的氣喘療法仍是當務之急。僅管間葉幹細胞近來已因其免疫抑制特性的優勢而有潛力成為新興氣喘療法，其潛在的異質性將削弱其治療效益。在這篇論文中，我們進行了一個利用類鐸受體 2 之配體刺激間葉幹細胞以增強其氣喘療效的實驗計畫。根據實驗結果，在尾靜脈注射投予間葉幹細胞前，用一個類鐸受體 2 之配體（Pam_3CSK_4）刺激間葉幹細胞，可增強其對已誘發氣喘的小鼠的療效。為確效此刺激方法，我們進一步分析其作用機轉，並證實在間葉幹細胞中，Pam_3CSK_4 可透過轉錄因子 NF-κB 的途徑，刺激細胞激素 IL-6 的產生，進而活化轉錄因子 STAT3。轉錄因子 STAT3 磷酸化而活化後，隨即刺激激發型一氧化氮合成酶（iNOS）的表現量，進而合成更多與間葉幹細胞免疫調控能力相關的關鍵因子一氧化氮（NO）。經 Pam_3CSK_4 刺激的間葉幹細胞最後便藉由誘發更多調控型 $CD4^+CD25^+Foxp3^+$ T 細胞而達到其免疫抑制能力增強的效果。本篇研究所提出的間葉幹細胞改良方法，除在細胞和小鼠氣喘模式上均可得到較為穩定的治療成效外，我們更進一步藉由研究其免疫調控機轉而達到確效的目的。藉由對間葉幹細胞的改良，未來可望能減少利用間葉幹細胞治療所產生的副作用，以利長期治療的規劃。

關鍵字：間葉幹細胞; 異質性; 類鐸受體 2; 一氧化氮; 調節性 T 細胞

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Abstract

Under current therapeutic strategies, the cure to asthma remains elusive; thus-novel approaches for treating asthma are desperately needed. Despite that mesenchymal stem cells (MSCs) have recently been established as potential candidates by virtue of their immunomodulatory properties, the underlying heterogeneity of MSCs diminishes their therapeutic efficacy. Here we evaluated a toll-like receptor (TLR) 2 ligation protocol of MSCs to augment their therapeutic benefits on asthma. We surmise that more effective MSCs were activated with a TLR2 ligand, Pam_3CSK_4, at low concentration (1 μg/mL) for a longer period (96 h) induction prior to intravenous administration for ameliorating asthma in mice in a post-treatment manner. We further validated this regimen by demonstrating that Pam_3CSK_4 activated STAT3 in BM-MSCs through IL-6, which was likely stimulated with NF-κB signaling. Nitric oxide (NO), the key suppressive molecule of Pam_3CSK_4-primed BM-MSCs, was later highly increased through upregulation of iNOS, which was in the downstream of STAT3 phosphorylation. The intensified suppressive functions of BM-MSCs were then executed by inducing $CD4^+CD25^+Foxp3^+$ regulatory T cells in a post-treatment manner. The consistent therapeutic outcomes and the valid suppressive mechanisms in the study might shed light on eliminating the proinflammation-prone uncertainties of MSCs, and enhancing the future feasibility of obtaining long-term effects with this regimen.

Key words: Mesenchymal stem cell; Heterogeneity; Toll-like receptor 2; Nitric oxide; Regulatory T cell

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Chapter I: Introduction

The facts and the unsolved problems of asthma

Asthma is a chronic inflammatory disease, affecting an estimated 300 million individuals of all ages worldwide. Annual worldwide deaths from asthma have been estimated at 250,000 (1). The etiological complexity of asthma is attributed to the interaction between heritable and environmental components. Chronic airway inflammation is induced to cause the chief complaints of asthma- recurring episodes of intermittent dyspnea, wheezing, and nighttime or early-morning coughing. Subsequently, the recurrent inflammation leads to airway remodeling which causes smooth muscle spasm and airway narrowing.

Asthma is diagnosed based on medical and family histories, a physical examination, and pulmonary function tests. Current pharmacotherapeutic strategies of asthma are categorized by the severity of the cases. According to the progression of the disease, short-term bronchodilators are prescribed for patients with mild asthma as needed, while a combination of long-term bronchodilators and steroids is prescribed for patients with severe cases (2). However, the standard strategies are still far from perfect. Two problems remain unsolved: asthma can only be controlled with current protocols- even the patient with mild asthma despairs of being cured. Moreover, around 5% to 10% of the difficult-to-treat population is refractory to the most aggressive standard treatment (1, 3). Therefore, new approaches to treat asthma are desperately needed.

The attempts and the failures of treating asthma based on its molecular mechanisms

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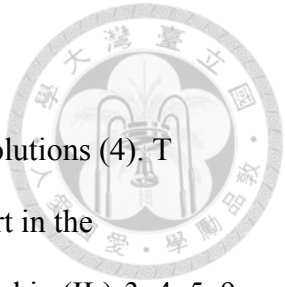

Molecular therapies have recently been suggested as potential solutions (4). T helper (Th) 2 lymphocyte has been considered the most important part in the pathogenesis of allergic asthma. Th2-type cytokines including interleukin (IL)-3, 4, 5, 9, 13, granulocyte-macrophage colony-stimulating factor (GM-CSF), thymic stromal lymphopoietin (TSLP) and their downstream immune responses contribute to varieties of pathological features. For example, IL-4 promotes differentiation and proliferation of Th2 cells which induces B cells into immunoglobulin (Ig)-E-producing plasma cells. IL-5 signals are critical for the growth, maturation and activation of eosinophils. IL-5 associated eosinophil infiltration in the airway is related to the induction of airway hyper-responsiveness. IL-13 is secreted by activated Th2 cells. IL-13 stimulates airway hyperactivity and mucous hyperproduction through IgE production, eosinophil recruitment and fibroblast proliferation in the lungs (5, 6). Hence, specific modulation of the Th2 profile has been suggested as a new strategy of asthmatic therapy. Except for Th2-derived cytokine antagonists or neutralizing antibodies, administration of several cytokines including IFN-γ, -α and -β and IL-12 have been trialed in attempts to suppress Th2 responses in asthma. However, the limited success with a particular target molecule is attributable to the complexity of asthmatic pathogenesis. In addition, no combination of different target molecules has achieved an advantageous benefit-risk ratio (6). Moreover, neither non-selective standard therapeutic strategies nor these specific approaches lead to prolonged periods of drug-free remission (7). Therefore, novel treatments with homeostatic properties may achieve more useful results.

Mesenchymal stem cells in the treatment of immune disorders

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Cell-based therapy is a follow-up to the small-molecule drugs and biologicals. In contrast to traditional drugs, cells are capable of sensing and responding to environmental signals, not to mention that the longevity of cells provides long-term treatment with the potential to restore immunological imbalances (8).

Resident mesenchymal stem cells (MSCs) and MSC-like cells exist in most postnatal tissues. They can be isolated from bone marrow, muscle, fat, hair follicles, tooth root, placenta, brain periosteum, dermis, perichondrium, umbilical cord, Wharton's jelly, lung, liver and spleen. However, bone-marrow (BM)-derived MSCs are the most characterized MSCs displaying distinct transcriptomes, and can thus be considered distinct cell types in a therapeutic setting (8). BM-MSCs are identified as multipotent non-hematopoietic progenitor cells with three distinct qualities. First, they express mesenchymal lineage surface markers like CD73, CD105 and CD44, but not hematopoietic ones like CD34, B220 and CD11b. Second, they are capable of differentiating into mesenchymal cell lineages including the adipogenic, osteogenic and chondrogenic lineages. And third, BM-MSCs perform their immunosuppressive properties on a variety of cell types mediating both innate and adaptive immunity (8).

MSCs, identified initially as multipotent nonhematopoietic progenitor cells, are considered attractive candidates in regenerative medicine because of their self-renewal and tri-lineage differentiating features (9). However, despite the clear therapeutic benefits of MSC-based therapies reported in previous studies, less than 1% of infused MSCs have been found at the target sites, even though their homing to the injured tissues was tracked immediately after administration. Since MSCs disappear shortly after infusion in the injured tissues, the repairable microenvironment they build is instead suggested as a more powerful facility.

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Consequently, the immunoprivileged properties and immunomodulatory molecular expression have been suggested as the main therapeutic mechanisms of MSCs, thus shedding light on the treatment of degenerative disorders, allograft rejection, and inflammatory and autoimmune diseases, even when cross-species applications are involved (10-12). In the treatment of multiple sclerosis (MS) and autoimmune type I diabetes, intravenous (i.v.) infused MSCs exhibit beneficial effects on experimental autoimmune encephalomyelitis (EAE) and non-obese diabetic (NOD) animal models, respectively. Allogenic MSCs protect *fas*$^{-/-}$ mice, a disease model of systemic lupus erythematosus (SLE), from autoantibody-induced renal dysfunction. In addition, even the xenogenic MSCs have been reported to significantly alleviate colon inflammation and body weight loss in inflammatory bowel disease (IBD) mice (13). Furthermore, despite that the precise MSC therapeutic mechanisms are unclear, many clinical trials are ongoing to evaluate its safety and efficacy (14). In SLE and Crohn's disease patients, both autologous and allogenic MSCs are able to suppress inflammation and reduce damage to kidneys and intestines, supposedly by the induction of regulatory T cells in patients. BM-MSCs can also exert therapeutic effects on patients suffering from diabetes, MS, stroke and liver cirrhosis, likely through immediate immunomodulatory effects. One or two infusions of MSCs after BM transplantation greatly improve the survival rate of both mice and humans with graft-versus-host disease (GvHD). And most encouragingly, MSCs successfully reverse steroid-resistant GvHD in patients receiving BM transplantation (15).

More recent studies on the immunomodulatory effects of MSCs have also evidenced their efficacy in animal models of lung injuries, chronic obstructive pulmonary disease (COPD), asthma and pulmonary hypertension (16, 17). Using an acute ovalbumin (OVA)-induced asthma murine model, both autologous and allogenic

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BM-MSCs ameliorate methacholine-induced airway hyper-reactivity. Th2-driven inflammatory signs such as circulating IgE and IgG1 levels, pulmonary eosinophil recruitment, and pulmonary IL-4 and IL-5 secretion are mitigated by allogenic BM-MSCs (18). Using a chronic OVA-induced asthma murine model, researchers have found that the airway remodeling can also be improved with infused BM-MSCs.

The immunosuppressive mechanisms of mesenchymal stem cells

Reports have revealed the correlation between the therapeutic effects of MSCs and their abilities to directly abrogate T cell, macrophage, dendritic cell (DC), neutrophil, and B cell proinflammatory functions (please see appendix for Fig. 1). The first evidence of the regulatory functions in MSCs *in vivo* came from models of GvHD. These studies demonstrate that MSCs reduce allograft rejection partly by modulating T lymphocytes (19). In addition, the timing of MSC administration is critical for natural killer (NK) cell and cytotoxic T cell modulation. It seems that MSCs have to be present when NK cells are stimulated. Contrarily, MSCs are effective at modulating cytotoxic T cell proliferation at the beginning of the mixed lymphocyte reaction (20). Further, MSCs prevent the differentiation and expansion of T cell subtypes which possess proinflammatory behaviors, Th1 and Th17 (21, 22); while promoting T cell subtypes which possess immunomodulatory behaviors, Th3 and Treg (23). To date, several reports support that the ability of MSCs to promote the Treg subtype is dependent on cell-cell contact as well as specific MSC to T cell ratios. With a low MSC to T cell ratio, the T cell proliferation is reduced, however the percentage of Treg in the population is elevated. (24) (please see appendix for Fig. 2).

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Next, MSCs are proved to maneuver macrophage plasticity towards regulatory macrophage (M2) behavior. In an experimental model of colitis, MSCs facilitate therapeutic effects by directly acting on M2 induction (25) (please see appendix for Fig. 3).

Third, MSCs have been shown to affect DC differentiation, maturation, and function in numerous *in vitro* coculture systems. MSCs inhibit $CD34^+$ and monocyte-derived DC through MSC-derived arrest of cell cycle in G0 (26). MSCs are as well observed to reduce major histocompatibility complex (MHC) class II and costimulatory molecules in DC, and consequently impair the stimulation of T cell proliferation (27). Moreover, they are indicated to enhance regulatory mature DC (DC2) function, while decrease proinflammatory mature DC (DC1) function (28) (please see appendix for Fig. 4).

Fourth, in both the reduced neutrophil recruitment in mouse bleomycin-injured lungs as well as the lowered levels of granulocytic enzymes in septic mice, the effects of MSCs on neutrophil invasion are likely to be cell-contact independent (29).

Finally, MSCs have been found to modulate a B cell-implicated autoimmune disease, a model of SLE (30). Reduced antibody production in non-differentiated B cells, arrested cell cycle in stimulated B cells, and reduced responses of B cells to chemotactic ligands are also observed when B cells are cocultured with MSCs (31, 32). Moreover, cell-cell contact is claimed to enhance MSC inhibitory effects on B cells. This result is found to be related to the interaction between programmed death 1 (PD-1) receptors and PD-1 ligands (33) (please see appendix for Fig. 1).

The indirect immunomodulatory effects of MSCs on T cells and neutrophils are also found. MSCs can modulate monocyte and DC functions, which ultimately result in the suppression of T cell proliferation. It is also reported that certain subpopulations of

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MSC produce IL-1 receptor antagonist (IL-1RA). IL-1RA may decrease neutrophil recruitment by blocking the production of proinflammatory cytokines from macrophages, which consequently diminishes the expression of chemokine and adhesion molecules in endothelial cells (29) (please see appendix for Fig. 1).

The immunoregulatory molecules of mesenchymal stem cells

Several candidates are identified to be essential for MSCs to perform immunosuppressive functions (34). MSC-secreted indoleamine 2, 3-dioxygenase (IDO) is a rate limiting enzyme in kynurenine-dependent catabolism of tryptophan, which halts T cell proliferation (22, 35). MSC secretion of IDO and nitric oxide (NO) respectively promotes T cell anergy, instead of T cell apoptosis (36). Enhanced IDO, IL-10, or prostaglandin E2 (PGE2) secretion of MSCs leads to regulatory T cell promotion and proinflammatory T cell inhibition (22). MSC secretion of IDO, PGE2, HLA-G5, and transforming growth factor-beta 1 (TGF-β1) have all been reported as the mediators which are responsible for modulating cytotoxic functions in NK cells (37, 38) (please see appendix for Fig. 2). PGE2, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) secreted by MSCs may increase the mannose receptor CD206 presenting and IL-10 secreting macrophages (39) (please see appendix for Fig. 3). IL-6 secreted by MSCs is suggested to regulate DC maturation by reducing the expression of MHC class II and costimulatory factors and the secretion of IL-12 in DCs (40) (please see appendix for Fig. 4). MSC secretion of IL-6 additionally has anti-apoptotic and reactive oxidative species downregulating effects on neutrophils (41) (please see appendix for Fig. 1). PD-1 ligand and vascular cell adhesion molecule

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(VCAM)-1 produced by MSCs can not only support cell-cell contact but also promote the immunomodulatory functions of MSCs (42).

Hence, the immunomodulatory effects of MSCs are likely mediated through soluble factors and cell contact-dependent mechanisms (42). The conditioned medium obtained from human palatine tonsil-derived MSCs inhibits fibrosis in the skeletal muscle cells through the counteraction of IL-1β by IL-1 receptor antagonist (IL-1RA) (43). On the other hand, several reports on culture systems have shown that cell-cell contact is a key factor involved in the immunomodulatory effects of MSCs. MSCs are found to decrease the survival and proliferation of T cells through cell contact-dependent mechanisms (44). Moreover, MSC-T cell contact is required for Treg induction (45). PGE2 and NO, despite their seemingly soluble properties, contribute to MSC-mediated suppressive functions in a cell-cell contact-dependent manner. High level production of PGE2 is initially dependent on a cell contact-dependent cross talk between MSCs and their target cells (46). MSC-secreted NO is able to inhibit T cell responses at its high concentration. However, the concentration of the active form of NO drops sharply within 100 μm because of its quick diffusive property. Therefore, MSCs require proximity for the effective inhibition of T cells through NO (36).

Heterogeneous mesenchymal stem cells

Although the immunosuppressive properties of BM-MSCs are well documented, studies on the immunomodulatory effects of MSCs produce conflicting data. Some studies indicate that MSCs are effective in suppressing excessive immune responses of GvHD and SLE in both animal models and human diseases. Whereas several other studies find that MSCs are unable to prolong GvHD remission *in vivo* even when they

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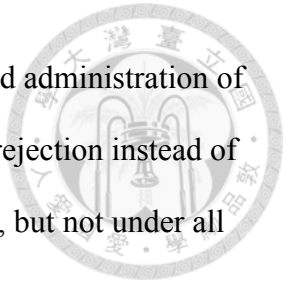

do suppress T lymphocyte proliferation *in vitro*. In addition, combined administration of MSCs and an immunosuppressant, cyclosporine A, accelerates graft rejection instead of the opposite. These studies reveal that MSCs are immunosuppressive, but not under all conditions.

Single cell-derived MSC studies create different clones with varied properties (47). Scientists therefore suppose that the applications of BM-MSCs are limited by their underlying heterogeneity (48, 49). Tissue stem cells are defined as undifferentiated cells being able to self-renew their own population and to guarantee the long-term production of differentiated cells. A single cell is apparently unable to fulfill these demands simultaneously. In this sense, it appears that the functional definition of stem cells refers to a population of heterogeneous cells. It is not known whether the immunoregulatory properties are common to all the different MSC subpopulations (50). It is also evident that contrary to the anti-inflammation and anti-scarring formation potential (51-54), BM-MSCs possess nurturing, chemotactic, proinflammatory, and wound-healing hazards (55, 56). MSCs support immune cells by secreting growth factors and home to damaged tissues by responding to chemokines. When the hosts are invaded by bacteria, MSC-like cells inhibit bacterial growth in two ways. They attack it by producing bactericidal factors directly, as well as enhancing proinflammatory responses from hosts (57-59). For instance, in the *E. coli* pneumonia mouse model, human BM-MSCs are suggested to impede the growth of bacteria partially by secreting antimicrobial peptide LL-37 (58). Paradoxically, the survival rates of both bacteria and host cells from bacterial-caused sepsis increase with immunomodulatory BM-MSCs; the host cells and bacteria must be saved or destroyed together. Therefore, MSCs are a double-edged sword.

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Mesenchymal stem cell polarization

Inspired by the special role of the hematopoietic niche for the maintenance of hematopoietic stem cells, the interactions between stem cells and the signaling context to which they are exposed allow for an adaptive control of the balance between self-renewal and differentiation, and a decision of which lineage they are committing to. This concept assumes that stem cell development is directly influenced by each cell's local microenvironment (50). Indeed, evidences do support that homogenously acting BM-MSCs can be polarized through microenvironmental licensing (36, 60).

In support of the "licensing" theory, first, MSCs are probably most effective when administered after the onset of inflammatory diseases. Several reports have indicated that after disease stabilization, the therapeutic efficacy of MSCs is not as apparent in mouse GvHD and EAE models. This is probably because the levels of inflammatory mediators in recipients with stabilized disease may be insufficient to elicit the immunomodulatory effect of MSCs. Second, pretreatment of MSCs with inflammatory factors has been reported to enhance the potential therapeutic efficacy of MSCs (36).

Being the licensing factors, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-1 α/β are recommended. Different single cell-derived MSC populations display varied immunosuppressive abilities. However, such varieties disappear when the monoclonal cell lines are pretreated with Th1-type cytokines, IFN-γ and TNF-α. IFN-γ and TNF-α treatment changes the proclivity of the poorly immunosuppression-acting clone. Its enhanced immunosuppressive function is then revealed by negatively affecting concanavalin A-induced T cell proliferation and alleviating OVA-induced delayed-type hypersensitivity response (61).

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MSCs have also been demonstrated to be primed by TLR ligands, leading to modulation of their differentiation, migration, proliferation, survival, and immunosuppressive capacities (35, 62). Cultured mouse BM-MSCs express TLR1 to TLR8. TLR2 has been shown to be involved in the cardioprotective effects of mouse BM-MSCs in an ischemia/reperfusion injury model despite that the immunosuppressive properties of mouse BM-MSCs in response to TLR2 activation remain elusive (63). TLR3 and TLR4 are among the most well-examined TLR subtypes of MSCs despite that their positions toward polarizing MSCs are varied in different experimental settings. TLR3 and TLR4 binding antagonizes MSC immunosuppressive activity by interfering with the Jagged1-Notch1 loop, while the other reports support the role of either TLR3 or TLR4 in promoting immunosuppressive MSCs (47).

Apparently, the role of proinflammatory cytokine and TLR ligand licensing in priming MSCs is more complex. MSCs acquire different functional properties depending on the level of stimulation. MSCs can be antigen presenting cells with upregulated MHCII if exposed to low-level IFNγ (10 U/mL), whereas high-level IFN-γ (100 U/mL) or TGF-β suppresses MHCII expression in MSCs (47). Low or high levels of specific TLR ligand exposure may also explain the different immunogenic characteristics of MSCs. Exposure of human MSCs to a high-level TLR3 ligand, poly (I:C) (1 μg/mL), polarizes MSCs into a IDO, TGF-β1, and IL-6-secreting phenotype which suppresses T cell proliferation and induces Treg generation. By contrast, exposure of human MSCs to a low-level TLR4 ligand, LPS (10 ng/mL), leads MSCs to secret chemokines, such as macrophage inflammatory protein-1a (MIP-1a), IFN-γ-induced protein-10 (IP-10), chemokine (C-C motif) ligand 5 (CCL5), and chemokine (C-X-C motif) ligand 9 (CXCL9), and consequently activate T lymphocytes (64). Moreover, MSCs acquire different functional properties with different patterns of

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stimulation. For instance, while IFN-γ alone induces IDO upregulation in MSCs, IFN-γ activates COX2 induction in MSCs synergistically when in combination with TNF-α (7).

Hence, the enhanced immunosuppressive properties of mouse BM-MSCs may be manipulated through their functional plasticity with specific priming factors. However, the outcome of MSC activation is dependent on the types of stimulating factors as well as the timing and levels of MSC exposure (65). In this study, we eventually selected a TLR2 ligand, Pam_3CSK_4, to be the priming factor for its better effects on enhancing immunosuppressive function in mouse BM-MSCs, and started establishing a priming protocol to optimize the beneficial effects of MSC treatment. OVA-induced asthmatic mice were used in this study to evaluate whether BM-MSCs preactivated with Pam_3CSK_4 (Mpam) could alleviate asthma symptoms more effectively than would control BM-MSCs (Mctrl).

Although MSCs are certainly functionally plastic, the unclear molecular mechanisms underlying MSC polarization into two distinct directions discourage their applications. By investigating the immunoregulatory mechanisms of modified BM-MSCs, not only would we be allowed to enlarge their therapeutic index with the understanding of properly administered conditions, but also, we would be able to select the validated BM-MSCs ahead of the administered time to prevent unwanted results. Since STAT3 promotes the production of immunosuppressive factors in many cell types (66, 67), we first investigated the role of STAT3 signaling in augmenting the immunomodulatory properties of BM-MSCs through TLR2 priming. Overall, the significance of this study is that we established a valid protocol to enhance the therapeutic effectiveness of BM-MSCs in asthma.

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$CD4^+CD25^+Foxp3^+$ Treg

The immune system is able to destroy exogenous and endogenous pathogens. Because its responses are potent enough to impair the integrity of the host, regulatory mechanisms must be essential to prevent the inappropriate harmful effects. One of the regulatory mechanisms is the clonal deletion that occurs in the thymus. Other key mechanisms in the maintenance of tolerance are the activation-induced T cell death and T cell anergy. In addition, a distinct subset of $CD4^+$ T cells, regulatory T cells, Treg in short, is now widely perceived as the mediator of permanent suppression of autoimmune responses in the periphery (68).

Although Treg cells comprise only 3-5% of the peripheral blood T cell pool, they are important for regulating the activation of the immune system. The most extensively studied Treg is characterized by expression of CD4, CD25, and the forkhead/winged-helix transcription factor Foxp3.

A subpopulation of $CD4^+$ T cells that expresses the α-chain of IL-2 receptors (IL-2Rα, CD25) plays an important function in peripheral tolerance by suppressing autoimmune disease in mice and humans (69). CD25-deficient mice develop massive lymphocyte accumulation and autoimmune disease. On the other hand, with age, IL-2-deficient mice develop fatal inflammatory bowel disease and lymphocyte proliferation. In addition, the peripheral T cell compartments of IL-2-deficient mice lack a well-defined population of $CD25^+$, which never exceeds 1-2% of the $CD4^+$ T cells.

Moreover, adoptive transfer of $CD4^+CD25^+$ T cells, instead of being hyperproliferative in mouse chimeras reconstituted with BM from CD25-defecient mice, rescues these chimera mice from chaotic lymphocyte accumulation. And the establishment of a stable and sizeable population of peripheral $CD4^+CD25^+$ regulatory

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T cells in these CD25-deficient mice is also achieved with adoptive transfer of $CD4^+CD25^+$ T cells from IL-2-deficient mice. This additionally indicates that IL-2/CD25 signal is required for the generation and peripheral expansion of a population of regulatory $CD4^+$ T cells essential for peripheral $CD4^+$ T cell homeostasis. Therefore, it is not the elusion from thymic negative selection, or the lack of sensitization to apoptosis in periphery that causes the autoimmunity in IL-2/IL-2R-deficient mice. Instead, it is the generation and maintenance of $CD4^+CD25^+$ Treg cells that are mediated through IL-2/IL-2R interaction (70).

Although most $CD4^+CD25^+$ T cells have regulatory properties in naïve mice, the isolation of a pure Treg population according to the expression of CD25 is not enough in diseased mice and for *in vitro* activated $CD4^+$ T cells. Because CD25 is upregulated in activated $CD4^+$ T cells, new additional molecules that allow a more precise definition of Treg are still needed.

Polyendocrinopathy enteropathy X linked syndrome (IPEX) is a severe multisystemic autoimmune disease whose onset usually occurs in the neonatal period. A frameshift mutation-resulting truncation of Foxp3 causes IPEX and its equivalent murine model, scurfy mice (71). Further, Foxp3 is demonstrated to be required for the development and the function of Treg: mice with either *foxp3*$^-$ or *foxp3*$^+$ reconstituted BM are used to demonstrate that $CD4^+CD25^+$ regulatory T cell development requires Foxp3. Moreover, retroviral-mediated ectopic expression of Foxp3 is sufficient to activate the suppressive function in peripheral $CD4^+CD25^-$ T cells (72). Although $CD4^+Foxp3^+$ cells are also characterized by a particular expression pattern of other cell surface molecules including cytotoxic T lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), etc., they are at present best identified by their expression of CD4, very high level of CD25,

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and Foxp3. Furthermore, several genes preferentially expressed by $CD4^+CD25^+$ Treg cells such as CD25, CTLA-4, and GITR are shown to be directly controlled by Foxp3 (73). Foxp3 therefore allows the most precise recognition of $CD4^+CD25^+$ Treg.

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Chapter II: Materials and methods

Materials

Animals

BALB/cByJNarl, C57BL/6JNarl, B6.129-Tlr2^{tm1kir}/J

Buffers

DPBS (Sigma), HBSS (Sigma), Golden lysis buffer [137 mM NaCl + 20 mM Tris-base + 5 mM EDTA + 1mM EGTA + 10% glycerol + 1% Triton X-100 + protease inhibitor cocktail (Roche) + phosphatase inhibitor cocktail (Roche)], ACK lysis buffer (150 mM NH_4Cl + 10 mM $KHCO_3$ + 0.1 mM Na_2EDTA in distilled water)

Culture medium

Mouse MSC culture medium (MesenCult^{TM} proliferation kit + 2 mM L-glutamine + 250 ng/mL amphotericin B), Adipogenesis kit (MesenCult), Osteogenesis kit (Gibco), Chondrogenesis kit (Gibco), Mouse $CD4^+$ lymphocytes/total splenocytes and MSC coculture medium [RPMI 1640 medium (Hyclone) + 10% FBS (Gibco) + 2 mM L-glutamine (Biological Industries) + PSA (Biological Industries) + 15 mM pH7.0 HEPES (Sigma)]

Reagents

Polyinosinic-polycytidylic acid [poly (I:C)] (Sigma), Lipopolysaccharide [LPS] (Sigma), Recombinant flagellin from *S. typhimurium* (Rec FLA-ST) (Invivogen), Recombinant TNF-α (PeproTech), Recombinant IFN-γ (PeproTech), Pam_3CSK_4

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(Invivogen), S3I-201 (Cayman), L-NMMA (Sigma), IL-6 neutralizing antibody (BD Pharmingen Cat. 554398), IgG1 κ isotype control antibody (BD Pharmingen Cat. 554682), Anti-mouse CD16/32 (BD Pharmingen Cat. 553142), Foxp3/Transcription Factor Staining Buffer Set (eBioscience Cat. 005523), FITC anti-mouse CD25 (BioLegend Cat. 102005), FITC Rat anti-mouse CD34 (BD Pharmingen Cat. 560238), FITC anti-mouse CD80 (eBioscience Cat. 11-0801), FITC anti-mouse CD86 (BD Pharmingen Cat. 553691), FITC anti-mouse CD90.2 (eBioscience Cat. 11-0903), FITC anti-mouse Sca-1 (BioLegend Cat. 108105), PerCP-Cyanine5 anti-mouse CD4 (BD Pharmingen Cat. 550954), PE-Cyanine5 anti-human/mouse B220 (eBioscience Cat. 15-0452), APC anti-mouse CD11c (eBioscience Cat. 17-0114), APC anti-mouse CTLA-4 (BioLegend Cat. 106310), APC anti-mouse/rat Foxp3 (eBioscience Cat. 17-5773), PE anti-mouse/rat CD29 (BioLegend Cat. 102208), PE anti-mouse CD44 (BD Pharmingen Cat. 553134), PE anti-mouse CD73 (eBioscience Cat. 12-0731), PE anti-mouse CD105 (BioLegend Cat. 120408), Recommended isotype control antibodies for flow cytometry (BD Pharmingen or eBioscience), Mouse $CD4^+$ T cell isolation kit (EasySep), Anti-CD3ε (BioLegend Cat. 100314), Anti-CD28 (BioLegend Cat. 102112), Mitomycin c (Sigma), ^{3}H-thymidine (PerkinElmer), DNA polymerase kit (GoTaq), SYBR Green system (Bio-genesis), Ovalbumin (Sigma), Alum (Thermo Scientific), Anti-IgE, IgG1, IgG2a antibody for ELISA (BD Pharmingen), Antibodies for cytokine levels (R&D), TMB substrate (Clinical), β-actin antibody (Thermo Scientific Cat. MA5-15739), IκBα antibody (Cell Signaling Cat. 9247), NF-κB p65 antibody (Cell Signaling Cat. 8242), Phospho-NF-κB p65 (Ser 536) antibody (Cell Signaling Cat. 13346), STAT3 antibody (Flarebio Cat. CSB-PA004174), Phospho-STAT3 (Ser727) antibody (Cell Signaling Cat. 9134), NOS2 antibody (eBioscience Cat. 14-5920), Acetyl-β-methylcholine chloride (Sigma), Griess reagent (Sigma), Anti-CD25 PE antibodies (BD

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Pharmingen Cat. 558642), Anti-PE enrichment set (BD IMag), Type II collagenase (Worthington), Ficoll solution (GE).

Methods

Passage numbers 6 to 13 of characterized mouse BM-MSCs were used in this study. A TLR2 ligand, Pam_3CSK_4, was selected to generate the conditioned BM-MSCs. [^{3}H]-thymidine incorporation assays and the acute OVA-induced asthma murine model were used to examine the immunosuppressive activity of the conditioned BM-MSCs *in vitro* and *in vivo*, respectively. To investigate the signaling pathways of the Pam_3CSK_4 induction in BM-MSCs, STAT3 inhibitor, S3I-201 (Cayman), iNOS inhibitor, L-N^G-monomethyl arginine citrate (L-NMMA) (Sigma), IL-6 neutralizing antibody (BD Pharmingen), or IgG1 κ isotype control antibody (BD Pharmingen) was added in addition to the Pam_3CSK_4 treatment. On the other hand, to investigate the suppressive mechanisms of Mpam, the conditioned $CD4^+CD25^+$ T cells were generated by coculturing with the conditioned BM-MSCs. [^{3}H]-thymidine incorporation assays were further performed to examine the immunomodulatory effects of the conditioned $CD4^+CD25^+$ T cells. The induction of $CD4^+CD25^+Foxp3^+$ cells through conditioned BM-MSCs were elucidated *in vitro* and further investigated *in vivo*.

Additional details are described as follow:

Isolation and culture of MSCs from mouse bone marrow

BM cells were obtained from femurs and tibias of 4-5 weeks old female Balb/c mice, flushed with mouse MSC culture medium (MesenCult). MSCs were obtained by

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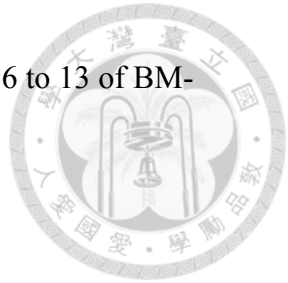
expansion of the BM plastic-adherent cell fraction. Passage numbers 6 to 13 of BM-MSCs were used in this study.



Flow cytometry

Cells were processed according to the manufacturer's protocols. Non-antigen-specific bindings of primary antibodies to the FcγII and FcγIII, and possibly FcγI receptors of cells were blocked by anti-mouse CD16/32 (BD Pharmingen Cat. 553142). Non-hematopoietic surface markers CD29, stem cell antigen (Sca)-1, CD73, CD44, and CD105, and hematopoietic surface markers CD11b, B220, CD34, and CD11c were used to identify the non-hematopoietic MSCs. We performed intracellular staining using Foxp3/Transcription Factor Staining Buffer Set (eBioscience Cat. 005523). The flow cytometric acquisition was performed on a BD FACSCalibur device. The cells were acquired using forward scatter and side scatter and gated to exclude debris. The cytometer then acquired 10,000 gated cell events. The fluorescence analysis was further conducted using BD CellQuest^{TM} Pro software.

Differentiation assays

The cells were differentiated into adipocytes, osteocytes, or chondrocytes with lineage induction medium (MesenCult, Gibco, and Gibco, respectively). The manufacturer's instructions were followed to stain the differentiated cells. Adipocytes were detected by Oil Red O (Merck Millipore), osteocytes by Alizarin Red S (2 % Alizarin Red S in distilled water, adjusted to pH4.1-4.3 with NH_4OH), and chondrocytes by Alcian Blue (1 % Alcian Blue in 0.1 N HCl) staining.

[^{3}H]-thymidine incorporation assays

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Spleens from 5-8 weeks old female Balb/c mice were dissected. Red blood cells in total splenocytes were lysed using Ammonium-Chloride-Potassium (ACK) lysing buffer. $CD4^+$ T cells in splenocytes were isolated by mouse $CD4^+$ T cell isolation kit (EasySep) according to the manufacturer's protocol. Total splenocytes or $CD4^+$ T cells were respectively preactivated with soluble or coated 2 μg/mL anti-CD3ε (BioLegend) and 2 μg/mL anti-CD28 (BioLegend) for 24 h before being cocultured with mitomycin c (Sigma)-induced mitotically inactive BM-MSCs for 48 h. The cell ratio of total splenocytes or $CD4^+$ T cells to BM-MSCs was 20 to 1. 1 μCi/mL ^{3}H-thymidine (PerkinElmer) was added into each well and the plate was incubated for 18 h. The cells were harvested and the thymidine incorporation was detected by a β-counter (Matrix^{TM} 96). The same process was applied with the transwell insertions in the transwell assays. In the transwell system, $CD4^+$ T cells were seeded in the bottom well, while BM-MSCs were placed in the upper insertions.

Semi-quantitative and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

In vitro conditioned primary cells were collected after 24-h-conditioned treatment. Total RNA was isolated from primary cultured cells and lung tissues using Trizol® reagent. The RNA templates were reversed into first strand cDNA for PCR analysis (Fermentas). A DNA polymerase kit (GoTaq) and a SYBR Green system (Bio-genesis) were respectively used in semi-quantitative and real-time RT-PCR. In real-time PCR, gene expression levels were normalized to the housekeeping gene, *gapdh*, and calculated using the $2^{\wedge(-\Delta\Delta Ct)}$ method. Please see appendix for the primer sequences used in this study.

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Conditioned BM-MSCs

After being seeded for 24 h, BM-MSCs were either nontreated as the Mctrl or treated with a TLR2 ligand, Pam_3CSK_4 (InvivoGen) as the Mpam; a TLR3 ligand, polyinosinic-polycytidylic acid [poly (I:C)] (Sigma) as the M3; a TLR4 ligand, lipopolysaccharide (LPS) (Sigma) as the M4; a TLR5 ligand, recombinant flagellin from *S. typhimurium* (Rec FLA-ST) (Invivogen) as the M5; a recombinant TNF-α as the Mα (PeproTech); and a recombinant IFN-γ as the Mγ (PeproTech). To investigate the signaling pathway of immunosuppression-prone Mpam, a STAT3 inhibitor, S3I-201 (Cayman), an iNOS inhibitor, L-NMMA (Sigma), an IL-6 neutralizing antibody, or an IgG1 κ isotype control antibody was added in addition to the Pam_3CSK_4 treatment to make Ms&p, Ml&p, M6&p, and Mic&p, respectively (Fig. 1A).

Acute OVA-induced asthma murine model

After acclimating to the Laboratory Animal Center for 1 week, 6-week-old female Balb/c mice were divided into four groups: the negative control (PBS), positive control (OVA), Mctrl treatment, and Mpam treatment groups. Each group originally had 9 mice in one experimental setting. Except for the negative control group, which was sensitized and challenged with PBS, they were intraperitoneally sensitized by 200 μl of 50 μg ova (Sigma)/2 mg Alum (Thermo Scientific) at day 0, 14, and 21, and then were intranasally challenged by 40 μl of 100 μg OVA at day 28, 29, and 30. After the third sensitization, OVA-sensitized mice with 0.5-0.7 ELISA units of serum OVA-specific IgE levels and negative control mice with less than 0.015 ELISA units of serum OVA-specific IgE levels were included in the following procedures. The OVA-sensitized groups were randomized into three groups based on their serum OVA-specific IgE levels. The treatment groups were intravenously infused once a day with 100 μl of 5×10^5 Mctrl and

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Mpam respectively, day 28 through day 30. The animals were either sacrificed or subjected to an airway resistance experiment on day 31 (Fig. 1B).

The composition of immune cells in lung

The bronchoalveolar lavage fluid (BALF) was collected from each sacrificed and tracheotomized mouse with 1 ml Hanks' Balanced Salts (HBSS) buffer (Sigma). After the BALF was centrifuged at 1500 rpm for 5 min, the supernatant was used to examine the cytokine levels, while the nucleated cell pellet was resuspended with HBSS containing 2% FBS to determine the cellular composition. The differential cell counts collected by Thermo Scientific^{TM} Cytospin^{TM} 4 Cytocentrifuge were discriminated with Liu's stain. The labels of the samples were covered by the other colleague during the counting processes, and a flow cytometric method was used to confirm the results (74).

Measurement of OVA-specific IgE levels in serum and OVA-specific cytokine levels in supernatants

The enzyme-linked immunosorbent assay (ELISA) protocol was followed to detect OVA-specific IgE (BD Pharmingen) and OVA-specific cytokine levels (R&D). The supernatants of the *in vitro* conditioned primary cells and the *ex vivo* restimulated splenocytes were collected after 72-h-conditioned treatment and 72-h-stimulation, respectively. The HRP-catalyzed oxidation of TMB substrate (Clinical) was finally conducted to detect the presence of the targets. And the optical density (O.D.) differences between 540 nm and 450 nm wavelengths of the converted TMB were read on a standard electroimmunoassay plate reader (Molecular Devices, VersaMax).

Noninvasive measurement of airway hyperresponsiveness

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The response of airway to aerosolized acetyl-β-methylcholine chloride (Sigma) in mice was detected using whole body plethysmography, and was analyzed using FinePoint^{TM} system. The response of airway (Penh) to aerosolized acetyl-β-methylcholine chloride was then divided by the response of airway to aerosolized Dulbecco's phosphate-buffered saline (DPBS) in each mouse, yielding Penh (%).

Invasive measurement of airway resistance

Airway function was measured by detecting changes in lung resistance (R_L) in response to increasing doses of aerosolized acetyl-β-methylcholine chloride (Sigma) in anesthetized and tracheotomized mice. The calculation of R_L was yielded by subtracting transpulmonary pressure (0.45 $cmH_2O.s.ml^{-1}$) from respiratory flow measurement. Data were presented as average R_L in the ratio of average R_L after PBS nebulization.

Histological evaluation

Mouse lung tissues were dissected and then fixed in a 10 % formaldehyde solution (Merck Millipore). Samples were embedded in paraffin blocks and the histological sections were stained by hematoxylin and eosin (H&E).

Conditioned $CD4^+CD25^+$ T cells

$CD4^+$ T cells were prestimulated with coated 2 μg/ml anti-CD3ε and 2 μg/ml anti-CD28 for 24 h before they were cocultured with mitomycin c-treated conditioned BM-MSCs in a 20:1 ratio. $CD25^+$ cells were then isolated from these $CD4^+$ T cells by anti-CD25 PE antibodies (BD Pharmingen Cat. 558642) and an anti-PE enrichment set (BD IMag) to make the Tctrl, Tpam, Ts&p, and Tl&p (Fig. 1C).

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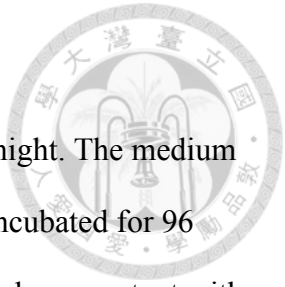

Griess assay

Mitotically inactive BM-MSCs were seeded as 1×10^5 cells overnight. The medium was then replaced by 250 μl of culture medium with treatments and incubated for 96 hours. The supernatants were assayed for nitric oxide by mixing 100 μl supernatant with 100 μl Griess reagent (Sigma) for 15 min at room temperature and were read on an electroimmunoassay plate reader at 550 nm. The concentration of nitrite in the culture supernatant was quantified by a standard curve of 100-0.003 μM $NaNO_2$ concentrations (Sigma).

Western blot analysis

Protein samples were collected using Golden lysis buffer (137 mM NaCl, 20 mM Tris, 10 mM NaF, 5 mM EDTA, 1 mM EGTA, 10 % (v/v) glycerol, 1 % Triton X-100, protease and phosphatase inhibitor cocktails, pH 7.9), separated on 10 % SDS-PAGE gels, and transferred onto NC membranes (Whatman). After the membranes were incubated with primary and secondary antibodies, protein bands were visualized using chemiluminescence reagent (BIOSCIENCES). IκBα (Cell Signaling Cat. 4814), NF-κB p65 (Cell Signaling Cat. 3034), p- NF-κB p65 (ser536) (Abcam Cat. ab76302), STAT3 (Flarebio Cat. CSB-PA004174), p-STAT3 (ser727) (Cell Signaling Cat. 9134), iNOS (eBioscience Cat. 14-5920), and β-actin (ThermoFisher Cat. MA5-15739) were used as primary antibodies.

Pulmonary mononuclear cell preparation

The protocol established by D.L. Wiesner et al. was referenced (75). Lungs were excised and minced to generate pieces in HBSS (Sigma) + 1.3 mM EDTA (Sigma) solution for 30 min at 37°C with agitation. The solution was transferred to RPMI 1640

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medium (Hyclone) supplemented with 10% FBS (Gibco) and 150 U/ml type II collagenase (Worthington), and incubated for 1 h at 37°C with agitation. The cells were then grounded through a 70 μm filter, pelleted and resuspended with DPBS (Sigma). Ficoll solution (GE) was finally used to separate mononuclear cells from the pulmonary homogenate.

Statistical analysis

Data were analyzed using GraphPad Prism v5 and are presented as mean and individual data points. To determine the significant variances between groups, Student's t test was used between two groups, while one-way ANONA was used for multiple comparisons and Newman-Keuls Multiple Comparison Test was used for post hoc analysis. The statistical significance was set at $p < 0.05$.

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Chapter III: Results

Characterization of mouse BM-MSCs

According to the criteria of the International Society for Cellular Therapy, mouse BM-MSCs have been characterized by their mesenchymal lineage surface markers, tri-lineage differentiation abilities, and immunosuppressive properties. The positive expression of CD29, stem cell antigen (Sca)-1, CD73, CD44, and CD105 and the negative expression of CD11b, B220, CD34, and CD11c exhibited no contamination of the stromal cells with hematopoietic cells (Fig. 2A). To establish that the stromal cells contained MSCs, the cells were cultured under a variety of conditions to assess their capacity to differentiate into mesodermal lineages. The isolated, purified stromal cells were able to differentiate into adipocytes, osteocytes, and chondrocytes when they were incubated with a specific induction medium, suggesting that these isolated stromal cultures certainly contained MSCs (Fig. 2B). We further ascertained the immunosuppressive functions of cultured plate-selected BM-MSCs through [^{3}H]-thymidine incorporation assays. The proliferative response of $CD4^+$ T cells was diminished when these cells were cocultured with BM-MSCs (Fig. 2C). In addition, the suppressive function of BM-MSCs was neither enhanced with lower $CD4^+$ T to BM-MSC ratio (2:1), nor diminished with higher $CD4^+$ T to BM-MSC ratio (200:1) (Fig. 2D).

Post-treatment as the more appropriate setting of BM-MSC treatment

We next used an acute OVA-induced murine model to examine whether BM-MSCs would be a potential therapeutic strategy for asthma. First of all, we examined which time point we should intravenously administer BM-MSCs. Measured using whole body

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plethysmography, the airway hyper-responsiveness of OVA-immunized mice was improved with BM-MSCs in the post-treatment setting (Mpost), but not in the pretreatment setting (Mpre) (Fig. 3A). In addition, less proliferative total splenocytes were observed in the Mpost group compared to those in the Mpre group from our *ex vivo* experiments (Fig. 3B).

Administering 5×10^5 of BM-MSCs for higher immunosuppressive activities *in vivo*

After the immunosuppressive properties of BM-MSCs had been demonstrated *in vivo*, we subsequently examined with how many BM-MSCs would they become more effective. We intravenously administered 1×10^5 (Ma), 5×10^5 (Mb), or 1×10^6 (Mc) BM-MSCs, separately into OVA-challenged mice. Although BM-MSCs could improve airway hyper-responsiveness by the same extent no matter how many cells were administered (Fig. 4A), different amounts of BM-MSCs decreased serum IgE level and Th2 cytokines in BALF by different degrees. Either 5×10^5 or 1×10^6 BM-MSCs would downregulate serum IgE level more effectively than that of 1×10^5 BM-MSCs (Fig. 4B). Only 5×10^5 BM-MSCs however, decreased IL-4 and IL-5 secretion detected in BALF more effectively than that of 1×10^5 or 1×10^6 BM-MSCs (Fig. 4C). Hence, we decided that 5×10^5 BM-MSCs would be appropriate for this model.

TLR mRNA expression of mouse BM-MSCs

The TLR mRNA expression of BM-MSCs was examined using semi-quantitative RT-PCR to reveal the potential TLR ligand candidates. Specific primers were used to amplify sequences from TLR1 to TLR9, and the RAW264.7 cell line was used as a positive control (Please see appendix for Table 1). TLR3 and TLR4 are the most studied

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MSC-priming TLR ligands, while the expression of TLR2, TLR4, and TLR5 was particularly high in the mouse BM-MSCs under our culture system (Fig. 5).

Pam_3CSK_4 as the comparatively effective stimulant for enhancing regulatory activities in BM-MSCs

TLR ligands can polarize MSCs according to other reports. We thus examined TLR2, 3, 4, 5 ligands for their possibilities to polarize MCSs or for their abundance in MSCs under our system. According to our results from [^{3}H]-thymidine incorporation assays, Pam_3CSK_4 at 1 μg/mL for 72 h further enhanced the suppressive function of BM-MSCs whereas a TLR3 ligand, poly (I:C) at 10 μg/mL; a TLR4 ligand, LPS at 10 μg/mL; and a TLR5 ligand, Rec-FLA-ST at 100 ng/mL for 72 h did not (Fig 6A). After we analyzed the regulatory factors expressed in BM-MSCs with different stimulation, we observed using qPCR that 1 μg/mL Pam_3CSK_4 and 10 μg/mL LPS substantially enhanced *inducible nitric oxide synthase* (*iNOS*) in BM-MSCs (Fig. 6B). The expression of *cox-2*, *il-1ra*, *il-10*, *tsg-6*, *hgf*, *ido*, and *tgf-β* in BM-MSCs, on the other hand, was affected through neither TLR ligands we examined (data not shown). At the protein level, Pam_3CSK_4 at 1 μg/mL and LPS at 10 μg/mL for 96 h enhanced NO secretion in BM-MSCs, in parallel with the results at the mRNA level (Fig. 6C).

In addition to TLR ligands, proinflammatory cytokines, such as TNF-α and IFN-γ, are well-known microenvironmental factors to stimulate MSCs. According to our results from [^{3}H]-thymidine incorporation assays, Pam_3CSK_4-activated BM-MSCs performed the most effective immunomodulatory function among the BM-MSCs activated with different stimulants (Fig. 7A). After we analyzed the regulatory factors expressed in BM-MSCs with different stimulation, we observed using qPCR that *iNOS* could be substantially enhanced through Pam_3CSK_4 in BM-MSCs, and *ido* could be

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enhanced through 200 ng/mL IFN-γ with or without 10 ng/mL TNF-α (Fig. 7B). In addition, IL-1RA expression detected by ELISA assays could not be induced through IFN-γ without TNF-α in BM-MSCs (Fig. 7C).

Deciding to treat BM-MSCs with 1 μg/mL Pam_3CSK_4 for 96 h

We subsequently examined the ideal Pam_3CSK_4 stimulation protocol for BM-MSCs. In OVA-induced asthma model, compared to 5 μg/mL (Mpam5), 1 μg/mL Pam_3CSK_4-treated BM-MSCs (Mpam1) alleviated airway hyper-responsiveness (Fig. 8A) and diminished IL-5 secretion in BALF (Fig. 8C) more effectively. Further, compared to 1 μg/mL Pam_3CSK_4 treated for 24 h, treating BM-MSCs with 1 μg/mL Pam_3CSK_4 for 96 h was a more effective regimen to enhance the immunosuppressive function of BM-MSCs (Fig. 9).

Enhanced immunosuppressive activities in Pam_3CSK_4-stimulated BM-MSCs

To examine whether Pam_3CSK_4 could enhance the immunoregulatory function of BM-MSCs, we performed the [^{3}H]-thymidine incorporation assays *in vitro*. The incorporated radioactivity increases proportionally with $CD4^+$ T cell growth because $CD4^+$ T cells use [^{3}H]-thymidine as a nucleoside resource during mitosis. Consequently, $CD4^+$ T cell proliferation was further inhibited significantly when they were cocultured with Mpam compared with when they were cocultured with Mctrl (Fig. 10).

Mpam as a more effective treatment in the asthma murine model

We further performed more experiments to compare the therapeutic effects of Mctrl and Mpam on OVA-induced allergic asthma murine model. The observations of serum IgE production, Th2 cytokine profiles on OVA restimulated splenocytes, Th2

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cytokine profiles on the BALF, eosinophil infiltration in BALF, airway resistance, and pathological changes in lung tissues are the main criteria for studying OVA-induced asthmatic mice. Based on our results, serum IgE level was significantly decreased by BM-MSCs, irrespective of the Pam_3CSK_4 treatment; however, serum IgG1/IgG2a level was further decreased in the Mpam-treated group, compared to that in the Mctrl-treated group (Fig. 11A). Th2-related IgG1 and Th1-related IgG2a represented whether immune homeostasis was achieved in our asthma murine model. In our results, the Mpam treatment decreased the serum IgG1/IgG2a levels more effectively than those did the Mctrl treatment, suggesting that the imbalanced phenomenon in asthmatic mice was corrected more effectively with the Mpam than with that of the Mctrl treatment. IL-13 secretion of the OVA restimulated splenocytes was significantly downregulated only by Mpam treatment (Fig. 11B). The decreased serum IgG1/IgG2a ratio and IL-13 secretion of the restimulated splenocytes might suggest the advanced systematic therapeutic effects of Mpam on asthmatic mice. For the cytokine profiles and the infiltrated cell compositions from BALF, less Th2 cytokines (such as IL-4 and IL-5) were secreted (Fig. 12A) and less eosinophil was accumulated (Fig. 12B) in the Mpam-treated group compared with those in the Mctrl-treated group. In addition, the cell compositions in BALF were confirmed through flow cytometry (Fig. 12C). The results supported that the pulmonary inflammation attributed to OVA sensitization was alleviated more effectively by Mpam than that by Mctrl. Evidenced by a plethysmograph with paralyzed and tracheotomized mice, the increased airway resistance in OVA-sensitized mice revealed that the recurrent inflammation made their airways susceptible to stimulants. Our results demonstrated that the airway resistance of the OVA-induced asthmatic mice could be ameliorated further with the Mpam treatment than with the Mctrl treatment (Fig. 13A). The susceptible airways of asthmatic mice aggravated the pathological

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effects of the recurrent inflammation, accordingly leading to the remodeled parenchymal lung tissues as the disease progressed. Among the lung histopathological sections of all groups, both Mctrl and Mpam apparently diminished the inflammatory cell infiltration and the bronchial epithelial thickness (Fig. 13B). Moreover, mucin 5AC, which is one of the major components of the mucus secreted by goblet cells in the respiratory tracts, was also measured (76). Further downregulated *muc5ac* expression in the lung tissues was observed in the Mpam-treated group, compared to that in the Mctrl-treated group. This result implied that the Mpam treatment might be able to abolish mucin hypersecretion more effectively than did Mctrl treatment (Fig. 13C). In summary, the therapeutic effectiveness of BM-MSCs on asthmatic mice can be enhanced with Pam_3CSK_4 preactivation.

Pam_3CSK_4 changed the multipotent stem cell properties of BM-MSCs

Mpam was found to form more colonies than Mctrl according to our colony formation assays (Fig 14A). In other words, Pam_3CSK_4 might help enhancing the self-renewal properties of BM-MSCs. In addition, the expression of the stem cell factor, Sca-1, was slightly enhanced in Mpam, compared to that in Mctrl (Fig 14B). Further, under the adipogenic condition, while Pam_3CSK_4 seemed to impair the adipogenic ability of normal mice-derived BM-MSCs, BM-MSCs isolated from TLR2 knockout mice were more prone to differentiate into adipocytes (Fig 14C).

STAT3 signals of the immunosuppression-prone BM-MSCs

To determine whether the STAT3 signaling pathway was involved in the Pam_3CSK_4-enhanced immunoregulatory function of BM-MSCs, we used S3I-201, a STAT3 inhibitor, to treat Mpam (Ms&p). The results of [^{3}H]-thymidine incorporation

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assays revealed that the $CD4^+$ T cell proliferative reduction affected by Mpam was reversed when the STAT3 activating process of Mpam was disturbed. This result supported that the STAT3 signaling pathway was involved in the enhanced regulatory function of BM-MSCs induced by Pam_3CSK_4. However, neither Pam_3CSK_4 nor S3I-201 affected the immunosuppressive function of BM-MSCs in the transwell system (Fig. 15A). It additionally suggested that the enhanced immunosuppressive function of Mpam through STAT3 signaling was cell-cell contact-dependent.

The role of STAT3 activation in enhancing the regulatory function of Mpam was also suggested *in vivo*. Compared to the Mctrl-treated group, less eosinophils infiltrated to lungs in the Mpam-treated asthmatic mice. However, such diminished eosinophil infiltration in the Mpam-treated group was reversed in the Ms&p-treated group. It might imply that the enhanced ameliorating effect of Mpam in asthma murine model was abolished by STAT3 inhibition (Fig 15B).

Nitric oxide as the dominant immunoregulatory factor of Mpam

To evaluate which regulatory molecules determined the enhanced immunosuppressive effects of Mpam, we screened the well-known candidates through qPCR. The mRNA upregulation of *iNOS*, *interleukin-1 receptor antagonist* (*il-1ra*), *tumor necrosis factor-stimulated gene sequence* (*tsg*)-*6*, and *hepatocyte growth factor* (*hgf*) observed in the TLR2 ligand-activated BM-MSCs was downregulated with STAT3 inhibition (Fig. 15C). By contrast, the mRNA expression of *cyclooxygenase* (*cox*)-*2*, *indoleamine 2,3-dioxygenase* (*ido*), and *transforming growth factor* (*tgf*)-*β* in BM-MSCs was not influenced by TLR2/STAT3 signaling (data not shown). The high expression of *iNOS* and *il-1ra* in Mpam was further confirmed at the protein level. In coherence with the patterns at the mRNA level, the increased NO secretion detected

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using Griess assays and the increased IL-1RA secretion detected using ELISA in Mpam were diminished in Ms&p (Fig. 15D, E). Therefore, because the NO and IL-1RA production in the Pam_3CSK_4-activated BM-MSCs were upregulated without STAT3 inhibition but were downregulated with STAT3 inhibition, STAT3 signaling pathway was involved in Pam_3CSK_4-induced NO and IL-1RA production. Although both *iNOS* and *il-1ra* were overexpressed in BM-MSCs through the TLR2/STAT3 signaling pathway, NO worked in a cell-cell contact-dependent manner, whereas IL-1RA worked as a soluble factor. The Pam_3CSK_4-enhanced immunosuppressive function in BM-MSCs and the diminished effects on Mpam through S3I-201 inhibition were observed using [^{3}H]-thymidine incorporation assays without transwell inserts; however, neither TLR2 activation nor STAT3 inhibition induced any differences in the immunosuppressive properties of BM-MSCs in the transwell system (Fig. 15A). The results suggested that cell contact dominated the enhanced regulatory effects of Mpam through the STAT3 pathway. Therefore, NO, rather than IL-1RA was the plausible Pam_3CSK_4-induced regulatory factor in BM-MSCs.

The mobility of BM-MSCs might be increased with Pam_3CSK_4 treatment

It is easy for MSCs to recognize and then migrate to inflammatory sites in hosts. It is therefore believed that MSCs are able to exert their proinflammatory/regulatory function because of such property. We thus examined whether Pam_3CSK_4 increased any chemokines and/or adhesion molecules in BM-MSCs. Among the chemoattractant which is induced in MSCs by TLR ligation, we found that the expression of *ccl7* (detected by qPCR), *mcp-1* (detected by qPCR), CCL3 (detected by ELISA), CCL5 (detected by ELISA), and VCAM-1 (detected by flow cytometry) was upregulated in Mpam. Further, the upregulated expression of *ccl7* and CCL5 in Mpam was reduced

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when we treated Mpam with either STAT3 or iNOS inhibitor. The upregulated expression of VCAM-1 in Mpam was reduced when we treated Mpam with STAT3 inhibitor. (Fig 16).

Unchanged immunoprivileged properties of the conditioned BM-MSCs

To determine the mechanism by which Mpam advanced its immunoregulatory function, we examined the immunoprivileged properties of the conditioned BM-MSCs. The unchanged expression of major histocompatibility complex and costimulatory molecules indicated that TLR2 activation did not significantly affect the immunoprivileged properties of BM-MSCs (Fig. 17).

The molecules indicating apoptosis and anergy in $CD4^+$ T cells were not induced by Mpam

To find out whether Pam_3CSK_4 enhanced the regulatory function of BM-MSCs through inducing T cell death, we examined molecules which are known to be related to apoptosis and anergy in conditioned $CD4^+$ T cells. The expression of FAS and PD-1 detected by flow cytometry showed no differences between all the different conditioned $CD4^+$ T cells (Fig 18A). The expression of *caspase-3* and *itch* detected by qPCR also showed no differences between all the different conditioned $CD4^+$ T cells (Fig 18B).

Regulatory T cells induced by nitric oxide as the immunosuppressive operators

The generation of regulatory T cells is one method by which MSCs control the T cell reactivity. Other reports have also shown that more regulatory T cells are induced through enhanced NO secretion (77). We next examined whether the excessively secreted NO of Mpam performed its immunoregulatory function by inducing regulatory

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T cells. To determine the role of NO as one of the downstream signals of TLR2 activation in BM-MSCs, we treated the Mpam with an iNOS inhibitor (Ml&p), L-NMMA. Furthermore, because STAT3 in the downstream of TLR2 was involved in the NO production in Mpam, we examined whether the STAT3 pathway was involved in the regulatory T cell induction. Through [^{3}H]-thymidine incorporation assays, the 24 h preactivated $CD4^+$ T cells were cocultured with the conditioned BM-MSCs (Fig. 1C). Our results demonstrated that Mpam diminished more proliferative $CD4^+$ T cells than did Mctrl, whereas Ms&p and Ml&p did not (Fig. 19A). The NO secretion of the conditioned BM-MSCs was confirmed using Griess assays. With TLR2 activation, more than ten times the amount of NO was secreted by the BM-MSCs. By contrast, both STAT3 and iNOS inhibitors diminished the NO secretion of Mpam (Fig. 19B). To determine whether the regulatory T cells educated by Mpam caused the differences, the 24 h preactivated responder $CD4^+$ T cells were cocultured with the $CD4^+CD25^+$ T cells, which were respectively preeducated by the different conditioned BM-MSCs (conditioned $CD4^+CD25^+$ T cells) (Fig. 1C). The results revealed that the Mpam-educated $CD4^+CD25^+$ T cells (Tpam) were able to inhibit the responder $CD4^+$ T cell proliferation more effectively than were Mctrl-educated $CD4^+CD25^+$ T cells (Tctrl). Furthermore, this phenomenon was reversed when the Mpam was treated with either the STAT3 inhibitor or the iNOS inhibitor (Fig. 19C). In conclusion, our results suggested that more immunoregulatory $CD4^+CD25^+$ T cells tended to be induced by Mpam than by Mctrl, and the STAT3 and iNOS inhibition appeared to diminish the immunoregulatory $CD4^+CD25^+$ T cell induction of Mpam.

Increased $CD4^+CD25^+Foxp3^+$ T cells induced by Mpam

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Next, to determine the phenotypes of the conditioned $CD4^+CD25^+$ T cells, we investigated the mRNA expression of regulatory molecules in the conditioned $CD4^+CD25^+$ T cells through qPCR and flow cytometry. Compared with the noneducated $CD4^+CD25^+$ T cells, the expression of *tgf-β*, *il-10* (Fig 20A), GITR, and CTLA-4 (Fig 20B) was not significantly changed in Tctrl, Tpam, Ts&p, and Tl&p. Compared with the noneducated $CD4^+CD25^+$ T cells, *foxp3* was upregulated only in Tpam (Fig. 20C). The upregulated Foxp3 expression of Tpam compared with that of Tctrl was also observed at the protein level through flow cytometry. Moreover, the increased $CD4^+CD25^+Foxp3^+$ T cells were induced by Mpam but not by Ms&p or Ml&p (Fig. 20D). Mononuclear cells were also examined in lungs of asthmatic murine model. With the Mpam treatment, less mononuclear cells were recruited, however, a higher percentage of $CD4^+CD25^+Foxp3^+$ cells was observed in the lung homogenate (Fig. 20E).

Sequentially activated NF-κB/STAT3/iNOS signaling in Mpam

The mediation of the seemingly independent mechanisms between TLR2 and STAT3 was examined. TLR2 ligation is known to lead to inhibitor of κB (IκB) kinase degradation then nuclear factor (NF)-κB transcriptional activation. In addition, our data suggested that the NO excessively generated by iNOS was the downstream product of Pam_3CSK_4-activated BM-MSCs through the STAT3 pathway. The sequential correlation between TLR2 stimulation and the downstream NF-κB/STAT3/iNOS activation was finally demonstrated using Western blot. The Western blot results suggested that the expression of pSTAT3 and iNOS in BM-MSCs was upregulated after Pam_3CSK_4 activation for 30 min and 24 h, respectively. In addition, the diminished expression of pSTAT3 and iNOS in Ms&p indicated that S3I-201 inhibited the STAT3

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activation and thus its downstream iNOS expression. The diminished expression of iNOS in Ml&p, however, was mainly attributed to the translation of *iNOS* mRNA, which was regulated by the availability of its substrate, l-arginine (Fig. 21).

Mediation between NF-κB and STAT3 through IL-6

Because STAT3 signaling is mainly induced by cytokine-mediated activation, we examined the cytokine profiles on BM-MSCs with different treatments (conditioned BM-MSCs). We observed that IL-6 production was increased by Pam_3CSK_4 induction irrespective of the inhibition of S3I-201 and L-N^G-monomethyl arginine citrate (L-NMMA), an iNOS inhibitor, suggesting that IL-6 secretion might occur upstream of STAT3 and iNOS activation (Fig. 22A). Moreover, detected using Western blot, TLR2 ligation no longer stimulated iNOS production in MSCs after being treated with IL-6 neutralizing antibody (M6&p), but not with IgG1 κ isotype control antibody (Mic&p) (Fig. 22B). The augmented immunosuppressive function of Mpam was then abolished by IL-6, but not by IgG1 κ isotype control neutralization (Fig. 22C). We thus suggested that IL-6 might play a critical role through the stimulation of iNOS to enhance the induction of regulatory T cells.

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Chapter IV: Discussion

This study established an appropriate priming protocol to enhance the immunosuppressive function of BM-MSCs.

Although MSCs have been proposed for the treatment of inflammatory lung diseases (17), the sustainable translation of MSC therapies to clinical settings has been hampered by the heterogeneity of MSCs and nonstandardized *in vitro* culture technologies (78). Therefore, the present study focused on enhancing the immunoregulatory function of mouse BM-MSCs by using the Pam_3CSK_4-polarizing protocol.

Heterogeneous MSCs can mediate their effects by sensing fluctuating proinflammatory cytokines and TLR ligands in the microenvironment (79). At the beginning of infectious challenges, TLR ligations link innate and adaptive immunity and trigger positive feedback of the immune system by activating proinflammatory cytokine and chemokine production. To maintain the health status of the host, proinflammation-prone MSCs help to eliminate pathogens by recruiting immune effector cells to the sites of infection. However, at the end of the challenges, proinflammatory damage and oxygen stress gradients are accumulated by resultant pathogen cell necrosis and tissue damage debris. To preserve host integrity, anti-inflammation-prone MSCs reduce effector immune cell infiltration but increase regulatory immune cell induction (48). Hence, plasticity in immunomodulatory function causes MSCs to be both advantageous and disadvantageous in the context of inflammation.

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The polarization of BM-MSCs is apparently susceptible to the stimuli conditions. This is why the discrepancies in the experimental settings both *in vitro* and *in vivo* led to several inconsistent and partially contradictory results related to TLR-polarized MSCs (35, 80). The differences in the source of MSCs, the concentration and timing of the stimuli used, and the culture conditions might explain the conflicting results (81). Given the uncertain effects of TLR polarization on MSCs, consistent therapeutic outcomes and immunoregulatory mechanism-based validations are required to establish the effectiveness of modified MSCs in asthma therapy.

We demonstrated that the immunomodulatory BM-MSCs were polarized by a TLR2 agonist, Pam_3CSK_4. According to both our *in vitro* and *in vivo* functional assays conducted using [^{3}H]-thymidine incorporation assays and acute OVA-induced asthmatic murine models, respectively, Pam_3CSK_4 augmented the immunosuppressive function of BM-MSCs. The results of our immunoregulation-prone BM-MSCs stimulated by Pam_3CSK_4 are consistent with the findings of Pevsner-Fischer et al. (82) but inconsistent with the findings of Lei et al (83). In those two studies, the mouse BM-MSCs were incubated with different concentrations (1 vs. 2 μg/mL) of Pam_3CSK_4 for the same period (48 h) before they were cocultured with different responder cells (T cell line specific to MOG p35-55 vs. splenocytes isolated from C57BL/6 and BALB/c spleens) in different MSC to responder cell ratios (1:20 vs. 1:10). The discrepant protocols might have caused the distinct results.

Based on our study, we observed that the immunosuppressive BM-MSCs should be administered in post-treatment instead of pretreatment settings. In other words, they should be cocultured with 24 h preactivated $CD4^+$ T cells *in vitro* and infused after asthmatic mice were OVA-challenged *in vivo*. The suggested modification of BM-MSCs in the appropriate therapeutic time point of asthma enabled the treatment of

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asthma by using BM-MSCs after the disease had developed. The ideal BM-MSC to responder $CD4^+$ T cell ratio was 1:2 to 1:200 for acting immunosuppressive function *in vitro*, and the ideal infused cell number of BM-MSCs was 5×10^5 cells per mouse. BM-MSCs treated with 1 μg/mL Pam_3CSK_4 exerted better immunosuppressive effects than those with 5 μg/mL Pam_3CSK_4 *in vivo*. Moreover, BM-MSCs treated with 1 μg/mL Pam_3CSK_4 for 96 h were more effective than those treated for 24 h *in vitro*.

According to our data, it was in contrast to the instinct that neither more BM-MSC administration (1×10^6 cells per mouse) nor BM-MSCs pretreated with higher Pam_3CSK_4 dosage (5 μg/mL) had better therapeutic effects. However, this may not be as surprising if we take the double-edged properties of the immunosuppressive molecules into account. The sustained production of NO endows macrophages with cytostatic or cytotoxic activities against pathogens and tumor cells (84). In contrast, suppression of iNOS activity by either chemical inhibitors or genetic ablation in MSCs largely reverses the therapeutic effect of MSCs in mouse models of GvHD and delayed-type hypersensitivity responses. A study has also shown that MSCs are the critical mediators of *Mycobacterium tuberculosis*-induced immunosuppression in an NO-dependent manner *in vitro* and *in vivo*, suggesting a function of the MSC-NO axis in endogenously induced immunosuppression (36). Hence, the discrepant outcomes are in fact discriminated by the concentration and source of NO, and by the exact stimulatory conditions which are used to induce NO production (85). The more NO production is therefore not necessary to guarantee the better immunosuppressive function at all time. Additionally, Pam_3CSK_4 was able to upregulate the expression of some adhesive molecules and chemokines in our BM-MSCs. The chemoattractant secreted by MSCs may stimulate the migration of immune cells in proximity of MSCs. It may consequently enable the local suppressive effect of MSCs through rapidly diffusing NO

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at one time, but induce inflammatory burden through recruiting proinflammatory mediators at another (36).

On the other hand, it was compatible with the instinct that the long-term stimulation (96 h) was more effective than the short-term stimulation (24 h) of Pam_3CSK_4 to enhance the suppressive activity of BM-MSCs. The same idea as we describe above: acute stimuli are likely to point to pathogen burden while chronic stimuli are likely to refer to hyper-responsive inflammation. Therefore, priming protocols must be adjusted before different applications.

In our *in vivo* experiments, the symptoms of allergic asthma, including proinflammatory cytokine production, airway resistance, and systemic IgG1/IgG2a levels on OVA-sensitized mice, were alleviated more significantly by BM-MSCs as long as they were coupled with Pam_3CSK_4 induction, whereas the serum IgE levels and histopathological changes were barely engaged. In our *in vivo* studies, when the Mpam treatment decreased the serum IgG1/IgG2a levels more effectively, it did not decrease the serum IgE levels at the same time. This result was possibly because serum IgE and IgG1 levels represent different stages of asthma. IgE was increased at the beginning of disease development in the murine asthma model. IgG1 was later increased while the Th2-mediated disease progressed while IgE was not increasing. Therefore, at certain time point, less amelioration in IgE levels, but more amelioration in IgG1/IgG2a levels would be observed in our Mpam-treated groups. On the other hand, the histopathological amelioration of BM-MSCs might take more time to develop because the thickness of basement membrane, epithelium, and subepithelial smooth muscle layer, and the number of mast and goblet cells gradually decreased with the longer duration of BM-MSC treatment in a chronic asthma murine model (86). Overall, we

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finally determined the unvarying protocol for the steady turnout of immunosuppressive BM-MSCs in treating the acute OVA-induced asthma murine model.

This study validated the Pam_3CSK_4-modified BM-MSCs with the understanding of their immunosuppressive mechanisms.

Increased NO secretion

The therapeutic advantages of Mpam were proved by its more effective asthma amelioration in the OVA-sensitized asthmatic mice. Next, to validate the enhanced immunosuppressive effects of the Pam_3CSK_4-stimulated BM-MSCs, we further studied the immunoregulatory mechanisms of the modified BM-MSCs.

In non-stimulated cells, STAT3, like other STAT proteins, is kept in an inactive cytoplasmic form. STAT3 activation is most commonly mediated by JAK1. Nuclear translocation of STAT3 is mediated by phosphorylation of a critical tyrosine residue (Tyr705). STAT3 transcriptional activity and DNA binding are further activated through serine (Ser727) phosphorylation (87). It is well-established that STAT3 signaling drives accumulation and activation of immunosuppressive cells. STAT3 is responsible for the activation of myeloid-derived immunosuppressive cells (MDSCs), differentiation of macrophages toward the M2 phenotype, and the absence of functional DCs (88, 89).

According to our [^{3}H]-thymidine incorporation assays, the augmented immunosuppressive functions of BM-MSCs induced by Pam_3CSK_4 were diminished with the incorporation of S3I-201, a STAT3 inhibitor. Evidence has shown that the constitutive activation of STAT3 in tumor cells is a crucial suppressive mechanism in

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immune reactions (90). In addition, STAT3 signaling not only upregulates proangiogenic genes, such as vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1-alpha (HIF-1α), and matrix metallopeptidase (MMP) 9, etc., but also mediates immunosuppressive function in regulatory B cells (91). Moreover, the enhanced suppressive activity through STAT3 signals in Mpam is supported by the responses of both mouse and human myeloid-derived suppressor cells (MDSCs) to tumor-derived exosomes. Tumor-derived exosomes triggered STAT3 activation in MDSCs through a TLR2/MyD88-dependent pathway (66).

NO is a rapidly diffusing molecule and an unstable oxidative species. Therefore, it can only act in close proximity to the cells producing it. The biological activity of NO diminishes markedly over a distance of a few cell diameters (36). This might explain that Pam_3CSK_4, which enormously stimulated NO secretion in BM-MSCs, enhanced the suppressive function of BM-MSCs in the cell-cell contact-dependent setting, but not in the transwell system. In addition, the highly-secreted NO and the enhanced suppressive function of BM-MSCs under Pam_3CSK_4 stimulation was diminished with STAT3 abrogation in a cell-cell contact-dependent manner. Further, NO production is catalyzed by NOS, nitric oxide synthase, for which there are three genes in humans and mice: iNOS, inducible primarily in macrophages; nNOS, in neurons; and eNOS, in endothelial cells. Among the three candidates, iNOS expression is inducible and plays a major role in immune regulation (36). Compatibly, other reports also demonstrate that iNOS transcription is downstream of the TLR and STAT signaling pathways in murine macrophages (92, 93). The unknown mechanism which regulates NO expression in the Pam_3CSK_4-induced BM-MSCs was thus hypothesized to be through the TLR2/STAT3/iNOS signaling pathway. To support this idea, our results demonstrated that the enhanced suppressive function of BM-MSCs by Pam_3CSK_4 was abolished by

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either the STAT3 or iNOS inhibitor. Diminished NO secretion because of STAT3 or iNOS inhibition in Mpam was therefore possibly related to the abrogation of the enhanced suppressive function of Mpam. Moreover, our Western blot results revealed that the STAT3 inhibitor impeded STAT3 phosphorylation, and subsequently diminished iNOS expression in Mpam.

How then can such labile NO make MSCs immunosuppressive? One of the characteristics of MSCs is their ability to migrate to sites of damaged tissue. MSCs have been shown to exert anti-inflammatory effects on almost all the cells of the innate and adaptive immune systems via a variety of mechanisms, notably cytokine and chemokine secretion. In addition, the chemoattractant profiles expressed by MSCs vary depending on the specific microenvironment which MSCs contact with. Through these chemokines and adhesion molecules, immune cells possibly accumulate in close proximity to the MSCs, where the high concentration of secreted NO can suppress the immune cells more efficiently (36, 94).

Our data suggested that Pam_3CSK_4 induced the expression of chemokines, such as CCL3, CCL5, *ccl7*, and *mcp-1*; and adhesion molecule, such as VCAM-1 in BM-MSCs. CCL3 and CCL5 are CCR5 ligands, and CCL7 is one of the CCR3 ligands. CCR5 is a chemokine receptor that is preferentially expressed on Treg cells. $CCR5^+$ T cells have been shown to play important roles in atherosclerosis, rheumatoid arthritis, and autoimmune encephalomyelitis. CCR5 expression is associated with Treg's homing to the damaged tissues and suppressing local inflammation (95). However, in lupus nephritis patients, CCR5 is one of the primary receptors in the mechanism of recruiting all T cells into the inflamed kidney (96). In addition, cancer cells can promote the recruitment of Treg cells into tumors and can facilitate their peripheral expansion and retention through secreting chemokines and cytokines. In 3-methylcholanthrene-induced

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fibrosarcoma mice, the receptor-ligand interactions for CCR3 and CCR5 have no preference for $Foxp3^+$ T cells over $Foxp3^-$ T cells (97). Therefore, it is uncertain whether the upregulated expression of CCL3, CCL5, and *ccl7* in BM-MSCs by Pam_3CSK_4 priming is associated with creating a better circumstance for the suppressive action of NO.

On the other hand, VCAM-1 is normally expressed on the surface of antigen presenting cells, mediating target cell binding by interaction with its receptor on leukocytes and lymphocytes. In an *in vitro* study, a blocking antibody against VCAM-1 in MSCs reduces the number of adhesive T cells to MSCs significantly. Moreover, although the production of NO by MSCs is unaffected by blockade of VCAM-1 in MSCs, the VCAM-1 antibody significantly diminishes the suppressive function of MSCs observed using T cell [H^3] incorporation assays (98). However, despite it is conceivable that the T lymphocytes may be held in place by VCAM-1 so that the effects of NO from MSCs can be attained *in vitro*, The interaction of T lymphocytes with VCAM-1 on MSCs has not been established *in vivo*.

MCP-1 is a CCR2 ligand. A report reveals that when *MCP-1*$^{-/-}$ BM-MSCs are transplanted into C57BL/6 mice, they fail to induce $CD3^+$ T cell apoptosis and Treg upregulation *in vivo*. However, the MCP-1 secretion of BM-MSCs is suggested to be regulated by FASL in that article. FASL-induced MCP-1 secretion in BM-MSCs recruits T cells for FASL-mediated apoptosis. The apoptotic T cells subsequently triggered macrophages to produce high levels of TGF-β, which in turn leads to the upregulation of $CD4^+CD25^+Foxp3^+$ Treg cells (99). In contrast, compared to Mctrl, Mpam neither expressed more FASL, nor triggered more *caspase-3* in T cells in accordance with our results. Therefore, Mpam-induced *mcp-1* expression was unlikely to pull T cells closer in our experimental setting.

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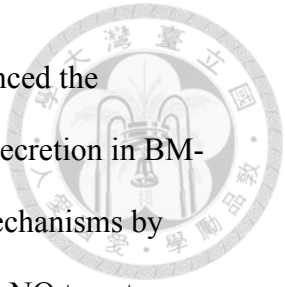

Apparently, our results could only suggest that Pam_3CSK_4 enhanced the immunosuppressive function of BM-MSCs through stimulating NO secretion in BM-MSCs. However, more experiments are still needed to explore the mechanisms by which Mpam could bring T cells closer enough to enable higher level NO to act.

More $CD4^+CD25^+Foxp3^+$ Treg cells

The peripheral tolerance mechanisms work naturally as a compensational strategy for the self-reactive immune cell eliminations (100). MSCs have been shown to inhibit macrophage and dendritic cell maturation, induce a shift in the T helper cell polarization, and promote regulatory T cell (Treg) differentiation (101). Antigen-specificity and longevity make Treg a more potent therapy with prolonged protection against recurrence of disease. Several T cell subsets with regulatory properties have been described, such as the IL-10-producing Tr1 cells and $CD8^+CD28^-$ T cells. However, the most studied cell in recent years is notably the naturally occurring $CD4^+CD25^+$ Tregs that express the transcription factor Foxp3 (8). IL-2R is composed of α-chain (CD25), β-chain (CD122), and the common cytokine receptor γ chain (CD132). Although all three chains contribute to IL-2 binding, only the β- and γ-chains are responsible for signal transduction following IL-2 binding to the receptor. Furthermore, because inducing the production of $CD4^+CD25^+Foxp3^+$ lymphocytes has been indicated as a potential mechanism of MSCs in ameliorating autoimmune disease and in immunoprivileging cancer cells (102, 103), Foxp3 expressing $CD4^+CD25^+$ T cells are primarily under the scope of our investigation.

Our *in vitro* results demonstrate that the production of NO, an immunoregulatory molecule of BM-MSCs, was highly induced by Pam_3CSK_4. NO secreted from Mpam

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might subsequently induce additional $CD4^+CD25^+Foxp3^+$ regulatory T cells, as shown in other reports, in a cell-cell contact-dependent manner (104). The $CD4^+CD25^+Foxp3^+$ cells were also detected in the lung tissues of the asthma murine model conducted in this report. In consistent with our *in vitro* study, the Mpam treatment induced more $CD4^+CD25^+Foxp3^+$ cells in the lung tissues than the Mctrl treatment did.

Our data suggested that cocultured BM-MSCs upregulated Foxp3 expressing $CD4^+CD25^+$ T cells. The immunosuppressive BM-MSCs polarized by Pam_3CSK_4 additionally educated more $CD4^+CD25^+Foxp3^+$ lymphocytes through iNOS activity in the downstream of STAT3 stimulation. However, NO has been implicated in the differentiation of various T cell subtypes, including the NO-dependent induction of $Foxp3^-$- or $Foxp3^+$ Treg. In addition, to help protect the organisms against exaggerated immune responses, iNOS-derived NO has also been shown to suppress T cells through versatile mechanisms other than Treg induction. For instance, NO promotes apoptotic signals while inhibits proliferative signals in T cells (105, 106). At high concentration, NO can inhibit TCR-induced T cell proliferation and cytokine production. Also, the high concentration of NO is found to suppress T cell proliferation through inhibiting STAT5 phosphorylation (36). Thereafter, inducing additional $CD4^+CD25^+Foxp3^+$ T cells might be just one mechanism through which Mpam performed its enhanced immunosuppressive function. Furthermore, because MSCs have been demonstrated to reduce allergic sensitization in asthmatic mice either through weakening the antigen presenting properties of dendritic cells or through skewing the suppressive phenotype of macrophages (107, 108), the transplantation of Mpam might have multiple effector targets to suppress asthmatic signs in the murine model. Furthermore, the therapeutic advantages of MSCs in chronic asthmatic felines led us to envision the notion of enhancing the long-term therapeutic benefits of BM-MSCs with Pam_3CSK_4

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modification (109). The induction of regulatory T cells by MSCs is additionally suggested as a crucial point for the long-term effects of MSCs after infusion (110).

$CD4^+CD25^+Foxp3^+$ Treg can be developed in the thymus as well as can be induced in the periphery. Thymus-derived and peripherally induced Treg cells are both critically involved in prevention of autoimmune diseases. However, despite thymic and peripherally induced Treg are phenotypically and functionally similar, their differential mechanisms are distinct. Treg development and Foxp3 induction are highly dependent on CD28 costimulation in thymus, whereas only when TGF-β is present, do $Foxp3^+$ Treg cells are generated in periphery (111).

In detail, at day 3 after birth, autoreactive Treg repertoire starts being shaped in the thymus through a two-step positive selection, combined with a reduced sensitivity to negative selection (69). The transgenic mice whose thymic cortical epithelial cells express a single MHC class II/peptide ligand are generated to clarify the differences between Treg and conventional T cells in positive selection process. Functional, single ligand-reactive Treg precursors, are preferentially selected by these transgenic mice. It indicates that positive selection of Treg precursors occurring in thymic cortical epithelium requires high avidity interactions. By contrast, positive selection of conventional T precursors is very MHC/peptide-specific and is mediated by nonagonist self-ligands (112). After high-avidity autoreactive Treg precursors are directly selected in thymic cortical epithelium, the second step of positive selection and the negative selection are taken place in thymic medullary epithelium. A transcription factor, autoimmune regulator (AIRE), is expressed in thymic medullary epithelium. AIRE promotes the expression of peripheral tissue antigens in thymic medullary epithelial cells. Autoimmune disorders which are developed either for AIRE deficiency or for MHC class II lack in thymic medullae demonstrate the idea that interactions of

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developing Treg cells with MHC class II medullae are sufficient to support Treg. Hence, after migration of developing Treg cells from the cortex to the medulla, interactions with cognate antigen on thymic medullary epithelium may then serve to selectively induce the survival and/or population expansion of otherwise short-lived Treg precursors (113).

The thymic medulla is packed with BM-derived antigen presenting cells and is permeable to circulating self-antigens entering from the blood stream. Thus, the medulla is the appropriate site for negatively selecting ubiquitous antigen-specific Treg precursors and autospecific conventional T cell precursors. Recognition of autoantigens presented by thymic DCs induces apoptosis of autospecific T cell precursors. On the other hand, recognition of autoantigen presented by thymic medullary epithelial cells mainly leads to induction of a reversible unresponsive state despite that thymic medullary epithelium can, to a limited extent, also induce deletion of autospecific T cell precursors. To spare the integrity of the host while the immune system is on its mission to attack, autoreactive conventional T precursors must be gotten rid of, but autoreactive Treg precursors must otherwise be preserved through negative selection. In BM chimeras which contain MHC-deficient hematopoietic cells, the increased generation of mature conventional T cells and Treg cells is observed. It indicates that conventional T precursors and Treg precursors are both sensitive to thymic DC-mediated deletion. However, although autospecific Treg precursors are negatively selected by BM-derived antigen-presenting cells, they appear relatively resistant to negative selection induced by thymic medullary epithelium. In mice in which superantigen is presented specifically in thymic medullary epithelium but not BM-derived antigen-presenting cells, superantigen-specific Treg precursors are substantially enhanced while conventional T cell precursors are limitedly deleted (114).

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It appears therefore that distinct subsets of antigen-presenting cells, presenting unique sets of self-peptides, contribute to shape the Treg repertoire in central tolerance. High avidity interaction is required for positively selecting Treg precursors in thymic cortical epithelial cells. In contrast, subsequent negative selection deletes the developing Treg repertoire of cells specific for ubiquitous antigens but spares cells specific for tissue-specific antigens in thymic medullary epithelial cells because the peripheral antigens are allowed to permeate into thymic medulla instead of cortex through circulation. Thymic DCs can cross-present tissue-specific antigens which are AIRE-mediated by thymic medullary epithelial cells, revealing that DCs are there to share the burden of thymic medullary epithelium (115).

The second pathway of Treg cell generation is in the periphery. Several immunomodulatory T cell populations induced in peripheral lymphoid organ and/or *in vitro*, such as Th3 and Tr1, have been described. Naïve $CD4^+Foxp3^-$ T cells encounter their cognate antigens have also been known to be able to differentiate into periphery-derived $CD4^+Foxp3^+$ Treg cells. Several reports have demonstrated this idea. Oral antigen administration, known to induce tolerance, is shown to lead to an increase in the proportion of $CD4^+CD25^+$ T cells with suppressive activity. In transgenic mice which do not express a TCR specific for an OVA-derived peptide presented by MHCII, they lack thymus-derived Treg for OVA-derived peptide. However, antigen feeding still leads to conversion of $CD4^+CD25^-Foxp3^-$ conventional T cells to $CD4^+CD25^+Foxp3^+$ regulatory T cells. In addition, the $Foxp3^-$ T cells isolated from RAG-deficient mice are generated to clarify this question. These $Foxp3^-$ T cells, which express a transgenic TCR specific for an influenza hemagglutinin-derived peptide presented by $I\text{-}E^d$ (HA-TCR), are adoptively transferred into normal mice presenting the influenza hemagglutinin peptide-recognized DCs. Two weeks later, substantial numbers of $Foxp3^+$ T cells are

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found among the HA-TCR$^+$ T cells. Several reports additionally indicate that antigen-specific Foxp3-expressing Treg can be induced from mature conventional T cells in presence of TGF-β *in vitro* and *in vivo* (69). It has been shown that in vivo TGF-β expression in the pancreatic β cells during the priming phase of diabetes is sufficient to inhibit disease onset via accumulation of Foxp3$^+$ Treg cells (116).

MSCs, which are able to secret TGF-β, may induce the development of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T lymphocytes. However, because the mRNA expression of *tgf-β* in BM-MSCs was not influenced by TLR2/STAT3 signaling according to our data, this might discourage the differentiation of naïve CD4$^+$Foxp3$^-$ T cells into periphery-derived CD4$^+$Foxp3$^+$ Treg cells by Mpam. On the other hand, despite the nonproliferative phenotype of Treg *in vitro*, these cells proliferate upon adoptive transfer into lymphopenic mice. Moreover, a sizeable proportion of Treg proliferates in the periphery of mice when they encounter cognate ligands (69). Therefore, our data might imply that BM-MSCs further induced peripheral expansion of thymic Treg, rather than peripheral induction of Treg with Pam_3CSK_4.

Via TLR2/NF-κB/IL-6/STAT3 signaling pathway

TLRs are broadly expressed in immune and non-immune cells. They are important sensors for the detection of foreign pathogens and endogenous danger signals (117). TLR2 is composed by the N-terminal extracellular domain, the transmembrane domain, and the C-terminal intracellular domain. Its extracellular domain, which is characterized by containing leucine rich repeat (LRR) motifs, displays high similarity with the corresponding sequences of TLR1 and TLR6. This is probably related to a cooperation of TLR2 with TLR1 or TLR6 in recognition of TLR2 agonists. The

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intracellular Toll/IL-1 receptor (TIR) domain formed with alternating β–sheets and α–helices contributes to a surface patch for interaction with the adapter molecule myeloid differentiation factor (MyD) 88, whereas the transmembrane domain of TLR2 is involved in canonical NF-κB pathway (please see appendix for Fig. 5A).

TLR2 signaling pathway starts with TLR2 ligands binding to TLR2. TLR2, which forms homodimers or heterodimers with TLR1 or TLR6, subsequently experiences an extracellular conformational change, altering the TIR domain interaction between TLR2 dimers. The alterations of the TIR domain interaction thus allow them to recruit MyD88. The recruitment of MyD88 to the receptor complex then leads to the recruitment of IL-1 receptor-associated kinase (IRAK) and IRAK2. Following IRAK recruitment, ubiquitinated TNF receptor-assiciated factor (TRAF) 6 interacts with TGF-β-activated kinase (TAK) 1 and TAK1 binding protein (TAB) 1. Activated TAK1 thereby starts kinase cascades as TAK1 can activate IκB kinase (IKK)-β (please see appendix for Fig. 5B) (118). IKK-β is responsible for IκB phosphorylation, leading to IκB ubiquitin-dependent degradation, thus dissociating IκB from NF-κB. The released NF-κB subsequently translocates into the nucleus and is further regulated through protein phosphorylation and acetylation.

NF-κB and STAT3 control numerous physiological processes including development, differentiation, immunity, and metabolism. They are rapidly activated in response to various stimuli. Beyond this, NF-κB and STAT3 control the expression of the other cytokines and inflammatory mediators while being activated by certain stimuli, thus serving as a regulatory hub that coordinates inflammatory responses. Quite often, cytokines whose expression is induced in response to NF-κB lead to STAT3 activation (119). MSCs are able to secret biologically active substances, such as growth factors, cytokines and chemokines. Cytokines including IL-6, IL-10 and TGF-β are

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among the most described soluble factors secreted by MSCs, while the actions of IL-6 and IL-10 are mediated by their interacting with the JAK-STAT system (94). On the other hand, the MyD88-dependent pathway in the downstream of TLR is known to lead to NF-κB transcriptional activation and consequent IL-6 secretion (120-122). Therefore, the mediation between NF-κB and STAT3 through IL-6 is suggested for the apparently independent signaling mechanics between TLR2 and STAT3 (123).

Based on our results, the iNOS inhibitor had a more influential role in responder T cell proliferation in response to the conditioned T cells than did the STAT3 inhibitor. This result might imply the synergic induction of iNOS through the TLR2/ NF-κB and IL-6/STAT3 pathways (124). In addition, our data revealed that IL-6 secretion of BM-MSCs under our condition was STAT3-independent, which might additionally eliminate the concern that the presence of active NF-κB in the nucleus was prolonged by STAT3 positive feedback (122). However, despite IL-6 was likely to be the mediator between NF-κB and STAT3 signaling, the possibility of the cooperation between NF-κB and STAT3 pathways was not excluded. In addition to binding to adjacent sites in the control regions of shared target genes, physically interactions are observed between NF-κB and STAT3 molecules (119).

Finally, the enhanced immunomodulatory function of Mpam demonstrated through IL-6/STAT3 pathway in our study may additionally inspire an assumption: directly using IL-6 to prime BM-MSCs may make things easier. IL-6-dependent secretion of PGE2 is the main mechanism by which the paw swelling in the collagen-induced arthritis (CIA) animal model is alleviated. MSC-derived PGE2 is reported to inhibit DC differentiation and maturation, and to increase IL-10 secretion from macrophages. As the consequences, T cell activation is reduced (125). However, according to our results, Pam_3CSK_4 induced IL-6 secretion without simultaneously upregulating the expression

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of *cox-2* in BM-MSCs. In addition, the amount of IL-6, which is able to induce enough PGE2 to suppress paw swelling in the CIA model, is 200 times more than that induced by our Pam_3CSK_4 priming protocol (100 ng/mL vs 500 pg/mL). It might imply that the enhanced immunoregulatory function of Mpam was unlikely to be attributed to IL-6-induced PGE2 secretion mainly. IL-6/STAT3 pathway might be one of the methods by which Pam_3CSK_4 induced substantial NO secretion in BM-MSCs. After all, the direct crosstalk between NF-κB and STAT3 could not be excluded. This is supported by another report which examines whether recombinant human IL-6 (5 ng/mL) improves the osteogenesis of human BM-MSCs as effectively as the low level LPS (10 ng/mL) does. Their results discourage the idea that recombinant IL-6 would work as well as LPS would in every way: recombinant IL-6 upregulates the chitinase-like protein YKL-40 (may prevent damage to bone extracellular matrix by reducing the harmful effects of proinflammatory cytokines), but fails on promoting osteogenic differentiation in human BM-MSCs (126). Hence, using Pam_3CSK_4 and recombinant IL-6 to stimulate mouse BM-MSCs may not promise the same effect despite there may be seemingly correlated mechanisms.

The limitations of this study suggested that more experiments might be needed to guarantee the successful applications of Pam_3CSK_4-priming BM-MSCs, especially, to the different cases. Based on the literature, in addition to the $CD4^+CD25^+Foxp3^+$ regulatory T cells, various suppressive effector cells induced by the modified BM-MSCs are expected to be effective in treating asthma. It possibly explained the discrepancy between strikingly NO level and the significant but less amount of $Foxp3^+$ cell induction with Mpam *in vitro* and *in vivo*. On the other hand, through chemoattractant, immune cells possibly accumulate in close proximity to the MSCs, where the high concentration of unstable NO can suppress the immune cells more

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efficiently. With more understanding of the varied chemoattractant profiles expressed by MSCs under specific microenvironment, we might have chances to exploit the benefit out of NO in Pam_3CSK_4-induced BM-MSCs. Finally, the priming protocol might be adjusted for different applications. More detailed studies are required to make sure that the excess production of NO is advantageous for the cases.

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Chapter V: Prospects

Our results raised several points that should be considered when applying clinically. Although our Pam_3CSK_4 priming protocol was demonstrated to enhance the therapeutic effects of BM-MSCs on asthma murine model, specific protocols must be established for different applications which are treated by MSCs derived from different sources and modified with different priming molecules.

Different sources of MSCs possess different TLR profiles. The characteristics of the same sort of MSCs are changed in response to different levels of the same cytokines or TLR ligands (36). Different effects stemmed from different MSCs in response to different stimulations thereafter would be detected.

The precise mechanisms based on specific candidate factors such as NO, IDO, IL-10, PGE2, chemoattractant, and growth factors, etc. from MSCs in specific diseases are needed to be fully described. MSCs, while creating an environment that favors endogenous tissue repair may be enough when applying to GvHD, their production of reparative growth factors and their ability to replace damaged cells are additively useful when treating cardiac diseases (127). To address these issues, more detailed studies are required to explore the production and functions of candidate factors individually and link their function with the cellular properties.

In addition, regarding homing to the site of tissue damage, it is important to find out which molecules and receptors are crucial. The different sources and different priming of MSCs may have differential homing mechanisms to various tissues in the body.

Most immunosuppressant drugs have severe side effects. They not only increase risk of infectious complications, but also induce nephrotoxicity, hepatotoxicity,

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neurotoxicity, and allergic reactions, etc. In contrast, MSCs are attractive because they are safe and induce little, if any, toxicity (34). Therefore, the Pam_3CSK_4-modified BM-MSCs are worthy being tested out in other autoimmune disease models despite the priming protocol may need to be adjusted to optimize the therapeutic effects. On the other hand, to expect a successful outcome, our understanding of the enhanced immunomodulatory mechanisms of Pam_3CSK_4-modified BM-MSCs in treating asthmatic mice possibly allows us to select the well-polarized BM-MSCs depending on their secretion of IL-6 and NO before their administration.

A question should be asked, then, that what kinds of diseases would plausibly get benefits from Mpam. The demonstrated immunosuppressive ability of MSC has translated to clinical trials currently being undertaken in GvHD, Crohn's disease, MS, SLE, COPD, insulin-dependent diabetes mellitus, and in the renal transplant setting. The tissue repair capability of MSC is being investigated in clinical trials for cardiac repair, bone disorders (osteogenesis imperfecta), bone fracture, and liver repair (cirrhosis), as well as for enhancing engraftment after haematopoietic stem cell transplantation (34). With Pam_3CSK_4 stimulation, BM-MSCs established better immunosuppressive function under our experimental setting. Mpam is more likely to be able to control diseases with chronic inflammatory burdens. However, because Pam_3CSK_4 seemed to increase stemness properties of BM-MSCs under our experimental setting, it is possible that Mpam might also have better tissue repair ability.

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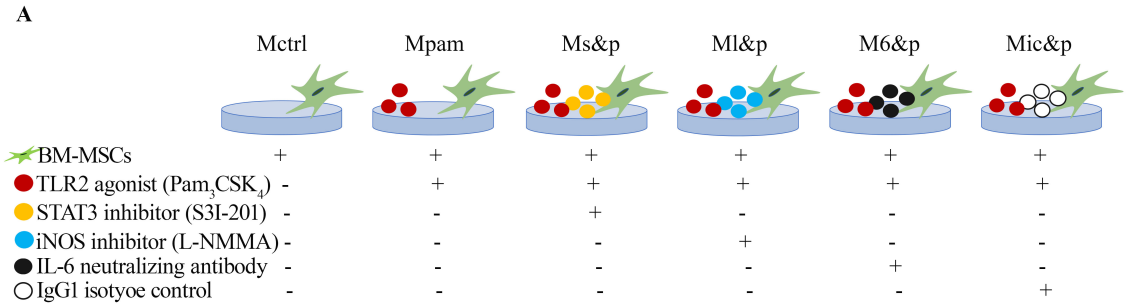
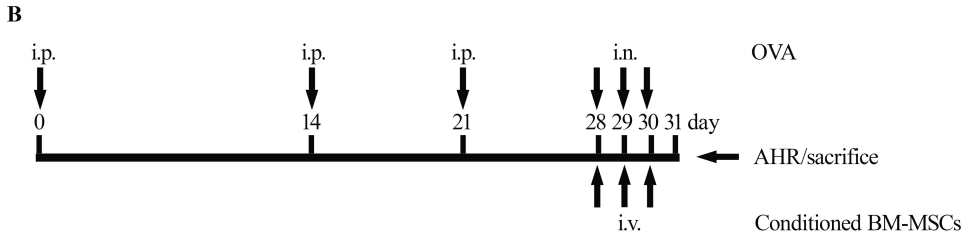
Chapter VII: Figures





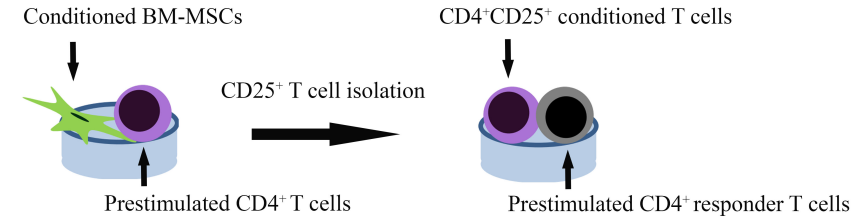


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Fig 1. Experimental designs

(A) The control BM-MSC is presented as Mctrl. The TLR2 agonist, Pam_3CSK_4, -treated BM-MSCs is presented as Mpam. The combination of Pam_3CSK_4 and the STAT3 inhibitor, S3I-201, -treated BM-MSC is presented as Ms&p. The combination of Pam_3CSK_4 and the iNOS inhibitor, L-NMMA, -treated BM-MSC is presented as Ml&p. The combination of Pam_3CSK_4 and the IL-6 neutralizing antibody-treated BM-MSC is presented as M6&p. The combination of Pam_3CSK_4 and the IgG1 κ isotype control antibody-treated BM-MSC is presented as Mic&p. (B) The timeline of OVA-induced asthma murine model. Mice were intraperitoneally
(i.p.) sensitized and intranasally (i.n.) challenged using PBS in the negative control (PBS) group but using OVA in the positive control (OVA) group, and Mctrl and Mpam treatment groups. Treatment groups were intravenously (i.v.) treated with Mctrl and Mpam. The cell number used to treat this asthma murine model was 5×10^5 / mouse. (C) Prestimulated $CD4^+$ T cells were cocultured with the mitomycin c-treated conditioned BM-MSCs in a 20:1 ratio. $CD25^+$ cells were then isolated from those $CD4^+$ T cells to make the conditioned $CD4^+CD25^+$ T cells, such as Tctrl, Tpam, Ts&p, and Tl&p. The mitomycin c-treated conditioned $CD4^+CD25^+$ T cells were finally cocultured with prestimulated $CD4^+$ responder T cells in a 1:1 ratio.

AHR: airway hyper-responsiveness

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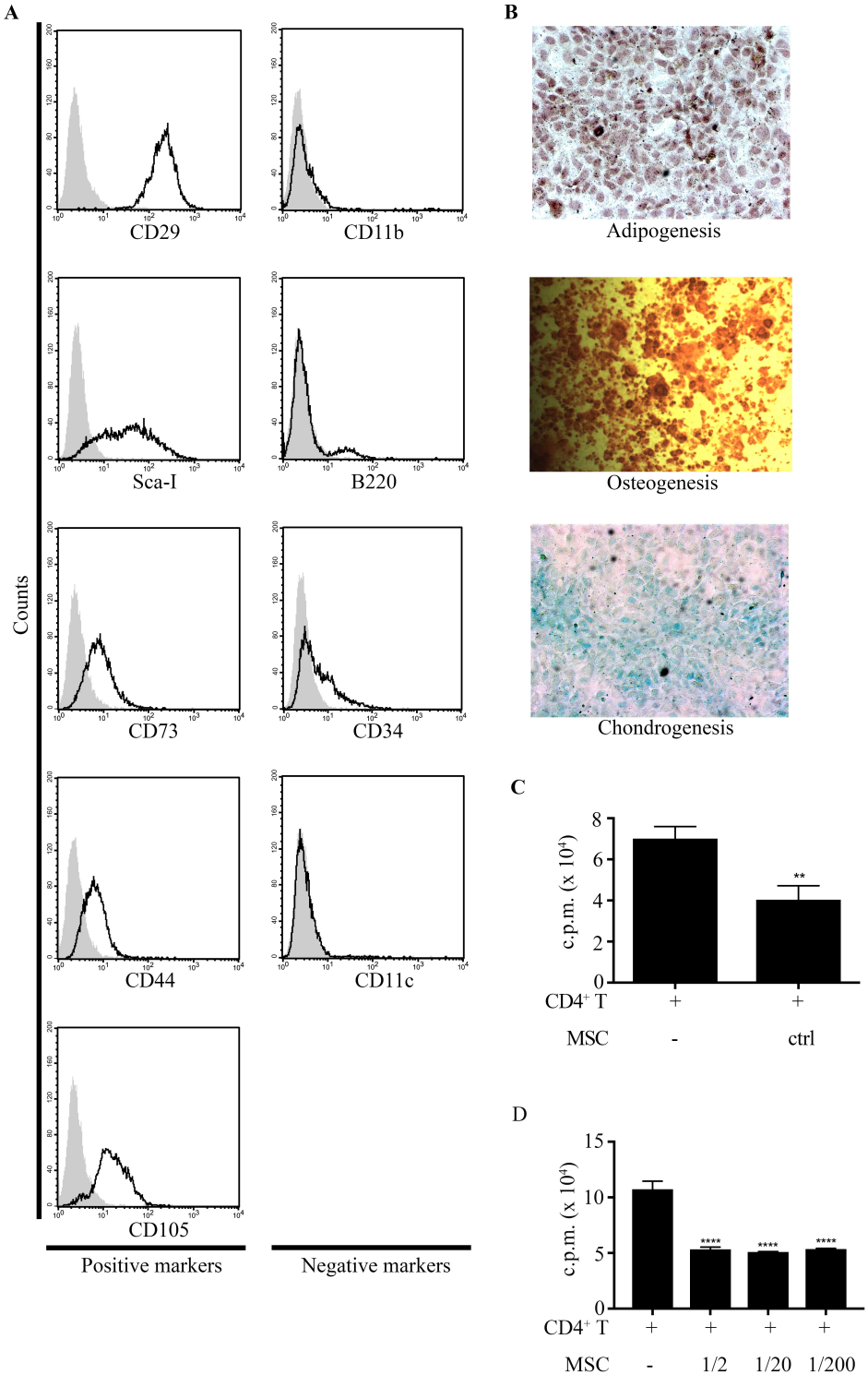

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Fig 2. Characterization of BM-MSCs

(A) Positive expression of mesenchymal lineage surface markers such as CD29, ScaI, CD73, CD44, and CD105 whereas negative expression of hematopoietic lineage surface markers such as CD11b, B220, CD34, and CD11c were determined in the mouse BM-MSCs. (B) The multipotent BM-MSCs could differentiate into adipocytes (neutral triglycerides and lipids were detected by Oil red O solution, presented as red color), osteocytes (calcium deposits were detected by Alizarin red S, presented as red color), and chondrocytes (acid mucosubstances and acetic mucins were detected by Alcian blue, presented as blue color). Results are presented using 200 X magnification. (C) The representative result from the [^{3}H]-thymidine incorporation assay demonstrated that the diminished $CD4^+$ T cell turnover was attributed to the suppressive effects of BM-MSCs. (D) The representative result from the [^{3}H]-thymidine incorporation assay demonstrated that the suppressive effects of BM-MSCs were independent of the $CD4^+$ T cell to BM-MSC ratio (mean ± s.d.; ** $p<0.005$, **** $p<0.00005$). More than three independent repeats were conducted in each experiment, and the reproducible results were observed in each experiment.

c.p.m.: counts per minute

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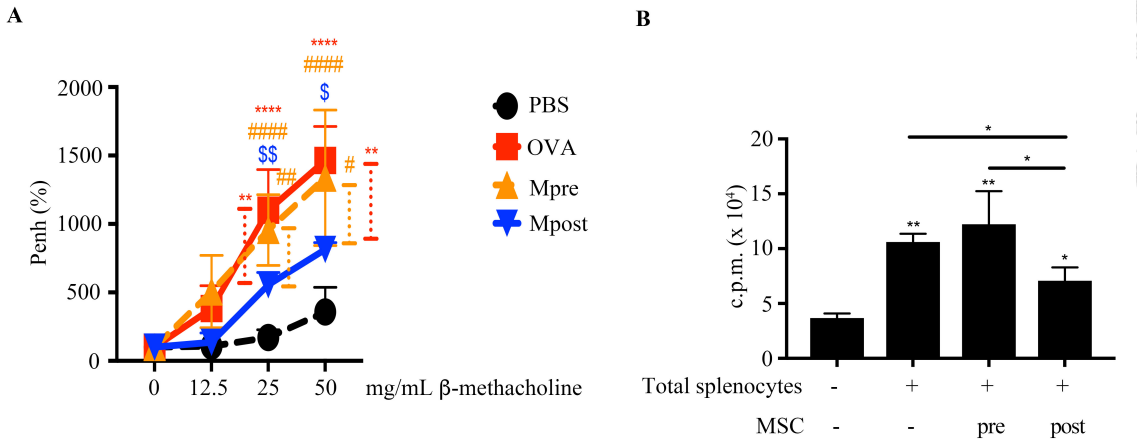

Fig 3. Post-treatment as a more appropriate setting of BM-MSC treatment

(A) The airway hyper-responsiveness of OVA-immunized mice was improved with BM-MSCs more in the post-treatment setting (Mpost) than that in the pretreatment setting (Mpre) (n=6). (mean ± s.d.; **, ****, comparisons between OVA and the other groups, $p<0.005$, and $p<0.00005$, respectively; #, ##, ####, comparisons between Mpre and the other groups, $p<0.05$, $p<0.005$, and $p<0.00005$, respectively; $, $$, comparisons between Mpost and PBS groups, $p<0.05$, and $p<0.005$, respectively) (B) Less proliferative total splenocytes were observed in the Mpost group compared to those in the Mpre group from our *ex vivo* experiments (n=6). (mean ± s.d.; * $p<0.05$, ** $p<0.005$).

Penh: enhanced pause, an index of airway hyperactivity, relating to ventilatory timing

Penh = (peak expiratory flow / peak inspiratory flow) – [(expiratory time / time for expiration of 65% volume) -1]

Penh%: the percentage increase in Penh over baseline Penh; c.p.m.: counts per minute

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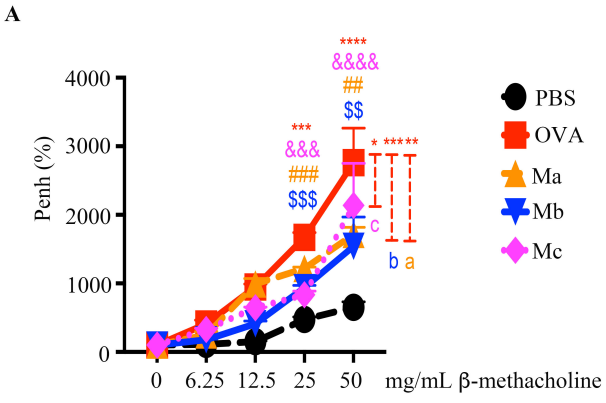

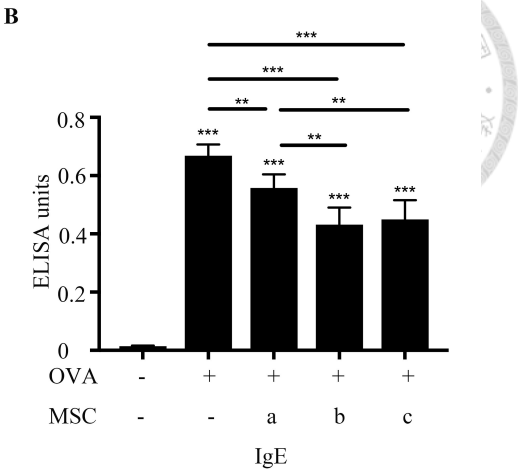

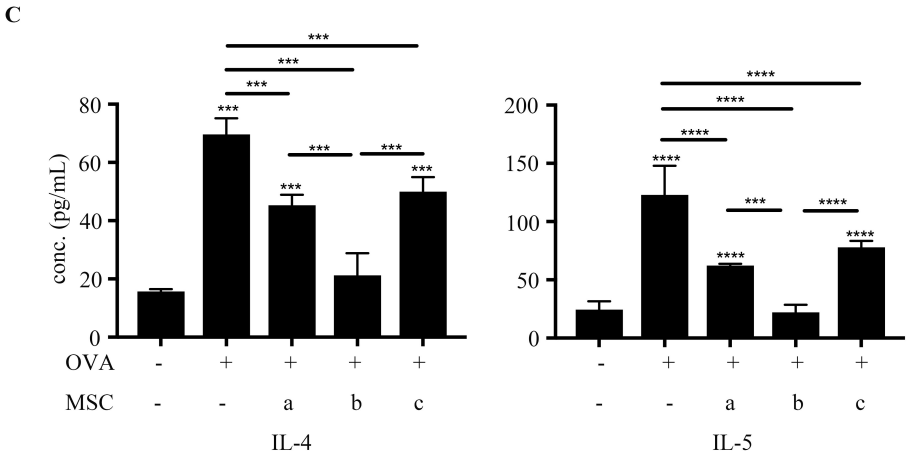

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Fig 4. Administering BM-MSCs in the amount of 5×10^5 cells resulted in higher immunosuppressive activities *in vivo*

(A) BM-MSCs alleviated airway hyper-responsiveness by the same extent when either 1×10^5 (Ma), 5×10^5 (Mb), or 1×10^6 (Mc) BM-MSCs was intravenously administered into OVA-challenged mice (n=6). (mean ± s.d.; *, **, ***, ****, comparisons between OVA and the other groups, $p<0.05$, $p<0.005$, $p<0.0005$, and $p<0.00005$, respectively; ##, ###, comparisons between Ma and the other groups, $p<0.005$, and $p<0.0005$, respectively; $$, $$$, comparisons between Mb and the other groups, $p<0.005$, and $p<0.0005$, respectively; &&&, &&&&, comparisons between Mc and PBS grouop) (B) Either 5×10^5 or 1×10^6 BM-MSCs would downregulate serum IgE level more effectively than that of 1×10^5 BM-MSCs (n=6). (mean ± s.d.; ** $p<0.005$, ***$p<0.0005$). (C) Only 5×10^5 BM-MSCs decreased IL-4 and IL-5 secretion detected in BALF more effectively than that of 1×10^5 or 1×10^6 BM-MSCs (n=6). (mean ± s.d.; *** $p<0.0005$, ****$p<0.00005$).

Penh%: the percentage increase in Penh over baseline Penh

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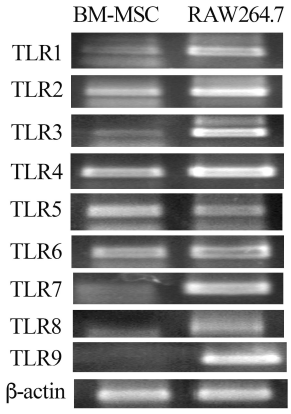

Fig 5. TLR expression in BM-MSCs

TLR expression of BM-MSCs was examined by semi-quantitative RT-PCR. RAW264.7 cells were used as positive controls. The BM-MSCs in our system expressed all TLR subtypes but TLR9. In addition, they especially expressed large amount of TLR2, 4, 5, and 6.

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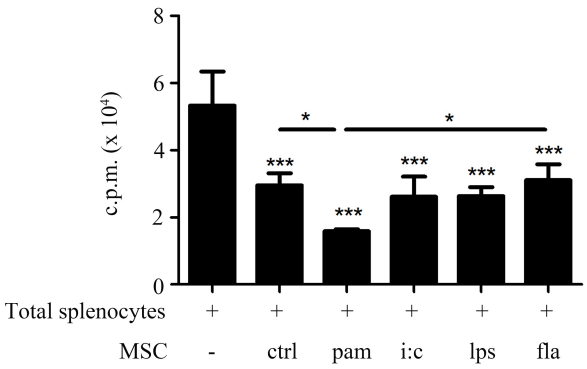
A



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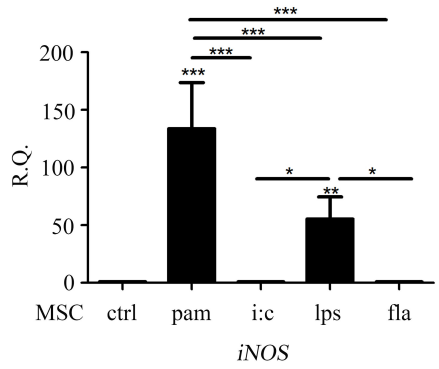

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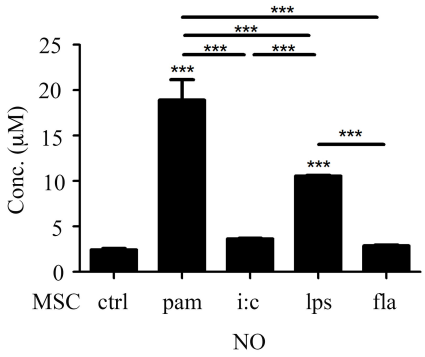

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Fig 6. Pam_3CSK_4 as the comparatively effective TLR ligand for enhancing regulatory activities in BM-MSCs

(A) The representative result from the [^{3}H]-thymidine incorporation assay suggested that incubating BM-MSCs with Pam_3CSK_4 at 1 μg/mL for 72 h further enhanced the suppressive function of BM-MSCs whereas incubating BM-MSCs with a TLR3 ligand, poly (I:C) at 10 μg/mL; a TLR4 ligand, LPS at 10 μg/mL; and a TLR5 ligand, Rec-FLA-ST at 100 ng/mL for 72 h did not. (mean ± s.d.; * $p<0.05$, ***$p<0.0005$). More than three independent repeats were conducted in this experiment, and the reproducible results were observed in this experiment. (B) The expression of regulatory factors in BM-MSCs stimulated with different TLR ligands was observed using qPCR. 1 μg/mL Pam_3CSK_4 and 10 μg/mL LPS substantially enhanced *iNOS* in BM-MSCs. Statistical results were calculated with three independent repeats. (mean ± s.d.; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$). (C) NO secretion in BM-MSCs was enhanced by incubating BM-MSCs with Pam_3CSK_4 at 1 μg/mL and with LPS at 10 μg/mL for 96 h. Statistical results were calculated with three independent repeats. (mean ± s.d.; *** $p<0.0005$).

c.p.m.: counts per minute; R.Q.: relative quantification

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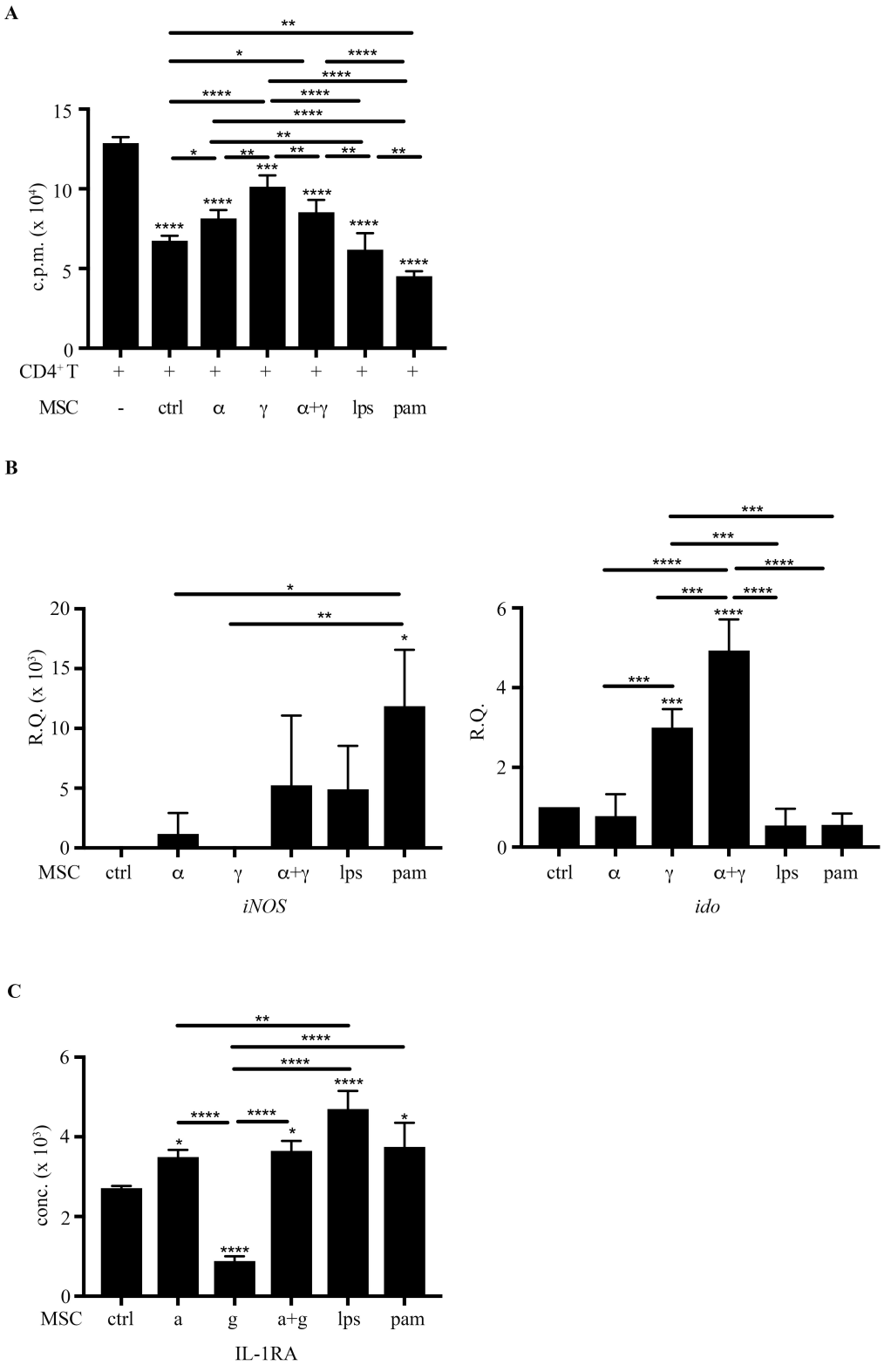

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Fig 7. Pam_3CSK_4 as the comparatively effective stimulant for enhancing regulatory activities in BM-MSCs

(A) According to the representative result from the [^{3}H]-thymidine incorporation assay, Pam_3CSK_4-activated BM-MSCs had the most effective immunomodulatory function among the BM-MSCs activated with different stimulants. (mean ± s.d.; * $p<0.05$, **$p<0.005$, ***$p<0.0005$, ****$p<0.00005$). More than three independent repeats were conducted in this experiment, and the reproducible results were observed in this experiment. (B) Using qPCR assays, we observed that *iNOS* could be substantially enhanced through Pam_3CSK_4 in BM-MSCs, while *ido* could be enhanced through IFN-γ at 200 ng/mL with or without TNF-α at 10 ng/mL in BM-MSCs. Statistical results were calculated with three independent repeats. (mean ± s.d.; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, ****$p<0.00005$). (C) IL-1RA expression detected by ELISA assays could not be induced through IFN-γ without TNF-α in BM-MSCs. Statistical results were calculated with three independent repeats. (mean ± s.d.; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.00005$).

c.p.m.: counts per minute; R.Q.: relative quantification

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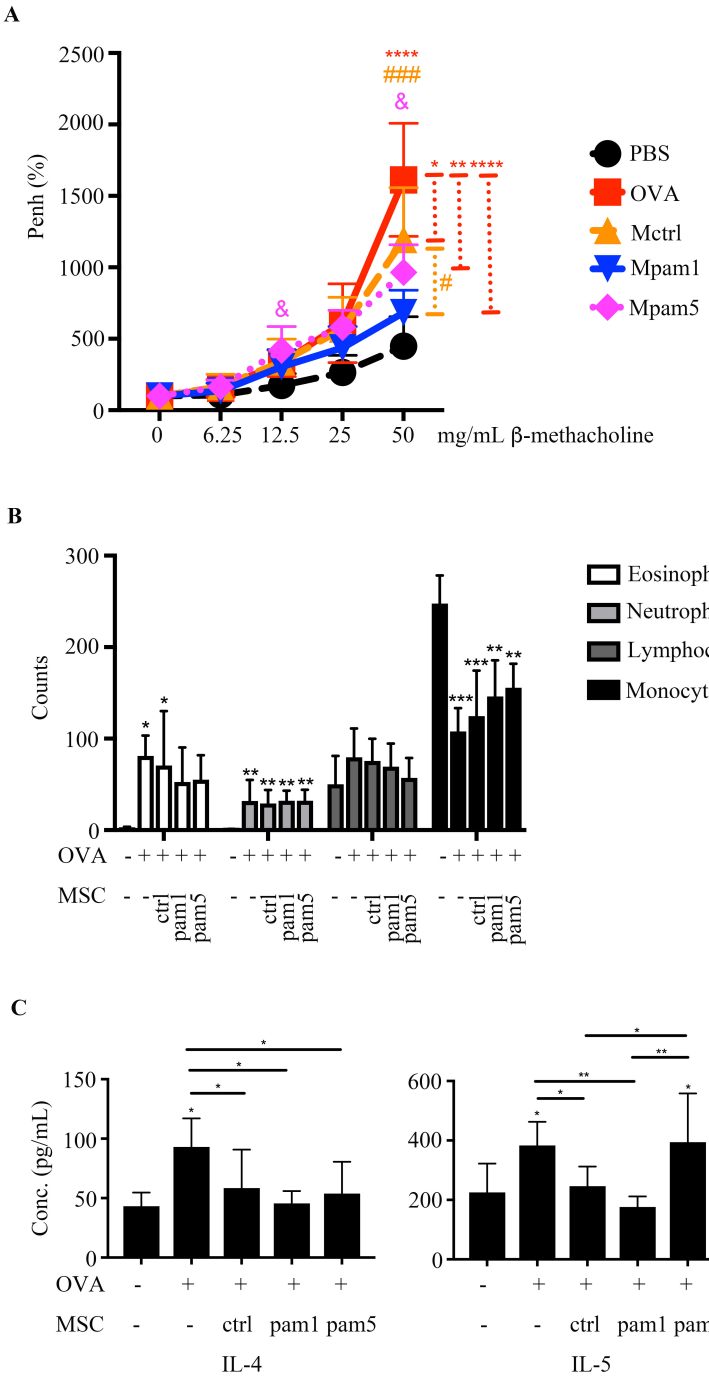

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Fig 8. Pam_3CSK_4 at 1 μg/mL was chosen to modify BM-MSCs

(A) In OVA-induced asthma model, compared to 5 μg/mL (Mpam5), 1 μg/mL Pam_3CSK_4-treated BM-MSCs (Mpam1) alleviated airway hyper-responsiveness more effectively (n=6). (mean ± s.d.; *, **, ****, comparisons between OVA and the other groups, $p<0.05$, $p<0.005$, and $p<0.00005$, respectively; #, ###, comparisons between Mctrl and the other groups, $p<0.05$, and $p<0.0005$, respectively; &, comparisons between Mpam5 and PBS groups, $p<0.05$) (B) There were no significant differences between Mpam1 and Mpam5 in pulmonary inflammatory cell infiltration (n=6). (mean ± s.d.; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$) (C) In OVA-induced asthma model, compared to 5 μg/mL, 1 μg/mL Pam_3CSK_4-treated BM-MSCs diminished IL-5 secretion in BALF more effectively (n=6). (mean ± s.d.; * $p<0.05$, ** $p<0.005$) (D) There were no significant differences between Mpam1 and Mpam5 in serum IgE level (n=6). (mean ± s.d.; *** $p<0.0005$)

Penh%: the percentage increase in Penh over baseline Penh

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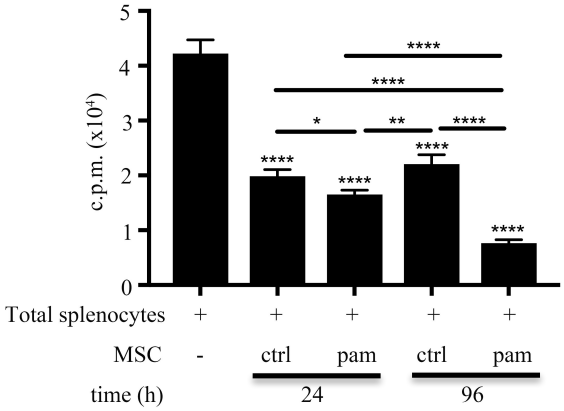

Fig 9. Pam_3CSK_4 at 1 μg/mL was chosen to treat BM-MSCs for 96 h

The representative result from the [^{3}H]-thymidine incorporation assay showed that compared to use 1 μg/mL Pam_3CSK_4 to treat BM-MSCs for 24 h, treating BM-MSCs with 1 μg/mL Pam_3CSK_4 for 96 h was a more effective regimen to enhance the immunosuppressive function of BM-MSCs. (mean ± s.d.; * $p<0.05$, ** $p<0.005$, **** $p<0.00005$). More than three independent repeats were conducted in this experiment, and the reproducible results were observed in this experiment.

doi:10.6342/NTU201800752

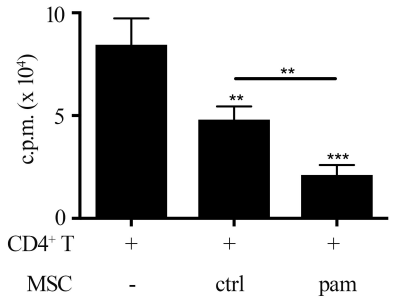

Fig 10. Immunosuppressive activities of BM-MSCs were enhanced by Pam_3CSK_4 stimulation

The representative result from the [^{3}H]-thymidine incorporation assay demonstrated that the diminished $CD4^+$ T cell turnover attributed to the suppressive effects of BM-MSCs was further diminished by the enhanced suppressive function of the Pam_3CK_4-treated BM-MSCs (mean ± s.d.; ** $p<0.005$, *** $p<0.0005$). More than three independent repeats were conducted in this experiment, and the reproducible results were observed in this experiment.

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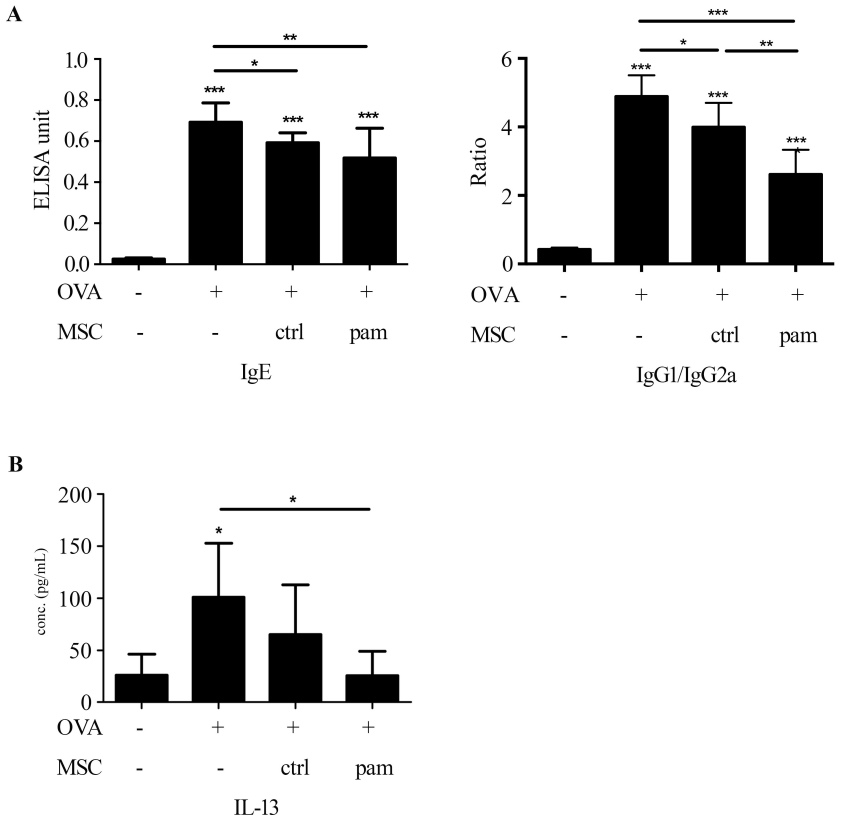

Fig 11. Mpam ameliorated systemic inflammation of asthmatic mice more effectively than did Mctrl

(A) Serum samples were collected from mice on day 31, immediately before sacrifices or invasive airway resistance measurements were performed. Serum IgG1/IgG2a levels were downregulated more effectively with Mpam than they were with Mctrl (n=5). (B) The IL-13 expression in the supernatants of OVA-stimulated splenocytes was significantly downregulated only in the Mpam treatment group (n=5). (mean ± s.d.; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$)

doi:10.6342/NTU201800752

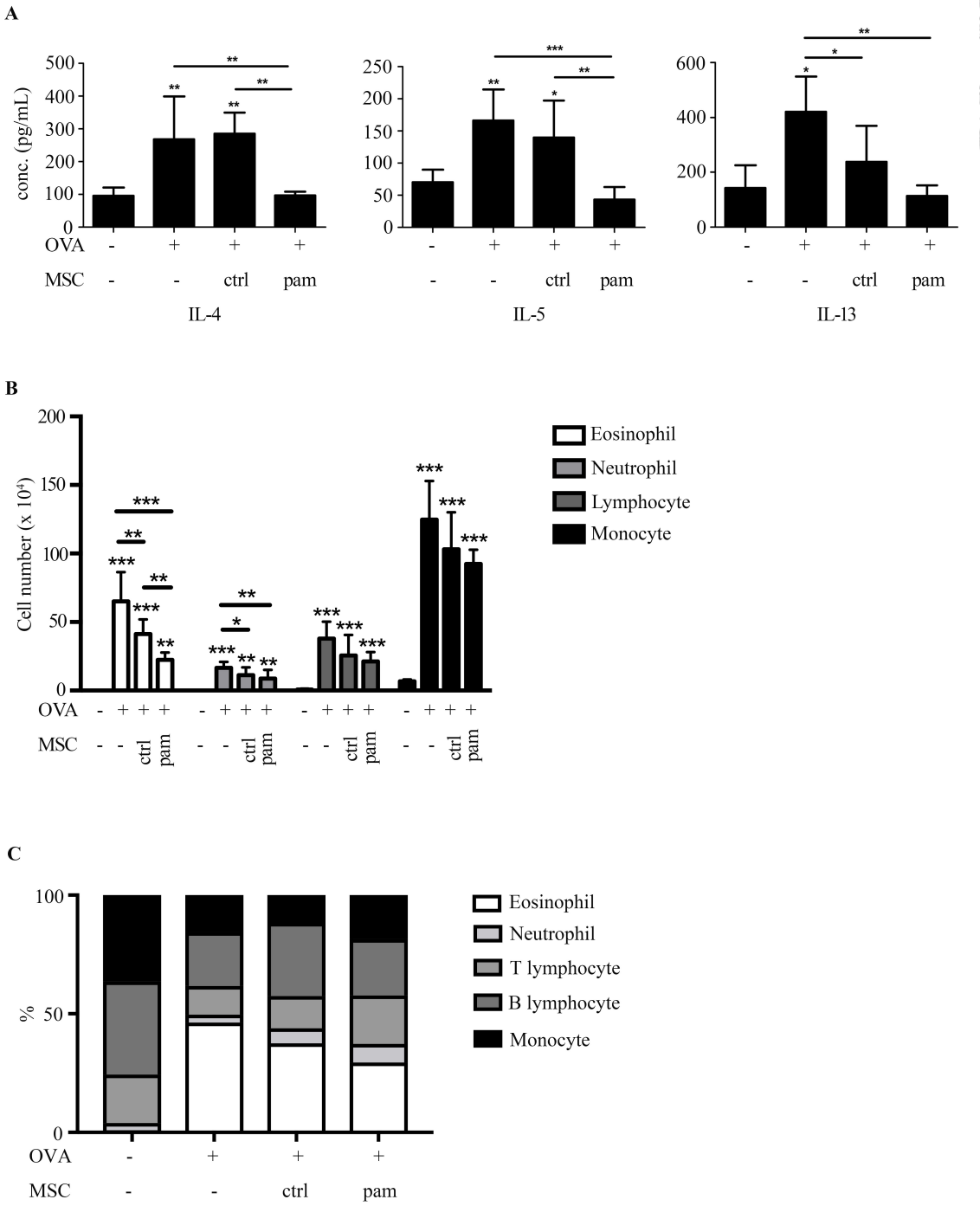

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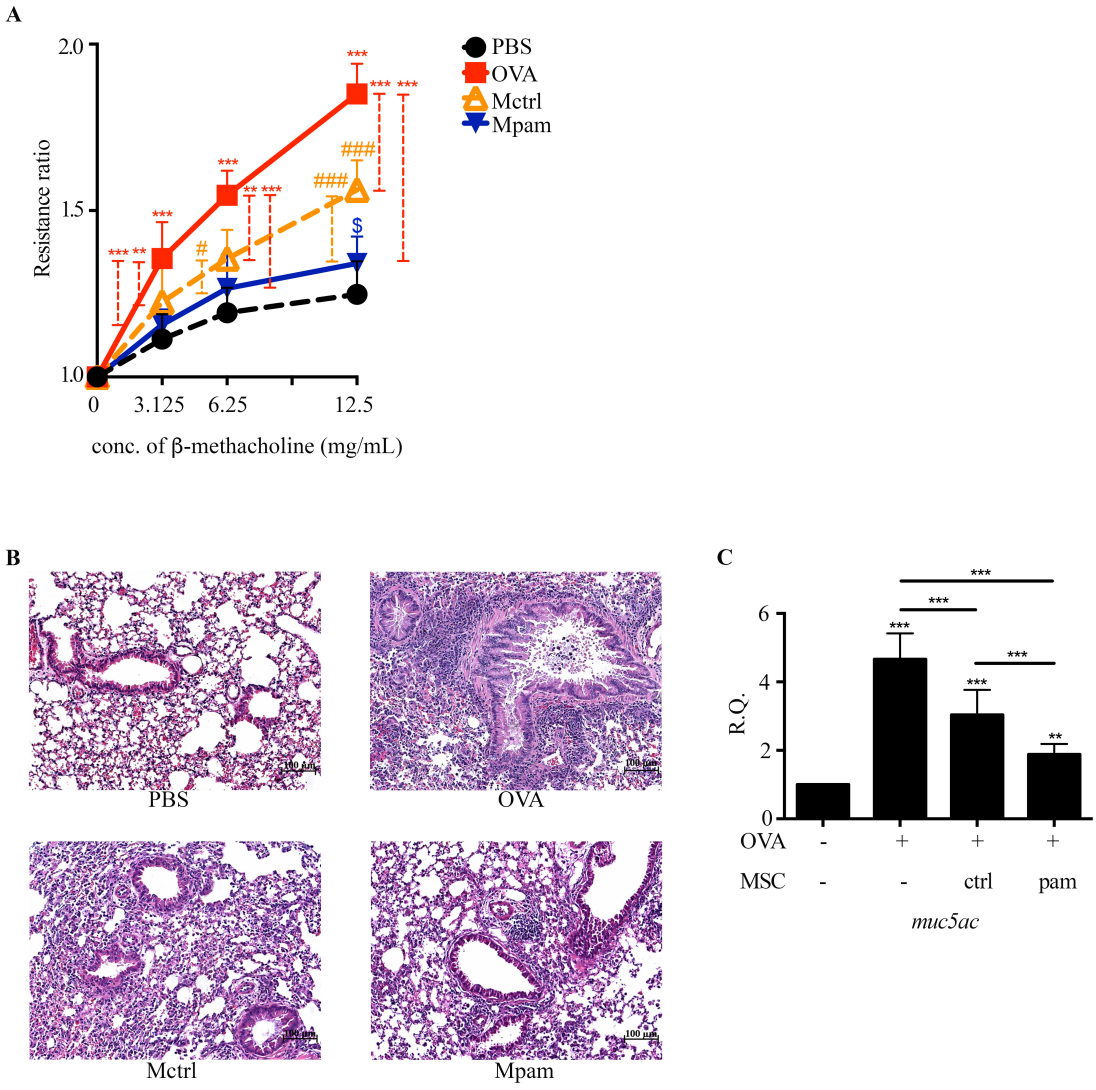
Fig 12. Mpam alleviated local symptoms of asthmatic mice more effectively than did Mctrl

(A) The expression of IL-4 and IL-5 in the BALF was further downregulated with Mpam than it was with Mctrl treatment (n=5). (B) Infiltrated cells were collected and counted from BALF. Eosinophil infiltration was much less in the Mpam treatment group than that in the Mctrl treatment group (n=5). (C) Less eosinophil infiltration in the Mpam treatment group than that in the Mctrl treatment group was confirmed using flow cytometry (n=5). (mean ± s.d.; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$)

doi:10.6342/NTU201800752



doi:10.6342/NTU201800752

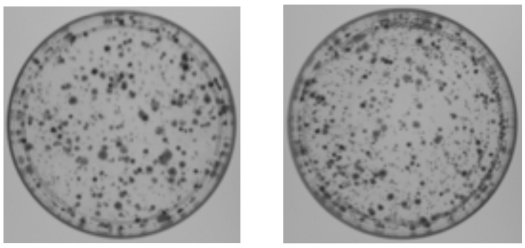
Fig 13. Compared to Mctrl, Mpam was more likely to improve airway remodeling in asthmatic mice

(A) Examined by the plethysmograph with paralyzed and tracheotomized mice, the airway resistance of OVA-induced asthmatic mice could be further alleviated with the Mpam therapy compared with the Mctrl therapy in response to 6.25 and 12.5 mg/mL of β-methacholine administrations (n=8). (mean ± s.d.; **, ***, comparisons between OVA and the other groups, $p<0.005$, and $p<0.0005$, respectively; #, ###, comparisons between Mctrl and the other groups, $p<0.05$, and $p<0.0005$, respectively; $, comparisons between Mpam and PBS groups, $p<0.05$) (B) Lung tissues were dissected and then made into paraffin sections. Hematoxylin was used as a positive stain colored basophilic nuclei violet, and eosin was used as a negative contrast stained acidophilic proteins in cytoplasm pink. Both Mctrl and Mpam treatments were demonstrated to diminish inflammatory cell infiltration and bronchial epithelial thickness. Results were presented using 100 X magnification. (C) Lung tissues were dissected and then RNA extraction and reverse transcription were performed sequentially. The expression of *muc5ac* was further downregulated in the Mpam more than it was in the Mctrl group, implying that Mpam might abolish mucin hypersecretion more effectively in the airways (n=8). (mean ± s.d.; $p<0.005$; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$)

R.Q.: relative quantification

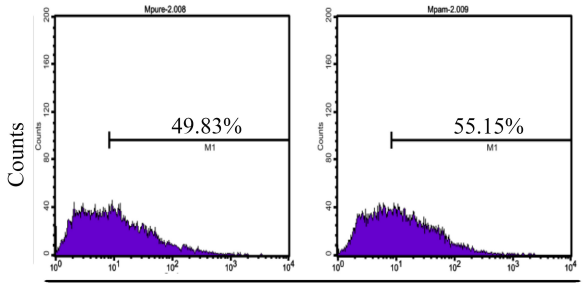
doi:10.6342/NTU201800752

A



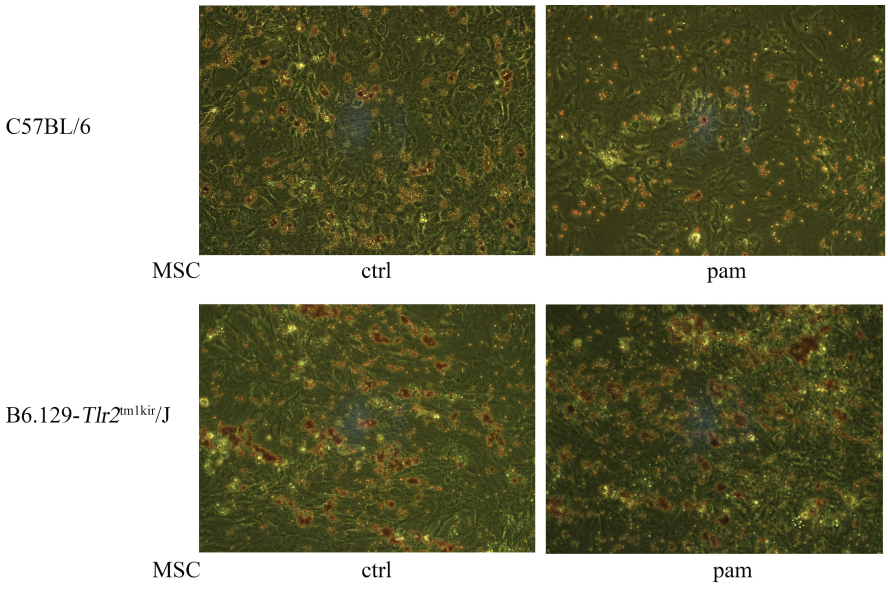
Mctrl Mpam

B



Mctrl Mpam

C



C57BL/6

MSC ctrl pam

B6.129-*Tlr2*^{tm1kir}/J

MSC ctrl pam

doi:10.6342/NTU201800752

Fig 14. Pam_3CSK_4 changed the multipotent stem cell properties of BM-MSCs

(A) Mpam was found to form more colonies than Mctrl according to our colony formation assays. The representative result presents that Mctrl and Mpam grew 482 and 662 colonies, respectively. (B) The expression of the stem cell factor, Sca-1, was slightly enhanced in Mpam, compared to that in Mctrl. (C) Under the adipogenic condition, while Pam_3CSK_4 seemed to impair the adipogenic ability of normal mice-derived BM-MSCs, BM-MSCs isolated from TLR2 knockout mice were more prone to differentiate into adipocytes. More than three independent repeats were conducted in each experiment, and the reproducible results were observed in each experiment.

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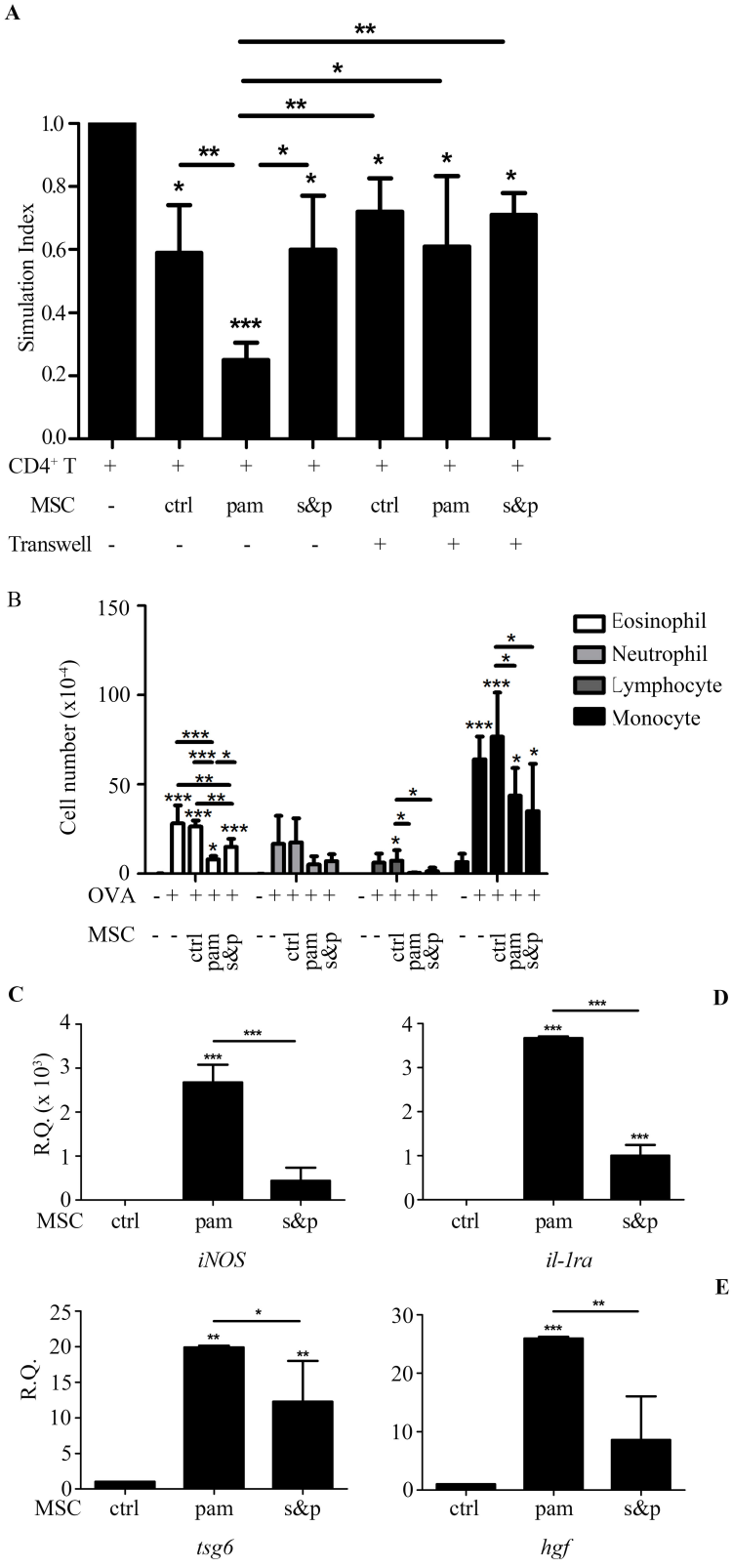

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Fig 15. The enhanced cell-cell contact-dependent suppressive effect of Mpam was through the TLR2/STAT3/iNOS signaling pathway

(A) The [^{3}H]-thymidine incorporation assays were done with or without transwell insertions. The immunosuppressive function of BM-MSCs was enhanced by Pam_3CSK_4 activation through STAT3 signaling pathway in a cell-cell contact-dependent manner. (B) Compared to the Mctrl-treated group, less eosinophils infiltrated to lungs in the Mpam-treated asthmatic mice. However, such diminished eosinophil infiltration in the Mpam-treated group was reversed in the Ms&p-treated group (n=5). (C) The upregulation of *iNOS*, *il-1ra*, *tsg-6*, and *hgf* expression stimulated with Pam_3CSK_4 in BM-MSCs was abolished by S3I-201 incorporation. (D)(E) The upregulation of NO and IL-1RA expression stimulated with Pam_3CSK_4 in BM-MSCs, consistent with the results at mRNA level, was abolished by S3I-201 incorporation. Statistical results were calculated with three independent repeats in *in vitro* experiments. (mean ± s.d.; * $p<0.05$, ** $p<0.005$, *** $p<00005$)

R.Q.: relative quantification

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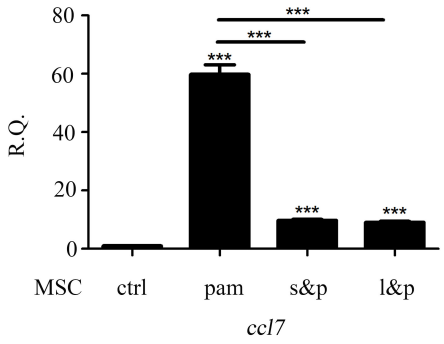

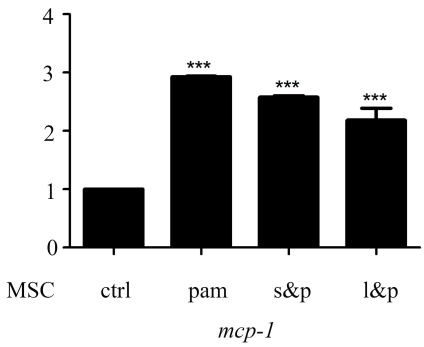

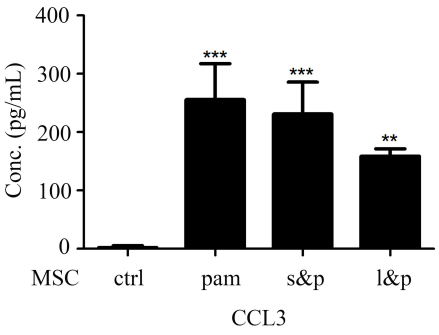

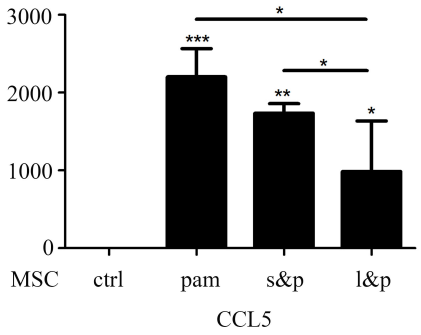

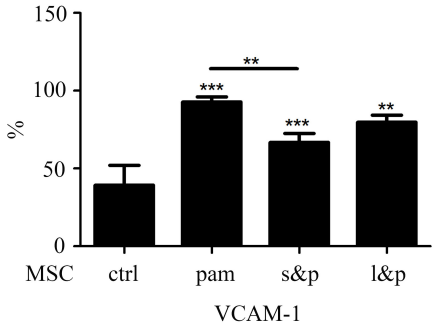

doi:10.6342/NTU201800752

Fig 16. The mobility of BM-MSCs might be increased with Pam_3CSK_4 treatment

The expression of *ccl7* (detected by qPCR), *mcp-1* (detected by qPCR), CCL3 (detected by ELISA), CCL5 (detected by ELISA), and VCAM-1 (detected by flow cytometry) was upregulated in Mpam. Further, the upregulated expression of *ccl7* and CCL5 in Mpam was reduced when we treated Mpam with either STAT3 or iNOS inhibitor. The upregulated expression of VCAM-1 in Mpam was reduced when we treated Mpam with STAT3 inhibitor. Statistical results were calculated with three independent repeats. (mean ± s.d.; * $p<0.05$, ** $p<0.005$, *** $p<00005$)

R.Q.: relative quantification

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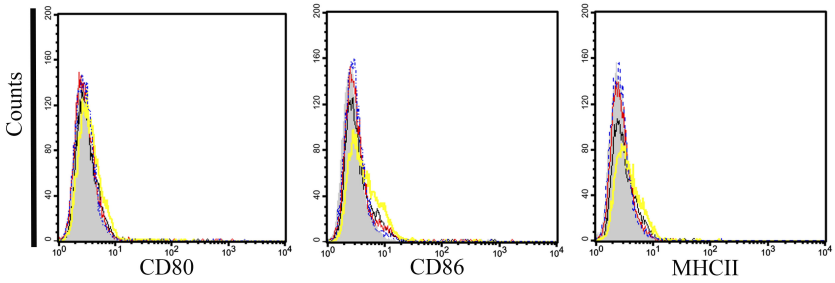

Fig 17. The immunoprivileged properties of conditioned BM-MSCs

TLR2 activation did not significantly affect the immunoprivileged properties of BM-MSCs. (Mctrl was presented using a black line, Mpam was presented using a red line, Ms&p was presented using a yellow line, and Ml&p was presented using a blue dotted line).

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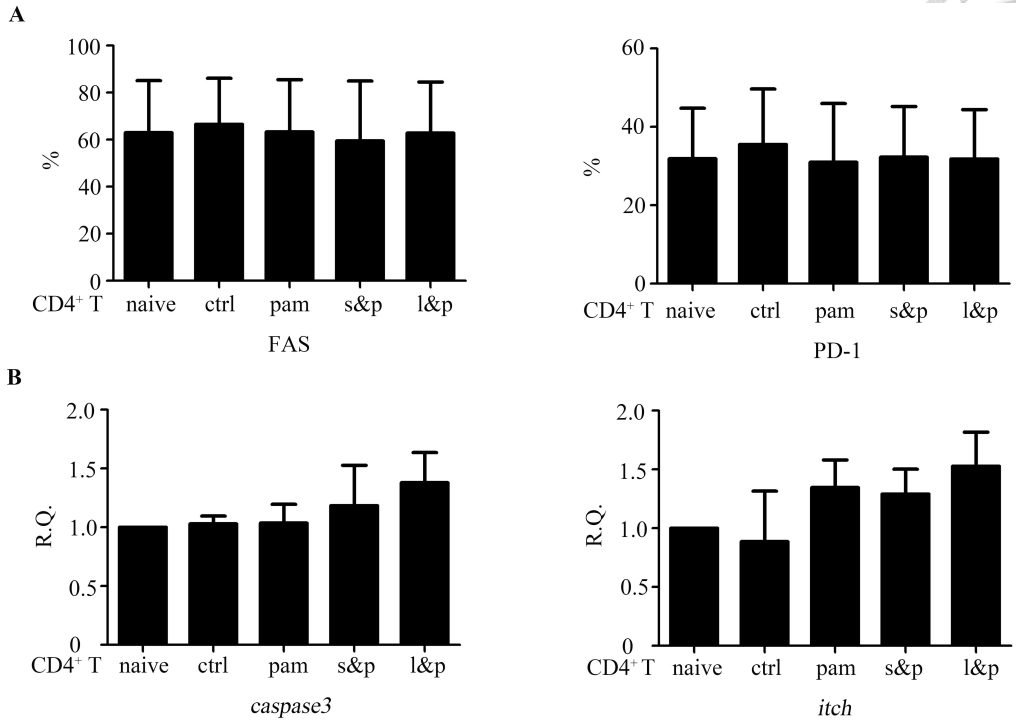

Fig 18. The molecules indicating apoptosis and anergy in CD4$^+$ T cells were not induced by Mpam

(A) The expression of FAS and PD-1 detected by flow cytometry showed no differences between all the different conditioned CD4$^+$ T cells. (B) The expression of *caspase-3* and *itch* detected by qPCR showed no differences between all the different conditioned CD4$^+$ T cells. Statistical results were calculated with three independent repeats.

R.Q.: relative quantification

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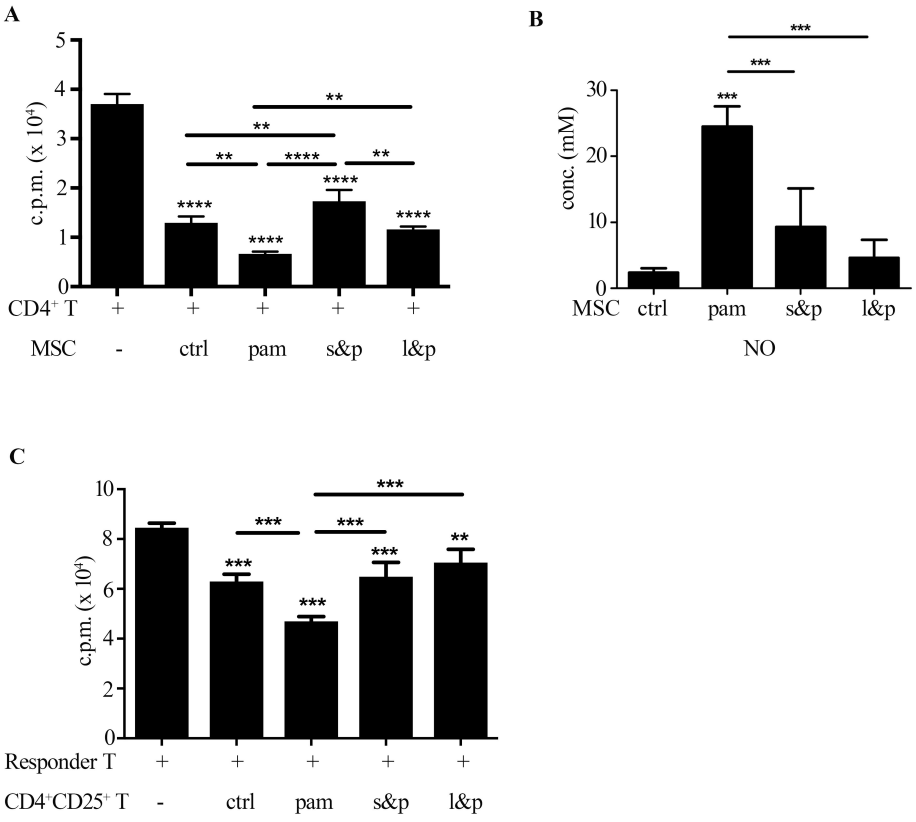

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Fig 19. Mpam induced more regulatory T cells

(A) Mpam further diminished $CD4^+$ T cell proliferation through STAT3/iNOS pathway. (mean ± s.d.; ** $p<0.005$, **** $p<0.00005$). The representative result from the [^{3}H]-thymidine incorporation assay is presented here. More than three independent repeats were conducted in this experiment, and the reproducible results were observed in this experiment. (B) The increased NO secretion of Mpam was diminished through STAT3 or iNOS inhibition. Statistical results were calculated with three independent repeats. (mean ± s.d.; *** $p<0.0005$). (C) Only the Mpam-educated $CD4^+CD25^+$ T cells were able to significantly inhibit the Tresp proliferation. Moreover, this phenomenon was reversed when the Mpam was treated with the iNOS inhibitor. (mean ± s.d.; ** $p<0.005$, *** $p<0.0005$, **** $p<0.00005$). The representative result from the [^{3}H]-thymidine incorporation assay is presented here. More than three independent repeats were conducted in this experiment, and the reproducible results were observed in this experiment.

c.p.m.: counts per minute

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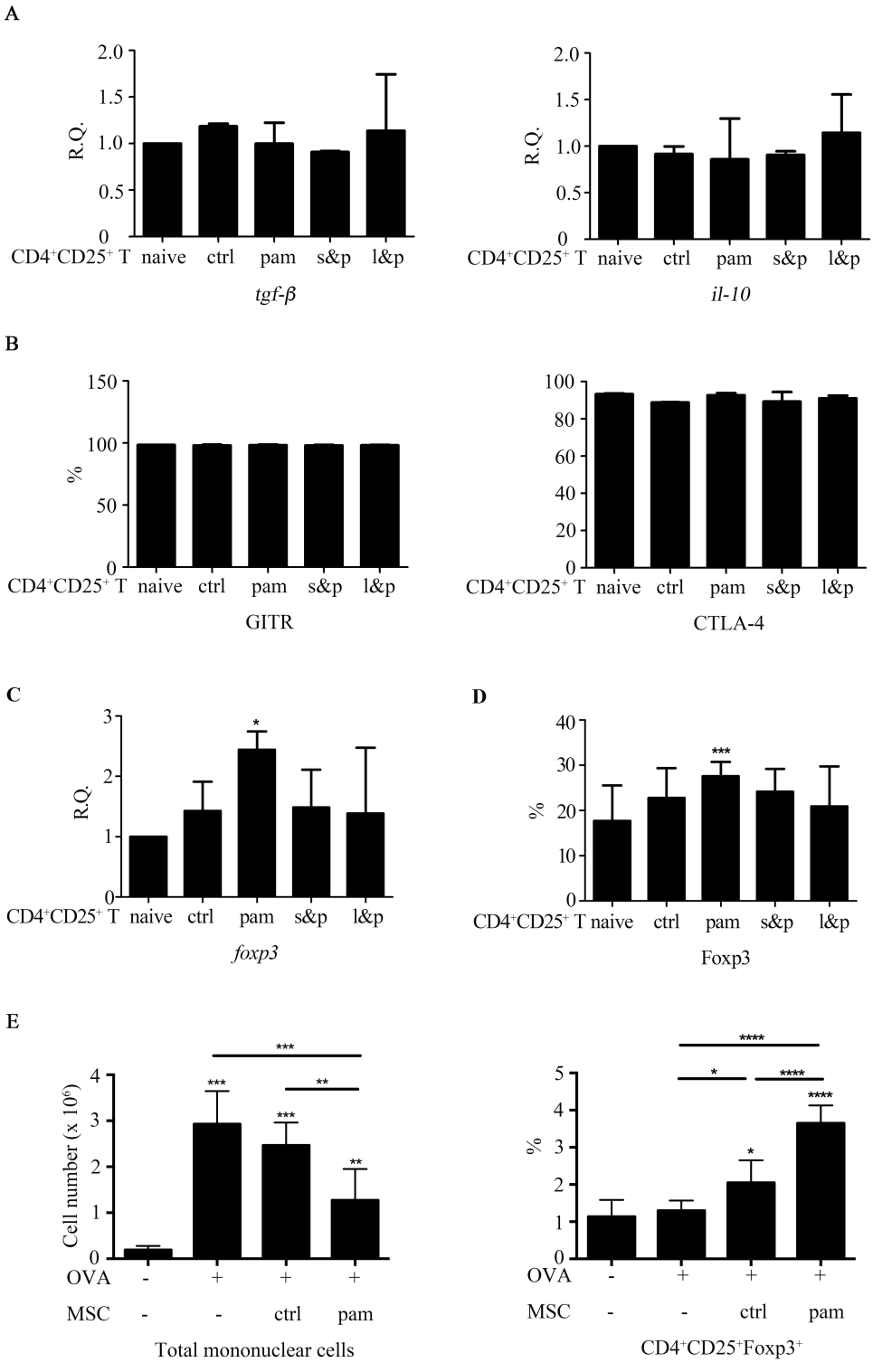

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Fig 20. More $CD4^+CD25^+Foxp3^+$ T cells were detected after the Mpam treatment *in vitro* and *in vivo*

(A) Compared with the noneducated $CD4^+CD25^+$ T cells (Tnaive), the expression of *tgf-β* and *il-10* was not significantly changed in Tctrl, Tpam, Ts&p, and Tl&p. Statistical results were calculated with three independent repeats. (B) Compared with the noneducated $CD4^+CD25^+$ T cells (Tnaive), the expression of GITR and CTLA-4 was not significantly changed in Tctrl, Tpam, Ts&p, and Tl&p. Statistical results were calculated with three independent repeats. (C)(D) Compared to the noneducated $CD4^+CD25^+$ T cells, the expression of *foxp3* was upregulated only in the Mpam-educated $CD4^+CD25^+$ T cells both at mRNA and protein levels. Statistical results were calculated with three independent repeats. (mean ± s.d.; * $p<0.05$, *** $p<0.0005$) (E) Total mononuclear cells and $CD4^+CD25^+Foxp3^+$ cells isolated from lung tissues were examined using flow cytometry. In the asthma murine model, compared to the Mctrl treatment, the Mpam treatment decreased total mononuclear cell recruitment to lung tissue, however, increased the percentage of $CD4^+CD25^+Foxp3^+$ cells in lung tissue (n=5). (mean ± s.d.; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.00005$)

R.Q.: relative quantification

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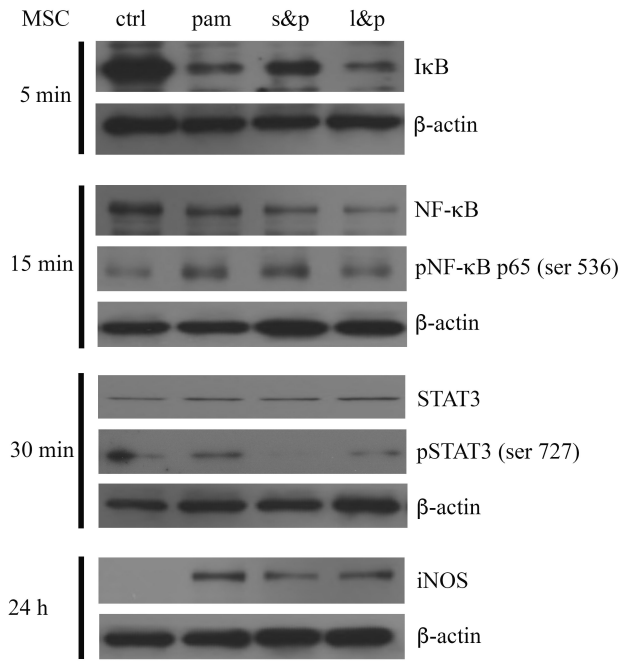

Fig 21. Pam_3CSK_4 induced NF-κB/STAT3/iNOS signals in BM-MSCs

Sequential activation of NF-κB, STAT3 and iNOS signaling was evidenced using Western blot. Pam_3CSK_4 induced IκB degradation, and consequently made NF-κB phosphorylated. The activation of the downstream STAT3/iNOS signaling pathway was established using a STAT3 inhibitor, S3I-201. Less iNOS expression was detected when the STAT3 phosphorylation was abolished through S3I-201in Ms&p.

doi:10.6342/NTU201800752

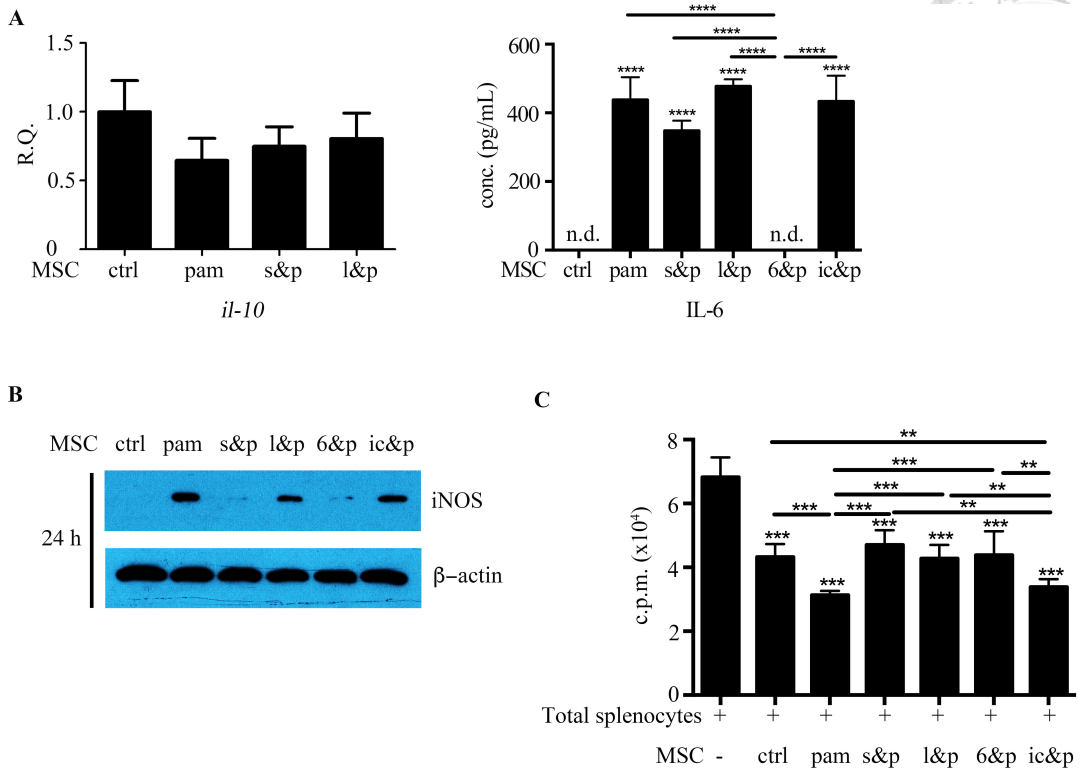

Fig 22. IL-6 Mediated NF-κB/STAT3 signaling in Mpam

(A) IL-6 over-secretion was induced with Pam_3CSK_4 irrespective of S3I-201 or L-NMMA inhibition. Statistical results were calculated with three independent repeats. (mean ± s.d.; **** $p<0.00005$; n.d.= not detected). (B) Using Western blot, enhanced iNOS expression by Pam_3CSK_4 induction was abolished when IL-6 neutralizing antibody was added. (C) The enhanced suppressive function of BM-MSCs with Pam_3CSK_4 treatment was diminished when IL-6 neutralizing antibody was added. (mean ± s.d.; ** $p<0.005$, *** $p<0.0005$, **** $p<0.00005$). The representative result from the [^{3}H]-thymidine incorporation assay is presented here. More than three independent repeats were conducted in this experiment, and the reproducible results were observed in this experiment.

R.Q.: relative quantification; c.p.m.: counts per minute

doi:10.6342/NTU201800752

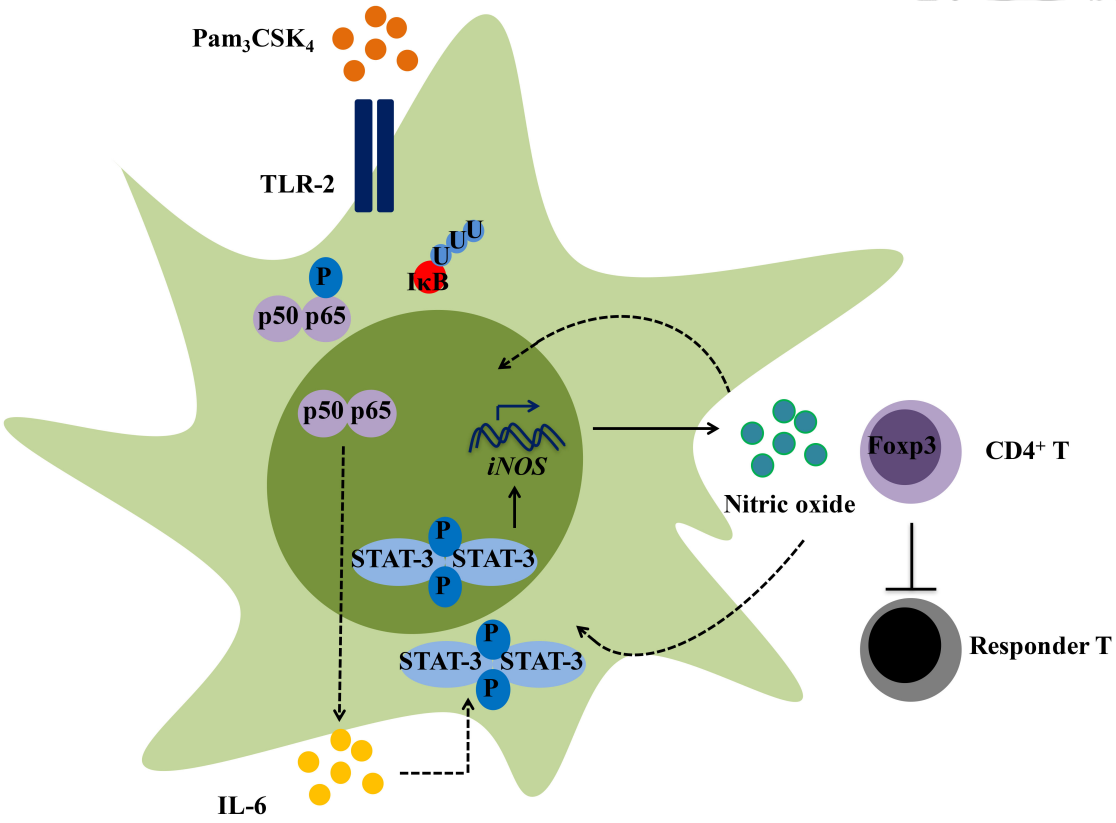

Fig 23. Proposed mechanisms of the enhanced immunosuppressive properties of Mpam

After IκB, an inhibitor of NF-κB, was degraded through Pam_3CSK_4 stimulation, IκB was dissociated from NF-κB. The released NF-κB was subsequently phosphorylated at S536 and was further translocated into the nucleus. In the downstream of NF-κB, IL-6 production was increased in Mpam. IL-6 in turn phosphorylated STAT3. NO, the key suppressive molecule of BM-MSCs, was later highly increased through upregulated iNOS expression, which was in the downstream of STAT3 phosphorylation. The intensified suppressive functions of BM-MSCs were finally executed by inducing $CD4^+CD25^+Foxp3^+$ regulatory T cells in a cell-cell contact-dependent manner.

doi:10.6342/NTU201800752

Chapter VIII: Appendix

Semi-quantitative PCR primers

TLR	Primer sequence	Size (bp)	Anealing temperature (℃)
tlr1	GGATGTGTCCGTCAGCACTA TGTAACTTTGGGGGAAGCTG	340	53
tlr2	CAGACGTAGTGAGCGAGCTG GGCATCGGATGAAAAGTGTT	390	50
tlr 3	GAGGGCTGGAGGATCTCTTT TGCCTCAATAGCTTGCTGAA	353	53
tlr 4	GCTTTCACCTCTGCCTTCAC CGAGGCTTTTCCATCCAATA	361	53
tlr 5	GCTTTGTTTTCTTCGCTTCG ACACCAGCTTCTGGATGGTC	342	50
tlr 6	GCAACATGAGCCAAGACAGA GTTTTGCAACCGATTGTGTG	349	53
tlr 7	ATTCAGAGGCTCCTGGATGA AGGGATGTCCTAGGTGGTGA	264	53
tlr 8	TCCTGGGGATCAAAAATCAA AAGGTGGTAGCGCAGTTCAT	302	50
tlr 9	ACCCTGGTGTGGAACATCAT GTTGGACAGGTGGACGAAGT	341	53
β-actin	AAGGTGTGATGGTGGGAATG ATGGCTACGTACATGGCTGG	286	50

doi:10.6342/NTU201800752

Real-time PCR primers

	Primer sequence	Size (bp)	Anealing temperature (℃)
muc5ac	CCATGCAGAGTCCTCAGAACA A TTACTGGAA AGGCCCAAGCA	106	60
inos	GGCAGCCTGTGAGACCTTTG GCATTGGAAGTGAAGCGTTTC	72	60
il-1ra	GACCCTGCAAGATGCAAGCC GAGCGGATGAAGGTAAAGCG	292	60
foxp3	TACCACAATATGCGACCC CTCAAATTCATCTACGGTCC	240	53
gapdh	GATGGGTGTGAACCACGAGA AGATCCACGACGGACACAT	339	60

Table 1. Primer sequences used in this study

doi:10.6342/NTU201800752

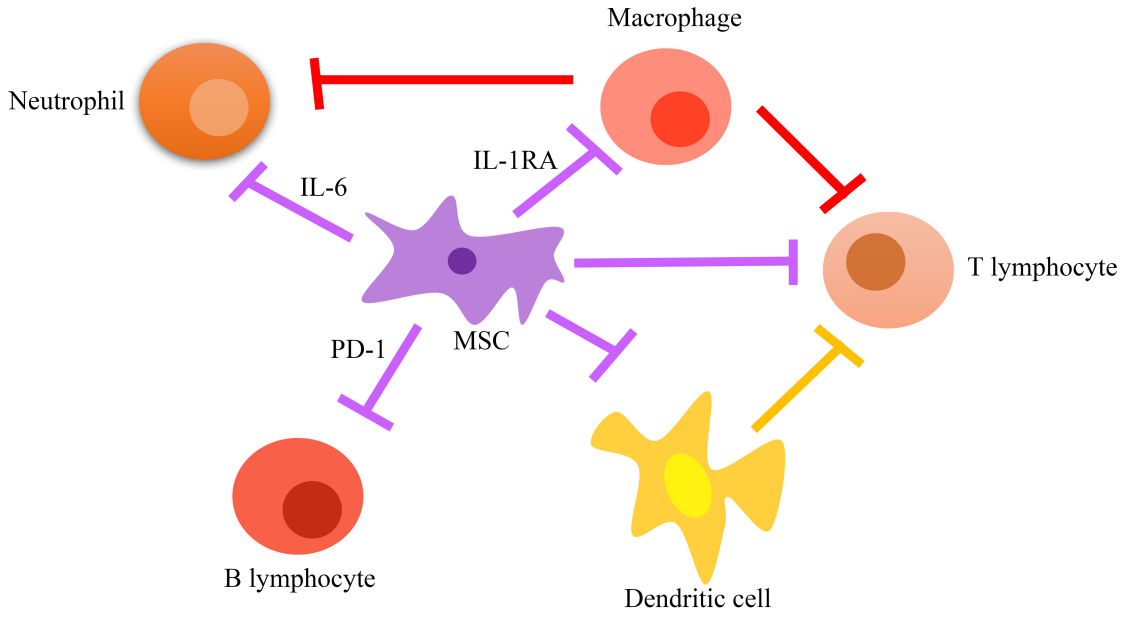

Fig 1. MSCs suppress immune cells directly and/or indirectly

MSCs suppress T cells, macrophages, dendritic cells (DCs), neutrophils, and B cells directly either through soluble regulatory factors or through cell-cell contact. In addition, indirectly mediated by macrophages and DCs, MSCs are able to suppress T cells and neutrophils.

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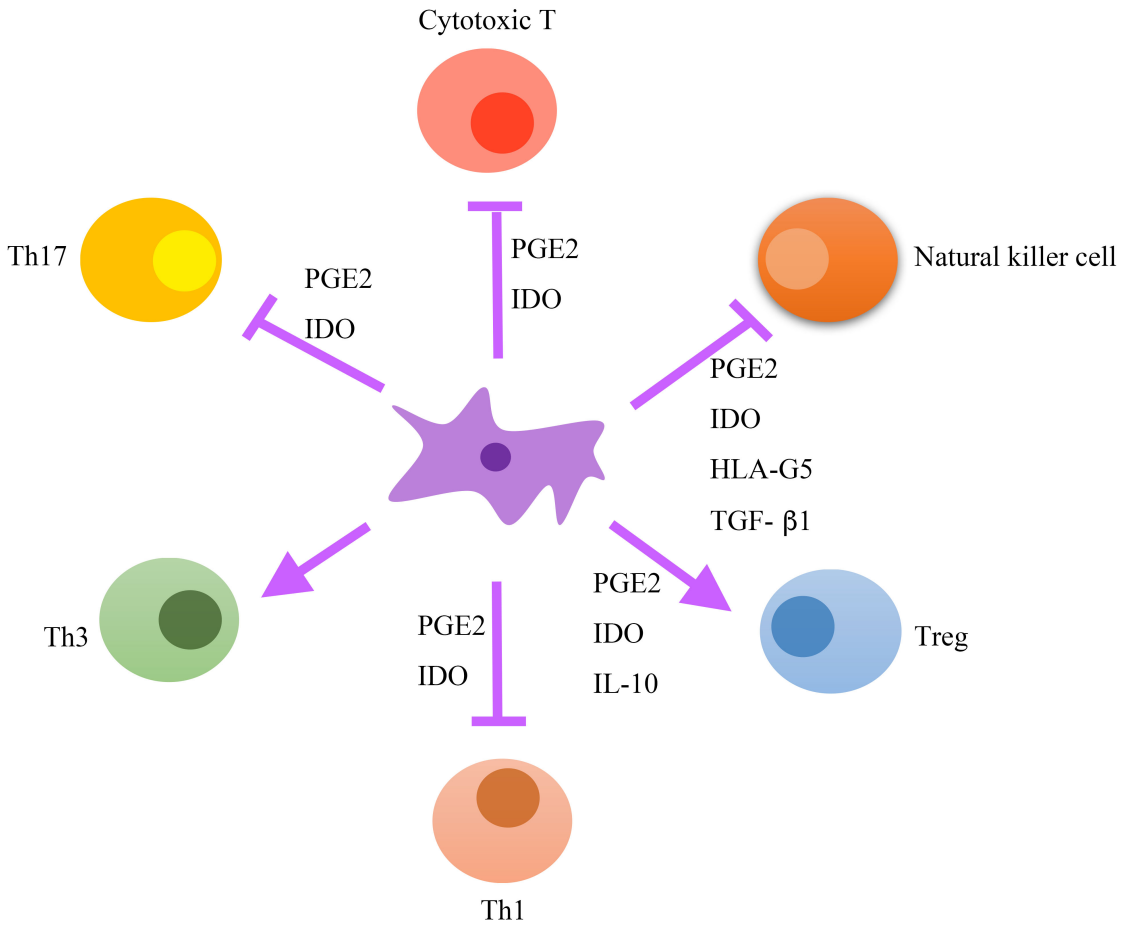

Fig 2. MSCs suppress proinflammatory T cell subsets and natural killer cells while stimulating regulatory T cell subsets

MSCs suppress proinflammatory natural killer cells and T cell subsets such as Th1, Th17, and cytotoxic T cells through soluble regulatory factors including PGE2 and IDO. However, MSCs can induce regulatory T cell subsets such as Th3 and Treg.

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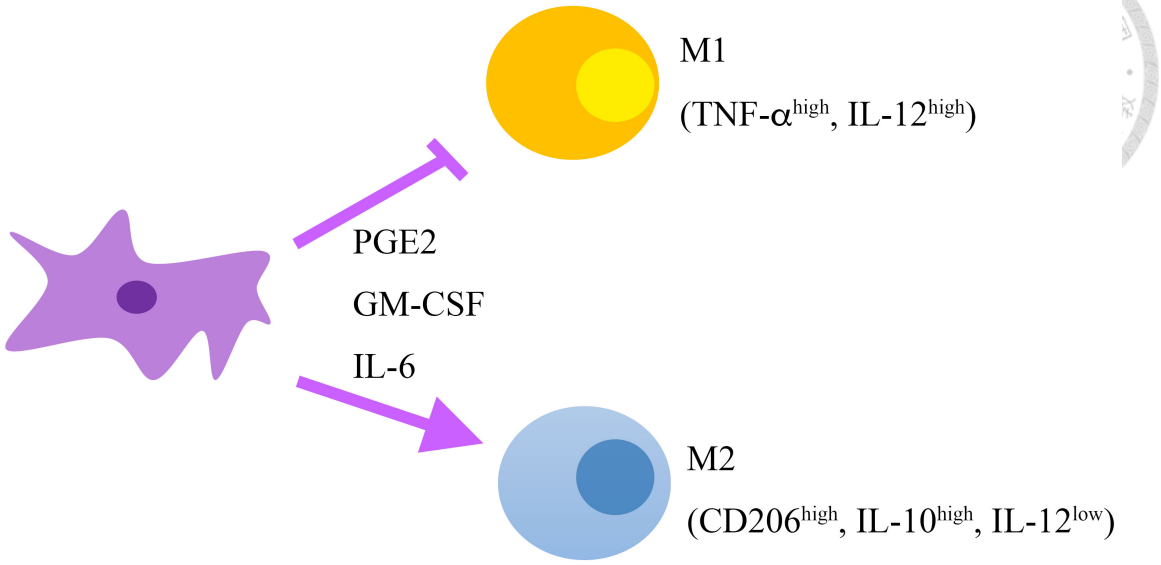

Fig 3. MSCs polarize macrophages into regulatory macrophages, instead of proinflammatory macrophages

When macrophages coculture with MSCs, they are polarized to regulatory macrophages which highly express mannose receptor CD206 and IL-10. In contrast, proinflammatory macrophages, which highly secrete TNF-α and IL-12, are unlikely to be induced by MSCs. This phenomenon is related to PGE2, GM-CSF, and IL-6.

doi:10.6342/NTU201800752

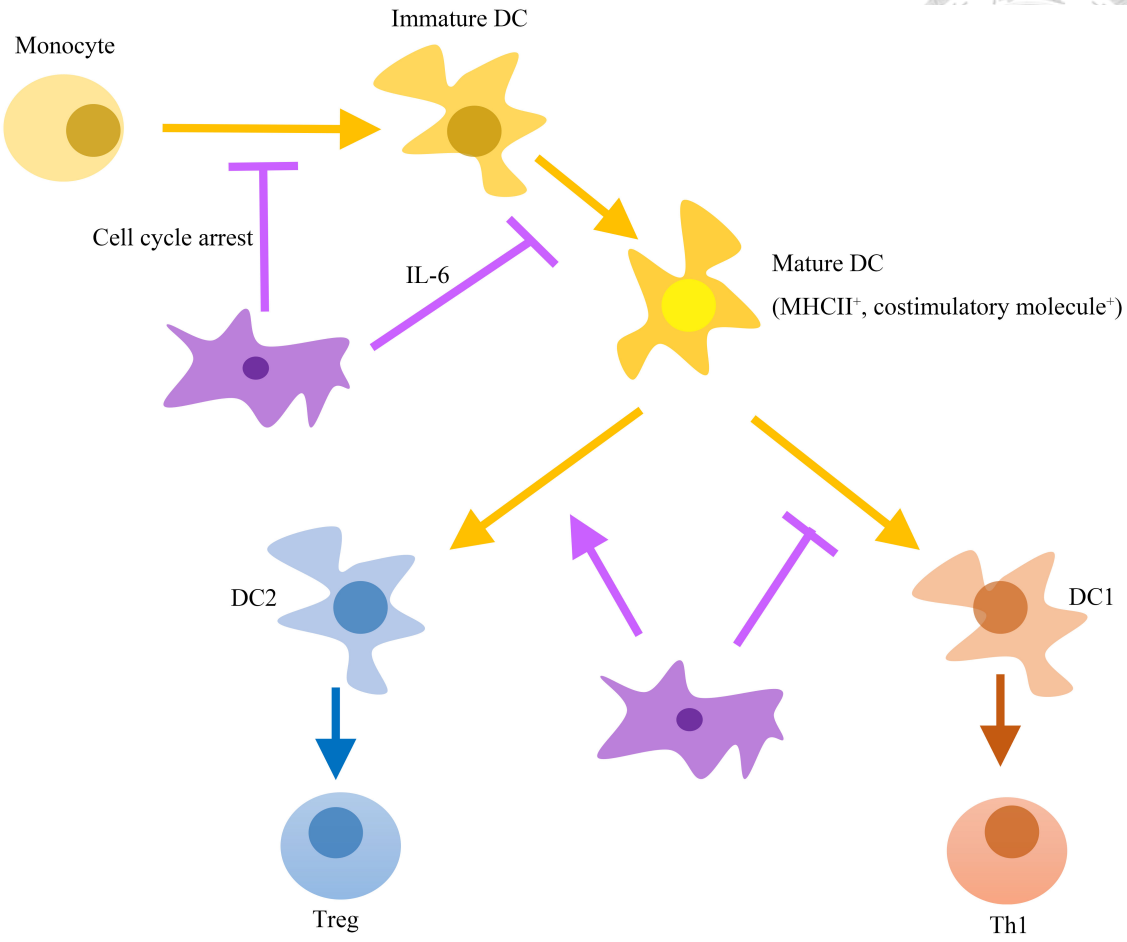

Fig 4. MSCs inhibit the differentiation, maturation, and proinflammatory polarization processes of DCs

MSCs arrest the cell cycle of monocytes in G0. The differentiation of DCs from peripheral blood monocytes is thus inhibited. IL-6 secreted by MSCs inhibits the maturation of DCs. Moreover, MSCs polarize DCs into regulatory DC2, rather than proinflammatory DC1.

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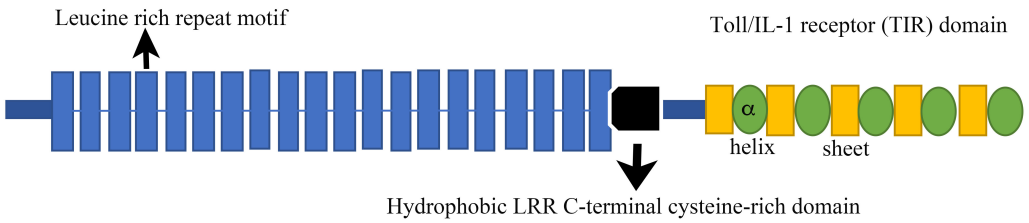
A



B

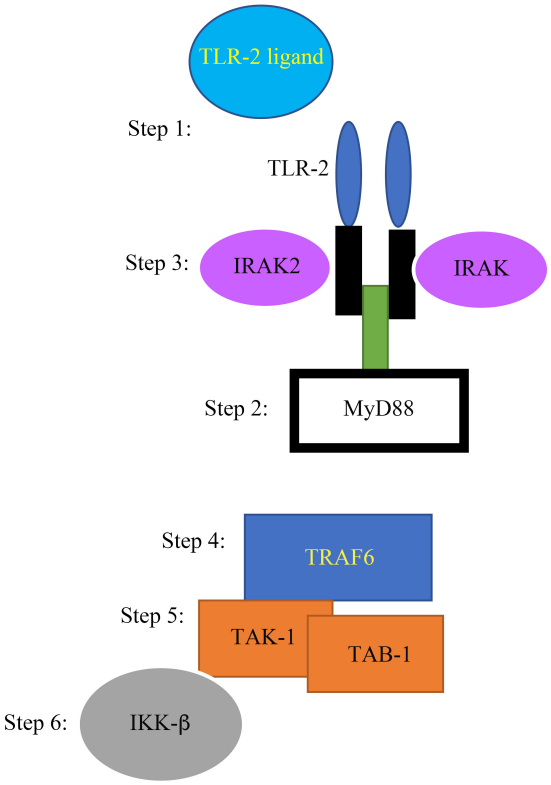


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Fig 5. Structure and signaling pathway of TLR2

(A) TLR2 is composed by the N-terminal extracellular domain, the transmembrane domain, and the C-terminal intracellular domain. (B) TLR2 signaling pathway starts with TLR2 ligands binding to TLR2. The recruitment of MyD88 to the receptor complex then leads to the recruitment of IL-1 receptor-associated kinase (IRAK) and IRAK2. Following IRAK recruitment, TNF receptor-assiciated factor (TRAF) 6, TGF-β-activated kinase (TAK) 1, and TAK1 binding protein (TAB) 1 were activated. Activated TAK1 thereby activates IκB kinase (IKK)-β.

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Chapter IX: Abbreviations

TLR: toll-like receptor

BM-MSC: bone marrow-derived mesenchymal stem cell

IκB: inhibitor of κB

NF-κB: nuclear factor-κB

IL-6: interleukin-6

STAT3: signal transducer and activator of transcription 3

iNOS: inducible nitric oxide synthase

NO: nitric oxide

OVA: ovalbumin

c.p.m.: counts per minute

qPCR: quantitative polymerase chain reaction

R.Q.: relative quantification

Mctrl: control MSCs

Mpam: Pam_3CSK_4-treated MSCs

Ms&p: the combination of Pam_3CSK_4 and S3I-201-treated MSCs

Ml&p: the combination of Pam_3CSK_4 and L-NMMA-treated MSCs

M6&p: the combination of Pam_3CSK_4 and IL-6 neutralizing antibody-treated MSCs

Mic&p: the combination of Pam_3CSK_4 and IgG1 κ isotype control antibody-treated MSCs

Tnaive: non-educated $CD4^+$ T cells

Tctrl: Mctrl-educated $CD4^+$ T cells

Tpam: Mpam-educated $CD4^+$ T cells

Ts&p: Ms&p-educated $CD4^+$ T cells

Tl&p: Ml&p-educated $CD4^+$ T cells

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