

terminal differentiation process and production of proinflammatory cytokines can be additional factors involved in PAR2-induced epidermal barrier impairment. Future studies are needed to uncover PAR2-mediated pathway(s) critical in TJ regulation in atopic skin.

In conclusion, increasing evidence has shown a role for PAR2 and proteases in AD particularly in pruritus, stratum corneum turnover/composition, and epithelial drive inflammation. Our study additionally revealed that PAR2 might contribute to epidermal barrier impairment of atopic skin, by compromising TJ integrity and composition. The observed effect of PAR2 activation on claudin-1 expression is very intriguing. We and others have shown reduced expression of claudin-1 in AD skin.⁸ Strong evidence from murine studies has highlighted a critical role played by this claudin in epidermis barrier function and more recently in inflammation. Tokumasu et al were able to reproduce features of AD in mice by reducing claudin-1 expression level (see reference E3 in in this article's Online Repository at www.jacionline.org). Our data suggesting a role of PAR2 in claudin-1 expression pave the way to new investigations in the pathogenesis of AD. Excitingly, over the past decade, there has been great effort toward the development of drugs (agonist/antagonist) targeting PAR2. All together a strategy to selectively block PAR2 downstream signaling could result in much needed therapeutic interventions for AD by targeting both barrier and inflammation/itch.

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REFERENCES

1. Guenther F, Melzig MF. Protease-activated receptors and their biological role—focused on skin inflammation. *J Pharm Pharmacol* 2015;67:1623-33.
2. Rothmeier AS, Ruf W. Protease-activated receptor 2 signaling in inflammation. *Semin Immunopathol* 2012;34:133-49.
3. De Benedetto A, Kubo A, Beck LA. Skin barrier disruption: a requirement for allergen sensitization? *J Invest Dermatol* 2012;132:949-63.
4. Lee SE, Jeong SK, Lee SH. Protease and protease-activated receptor-2 signaling in the pathogenesis of atopic dermatitis. *Yonsei Med J* 2010;51:808-22.
5. Voegelé R, Rawlings AV, Breternitz M, Doppler S, Schreier T, Fluhr JW. Increased stratum corneum serine protease activity in acute eczematous atopic skin. *Br J Dermatol* 2009;161:70-7.
6. Steinhoff M, Corvera CU, Thoma MS, Kong W, McAlpine BE, Caughey GH, et al. Proteinase-activated receptor-2 in human skin: tissue distribution and activation of keratinocytes by mast cell tryptase. *Exp Dermatol* 1999;8:282-94.
7. Roelandt T, Heughebaert C, Verween G, Giddel C, Verbeken G, Pirnay JP, et al. Actin dynamics regulate immediate PAR-2-dependent responses to acute epidermal permeability barrier abrogation. *J Dermatol Sci* 2011;61:101-9.
8. De Benedetto A, Rafaels NM, McGirt LY, Ivanov AI, Georas SN, Cheadle C, et al. Tight junction defects in patients with atopic dermatitis. *J Allergy Clin Immunol* 2011;127:773-86.
9. Jacob C, Yang PC, Darmoul D, Amadesi S, Saito T, Cottrell GS, et al. Mast cell tryptase controls paracellular permeability of the intestine: role of protease-activated receptor 2 and beta-arrestins. *J Biol Chem* 2005;280:31936-48.

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Toll-like receptor 2 ligation of mesenchymal stem cells alleviates asthmatic airway inflammation



To the Editor:

Under current therapeutic strategies, the cure to asthma remains elusive. Although molecular therapies have recently been suggested as the potential solutions, the limited success with a particular target molecule is attributable to the complexity of asthmatic pathogenesis. In addition, a combination of different target molecules has not achieved advantageous balanced benefit-risk ratios.¹ Hence, novel approaches for treating asthma are definitely needed. Mesenchymal stem cells (MSCs) have recently been established as potential candidates to treat asthma by virtue of their immunomodulatory properties.² However, the sustainable translation of MSC therapies to clinical settings has been hampered by the heterogeneity of MSCs. Therefore, on the basis of the concept of polarizing homogeneously acting MSCs with microenvironmental factors,³ we suggested a Toll-like receptor (TLR) 2 ligand, Pam₃CSK₄-treated MSCs (Mpam), as a possible approach.

Experimental procedures were presented in this article's Online Repository at www.jacionline.org.

Mouse bone marrow (BM)-derived MSCs have been characterized by their mesenchymal lineage surface markers, trilineage differentiation abilities, and immunosuppressive properties (please see Fig E1, A-C, in this article's Online Repository at www.jacionline.org). TLR2, one of the influential molecules in BM-MSCs, was selected for modification (please see Fig E1, D). Mpam was demonstrated to attenuate CD4⁺ T-cell proliferation more effectively than did control MSCs (Mctrl) (Fig 1, A). To study whether the advanced therapeutic effects of Mpam would be observed *in vivo*, we next compared the alleviative effect of Mctrl and Mpam on airway inflammation with the ovalbumin-induced asthma murine model (the timeline is presented in Fig E2, A, in this article's Online Repository at www.jacionline.org).

Furthermore, given several inconsistent results related to TLR-polarized MSCs, we examined the immunoregulatory mechanisms to validate the modified MSCs in asthma therapy. The previous results suggested that induction of signal transducer and activator of transcription (STAT) 3 promotes the production of suppressive factors downstream of TLR2 activation in many cell types.⁴ We thus hypothesized that Pam₃CSK₄ modification could enhance the immunosuppressive functions of MSCs through STAT3 signaling.

In mice with asthmatic inflammation, compared with Mctrl, the Mpam treatment further decreased IgG₁/IgG_{2a} level in serum (n = 5) (Fig 1, B), further decreased IL-4 and IL-5 secretion in bronchoalveolar lavage fluid (n = 5) (Fig 1, C), further diminished infiltrated eosinophils in lungs (n = 8) (Fig 1, E), further downregulated pulmonary *muc5ac* expression (n = 8) (Fig 1, F), and further attenuated airway resistance of tracheotomized mice in response to β -methacholine administrations (n = 8) (Fig 1, G). In contrast, pulmonary histopathology revealed that MSCs diminished the inflammatory cell infiltration and the bronchial epithelial thickness irrespective of Pam₃CSK₄ induction (Fig 1, H). The results suggested that the airway hyperresponsiveness was alleviated more effectively with Mpam than

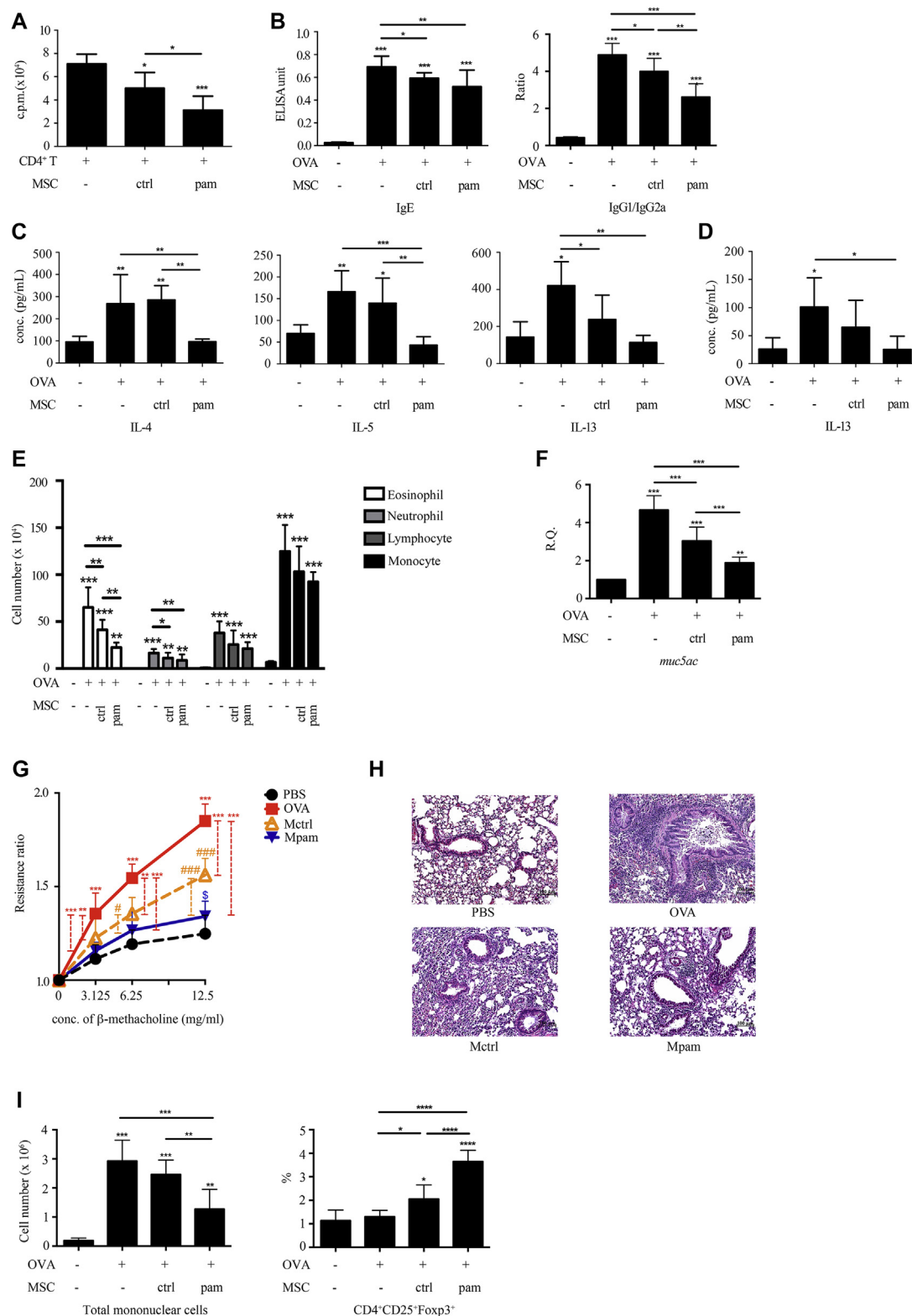


FIG 1. The immunosuppressive function was demonstrated *in vitro* using (A) [³H]-thymidine incorporation assays. In OVA-induced asthma murine model, ELISA was used to detect (B) serum IgE, IgG₁, and IgG_{2a} levels, (C) IL-4, IL-5, and IL-13 expression in BALF, and (D) IL-13 expression in the supernatants of OVA-stimulated splenocytes (n = 5). E, Infiltrated eosinophils in BALF were counted using microscope slide smear technique. F, The expression of *muc5ac* in lung tissues was observed using quantitative PCR. G, Airway resistance was examined using plethysmograph (mean $\pm$ SD; **, *** comparisons between OVA and the other groups, P < .005 and P < .0005, respectively; #, ###, comparisons between Mctrl and the other groups, P < .05 and P < .0005, respectively; \$, comparisons between Mpam and PBS groups, P < .05). H, Hematoxylin and Eosin staining results were presented using 100 $\times$ magnification. I, CD4⁺CD25⁺Foxp3⁺ cells isolated from lung tissues were examined using flow cytometry (mean $\pm$ SD; *P < .05, **P < .005, ***P < .0005, ****P < .00005). BALF, Bronchoalveolar lavage fluid; OVA, ovalbumin.

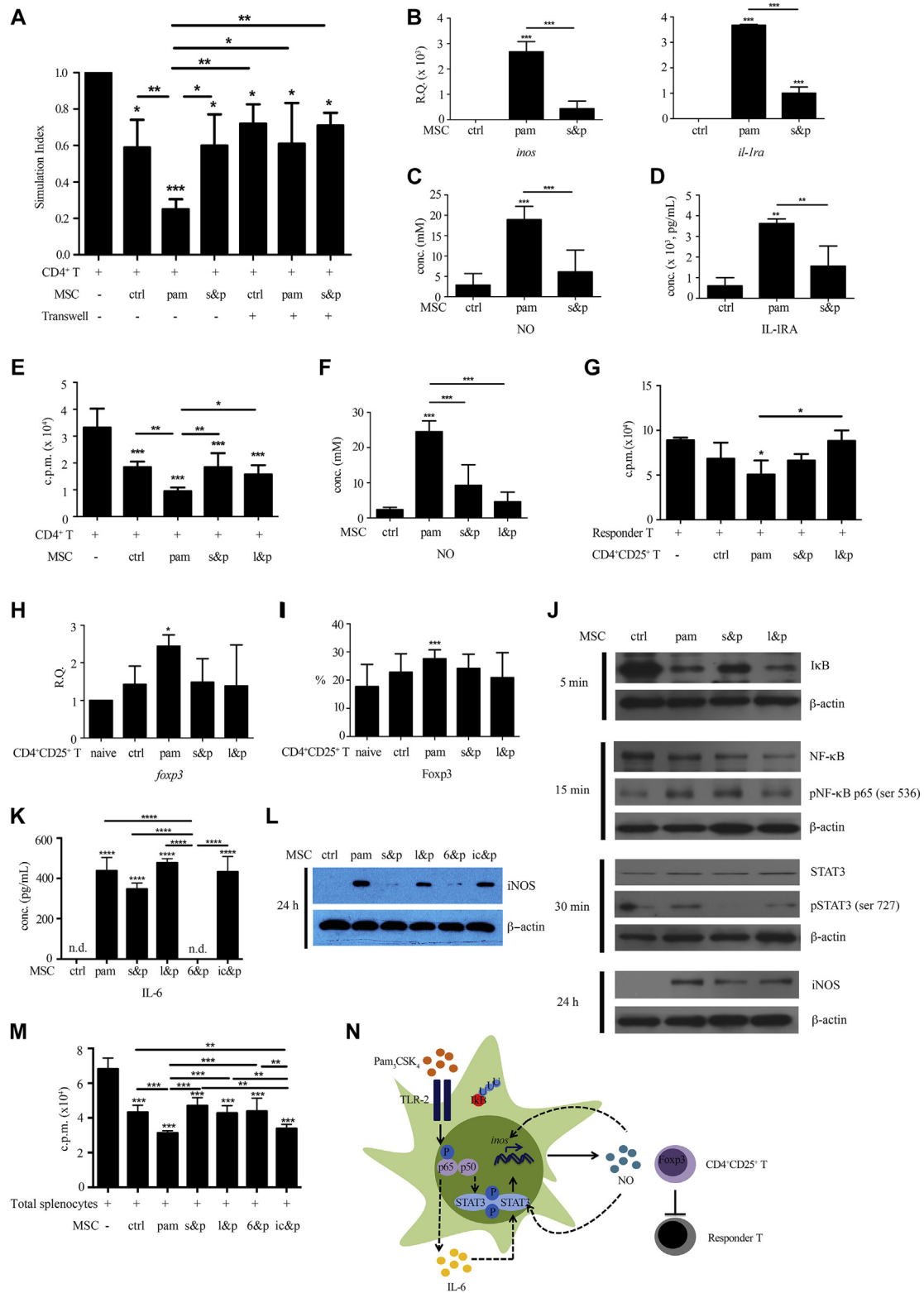


FIG 2. TLR2/STAT3/iNOS signaling pathway in Mpam was demonstrated with inhibitor treatment. **A** and **E**, The suppressive effects were examined using [³H]-thymidine incorporation assays with or without transwell insertions. **B**, The possible suppressive factors in Mpam were screened using quantitative PCR, and confirmed at the protein level with (**C** and **F**) Griess assays and (**D**) ELISA. **G**, The suppressive effects of the CD4⁺CD25⁺ conditioned T cells were examined using [³H]-thymidine incorporation assays. CD4⁺CD25⁺Foxp3⁺ T-cell population was further demonstrated using (**H**) quantitative PCR and (**I**) flow cytometry. **J**, I-κB, NF-κB, STAT3, and iNOS were sequentially detected using Western blotting (WB). The possible mediating role of IL-6 between NF-κB and STAT3 was examined using (**K**) ELISA, (**L**) WB, and (**M**) [³H]-thymidine incorporation assays with M6&p or Mic&p. **N**, Immunosuppressive mechanism-oriented validation of Mpam (mean ± SD; **P* < .05, ***P* < .005, ****P* < .0005, *****P* < .00005). I-κB, Inhibitor of κB; M6&p, IL-6 neutralizing antibody and Mpam; Mic&p, IgG₁ κ isotype control antibody and Mpam.

with Mctrl. However, the further histopathological amelioration of Mpam might take more time to develop.⁵

Using [³H]-thymidine incorporation assays, we subsequently demonstrated that the TLR2 ligation-enhanced suppressive function in MSCs was reversed with a STAT3 inhibitor, S3I-201 (S3I-201 and Mpam [Ms&p]), in the cell-cell contact-dependent, but not in the transwell system (Fig 2, A). Therefore, compared with Mctrl, despite the fact that the expression of both inducible nitric oxide synthase (*iNOS*) and interleukin-1 receptor antagonist (*il-1ra*) was increased in Mpam and diminished in Ms&p, nitric oxide (NO), which works in a cell-proximity manner, was more likely to be the plausible Pam₃CSK₄-induced regulatory factor in MSCs (Fig 2, B-D). Moreover, because Mpam compressed more proliferative CD4⁺ T cells than did Mctrl, whereas Ms&p and *iNOS* inhibitor, L-NMMA, -treated Mpam (L-NMMA and Mpam [Ml&p]) did not (Fig 2, E), we suggested the TLR2/STAT3/*iNOS* signaling by which Mpam advanced its immunoregulatory function.

Next, other reports have shown that more regulatory T cells are induced through enhanced NO secretion.⁶ Using Griess assays, higher NO was detected only in the supernatant of Mpam (Fig 2, F). It possibly explained how the CD4⁺CD25⁺ T cells cocultured with Mpam inhibited the responder CD4⁺ T-cell proliferation more effectively than those cocultured with Mctrl, Ms&p, and Ml&p (Fig 2, G). Furthermore, compared with the CD4⁺CD25⁺ T cells alone (Tnaive), Foxp3 was upregulated only in the Mpam-cocultured CD4⁺CD25⁺ T cells (Tpam), but not in the Mctrl-, Ms&p-, and Ml&p-cocultured CD4⁺CD25⁺ T cells (Tctrl, Ts&p, and Tl&p) both at mRNA and protein levels (Fig 2, H and I). Mononuclear cells were also examined in lungs of asthmatic murine model. With the Mpam treatment, less mononuclear cells, however, a higher percentage of CD4⁺CD25⁺Foxp3⁺ cells was observed in the lung homogenate (Fig 1, J). Therefore, we suggested that CD4⁺CD25⁺Foxp3⁺ regulatory T cells were possibly induced to exert the better suppressive functions of Pam₃CSK₄-modified MSCs through TLR2/STAT3/*iNOS* signaling.

Finally, the mediation of the seemingly independent mechanisms between TLR2 and STAT3 was examined. The TLR2 ligation is known to lead to inhibitor of κ B kinase degradation and then nuclear factor (NF)- κ B transcriptional activation. As proven by our results established using Western blotting, compared with Mctrl, the sequential degradation of inhibitor of κ B and activation of NF- κ B/STAT3/*iNOS* in Mpam suggested its TLR2 ligation-induced signaling pathway. In these data, TLR2 ligation failed to phosphorylate STAT3 and consequently produced less *iNOS* in Ms&p. The diminished expression of *iNOS* in Ml&p, otherwise, was mainly attributed to the translation of mRNA, which was regulated by the availability of its substrate, L-arginine (Fig 2, J). Despite the fact that the possibility of the interaction between NF- κ B and STAT3 pathways was not excluded,⁷ upregulated IL-6 production of Mpam irrespective of the inhibition by neither STAT3 nor *iNOS* inhibitor suggested the possible mediating role of IL-6 between TLR2 and STAT3 (Fig 2, K). TLR2 ligation no longer stimulated *iNOS* production in MSCs after treatment with IL-6 neutralizing antibody (IL-6 neutralizing antibody and Mpam [M6&p]) (Fig 2, L). The augmented immunosuppressive function of Mpam was then abolished by IL-6, but not by IgG₁ κ isotype control neutralization (Fig 2, M). We thus suggested that

IkB/NF- κ B/IL-6 might play the critical roles in mediating TLR2/STAT3 signaling.

In summary, we used Pam₃CSK₄ to enhance the immunomodulatory functions of MSCs, and high levels of *iNOS* and NO were noted in Pam₃CSK₄-stimulated MSCs. In addition, involvement of NO-stimulated Foxp3⁺ regulatory cell was proposed (Fig 2, N). However, *iNOS*-derived NO might suppress T-cell function through versatile mechanisms.^{8,9} It possibly explained the discrepancy between the strikingly NO level and the significant but less amount of Foxp3⁺ cell induction with Mpam *in vitro* and *in vivo*. More experiments might be needed to clarify the molecular mechanism involved in the immunomodulatory functions induced by MSCs stimulated by Pam₃CSK₄.

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REFERENCES

- Schuijs MJ, Willart MA, Hammad H, Lambrecht BN. Cytokine targets in airway inflammation. *Curr Opin Pharmacol* 2013;13:351-61.
- Bassi EJ, de Almeida DC, Moraes-Vieira PM, Camara NO. Exploring the role of soluble factors associated with immune regulatory properties of mesenchymal stem cells. *Stem Cell Rev* 2012;8:329-42.
- Schafer R, Spohn G, Baer PC. Mesenchymal stem/stromal cells in regenerative medicine: can preconditioning strategies improve therapeutic efficacy? *Transfus Med Hemother* 2016;43:256-67.
- Yu J, Du W, Yan F, Wang Y, Li H, Cao S, et al. Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer. *J Immunol* 2013;190:3783-97.
- Trzil JE, Maseau I, Webb TL, Chang CH, Dodam JR, Cohn LA, et al. Long-term evaluation of mesenchymal stem cell therapy in a feline model of chronic allergic asthma. *Clin Exp Allergy* 2014;44:1546-57.
- Yu C, Fitzpatrick A, Cong D, Yao C, Yoo J, Turnbull A, et al. Nitric oxide induces human CLA+CD25+Foxp3+ regulatory T cells with skin-homing potential. *J Allergy Clin Immunol* 2017;140:1441-4.
- Fan Y, Mao R, Yang J. NF- κ B and STAT3 signaling pathways collaboratively link inflammation to cancer. *Protein Cell* 2013;4:176-85.
- Bogdan C. Regulation of lymphocytes by nitric oxide. *Methods Mol Biol* 2011;677:375-93.
- Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, et al. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood* 2007;109:228-34.

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The nasal microbiome in patients with chronic rhinosinusitis: Analyzing the effects of atopy and bacterial functional pathways in 111 patients



To the Editor:

Chronic rhinosinusitis (CRS) is a chronic disease characterized by sinonasal mucosal inflammation. The inflammatory milieu in patients with CRS is thought to be affected by or even possibly initiated by commensal microbes, pathogens, and their products.^{1,2}

To date, only a few studies have evaluated the nasal microbiome in patients with CRS. There has been a lack of

METHODS

BM cells were obtained from femurs and tibias of 4- to 5-week-old female Balb/c mice. MSCs were obtained by expansion of the BM plastic-adherent cell fraction with mouse MSC culture medium (MesenCult, Vancouver, British Columbia, Canada). Passage numbers 6 to 13 of BM-MSCs were used in this study. Mouse BM-MSCs have been characterized by their mesenchymal lineage surface markers using flow cytometry (BD Pharmingen), trilineage differentiation abilities using induction medium (MesenCult, Gibco), and immunosuppressive properties using [^{3} H]-thymidine incorporation assays.

The conditioned BM-MSCs were generated under the following instructions: after being seeded for 24 hours, BM-MSCs were either nontreated as the Mctrl or treated with a TLR2 ligand, Pam₃CSK₄ (InvivoGen), for 96 hours as the Mpam. To investigate the signaling pathways of immunosuppression-prone Mpam, 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 Pam₃CSK₄ treatment to make Ms&p, MI&p, IL-6 neutralizing antibody and Mpam (M6&p), and IgG₁ κ isotype control antibody and Mpam (Mic&p), respectively (Fig E2, B). The final concentrations of the treatments are listed as following: 1 μ g/mL Pam₃CSK₄ was used to stimulate MSCs; 125 μ Mol S3I-201, 1000 μ Mol L-NMMA, 10 μ g/mL IL-6 neutralizing antibody, and 10 μ g/mL IgG₁ κ isotype control antibody were used to block the activated signaling in Pam₃CSK₄-treated MSCs. On the other hand, the conditioned CD4⁺CD25⁺ T cells were generated to demonstrate the suppressive mechanisms of Mpam. CD4⁺ T cells were prestimulated with 2 μ g/mL anti-CD3e and 2 μ g/mL anti-CD28 for 24 hours before they were cocultured with mitomycin c-treated conditioned BM-MSCs in a 20:1 ratio for 96 hours. CD25⁺ cells were then isolated from these CD4⁺ T cells by anti-CD25 Phycoerythrin antibodies (BD Pharmingen) and an Anti-R-Phycoerythrin enrichment set (BD IMag) to make Tctrl, Tpam, Ts&p, and TI&p (Fig E2, C).

To determine whether Pam₃CSK₄ could enhance the immunoregulatory functions of BM-MSCs, we performed the [^{3} H]-thymidine incorporation assays *in vitro* and the acute ovalbumin (OVA)-induced asthma murine model *in vivo*. In the animal studies, 6-week-old female Balb/c mice were divided into 4 groups: the negative control (PBS), the positive control (OVA), the Mctrl treatment (Mctrl), and the Mpam treatment (Mpam) groups. The main criteria for studying OVA-induced asthmatic mice were followed to examine the therapeutic effects of Mctrl and Mpam.^{E1}

The molecular mechanisms of Mpam were elucidated *in vitro*. The potential TLR ligand candidates for BM-MSC induction were demonstrated using semi-quantitative RT-PCR. The sequentially related signaling pathways were confirmed using Western blotting analysis. Potential immunoregulatory molecules in conditioned BM-MSCs and conditioned CD4⁺CD25⁺ T cells were examined using real-time quantitative PCR in an SYBR Green system (Bio-genes) at the mRNA level, and confirmed using Griess assays (Sigma), ELISA, or intracellular staining (eBioscience) at the protein level. PCR primer sequences are presented in Table E1.

Data were analyzed using GraphPad Prism v5 and were presented as mean and individual data points (mean $\pm$ SD). To determine the significant variances between groups, Student *t* test was used between 2 groups, whereas 1-way ANOVA was used for multiple comparisons and Newman-Keuls multiple comparison test was used for post hoc analysis. *In vitro* statistic results were calculated with at least 3 independent repeats. The statistical significance was set at *P* < .05.

Additional details on [^{3} H]-thymidine incorporation assay, acute OVA-induced asthma murine model, invasive measurement of airway resistance, pulmonary mononuclear cell preparation, specific primers, Griess assay, and intracellular staining are described as follows.

[^{3} H]-thymidine incorporation assays

Splenocytes from 5- to 8-week old female Balb/c mice were isolated by mouse CD4⁺ T-cell isolation kit (EasySep, Vancouver, British Columbia, Canada) according to the manufacturer's protocol. Total splenocytes or

CD4⁺ T cells were preactivated with 2 μ g/mL anti-CD3e (BioLegend) and 2 μ g/mL anti-CD28 (BioLegend) for 24 hours before being cocultured with mitomycin c (Sigma)-induced mitotically inactive BM-MSCs for 48 hours. The cell ratio of CD4⁺ T cells to BM-MSCs was 20 to 1. [^{3} H]-thymidine (1 μ Ci/mL; PerkinElmer) was added to each well and the plate was incubated for 18 hours. The cells were harvested and the thymidine incorporation was detected by a β -counter (Matrix 96). The same process was performed in the transwell assays.

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 4 groups: PBS, OVA, Mctrl, and Mpam groups. Each group originally had 9 mice at most in 1 experimental setting. Except for the negative control group, which was sensitized and challenged with PBS, they were intraperitoneally sensitized by 200 μ L of 50 μ g OVA (Sigma)/2 mg alum (Thermo Scientific) at day 0, 14, and 21, and then were intranasally challenged by 40 μ L of 100 μ g OVA at day 28, 29, and 30. After the third sensitization, OVA-sensitized mice with 0.5 to 0.7 ELISA units of serum IgE levels and negative control mice with less than 0.015 ELISA units of serum IgE levels were included in the following procedures. The OVA-sensitized groups were randomized into 3 groups on the basis of their serum IgE levels. The treatment groups were intravenously infused once a day by 100 μ L of 5×10^5 Mctrl and Mpam, respectively, through day 28 to 30. The animals were either sacrificed or were used in airway resistance experiment at day 31 (Fig E2, A). Animal experiments were undertaken with the explicit approval of the Institutional Animal Care and Use Committee before initiation.

Invasive measurement of airway resistance

Airway function was measured by detecting changes in lung resistance in response to increasing doses of aerosolized acetyl- β -methylcholine chloride (Sigma) in anesthetized and tracheotomized mice. The calculation of lung resistance was yielded by subtracting transpulmonary pressure (0.45 cmH₂O.s.ml⁻¹) from respiratory flow measurement. Data were presented as average lung resistance in the ratio of average lung resistance after PBS nebulization.

Pulmonary mononuclear cell preparation

The protocol established by Wiesner et al^{E2} was referenced. Lungs were excised and minced to generate pieces in HBSS (Sigma) + 1.3 mM EDTA (Sigma) solution for 30 minutes at 37°C with agitation. The solution was transferred to RPMI 1640 medium (Hyclone) supplemented with 10% FBS (Gibco) and 150 U/mL type II collagenase (Worthington), and incubated for 1 hour at 37°C with agitation. The cells were then grounded through a 70- μ m filter, pelleted, and resuspended with Dulbecco's Phosphate-Buffered Saline (Sigma). Ficoll solution (GE) was finally used to separate mononuclear cells from the pulmonary homogenate.

Griess assay

Mitotically inactive BM-MSCs were seeded as 6×10^4 cells overnight. The medium was then replaced by 250 μ L of culture medium with treatments and incubated for 96 hours. The supernatants were assayed for NO by mixing 100 μ L supernatant with 100 μ L Griess reagent (Sigma) for 15 minutes 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 to 0.003 μ M NaNO₂ concentrations (Sigma).

Intracellular staining

Cells were processed according to the manufacturer's protocols. Non-antigen-specific bindings of primary antibodies to Fc γ II and Fc γ III, and

possibly FcγI receptors of cells, were blocked by antimouse CD16/32 (BD Pharmingen). Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used to do the intracellular staining. 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 Pro software.

REFERENCES

- E1. [Huang MT, Chen YL, Lien CI, Liu WL, Hsu LC, Yagita H, et al. Notch ligand DLL4 alleviates allergic airway inflammation via induction of a homeostatic regulatory pathway. *Sci Rep* 2017;7:43535.](#)
- E2. [Wiesner DL, Smith KD, Kotov DI, Nielsen JN, Bohjanen PR, Nielsen K. Regulatory T cell induction and retention in the lungs drives suppression of detrimental type 2 Th cells during pulmonary cryptococcal infection. *J Immunol* 2016;196:365-74.](#)

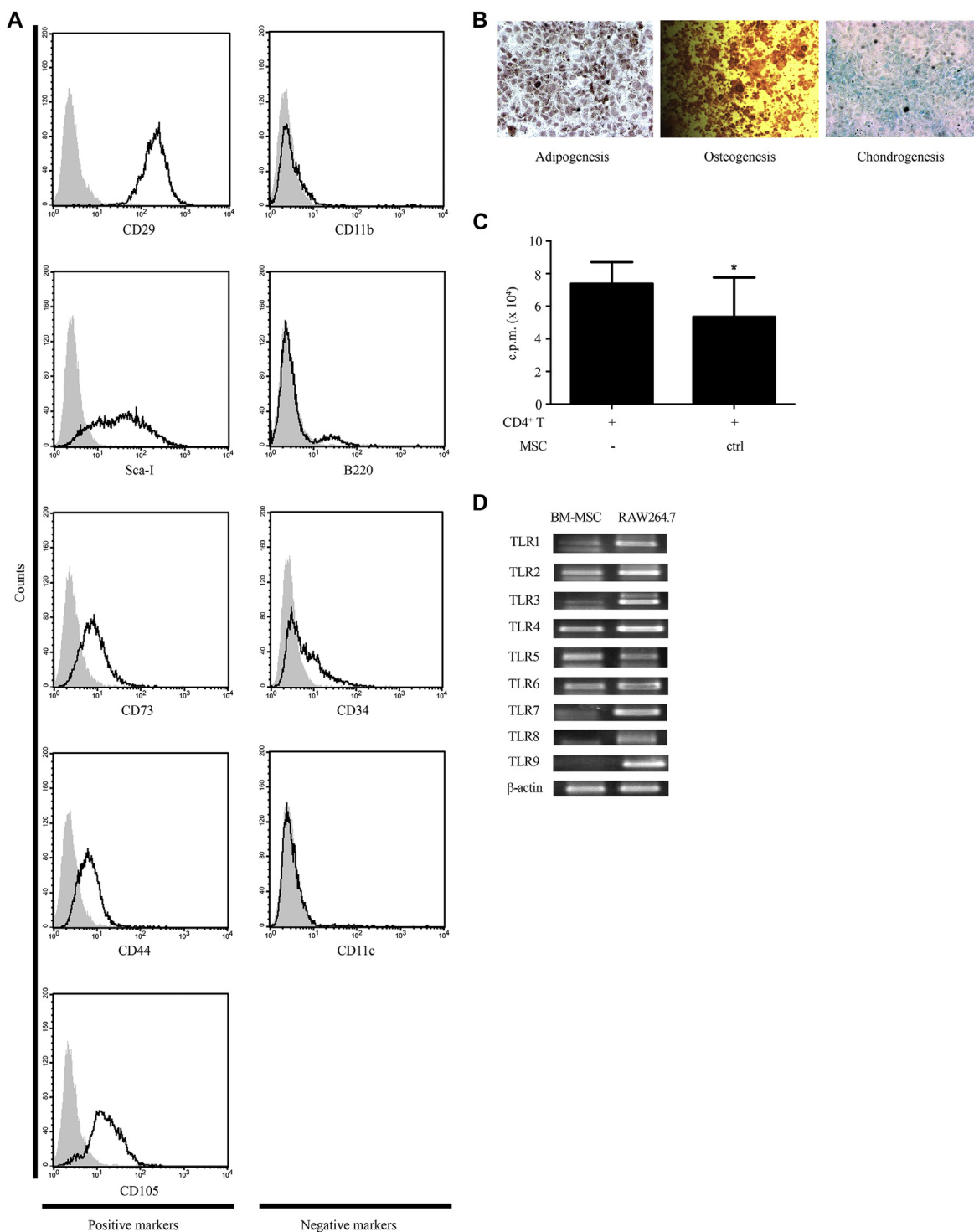


FIG E1. Mouse BM-MSCs were characterized through **(A)** positive expression of mesenchymal lineage surface markers such as CD29, Sca-1, CD73, CD44, and CD105, whereas negative expression of hematopoietic lineage surface markers such as CD11b, B220, CD34, and CD11c, **(B)** adipogenesis (neutral triglycerides and lipids were detected by Oil red O solution, presented as red color), osteogenesis (calcium deposits were detected by Alizarin red S, presented as red color), and chondrogenesis (acid mucosubstances and acetic mucins were detected by Alcian blue, presented as blue color) properties are presented using 200 $\times$ magnification, and **(C)** immunosuppressive functions proved using [^{3} H]-thymidine incorporation assays. **D**, TLR expression of MSCs was examined by semi-quantitative RT-PCR. RAW264.7 cells were used as positive controls. Statistic results were calculated with 6 independent repeats (mean $\pm$ SD; * $P < .05$).

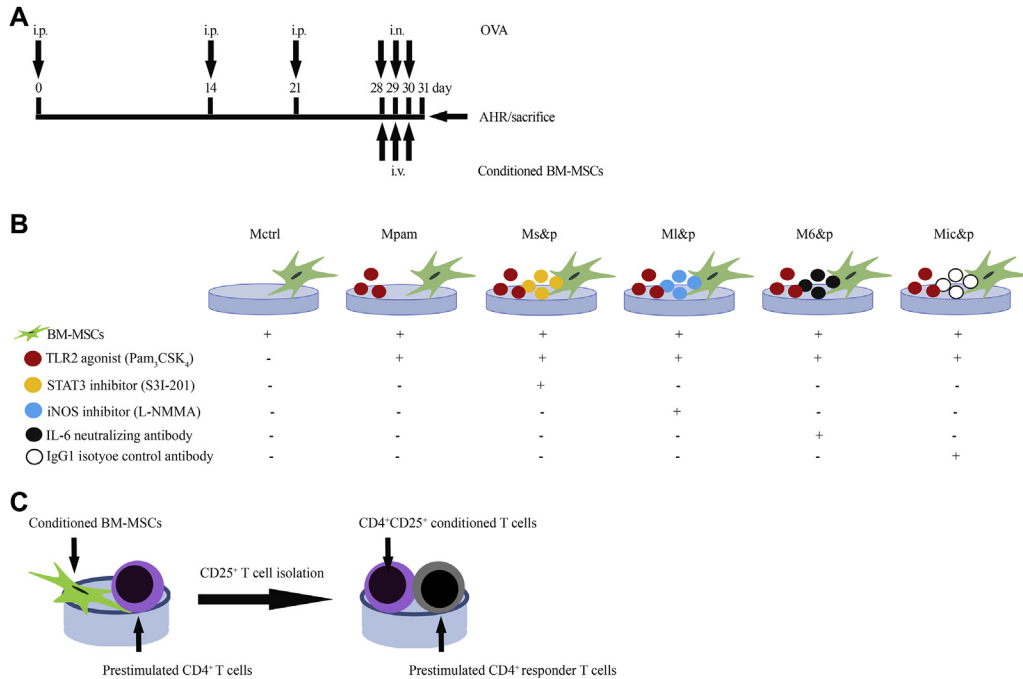


FIG E2. A, Mice were intraperitoneally (i.p.) sensitized and intranasally (i.n.) challenged using PBS in the PBS group but using OVA in the OVA, Mctrl, and Mpam treatment groups. Treatment groups were intravenously (i.v.) treated using 5×10^5 /mouse of Mctrl and Mpam, respectively. **B,** The nontreated MSCs were presented as Mctrl. The TLR2 agonist, Pam₃CSK₄, -treated MSCs were presented as Mpam. The STAT3 inhibitor, S3I-201-, iNOS inhibitor, L-NMMA-, and IL-6-neutralizing antibody- and IgG₁ κ isotype control antibody-treated MSCs in addition to Pam₃CSK₄ treatments were presented as Ms&p, Ml&p, M6&p, and Mic&p, respectively. **C,** Prestimulated CD4⁺ T cells were cocultured with the mitomycin c-treated conditioned 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; *M6&p*, IL-6 neutralizing antibody and Mpam; *Mic&p*, IgG₁ κ isotype control antibody and Mpam.

TABLE E1. PCR primers used in this study

Semi-quantitative PCR primers			
TLR	Primer sequence	Size (bp)	Annealing temperature (°C)
<i>tlr1</i>	GGATGTGTCCGTCAGCACTA TGTAACCTTTGGGGGAAGCTG	340	53
<i>tlr2</i>	CAGACGTAGTGAGCGAGCTG GGCATCGGATGAAAAGTGTT	390	50
<i>tlr3</i>	GAGGGCTGGAGGATCTCTTT TGCCTCAATAGCTTGCTGAA	353	53
<i>tlr4</i>	GCTTTCACCTCTGCCTTCAC CGAGGCTTTTCCATCCAATA	361	53
<i>tlr5</i>	GCTTTGTTTTCTTCGCTTCG ACACCAGCTTCTGGATGGTC	342	50
<i>tlr6</i>	GCAACATGAGCCAAGACAGA GTTTTGCAACCGATTGTGTG	349	53
<i>tlr7</i>	ATTCAGAGGCTCCTGGATGA AGGGATGTCCTAGGTGGTGA	264	53
<i>tlr8</i>	TCCTGGGGATCAAAAATCAA AAGGTGGTAGCGCAGTTCAT	302	50
<i>tlr9</i>	ACCCTGGTGTGGAACATCAT GTTGGACAGGTGGACGAAGT	341	53
<i>β-Actin</i>	AAGGTGTGATGGTGGGAATG ATGGCTACGTACATGGCTGG	286	50
Quantitative PCR primers			
Gene	Primer sequence	Size (bp)	Annealing temperature (°C)
<i>muc5ac</i>	CCATGCAGAGTCCTCAGAACAA TTACTGGAAAGGCCCAAGCA	106	60
<i>inos</i>	GGCAGCCTGTGAGACCTTTG GCATTGGAAGTGAAGCGTTTC	72	60
<i>il-1ra</i>	GACCCTGCAAGATGCAAGCC GAGCGGATGAAGGTAAAGCG	292	60
<i>foxp3</i>	TACCACAATATGCGACCC CTCAAATTCATCTACGGTCC	240	53
<i>gapdh</i>	GATGGGTGTGAACCACGAGA AGATCCACGACGGACACAT	339	60