

Passive Transfer of Tumour-Derived MDSCs Inhibits Asthma-Related Airway Inflammation

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Abstract

Myeloid-derived suppressor cells (MDSCs), a heterogeneous population including myeloid progenitor and immature myeloid cells, are known to inhibit T cell responses. The issue of whether tumour-derived MDSCs regulate the immune response in an asthma environment is currently unclear. Here, we have reported that tumour-derived MDSCs shift the balance back to normal in a Th2-dominant asthmatic environment. In an ovalbumin (OVA)-induced mouse asthma model, injected tumour-derived MDSCs were recruited to the lungs of asthmatic mice by CC chemokine ligand 2 (CCL2). MDSCs transferred into asthmatic mice via i.v. injection suppressed the infiltration of inflammatory cells into the lung, the Th2 cytokine, IL-4, concentration in bronchial lavage fluid and the serum level of OVA-specific IgE. Increased TGF- β 1 production in the lung was detected after transfer of MDSCs. The inhibitory effects of MDSCs were reversed upon treatment with an anti-TGF- β 1 antibody, suggesting dependence of these activities on TGF- β 1. Our findings imply that tumour-derived MDSCs inhibit the Th2 cell-mediated response against allergen in a TGF- β 1-dependent manner. Based on the collective results, we propose that asthma may be effectively targeted using a novel MDSC-based cell therapy approach.

Introduction

Asthma is a chronic airway inflammation associated with wheezing, breathlessness, chest tightening and coughing. In recent years, the incidence of asthma has increased markedly, particularly in developed countries [1]. At present, more than 39 million asthma patients are recorded in China alone and approximately 300 million asthma patients worldwide [2, 3]. Current treatments for asthma mainly rely on drug interventions, such as antihistamines or glucocorticoids. Although these interventions are effective for controlling symptoms in some patients, up to 30% of individuals do not achieve effective disease control [4]. Asthma-related deaths are relatively uncommon, but appear to be on the increase, with more than 100,000 deaths reported annually worldwide [5]. Thus, there is an urgent medical need to develop new treatments for therapy-resistant patients.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group including myeloid progenitor cells and immature myeloid cells [6]. In healthy individuals, MDSCs derived from bone marrow rapidly differentiate into mature granulocytes, macrophages or dendritic cells. The MDSC numbers increase with partial blocking of differentiation into mature myeloid cells in disease states, such as tumours, infectious diseases, trauma and autoimmune

disease [7]. Accumulating evidence has shown that MDSCs negatively regulate immune responses by inhibiting T cell function in tumours and other diseases [8], although Arora *et al.* and Deshane *et al.* have demonstrated the suppressive function of MDSC in Th2-dominant allergic inflammation [9, 10]. However, it is unclear whether tumour-derived MDSCs have the ability to regulate the immune response in such conditions. Asthma is caused by an imbalance of Th1/Th2 cells. Th2 cells and associated cytokines, such as IL-4, IL-5 and IL-13, are known to play a leading role in asthmatic conditions. Th2 cells recruit other immune cells to the lungs, which are in favour of allergic airway inflammation [11, 12]. In the current study, we investigated whether tumour-derived MDSCs are able to shift the balance back to normal in an asthmatic environment with existing Th2-dominant responses. Our results clearly showed that tumour-derived MDSCs inhibit the Th2-dominant immune response to allergen in a TGF- β 1-dependent manner *in vivo*.

Materials and methods

Mice and cell lines. BALB/c mice (4-to 6-week-old female) were purchased from the Center of Medical Experimental Animals of Hubei Province (Wuhan, China). This study was

approved by the Animal Care and Use Committee of Tongji Medical College. The BALB/c background H22 hepatocarcinoma cell line was provided by the China Center for Type Culture Collection (CCTCC, Wuhan, China), and culture conditions were in accordance with the guidelines.

Animal model and treatment protocol. To establish the asthma model, BALB/c mice were treated using the protocol shown in Fig. 1. Mice (4–6 weeks old) were sensitized via i.p. injection of 200 μ l ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO, USA, 0.5 mg/ml) bound to Imject[®] Alum (Pierce Biotechnology, Rockford, IL, USA) on d0, d7, d14. From d17 to d23, sensitized mice were challenged using aerosolized 5% ovalbumin for 30 min once a day. Sensitized control mice received mock challenge with PBS. For MDSC treatment, mice received an i.v. injection of 6×10^6 MDSCs two times, 1 day before and 2 days after starting the antigen challenge, respectively. Control mice received an intravenous injection of the same volume of PBS. In some experiments, mice were injected i.p. with 10 μ g anti-TGF- β 1-blocking Ab or isotype control antibody when MDSCs were injected.

Preparation of MDSCs. BALB/c mice were inoculated via subcutaneous injection of 10^5 H22 cells to the left flank. Three weeks later, mice with tumour sizes of approximately $1 \times 1 \text{ cm}^2$ were sacrificed. Bone marrow cells were obtained from femora and tibia of mice, centrifuged and fractionated using Percoll density gradient centrifugation, as described previously [13]. Cells were harvested from gradient interfaces after centrifugation at $1800 \times g$ for 30 min. Cells between 50% and 60% banding (1.063–1.075 g/ml) were designated fraction (Fr) II. MDSCs (Gr1⁺CD11b⁺ cells) were isolated from Fr II using FITC-anti-Gr1 Ab, PE-anti-CD11b Ab, magnetic microbeads and MiniMACS columns (Miltenyi Biotec, Auburn, CA, USA), in accordance with the manufacturer's protocol.

In vitro assessment of suppressive capacity. Splenocytes were isolated from asthmatic BALB/c mice and stained using carboxyfluorescein diacetate succinimidyl ester (CFSE),

according to the manufacturer's instructions. CFSE-labelled splenocytes were seeded into 96-well plates at a concentration of 2×10^5 cells/well in triplicate. Cells were stimulated with anti-CD3 and anti-CD28 Abs (1 μ g/ml each; eBioscience, San Diego, CA, USA) or left untreated in the absence or presence of 1×10^5 MDSCs. After 72-h culture, cells were harvested, and proliferation assessed using flow cytometry. The cell proliferation index was calculated with MODFIT software (Verity Software House, Topsham, ME, USA) based on the reduction of CFSE.

Analysis of infiltration of MDSCs in lung tissues. Of 24 h after final antigen or mock challenge, mice were subjected to i.v. injection of CFSE-labelled MDSCs (6×10^6) with or without the indicated antibodies. Lung tissues were surgically obtained from mice 24 h after the injection. Frozen sections were prepared and observed using fluorescence microscopy. Single-cell suspensions were prepared from lung tissues to analyse CFSE⁺ cells using a flow cytometer (BD LSR II; BD Bioscience, Franklin Lakes, NJ, USA).

Preparation of BALF. Mice were anesthetized with 1% pentobarbital, and their lungs lavaged with PBS. Bronchoalveolar lavage fluid (BALF) was obtained by aspiration six times (total 4.8 ml) via endotracheal intubation. The supernatant from BALF was stored at -70°C for enzyme-linked immunosorbent assay (ELISA). After lysis of RBC, cell pellets were suspended in 0.5 ml of PBS, and the total numbers of BALF cells were counted. Slides were prepared and stained with Wright-Giemsa staining solution. Differential cell counting was conducted according to standard morphological criteria.

Lung histology. For histopathological determination, lungs of mice were removed and fixed in 4% formalin for 24 h. After paraffin embedding, tissues were cut into 5- μ m-thick slices and stained with haematoxylin and eosin.

Cytokine analysis. Cytokines (IL-4, IFN- γ , CCL2, TGF- β 1) in BALF and/or lung tissue homogenate supernatants were determined using ELISA kits (CUSABIO, Wuhan, Hubei, China), following the manufacturer's instructions.

Determination of serum OVA-specific IgE. OVA-specific IgE levels in serum samples were measured using IgE-specific ELISA, in keeping with the manufacturer's instructions (Usen Life Science Inc, Wuhan, Hubei, China). O.D. values at 450 nm of samples were converted to U/ml, using a standard curve generated with standard substance reconstituted with sample diluent.

Statistical analysis. Data were expressed as mean values $\pm$ SD. Statistical interpretation of data was performed with Student's *t*-test or one-way ANOVA. $P < 0.05$ was considered statistically significant.

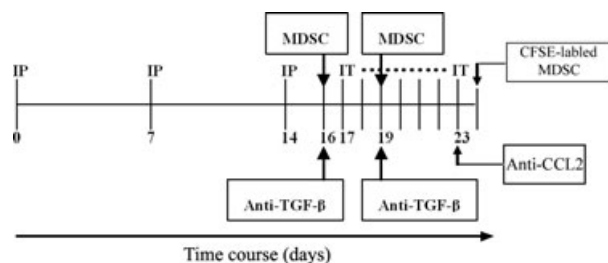


Figure 1 Experimental model. BALB/c mice were sensitized (S) via i.p. injection of OVA bound to Imject[®] Alum and challenged (C) with either aerosolized OVA (allergen challenge) or PBS (mock challenge). MDSCs were administered on the indicated day to determine their specific roles. CFSE-labelled MDSCs were only done in experiments shown in Fig. 2 and the transfers of unlabelled MDSCs done in expts. shown in Figs. 3–5. In some experiments, anti-TGF- β 1 or anti-CCL2 and its isotype Ab were administered on the indicated day.

Results

CCL2 mediates migration of tumour-derived MDSCs to the lungs of asthmatic mice

To determine whether tumour-derived MDSCs influence pulmonary inflammation of asthmatic mice, we initially

investigated whether the injected cells migrate to the lung. To this end, CFSE-labelled MDSCs were transferred into asthmatic mice 24 h after the last antigen challenge. MDSCs that migrated to lung tissues were analysed 24 h after the injection. Flow cytometric analysis of lung tissues revealed higher numbers of CFSE-labelled MDSCs (infiltrated) in asthmatic mice than mock challenge groups (Fig. 2A,B). In view of the previous finding that CCL2 plays a key role in the recruitment of MDSCs [14], we analysed CCL2 levels in lung tissues. Notably, CCL2 levels in the lungs of asthmatic mice were higher than those of control mice (Fig. 2C), leading to the suggestion that CCL2 recruits injected MDSCs to inflammatory lung tissues. To confirm this finding, we injected MDSCs in conjunction with an anti-CCL2 antibody into asthmatic mice. Infiltration of MDSCs into lung tissues was effectively impaired by the anti-CCL2 antibody (Fig. 2D, E). The data suggest that CCL2 is produced in an allergy-related inflammatory environment, and injected MDSCs, are recruited, at least in part, by CCL2 into inflammatory lung tissues of asthmatic mice.

Th2 response to allergen challenge is modulated by tumour-derived MDSCs

Next, we determined whether tumour-derived MDSCs influence the balance of Th1/Th2 responses in asthmatic

lung tissues. Levels of IL-4, a characteristic Th2 cytokine, were significantly higher (Fig. 3A), whereas those of the Th1-specific cytokine, IFN- γ , were markedly lower (Fig. 3B) in BALF from asthmatic mice relative to control mice, indicating a typical Th2 response in asthmatic lung. Upon i.v. injection of animals with MDSCs (Fig. 3A), the IL-4 level in BALF was significantly reduced (Fig. 3A), whereas IFN- γ was significantly increased (Fig. 3B). In addition to its local effects in lung, i.v. injection of tumour-derived MDSCs resulted in systemic effects. High concentrations of OVA-specific IgE were detected in sera of asthmatic mice (Fig. 3C). Treatment with MDSCs led to a significant decrease in the OVA-specific IgE level in the circulation. We additionally observed inhibition of T cell proliferation by MDSCs *in vitro*. T cells in lymphocytes were activated by stimulation with antibodies, including anti-CD3 and anti-CD28. MDSCs significantly suppressed the proliferation of T cells from asthmatic mice (Fig. 3D, E). These results collectively indicate that tumour-derived MDSCs are able to suppress the T cell response, both *in vivo* and *in vitro*.

Tumour-derived MDSCs suppress allergen-induced airway inflammation in asthmatic mice

Asthmatic mice exhibit some of the characteristic histopathological signs of human asthmatic lungs, most

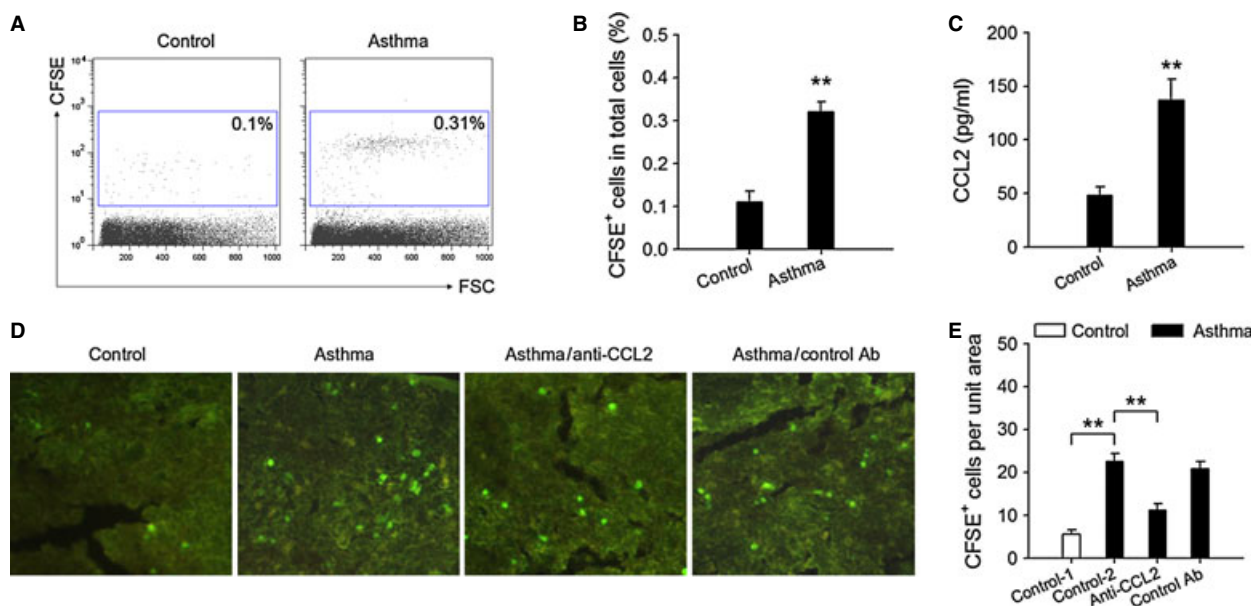


Figure 2 CCL2 mediates migration of MDSCs to the lungs of asthmatic mice. (A) and (B) single-cell suspensions of lung were prepared, and CFSE-labelled MDSCs were analysed using flow cytometry. The representative data are shown in (A). The percentage of CFSE⁺ cells in total cells was calculated (B). (C) ELISA analysis of CCL2. Lysates were prepared from lung tissues of asthmatic and control mice and analysed for CCL2 expression using ELISA. (D) and (E) CFSE-labelled MDSCs with or without antibodies were injected into asthmatic or control mice via the tail vein. Lung tissues were surgically excised from mice 24 h after the injection, and frozen sections were prepared and analysed via fluorescence microscopy. MDSCs were detected as CFSE⁺ cells in lung tissues (D). CFSE⁺ cells within the unit area were counted (E). Results are mean $\pm$ SD, $n = 5$ mice/group. Data are representative of two to three independent experiments. P -values, ** $P < 0.01$.

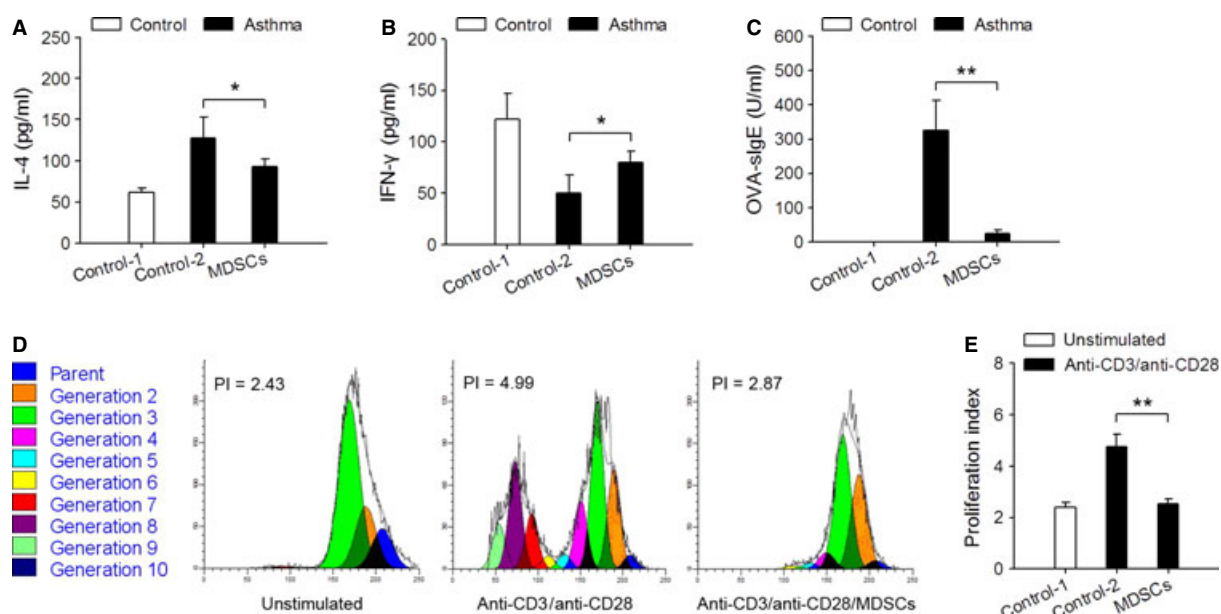


Figure 3 MDSCs suppress T cell responses *in vivo* and *in vitro*. (A–C) Transfer of MDSCs reduces Th2 cell responses in the lung after allergen challenge. IL-4 (A) and IFN- γ (B) in BALF and OVA-specific IgE in serum from control and asthmatic mice with or without injection of MDSCs were detected using ELISA. (D) and (E) MDSCs suppress T cell proliferation *in vitro*. Splenocytes from asthmatic mice were stained with CFSE. Cells were left unstimulated or stimulated with anti-CD3/anti-CD28 (1 μ g/ml) in absence or presence of MDSCs. Cell proliferation was analysed using flow cytometry (D). The proliferation index (PI) was calculated (E). Control 1 means mice sensitized and challenged using PBS. Control 2 refers to asthmatic model mice. Results are mean $\pm$ SD, $n = 6$ mice/group. Experiments were repeated three times. P -values, * $P < 0.05$, ** $P < 0.01$.

notably, infiltration of inflammatory cells. There is minimal or no inflammation in normal mice upon microscopic examination. In contrast, asthmatic mice showed extensive inflammation with severe perivascular and peribronchial cuffing. Asthmatic mice treated with tumour-derived MDSCs displayed significantly reduced lung pathology with few infiltrated inflammatory cells (Fig. 4A). In addition to improved lung pathology, transfer of MDSCs significantly lowered the total cell numbers, including macrophages, neutrophils and eosinophils, in BALF (Fig. 4B). Our findings clearly demonstrate that infiltration of inflammatory cells into lung tissues is strongly inhibited by tumour-derived MDSCs.

Suppression of allergic inflammation by tumour-derived MDSCs is TGF- β 1-dependent

Among the various cytokines shown to suppress immune responses, the anti-inflammatory action of TGF- β 1, which could be produced by MDSCs [15, 16], is well established. To determine whether TGF- β 1 is involved in the effects of MDSCs on asthma-related inflammation, we examined the levels in lung homogenate supernatant and BALF. As shown in Fig. 5A,B, treatment with tumour-derived MDSCs led to significantly elevated concentrations of TGF- β 1 in BALF and lung homogenate supernatants of asthmatic mice. Mice were subjected to i.v. injection of tumour-derived MDSCs with or without neutralizing antibody against TGF- β 1. Blockage of TGF- β 1 suppressed

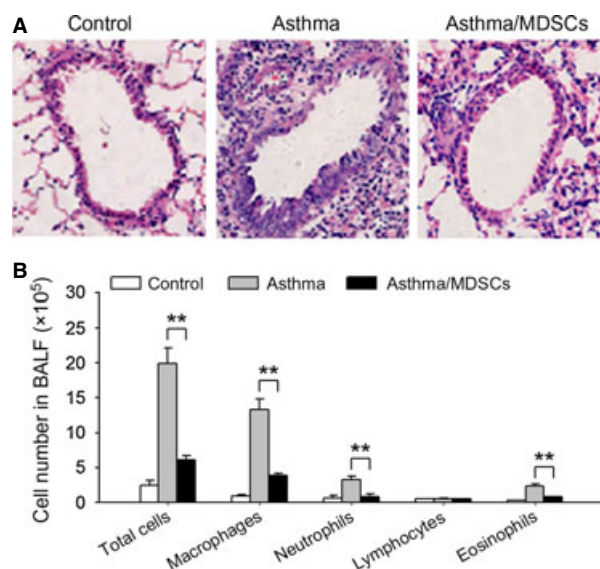


Figure 4 MDSCs suppress allergen-induced airway inflammation of asthmatic mice. Asthmatic mice received an injection of either MDSCs or an equivalent volume of PBS. Lungs were fixed, sectioned and stained with haematoxylin and eosin. Representative sections are shown (A). BALF cells were isolated and counted, as described in Materials and Methods (B). Data are shown as mean $\pm$ SD, $n = 6$ mice/group. Results are representative of three independent experiments. P -values, ** $P < 0.01$.

the effects of MDSCs, evaluated based on the lower extent of decrease in total cell numbers and eosinophil counts in BALF (Fig. 5C,D). The results collectively indicate that

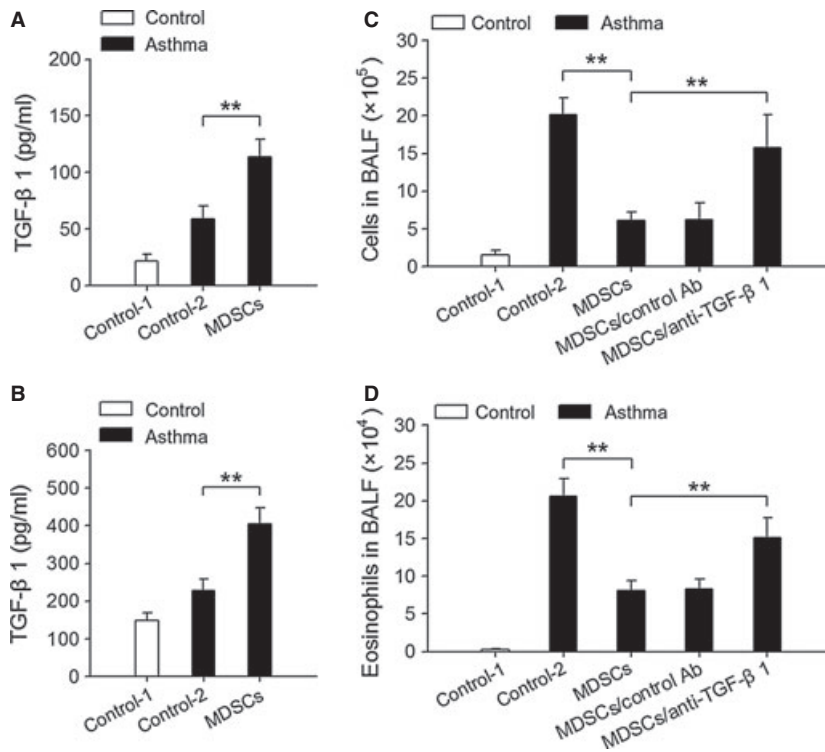


Figure 5 Suppression of allergic inflammation by MDSCs is TGF- β 1-dependent. (A) and (B) MDSC treatment results in elevation of TGF- β 1 levels. TGF- β 1 levels in BALF (A) and lung homogenate supernatant (B) from sensitized and asthmatic mice with or without injection of MDSCs were measured using ELISA. (C) and (D) blockage of TGF- β 1 suppresses the effects of MDSCs. Before and during the allergen challenge phase, MDSCs were injected with or without anti-TGF- β 1 antibody or control Ab, as shown in Fig. 1. Total cells in BALF (C) and eosinophils in BALF (D) were quantified, as described in Materials and Methods. Results are mean $\pm$ SD, $n = 5$ mice/group. Data are representative of three independent experiments. P -values, ** $P < 0.01$.

transfer of tumour-derived MDSCs promotes pulmonary TGF- β 1 expression, and MDSC-mediated suppression of allergic inflammation is TGF- β 1-dependent.

Discussion

Myeloid-derived suppressor cells represent a heterogeneous cell population including myeloid progenitor and immature myeloid cells. These cells can be distinguished in mice by surface expression of CD11b and Gr1 [6]. MDSCs exert immunosuppressive effects in both antigen-specific and non-specific ways in various diseases, including cancer, traumatic stress, burns and infection [17, 18]. In the current study, we have shown that tumour-derived MDSCs inhibit the pathological features of allergic asthma in mice. Specifically, transfer of tumour-derived MDSCs to asthmatic mice led to reduced recruitment of inflammatory cells and suppressed production of IgE and Th2 cytokines in a TGF- β 1-dependent manner.

Although MDSCs are known to accumulate in local tissues in different diseases, the means by which these cells are recruited to asthma-related inflammatory environments and the specific signals directing recruitment are unclear. We previously reported that the CCL2/CCR2 pathway plays a key role in MDSC infiltration into tumours [14]. Here, we have shown that CCL2 is elevated in the lungs of asthmatic mice and recruits MDSCs into lung tissues. In accord with our results, Qiu *et al.* and Wohlleben *et al.* also demonstrated the increased expression of CCL-2 in

mouse allergy models [19, 20]. Although other chemokines may also be involved, our data clearly indicate that injected tumour-derived MDSCs are efficiently recruited to asthma-related inflammatory lung tissues.

Several factors are implicated in MDSC-mediated immune suppression, including arginase, reactive oxygen and nitrogen species, as well as inhibitory surface molecules [21–23]. Our experiments demonstrated that transfer of MDSCs *in vivo* significantly elevates TGF- β 1 release in BALF and production in the lungs of asthmatic mice. Several types of cells produce TGF- β 1, including endothelial cells, vascular smooth muscle cells, regulatory T cells (Treg) and fibroblasts [24, 25]. In addition, MDSCs have been shown to produce TGF- β 1 [15, 16]. In our mouse asthma model, MDSCs may be a significant source of TGF- β 1, consistent with a recent report that increased focal TGF- β 1 production *in vivo* is correlated with enhanced MDSC recruitment [26]. TGF- β 1 has a negative effect on the immune response through a number of mechanisms, such as suppression of cell proliferation, cytokine production and signalling, as well as induction of apoptosis [27]. TGF- β 1 appears to suppress a wide variety of inflammatory responses *in vivo*. This is evident in TGF- β 1-deficient mice, which exhibit pathological signs characterized by large inflammatory cells in several organs [28, 29]. However, the mechanisms by which TGF- β 1 negatively regulates inflammatory responses in the lung are poorly understood. Data from the current study indicate that TGF- β 1 is a pivotal negative regulator of inflammation in lungs of asthmatic mice.

Recent studies have reported that MDSCs accelerate the development of Treg cells *in vivo* [30, 31]. Induction of Treg cell production by MDSCs requires the presence of IFN- γ and IL-10 [31]. MDSCs may act in concert with Treg cells to inhibit allergic inflammation in our asthmatic model mouse. It is possible that MDSCs trigger the inhibitory process by secreting TGF- β 1 to suppress Th2 inflammation and simultaneously induce Treg cell production [32]. Treg cells may be maintained to alleviate asthma pathology after the MDSCs disappear.

Notably, transfer of tumour-derived MDSCs induced elevation of IFN- γ in lung tissue homogenate supernatants in our mouse asthma model. IFN- γ is considered a promising candidate for asthma therapy, owing to its ability to reduce goblet cell hyperplasia, LTC₄ production and eosinophilia [33, 34]. Therefore, elevation of IFN- γ induced by transfer of tumour-derived MDSCs is in favour of an inhibitory role of MDSCs in airway inflammation. On the other hand, the increase in IFN- γ production suggests that the Th1 response is augmented, possibly due to inhibition of Th2 response. IFN- γ facilitates Th1 responses, which suppress Th2 immune responses that mediate allergic diseases, including asthma [35]. The increase in IFN- γ and decrease in IL-4 concentrations signify that MDSCs shift the balance back to normal in an asthmatic environment with an existing Th2-dominant response. In our study, OVA-specific IgE was greatly reduced; however, the reduction in IL-4 is a small amplitude after transfer of tumour-derived MDSCs in asthmatic mice. It is possible that MDSC has a direct effect on B cells.

Asthma is a chronic airway inflammation characterized by infiltration of inflammatory cells into lung tissues, such as macrophages, neutrophils and eosinophils. These cells are attracted to the airways by a number of chemokines, including eotaxin, IL-1, IL-8, RANTES and CCL2 [36]. Infiltrated inflammatory cells secrete various chemical mediators that trigger constriction of the bronchi and secretion of mucus [37, 38]. Therefore, these cells are key players in the inflammatory process of asthma and present important targets for therapeutic strategies. Our results demonstrated that i.v.-injected tumour-derived MDSCs suppress Th2-dominant inflammation, confirming that transfer of MDSCs *in vivo* significantly diminishes infiltration of inflammatory cells into lungs of asthmatic mice. It is difficult for a drug or combination of drugs to respond in this way. Although no substantial approaches to gain sufficient levels of MDSCs have been documented to date, a recent report by Marigo and colleagues showed that the cytokine IL-6, together with GM-CSF, induces rapid development of MDSCs from mouse or human bone marrow precursors [39]. Once an approach to obtain large amounts of MDSCs *in vitro* is established, an effective strategy designed to transfer MDSCs could be used as a potential treatment for allergic asthma.

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