Passive transfer of lipopolysaccharide-derived myeloid-derived suppressor cells inhibits asthma-related airway inflammation

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Abstract. – **OBJECTIVE:** Myeloid-derived suppressor cells (MDSCs) have recently been implicated in the pathogenesis of asthma through inhibiting T cell response. However, the issue of whether Lipopolysaccharide (LPS)-derived MDSCs regulate the immune response in an asthma environment is currently unclear. We sought to characterize the pathogenic function of various subtypes of MDSCs in asthma mediated by ovalbumin in mice model, in order to show that LPS-induced MDSCs can shift the balance back to normal in a Th2-dominant asthmatic environment.

MATERIALS AND METHODS: Subgroups of MDSCs with Ly6C+Ly6G+, Ly6C−Ly6G+, Ly6C+Ly6G− or Ly6C−Ly6G− expression were isolated by flow cytometry and were co-cultured with spleen lymphocytes. The proportion of Th1, Th2, or Treg cells in the treated spleen lymphocytes were analyzed by flow cytometry. In an ovalbumin (OVA)-induced mouse asthma model, mice were intravenously injected (tail vein) by MDSCs with specific marker, then the lung function and tissue pathology, IL-4 content in bronchoalveolar lavage fluid (BALF) and peripheral blood, and proportion of Th1, Th2, or Treg cells in peripheral blood were analyzed.

RESULTS: Ly6C+Ly6G+ MDSCs transferred into asthmatic mice via intravenous injection suppressed the infiltration of inflammatory cells into the lung and Th2 cytokine in BALF and blood. We observed a significant increase of Treg cells in the spleen lymphocytes co-cultured with Ly6C+Ly6G+, Ly6C−Ly6G+, Ly6C+Ly6G−, Ly6C−Ly6G− or CD11b+ MDSCs. The adoptive transfer of Ly6C+Ly6G+, Ly6C−Ly6G+, CD11b+ MDSCs resulted in decrease of Penh, total cell number, eosinophil and neutrophil percentage in BALF, and concentration of IL-4 in BALF and serum, thus improving the inflammatory injury, histopathology and lung function in the mice with asthma. The up-regulation of the Th1/Th2 ratio and Treg frequency were observed after adoptive transfer of Ly6C+Ly6G+, Ly6C−Ly6G+, Ly6C+Ly6G−, Ly6C−Ly6G− and CD11b+ MDSCs.

CONCLUSIONS: The LPS-derived MDSCs with specific markers were able to suppress natural inflammatory response and improve inflammatory injury through reversing Th1/Th2 ratio, increasing Treg proportion and decreasing IL-4 concentration. These findings imply that LPS-derived MDSCs inhibit Th2 cell-medicated response against allergen. We propose that asthma may be effectively targeted using a novel MDSC-based cell therapy approach.

Key Words:
   Myeloid-derived suppressor cells (MDSCs), Asthmatic mice, Airway inflammation, Th1/Th2, Regulatory T cell.

Introduction

Asthma has become a major epidemic affecting up to one third people in developed countries over the last decades, and making a crucial impact on morbidity rates[1]. In 2011, 235-300 million people globally have been diagnosed with asthma, among which 250,000 died due to asthma-related cause. Bronchial asthma is a respiratory disease that affects nearly one in ten individuals in the developed world[2,3], and the most common chronic disease of childhood[4]. Although current therapies are effective in suppressing disease symptoms, to date, there are no preventive treatments or cures for asthma. Current treatments for asthma mainly rely on drug interventions. Although these interventions are effective for controlling symptoms in some patients, up to 30% of individuals do not achieve effective disease control[5-7]. The mechanisms of asthma onset and development are still unclear, thus, there is an urgent medical need to develop new treatments for therapy-resistant patients.

Studies indicated that asthma is an atopic disorder of the airways which involves activated T helper lymphocytes, specifically Th2 cells. It was observed that asthmatic patients showed an infil-

tration of Th2 lymphocytes, eosinophils and de-granulated mast cells[8-10]. Exposure to allergen will activate Th2 cells and their cytokines, such as IL-4, IL-5, IL-9 and IL-13, orchestrate eosinophilic airway inflammation as well as B-cells to generate allergen-specific IgE antibody, all these events combined to ultimately lead to the release of preformed or newly synthesized inflammatory mediators from mast cells[11,12].

It is widely believed that Th2 cells initiate and perpetuate asthma. Th2 cells were found to be present in the lungs of asthmatic patients, particularly those with allergic asthma[10,13]. The specific contribution of Th2 lymphocytes has also been documented[14]. Mouse models of antigen-induced airway inflammation[15,16] have also reinforced the central role for Th2 lymphocytes and their cytokines in asthma. As a result of activation of allergen-specific Th2 cells, allergen-specific IgE is produced and binds to IgE receptors on the surface of mast cells. Then, mast cell activation results in release of histamine and leukotrienes, which directly affect airway smooth muscle and mucous glands and ultimately caused AHR[17-19]. On the other hand, Th1 cells have been shown to provide beneficial effect by inhibit development of Th2 cells[20]. It was shown that IFN-γ, the hallmark cytokine produced by Th1 cells abrogated IgE production and eosinophilia[21,22], and exogenous administration of IFN-γ can result in suppression of allergic airway inflammation in animal models[23].

Treg cells are characterized by the expression of Foxp3 (Forkhead box p3) and the CD25 (IL-2 receptor), and are known to produce the inhibitory cytokines IL-10 and TGF-β[24]. Treg cells have been strongly associated with suppression of allergic responses in murine models of asthma. Adoptive transfer of Treg cells following the onset of allergic airway inflammation down-regulated established inflammation and prevent airway remodeling[25]. It was demonstrated that the suppressive ability of Treg cells was dependent on IL-10. Not only did the adoptively transferred Treg cells produce IL-10, but also induced IL-10 production from bystander recipient CD4+ T cells[26]. In agreement with these findings, depletion of Treg cells before allergen sensitization was found to enhance the severity of airway inflammation and AHR[27]. There is also strong evidence in humans that Treg cells inhibit Th2 cell responses, which suggests that atopy can result from an imbalance between Th2 cells and Treg cells[28]. Interestingly, there are substantial de-crease in the frequencies of allergen-specific IL-10-producing Treg cells, and increase in IL-4-producing T cells, in allergic individuals compared to healthy, nonatopic individuals[29], which further highlight the close interplay between Treg and T effectors cells in asthma. In the balance of Th1/Th2, IL-10 enhances the differentiation of Th0 to Th2 through inhibiting the proliferation of Th1 or directly activating Th2[30].

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that consist of 2 distinct subsets: Ly6G+Ly6C int polymorphonuclear MDSCs (PMN-MDSCs) andLy6G-Ly6C high monocytic MDSCs (Mo-MDSCs)[31]. In healthy individuals, MDSCs derived from bone marrow rapidly differentiate into mature granulocytes, dendritic cells or macrophages. The MDSCs numbers increase with partial blocking of differentiation into mature myeloid cells in disease states, such as tumors, infections, trauma, and chronic inflammatory diseases, such as diabetes mellitus and inflammatory bowel disease[31,32]. MDSCs have recently been implicated in the pathogenesis of asthma, but their regulation in patients with asthma remains unclear.

Recently, lipopolysaccharide (LPS) was shown to induce the expansion of CD11b+ Gr1 int F4/80+ immature myeloid cells in patients with allergic airway inflammation[33]. These cells alleviated asthma by suppressing the dendritic cell-mediated reaction of primed TH2 cells[33,34], suggesting a potential protective effect of MDSCs in the development of asthma. However, it is unclear whether LPS-derived MDSCs have the ability to regulate the immune response in bronchial asthma conditions. In the current study, we investigated whether LPS-derived MDSCs are able to shift the balance back to normal in asthmatic environment with existing Th2-dominant responses.

Materials and Methods

Mice

Four- to 6-week-old female mice were used in all experiments in the study. All mice were maintained on a mixed BALB/c genetic background for more than 20 generations in pathogen-free conditions. The study was approved by the Institutional Animal Care and Use Committee of the Institution for Southern medical university, Guangzhou, China.

Allergy-Induced Airway Inflammation Model in Mice

To establish the asthma model, mice were intraperitoneally sensitized with 100 mg of OVA (Grade II-V, Sigma-Aldrich, St. Louis, MO, USA) emulsified in 20 mg of aluminum hydroxide (200-mL total volume, Sigma-Aldrich, St. Louis, MO, USA) on days 0 and 14, followed by intranasal challenge with OVA (80 mg in 40 mL of PBS) once on days 21, 22, 23, 24, 25, 26 and 27. Sensitized control mice received mock challenge with PBS. One day after the last challenge, mice were killed, and peripheral blood, bronchoalveolar lavage fluid (BALF) and lung tissue were collected for further analysis. The peripheral blood was treated with red blood cell lysis buffer to eliminate erythrocyte, and the cells were re-suspended in staining buffer (eBioscience, San Diego, CA, USA).

Isolation of MDSCs

Mice were intraperitoneally injected with 2 μg of LPS (lipopolysaccharides, LPS, *E. coli* 0111:B4, Sigma-Aldrich, St. Louis, MO, US) on days 1 and 2, followed by intraperitoneal injecion with 16 μg of LPS on days 3. Ten days after the first injection, mice were killed for spleen excision. Then splenocyte suspensions were prepared by 200-mesh sieve. The crude suspensions were further filtered through 60 μm cell strainers to obtain single-cell suspensions. Single-cell suspensions from spleens were marked by FITC-CD11b antibody (eBioscience, San Diego, CA, USA), APC-Ly-6G antibody (eBioscience, San Diego, CA, USA), PE-Ly-6C antibody (eBioscience, San Diego, CA, USA). Ly6C$^+$Ly6G$^+$, Ly6C$^-$Ly6G$^+$, Ly6C$^+$Ly6G$^-$, Ly6C$^-$Ly6G$^-$ and CD11b$^+$ MDSCs were subsequently isolated through flow cytometric sorting.

Adoptive Transfer of MDSCs

Ly6C$^+$Ly6G$^+$, Ly6C$^-$Ly6G$^+$, Ly6C$^+$Ly6G$^-$, Ly6C$^-$Ly6G$^-$ and CD11b$^+$ MDSCs from the spleens of allergic mice were purified by means of flow cytometric cell sorting. For adoptive transfer 0.3 x 10^6 isolated cells were injected through the tail vein 14 and 21 days after OVA challenge. Control mice received an intravenous injection of the same volume of PBS.

Airway Resistance Analysis in Mice

Airway responsiveness to acetylcholine chloride was evaluated with a noninvasive mouse lung function analysis system (Buxco, Troy, NY, USA) 24 hours after the last OVA challenge. Mice were anesthetized with chloral hydrate (400 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) and then subjected to endotracheal intubation and placed in a rodent plethysmograph for passive ventilation at a tidal volume of 6 mL/kg and 90 breaths/min. After reaching a stable tracing, lung resistance was measured for 3 minutes at baseline and after aerosol inhalation of 300 μl acetylcholine chloride (Sigma-Aldrich, St. Louis, MO, USA) at different concentration (0, 3.125 mg/ml, 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, 50 mg/ml). The signals of lung resistance were collected continuously, and the maximal values for airway resistance were expressed as the fold change relative to baseline.

Lung Histology

Lungs were fixed in 4% neutral paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), embedded in paraffin and sectioned. Specimens were stained with hematoxylin and eosin for examining cell infiltration. Peribronchiolar and perivascular inflammation in hematoxylin and eosin-stained slides was graded: 0, normal; 1, infrequent inflammatory cells; 2, a ring of 1 layer of inflammatory cells; 3, a ring of 2 to 4 layers of inflammatory cells; and 4, a ring of more than 4 layers of inflammatory cells. Scoring was performed at 400 magnifications by examining at least 40 consecutive fields.

ELISA

IL-4 levels in serums and BALF supernatants were assayed by using IL-4 ELISA kit according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

Analysis of in vitro Immunosuppressive Function of MDSCs

Splenocytes were isolated from naive BALB/c mouse spleen by 200-mesh sieve. The crude suspensions were further filtered through 60 μm cell strainers to obtain single-cell suspensions. Ly6C$^+$Ly6G$^+$, Ly6C$^-$Ly6G$^+$, Ly6C$^+$Ly6G$^-$, Ly6C$^-$Ly6G$^-$ and CD11b$^+$ MDSCs were purified from spleens as described above. Splenocytes were seeded in 6-well plates at 2×10^6 cells per well and co-cultured separately with Ly6C$^+$Ly6G$^+$, Ly6C$^-$Ly6G$^+$, Ly6C$^+$Ly6G$^-$, Ly6C$^-$Ly6G$^-$ and CD11b$^+$ MDSCs (1:1 ratio) for 72h. The mixed cells were collected for analyzing of Th1, Th2 and Treg cells.

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Table I. Immunosuppressive function of MDSCs *in vitro*.

Treatment	Th1 frequency (%)	Th2 frequency (%)	Th1/Th2 ratio	Treg frequency (%)
Control	1.60 ± 0.36	6.25 ± 0.98	0.30 ± 0.14	0.29 ± 0.06
Ly6C$^+$Ly6G$^+$	24.46 ± 0.72	27.32 ± 0.91	0.90 ± 0.01**	2.56 ± 0.21**
Ly6C$^-$Ly6G$^+$	3.45 ± 0.39	14.27 ± 0.53	0.24 ± 0.03	1.61 ± 0.20**
Ly6C+Ly6G$^-$	3.68 ± 0.32	12.2 ±1.55	0.31 ± 0.04	1.09 ± 0.16**
Ly6C$^-$Ly6G$^-$	2.61 ± 0.36	8.58 ± 0.75	0.30 ± 0.02	1.29 ± 0.04**
CD11b$^+$	5.03 ± 0.72	13.68 ± 1.12	0.37 ± 0.08	0.90 ± 0.19**

Normal splenocytes were treated with control, Ly6C$^-$Ly6G$^+$, Ly6C$^+$Ly6G$^-$, Ly6C$^-$Ly6G$^-$ and CD11b$^+$ MDSCs, respectively. The frequency of Th1, Th2, and Treg cells in co-culture were analyzed. Values are mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

Flow Cytometric Analysis

Cells were stained with fluorochrome-conjugated antibodies, according to the manufacturer's protocols. Flow cytometry was performed with a BD FACS Aria flow cytometry system (BD Biosciences, San Jose, CA, USA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA). Treg cells were identified by CD4, CD25 and FoxP3 positive staining. Th1 cells were identified by staining with CD4 and IFN-γ. Th2 cells were identified by CD4 and IL-4 positive staining. The fluorescent-conjugated anti-mouse antibodies of FITC-CD4, APC-IFN-γ, PE-IL-4, APC-CD25, PE—Foxp3 and their corresponding isotype controls were purchased from eBioscience (San Diego, CA, USA).

Statistical Analysis

Data analysis was performed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Results were expressed as means ± SEM and analyzed with the Student *t*-test, Wilcoxon test, and 1- or 2-way ANOVA, as appropriate, followed by the relevant post hoc *t*-test to determine *p* values. A linear correlation between 2 continuous variables was tested with the R^2 coefficient of determination. *p* values of less than 0.05 were considered statistically significant.

Results

Immunosuppressive Function of MDSCs in vitro

The Th1/Th2 balance and Treg regulation plays a vital role in the development of asthma. We initially examined the immunosuppressive function of different subtypes of MDSCs *in vitro*. It was observed that only the Ly6C$^+$Ly6G$^+$ MDSCs significantly increased Th1/Th2 ratio in the co-culturing of normal splenocytes and different subtypes of MDSCs (2:1 ratio) for 72h ($p < 0.01$, Table I, Figure I). However, it was Ly6C$^+$Ly6G$^+$, Ly6C$^-$Ly6G$^+$, Ly6C$^+$Ly6G$^-$, Ly6C$^-$Ly6G$^-$and CD11b$^+$ MDSCs still showed to be capable of up-regulating Treg cell frequency *in vitro* ($p < 0.01$, Table I, Figure 1).

The Impact of MDSCs Adoptive Transfer

We further verified the *in vivo* immunosuppressive effect of different subtypes of MDSCs with allergy-induced airway inflammation mice models. Ly6C$^+$Ly6G$^+$, Ly6C$^-$Ly6G$^+$, Ly6C$^+$Ly6G$^-$, Ly6C$^-$Ly6G$^-$ and CD11b$^+$ MDSCs were adoptively transferred on day 14 and 21 after OVA
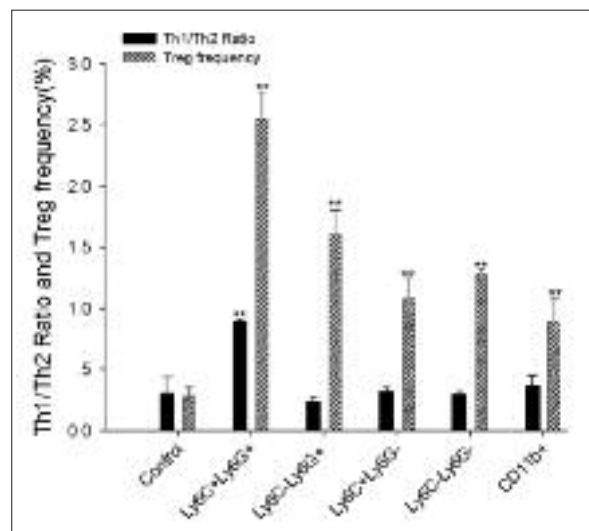
Figure 1. Immunosuppressive function of MDSCs *in vitro*. The columns of control, Ly6C$^-$Ly6G$^+$, Ly6C$^+$Ly6G$^-$, Ly6C$^-$Ly6G$^-$ and CD11b$^+$ were represented respectively for normal splenocytes and co-culture with Ly6C$^-$Ly6G$^+$, Ly6C$^+$Ly6G$^-$, Ly6C$^-$Ly6G$^-$ and CD11b$^+$ MDSCs. The frequency of Th1, Th2, and Treg cells in co-culture were analyzed. Results are mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

Table II. The effect of adoptive transfer of MDSCs on airway responsiveness.

Treatment	Acetylcholine chloride (mg/ml)					
	0	3.125	6.25	12.5	25	50
Model	0.61 ± 0.21	0.90 ± 0.69	4.57 ± 0.37	6.45 ± 0.92	8.63 ± 1.08	10.45 ± 0.64
Control	0.50 ± 0.98	0.79 ± 0.13	1.12 ± 0.40**	1.70 ± 0.54**	2.14 ± 0.81**	2.71 ± 0.67**
Ly6C⁺Ly6G⁺	0.63 ± 0.11	0.89 ± 0.05	2.24 ± 0.50*	2.83 ± 0.36*	3.32 ± 0.32*	4.29 ± 0.49*
Ly6C⁻Ly6G⁺	0.55 ± 0.10	0.91 ± 0.13	2.06 ± 0.07*	2.65 ± 0.33 *	3.48 ± 0.38 *	4.03 ± 1.02*
Ly6C⁺Ly6G⁻	0.53 ± 0.10	0.93 ± 0.09	4.14 ± 0.58	6.44 ± 0.74	8.28 ± 0.82	9.97 ± 0.75
Ly6C⁻Ly6G⁻	0.53 ± 0.42	0.90 ± 0.06	3.82 ± 0.70	6.39 ± 0.94	8.08 ± 0.48	10.05 ± 0.63
CD11b⁺	0.56 ± 0.18	0.88 ± 0.07	2.22 ± 0.13*	2.61 ± 0.36 *	3.31 ± 0.37*	4.35 ± 0.63*

The treatment of model, Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ represented asthmatic mice treated with normal saline and Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ MDSCs, respectively, with normal mice used as negative control. Results are mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

challenge. Airway responsiveness to acetylcholine chloride was evaluated with a noninvasive analysis system (Buxco) 24 hours after the last OVA challenge. Penh, which is able to represent airway responsiveness of asthmatic mice, showed significant difference between asthmatic model and control, indicating that the allergy-induced airway inflammation mice model was successful ($p = 0.000$, Table II, Figure 2). Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺ and CD11b⁺ MDSCs treatments, but not with Ly6C⁺Ly6G⁻ and Ly6C⁻Ly6G⁻ MDSC treatments significantly decreased Penh after aerosol inhalation of acetylcholine chloride at concentration of 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml or 50 mg/ml, compared with saline treated control ($p < 0.05$, Table II, Figure 2). No significant difference of Penh was detected between MDSCs-treated asthmatic mice, saline-treated asthmatic mice and untreated control after aerosol inhalation of acetylcholine chloride at concentration of 0 or 3.125 mg/ml (p > 0.05). The observations above suggested that adoptive transfer of Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺ and CD11b⁺ MDSCs improved airway responsiveness of asthmatic mice.

Our resulted indicated that total cell number, eosinophil and neutrophil percentage in BALF significantly increased in OVA induced-asthmatic mice model ($p = 0.000$, Table III, Figure 3). We further examined whether the MDSCs regulated infiltration of granulocyte in the development of asthma. BALF was collected for counting of eosinophil and neutrophil one day after the last OVA challenge, and there was a significant decrease of total cell number, eosinophil and neutrophil percentage in asthmatic mice treated with Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺

and CD11b⁺ MDSCs compared with saline treated asthmatic mice ($p < 0.01$, Table III, Figure 3). Ly6C⁺Ly6G⁻ and Ly6C⁻Ly6G⁻ MDSC treated asthmatic mice did not showed an improvement of infiltration of granulocyte ($p > 0.05$, Table III, Figure 3). No significant difference was detected for lymphocyte percentage among mice treated with MDSCs, normal saline and the untreated control ($p > 0.05$, Table III, Figure 3). These ob-
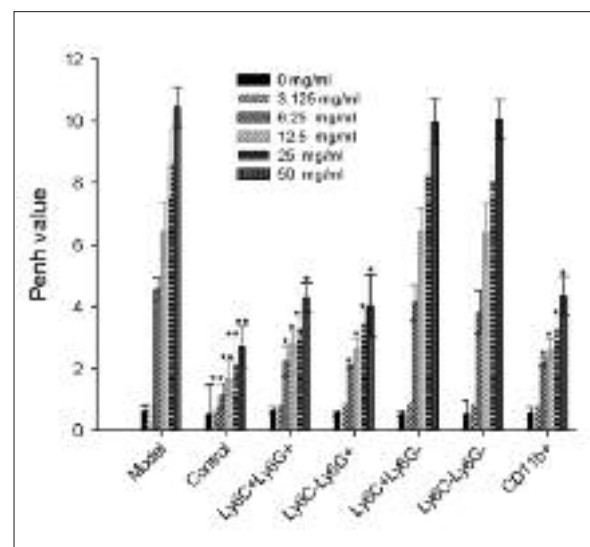

Figure 2. The effect of adoptive transfer of MDSCs on airway responsiveness. The treatments of model, Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b+ corresponded to asthmatic mice treated with normal saline and Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ MDSCs, respectively, with normal mice used as negative control. The Penh values were measured after aerosol inhalation of acetylcholine chloride at concentration of 0, 3.125, 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml or 50 mg/ml. Results are mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

Table III. The regulation of adoptive transfer of MDSCs on infiltration of granulocyte.

Treatment	Total cell number (×10⁵/ml)	Eosinophil percentage (%)	Neutrophil percentage (%)	Lymphocyte percentage (%)
Model	5.23 ± 0.70	19.49 ± 2.39	13.85 ± 1.95	66.30 ± 1.82
Control	1.19 ± 0.17**	6.22 ± 0.80**	6.30 ± 0.82**	73.28 ± 4.16
Ly6C⁺Ly6G⁺	3.18 ± 0.45**	8.20 ± 1.17**	9.15 ± 0.68**	70.33 ± 4.83
Ly6C⁻Ly6G⁺	3.16 ± 0.22**	8.25 ± 1.26**	8.29 ± 0.99**	69.32 ± 4.20
Ly6C⁺Ly6G⁻	5.67 ± 0.65	19.27 ± 1.42	12.52 ± 2.19	67.08 ± 1.16
Ly6C⁻Ly6G⁻	5.61 ± 0.5	18.93 ± 1.96	12.16 ± 1.48	66.58 ± 1.07
CD11b⁺	3.41 ± 0.44**	8.36 ± 1.79**	8.79 ± 1.30**	68.86 ± 3.14

The treatment of model, Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ represented asthmatic mice treated with normal saline and Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ MDSCs, respectively, with normal mice used as negative control. Results are mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

servations were further verified by the histology analysis of lung tissue (Figure 4), which all combined, suggested about the potential of these cells in therapeutic intervention of allergic airways disease.

Adoptive Transfer of MDSCs Up-Regulated the Th1/Th2 Ratio and Treg frequency

Th1/Th2 cytokine balance has been already characterized in asthmatic and Treg cells strongly associated with suppression of allergic re-

Figure 3. The regulation of adoptive transfer of MDSCs on infiltration of granulocyte. The treatment of model, Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ represented asthma mice treated with normal saline and Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ MDSCs, respectively, with normal mice used as negative control. The control means normal mice. The total cell number, eosinophil, neutrophil and lymphocyte percentage in BALF were measured. Values are mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

sponses in murine models of asthma. Our resulted indicated that the Th1/Th2 ratio and Treg frequency in peripheral blood were significantly down-regulated in OVA induced- asthmatic mice model ($p < 0.01$, Table IV, Figure 5). Peripheral blood was collected one day after the last OVA challenge for flow cytometric analysis, and the results showed that adoptive transfer of Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ MDSCs resulted in significant up-regulation of the Th1/Th2 ratio and Treg frequency compared with asthmatic mice treated with normal saline ($p < 0.01$, Table IV, Figure 5).

Adoptive Transfer of Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺ and CD11b+ MDSCs Down-Regulated IL-4 Level in Serum and BALF

Th2 cells and B-cell IgE production have been shown to be markedly affected by IL-4[35-38]. Our results indicated that IL-4 level in peripheral blood and BALF significantly up-regulated in OVA induced-asthmatic mice model ($p < 0.01$, Table V, Figure 6). Peripheral blood and BALF were collected for ELISA analysis one day after the last OVA challenge. There was a significant decrease of IL-4 level in peripheral blood and BALF in asthmatic mice treated with Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺ and CD11b⁺ MDSCs, indicating that LPS-derived MDSCs were involved ($p < 0.01$, Table V, Figure 6). In contrast, asthmatic mice treated with Ly6C⁺Ly6G⁻ and Ly6C⁻Ly6G⁻ MDSC, and control treated with saline, did not showed a significant decrease of IL-4 concentration ($p > 0.05$, Table V, Figure 6). Our findings clearly demonstrated that infiltration of inflammatory cells into lung tissues was strongly inhibited by LPS-derived MDSCs.
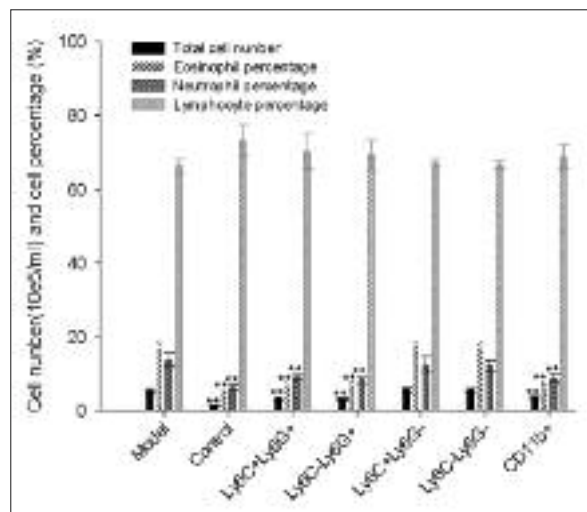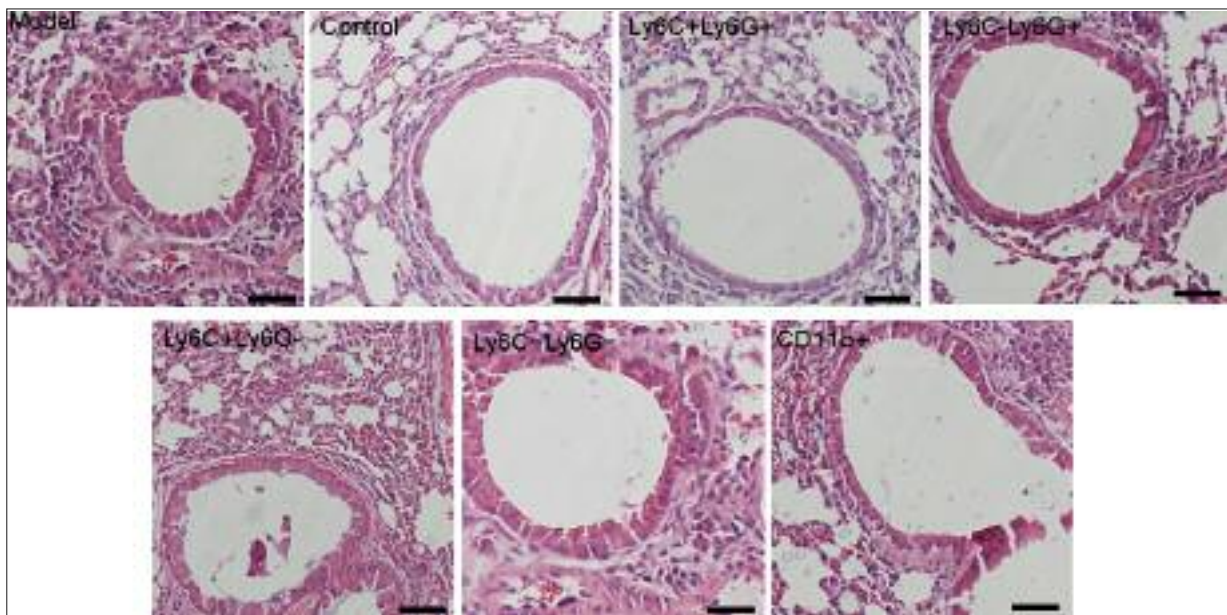
Figure 4. Haemotoxylin and eosin staining of lung sections and inflammatory scores. MDSCs suppress allergen-induced airway inflammation of asthmatic mice. Asthmatic mice received an injection of $Ly6C^-Ly6G^+$, $Ly6C^+Ly6G^-$, $Ly6C^-Ly6G^-$ and $CD11b^+$ MDSCs, respectively, with normal mice used as negative control. Lungs were fixed, sectioned and stained with haemotoxylin and eosin. Representative sections were shown. Scale bar = 50 mm. Magnification = × 400. *$p < 0.05$ and **$p < 0.01$.

Discussion

Myeloid-derived suppressor cells are the most abundant and heterogeneous population of leukocytes including myeloid progenitor and immature myeloid cells. They can be rapidly recruited from blood to areas of inflammation to perform various biological functions. The pathologically significance of these activated cells have been increasingly recognized as important players in autoimmunity for their abilities to modulate innate and adaptive immune responses. The adoptive transfer of LPS-induced $CD11b^+Gr1intF4/80^+$ cells suppressed allergen-induced airway inflammation, suggesting that these cells may have regulatory functions in asthma[33].

Although MDSCs are known to accumulate in local tissues in various diseases, the means by how these cells are implicated in asthma-related inflammatory environments was still unclear. In the current study, we established mice asthma model to test the suppressive effect of myeloid-derived suppressor cells (MDSCs) derived by LPS stimulation against asthma-related airway inflammation. Especially $Ly6C^+Ly6G^+$, $Ly6C^-Ly6G^+$ and $CD11b^+$ MDSCs showed better

Table IV. Adoptive transfer of MDSCs up-regulated the Th1/Th2 ratio and Treg frequency.

Treatment	Th1 frequency (%)	Th2 frequency (%)	Th1/Th2 Ratio	Treg frequency (%)
Model	5.37 ± 0.30	9.78 ± 0.95	0.55 ± 0.04	0.013 ± 0.005
Control	4.99 ± 1.06	6.93 ± 1.34	$0.72 \pm 0.05^{**}$	$0.072 \pm 0.007^{**}$
$Ly6C^+Ly6G^+$	8.47 ± 1.42	2.68 ± 0.64	$3.26 \pm 0.65^{**}$	$9.953 \pm 2.536^{**}$
$Ly6C^-Ly6G^+$	8.62 ± 1.52	3.47 ± 1.83	$2.74 \pm 0.62^{**}$	$4.33 \pm 1.559^{**}$
$Ly6C^+Ly6G^-$	6.08 ± 0.74	4.05 ± 0.64	$1.52 \pm 0.22^{**}$	$4.118 \pm 1.76^{**}$
$Ly6C^-Ly6G^-$	5.40 ± 0.97	2.56 ± 0.36	$2.12 \pm 0.32^{**}$	$4.787 \pm 0.87^{**}$
$CD11b^+$	5.54 ± 0.81	2.05 ± 0.67	$2.92 \pm 0.95^{**}$	$5.587 \pm 1.276^{**}$

The treatment of model, $Ly6C^-Ly6G^+$, $Ly6C^+Ly6G^-$, $Ly6C^-Ly6G^-$ and $CD11b^+$ represented asthmatic mice treated with normal saline and $Ly6C^-Ly6G^+$, $Ly6C^+Ly6G^-$, $Ly6C^-Ly6G^-$ and $CD11b^+$ MDSCs, respectively, with normal mice used as negative control. Results are mean ± SEM, *$p < 0.05$ and **$p < 0.01$.
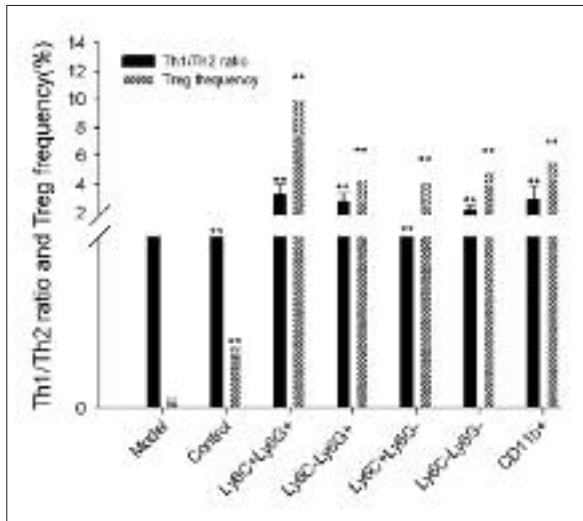
Figure 5. Adoptive transfer of MDSCs up-regulated the Th1/Th2 ratio and Treg frequency. The treatment of model, Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ represented asthmatic mice treated with normal saline and Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ MDSCs, respectively, with normal mice used as negative control. The frequency of Th1, Th2, and Treg cells in peripheral blood were analyzed. Results are mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

outcome than the rest subpopulations. The adoptive transfer of Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺ and CD11b⁺ MDSCs decreased Penh value and inhibited allergen-induced airway inflammation, such as significant decrease of total cell number, eosinophil and neutrophil percentage.

Suppressive factors expressed by MDSCs such as arginase-1, reactive oxygen species and in-

Table V. Adoptive transfer of MDSCs down-regulated the IL-4 level.

Treatment	IL-4 level	
	Serum	BALF
Model	53.85 ± 1.88	29.01 ± 0.93
Control	8.19 ± 0.65**	3.60 ± 0.96**
Ly6C⁺Ly6G⁺	19.76 ± 0.61**	7.83 ± 0.67**
Ly6C-Ly6G⁺	26.13 ± 1.04**	10.85 ± 0.48**
Ly6C⁺Ly6G⁻	58.40 ± 1.27	28.29 ± 1.43
Ly6C⁻Ly6G⁻	59.13 ± 2.12	27.64 ± 3.69
CD11b⁺	20.55 ± 0.91**	8.73 ± 1.43**

The treatment of model, Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ represented asthmatic mice treated with normal saline and Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ MDSCs, respectively, with normal mice used as negative control. Results are mean ± SEM, *p < 0.05 and **$p < 0.01$.

ducible nitric oxide synthase, have the ability to inhibit T cell proliferation and cytoxicity, induce the expansion of Treg cells, and block natural killer cell activation[39]. In our study, we separated typical subtypes of MDSCs and the results with those subtypes suggested that adoptive transfer of Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ MDSCs resulted in up-regulation of the Th1/Th2 ratio and Treg frequency in peripheral blood and BALF. Yet, the Ly6C⁺Ly6G⁻ and Ly6C⁻Ly6G⁻ MDSCs did not improve asthma in terms of airway responsiveness and inflammation. These observations were able to be reproduced using *in-vitro* assays. Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ MDSCs up-regulated Treg cell frequency when co-cultured with normal splenocytes. However, only the Ly6C⁺Ly6G⁺ MDSCs can increase Th1/Th2 ratio *in vitro* when co-culturing with normal splenocytes.

It was observed that IL-4 level decreased significantly in peripheral blood and BALF in asthmatic mice treated with Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺ and CD11b⁺ MDSCs, but not in asthmatic mice treated with Ly6C⁺Ly6G⁻ and Ly6C⁻Ly6G⁻ MDSCs, therefore, it may be concluded that Ly6C⁺Ly6G⁺, Ly6C⁻Ly6G⁺ and
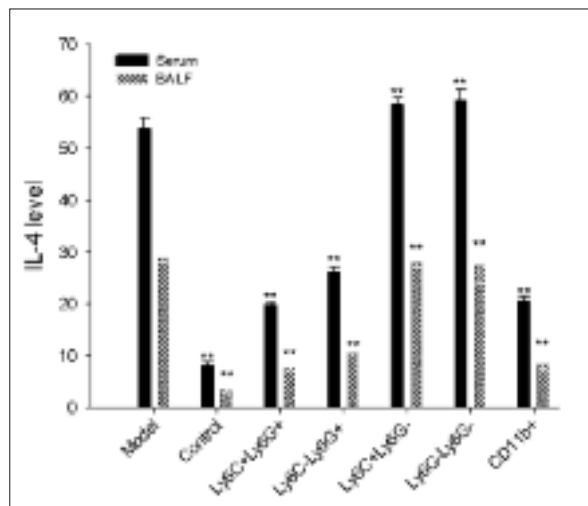

Figure 6. Adoptive transfer of MDSCs down-regulated the IL-4 level. The treatment of model, Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b+ represented asthmatic mice treated with normal saline and Ly6C⁻Ly6G⁺, Ly6C⁺Ly6G⁻, Ly6C⁻Ly6G⁻ and CD11b⁺ MDSCs, respectively, with normal mice used as negative control. IL-4 concentration in peripheral blood and BALF was measured. Results are mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

CD11b^{+} MDSCs attributed to the regulating effect of MDSCs through inhibiting Th2 cell proliferation.

Indeed, Th1 and Th2 cells may not simply serve to counterbalance each other in a dichotomous manner, but rather may act together in a harmful manner in asthmatic individuals. It was the regulatory pathways in addition to Th2 cells and eosinophils that may contribute to the development of the asthma phenotype. In these forms of asthma, Th2 directed therapies have shown to be effective[40]. However, it is clear that asthma is complex and that Th2 immunity does not explain all forms of the disorder. Clearly the Th2 paradigm does not explain non-allergic asthma[14]. Although Th2 paradigm predicts that eosinophilic infiltration and subsequent inflammation should provoke AHR, no clear cause or effect exists[41,42]. Besides, bronchial biopsies from asthmatic patients provided evidence of neutrophilic inflammation, and therapeutics targeted to Th2 cells and their cytokines, which are often effective in Th2 animal disease models, have been found to have minimal effectiveness in the clinical setting. Moreover, asthmatic patients exhibit elevated levels of non-Th2 cytokines and factors, such as interferon (IFN)-γ[43,44] and IL-17[45,46]. These findings suggested that immunological factors in addition to Th2 cells participated in the course of asthma.

The exact nature and function of MDSCs remain elusive; however, we have seen several articles reporting that MDSCs of certain subtypes were involved in development of disease conditions. Initial infiltration of Ly6C (+) Ly6G (-) immature monocyte fraction exhibited the same characteristics as MDSCs, and played a critical role in the resolution of acute inflammation and in the subsequent tissue repair by using mice spinal cord injury model[47]. Assorted tumor-secreted factors drive the accumulation of multiple immune suppressive mechanisms, and tumor-derived MDSCs have been proven to inhibit asthma-related airway inflammation. However, whether MDSCs generated by LPS stimulation would provide similar effect remained unclear. To our knowledge, our study first showed that certain subtypes of LPS-derived MDSCs can be used to develop new treatment for asthma, and initially explored the underlying mechanist base on the Th2 paradigm. However, limits remained in the study that some contradictions between the effect and the regulation of Th1/Th2 and Treg

cells mediated by MDSCs subtypes were recorded *in vitro* and *vivo*. Although no substantial approaches to gain sufficient levels of MDSCs have been documented to date, an effective strategy designed to transfer MDSCs could be used as a potential treatment for allergic asthma once an approach to obtain MDSCs *in vitro* is established. Meanwhile in the future we will further illustrate the regulation between the different subtypes of MDSCs and the innate and adaptive immune system, and examined the effect of different subtypes of MDSCs on asthma caused by various etiologies.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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