Letter to the Editor

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, Pam_3CSK_4-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 Pam_3CSK_4 modification could enhance the immunosuppressive functions of MSCs through STAT3 signaling.

In mice with asthmatic inflammation, compared with Mctrl, the Mpam treatment further decreased IgG_1/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 Pam_3CSK_4 induction (Fig 1, *H*). The results suggested that the airway hyperresponsiveness was alleviated more effectively with Mpam than with Mctrl. However, the further histopathological amelioration of Mpam might take more time to develop.[5]

Using [^{3}H]-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 Pam_3CSK_4-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, *I*). Therefore, we suggested that $CD4^+CD25^+Foxp3^+$ regulatory T cells were possibly induced to exert the better suppressive functions of Pam_3CSK_4-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 κB kinase degradation and then nuclear factor (NF)-κB transcriptional activation. As proven by our results established using Western blotting, compared with Mctrl, the sequential degradation of inhibitor of κB and activation of NF-κB/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-κB 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 IgG_1

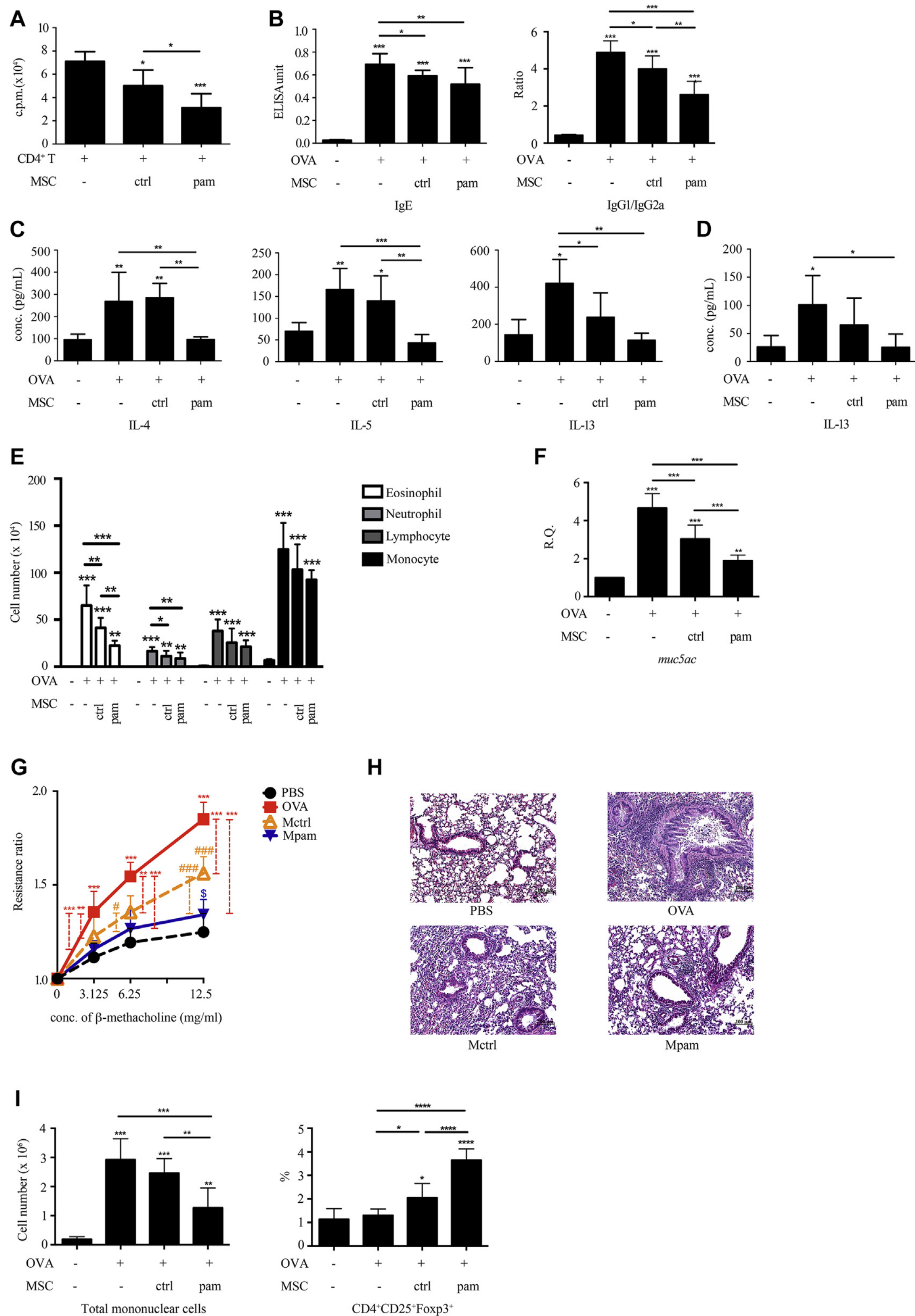

FIG 1. The immunosuppressive function was demonstrated *in vitro* using **(A)** $[^3H]$-thymidine incorporation assays. In OVA-induced asthma murine model, ELISA was used to detect **(B)** serum IgE, IgG_1, and IgG_{2a} levels, **(C)**, IL-4, IL-5, and IL-13 expression in BALF, and **(D)** IL-13 expression 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 ± 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× magnification. **I,** $CD4^+CD25^+Foxp3^+$ cells isolated from lung tissues were examined using flow cytometry (mean ± SD; *$P < .05$, **$P < .005$, ***$P < .0005$, ****$P < .00005$). *BALF*, Bronchoalveolar lavage fluid; *OVA*, ovalbumin.

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