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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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## Myeloid-derived suppressor cells: Roles and relations with Th2, Th17, and Treg cells in asthma

To the Editor:

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of cells that expand during many pathogenic conditions.<sup>1</sup> These cells have a remarkable ability to suppress various T cell responses by perturbing immune responses through secreted soluble mediators and induction of regulatory T cells (Treg) expansion, constituting a unique regulatory component of the immune system in health and disease.<sup>1</sup> Allergic asthma is a chronic inflammatory airway disorder immunologically characterized by the imbalance of Th2, Th17 and Treg cells, in which Th2 and, in severe asthma, Th17 responses are overexpressed, but the function of Treg is impaired.<sup>2</sup> To date, there are few groups that have studied MDSC in asthma patients and the results are conflicting. Two papers reported that the percentage of MDSC in peripheral blood mononuclear cells (PBMC) was significantly increased in asthma patients.<sup>3,4</sup> However, another study reported that there was no difference in the percentage of MDSC in PBMC between patients with aspirin-intolerant asthma and those with aspirin-tolerant asthma.<sup>5</sup> Deshane et al identified several subsets of MDSC in the bronchoalveolar lavage fluids of patients with mild asthma and found that CD11b<sup>+</sup>CD14<sup>+</sup>CD16<sup>+</sup>HLA-DR<sup>-</sup> nitric oxide-producing MDSC subset was significantly increased in the airways of patients with mild asthma but not in patients with chronic obstructive pulmonary disease or healthy controls.<sup>6</sup> In these studies, the phenotypic, morphological and suppressive function of MDSC and/or relationship of MDSC with other immune regulatory cells have not been fully studied.

In the present study, using a recently published characterization standard,<sup>7</sup> we examined the presence and function of monocytic MDSC (M-MDSC) and polymorphonuclear (PMN-MDSC) in fresh peripheral blood of asthmatic patients, and analyzed the relationships among MDSC, Th2, Th17 and Treg cells.

After approval by the University of Manitoba Research Ethics Board, twenty-four asthmatic patients assessed based on National Asthma Education and Prevention Program's guideline (Table S1) and eighteen age-matched healthy donors without an asthma history were recruited. Blood was drawn from each individual. White blood cells (WBC) were isolated to identify MDSC subsets and Treg, while fresh PBMC were isolated to study Th2 and Th17 cells by flow cytometer. Morphology and in vitro suppressive function of MDSC subsets were also examined. These methods were described previously<sup>8</sup> and/or in "Supporting information Materials and methods."

The gating strategy of MDSC subsets was shown in Figure 1A. The results showed the percentage of CD45<sup>+</sup>CD33<sup>+</sup>CD14<sup>+</sup>HLA-DR<sup>low</sup>CD15<sup>-</sup> M-MDSC in WBC was significantly decreased in asthma patients when compared to controls (Figure 1B), which was different to previous reports, where MDSC were increased.<sup>3,4</sup> The difference may be due to the fact that previous reports used CD33<sup>+</sup>CD11b<sup>+</sup> or Lin<sup>-</sup>HLA-DR<sup>+</sup>CD33<sup>+</sup> to identify MDSC, while we used newly recommended markers CD45, CD33, CD14, HLA-DR, CD66b and CD15 to identify MDSC. These six markers we used have reached consensus as a standard characterization of MDSC.<sup>7</sup> There was no

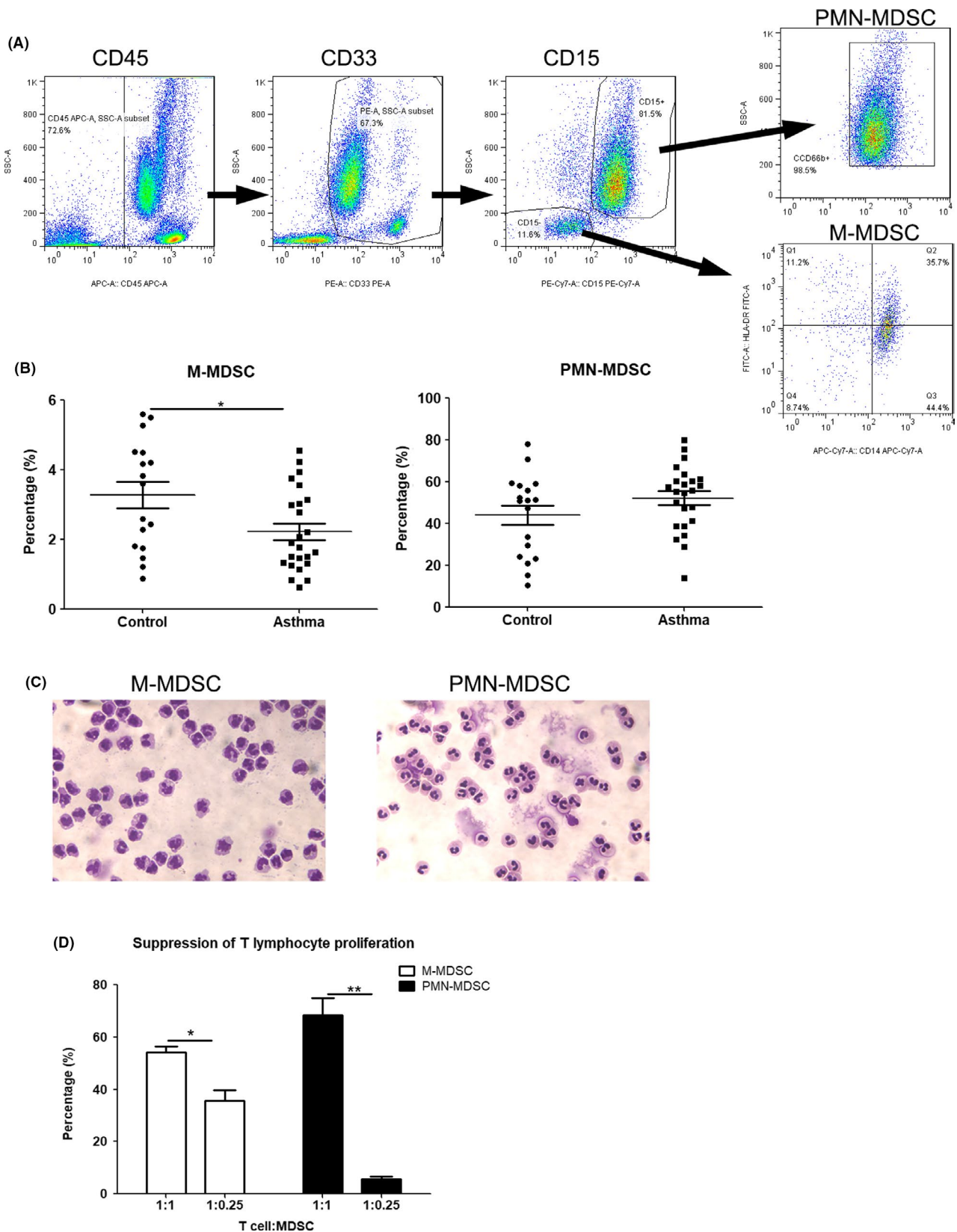


**FIGURE 1** The presence and suppressive function of M-MDSC and PMN-MDSC in the peripheral blood of asthmatic patients. A, Flow gating strategy of MDSC subsets. B, Percentages of M-MDSC and PMN-MDSC in CD45<sup>+</sup> WBC of peripheral blood ( $n = 24$  in patients and 18 in healthy controls). C, MDSC morphology. Sorted M-MDSC and PMN-MDSC from asthma patient were stained to evaluate the morphology by using HEMA-3 staining kit. D, Suppressive function of M-MDSC and PMN-MDSC on CD3<sup>+</sup>T cell proliferation (The results represent one of two patients' experiments). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$

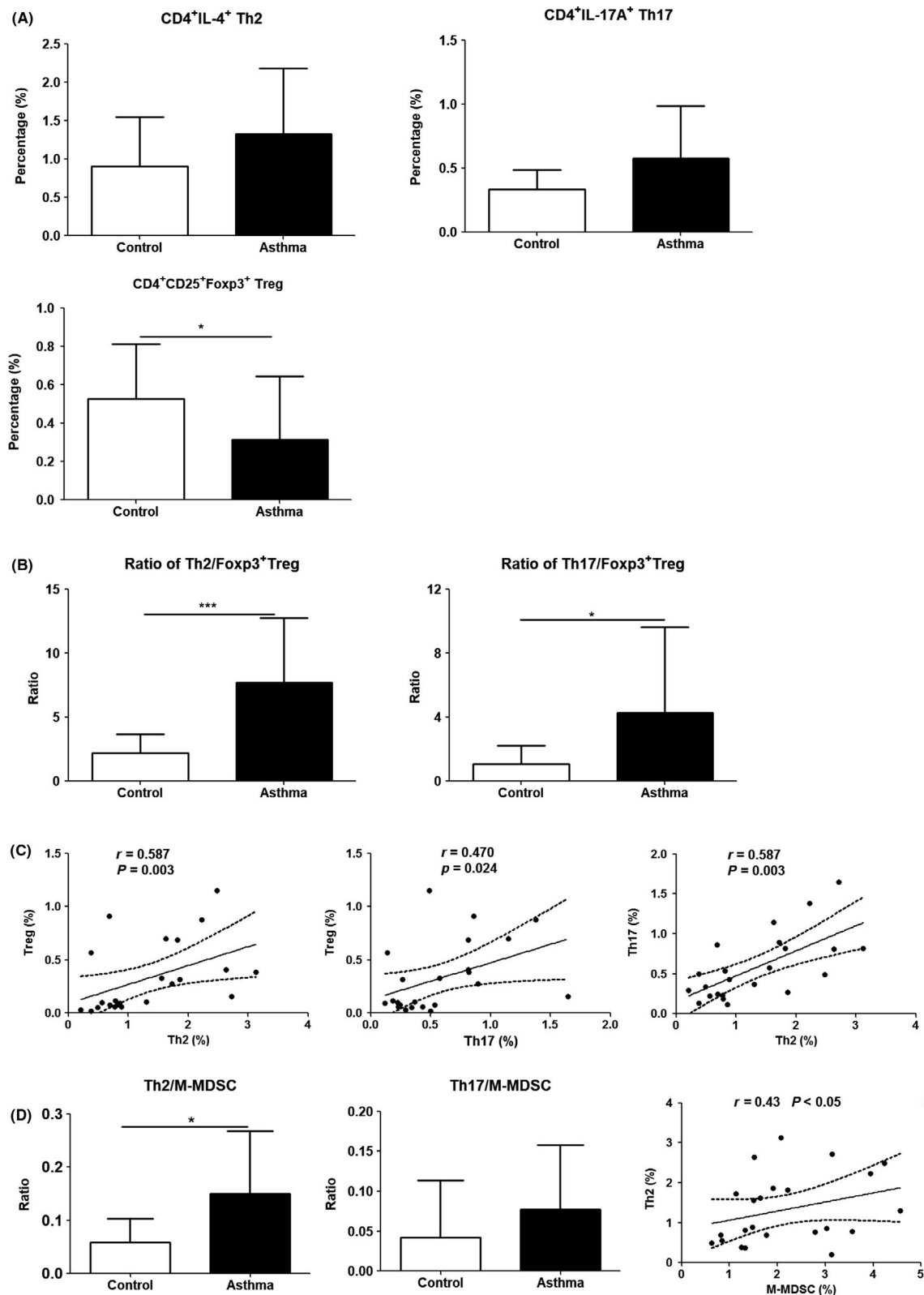


**FIGURE 2** The relationships among Th2, Th17, Treg and M-MDSC in asthmatic patients ( $n = 23$ ). A, Percentages of Th2, Th17, Treg in CD3<sup>+</sup>T cells. B, Ratios of Th2/Treg and Th17/Treg. C, Co-relationship of Treg, Th2, and Th17 in asthmatic patients. D, Relationships of M-MDSC with Th2 and Th17 in asthmatic patients. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$

significant difference on the percentage of CD45<sup>+</sup>CD33<sup>+</sup>CD14<sup>-</sup>CD15<sup>+</sup>CD66b<sup>+</sup> PMN-MDSC in WBC between controls and asthma patients (Figure 1B).

Through staining the flow cytometer-sorted M-MDSC and PMN-MDSC, the cells presented a typical mononuclear and polymorphonuclear type, respectively (Figure 1C). To evaluate whether MDSC

can suppress T cell proliferation, CFSE-labeled PBMC from healthy third parties were stimulated with anti-CD3/CD28 microbeads for 5 days in the presence of different ratios of MDSC sorted from asthmatic patient. The proliferation of CD3<sup>+</sup> T lymphocytes was analyzed using flow cytometry. The results showed that both M-MDSC and PMN-MDSC could suppress the proliferation of T lymphocytes, and M-MDSC still maintained the suppressive abilities even at a lower ratio of T:MDSC (Figure 1D). Our preliminary data also indicated that M-MDSC had increased expressions of Arginase-1 and iNOS, while PMN-MDSC had an increased expression of Arginase-1 in asthma patients (Figure S1). In the future, we will evaluate whether Arginase-1 and/or iNOS mediate the immunosuppression of MDSC in asthma.

To evaluate the effector T cells, fresh PBMC were stimulated with PMA/ionomycin/BFA for 5 hours to examine the presence of CD4<sup>+</sup>IL-4<sup>+</sup> Th2 and CD4<sup>+</sup>IL-17A<sup>+</sup>Th17 using flow cytometry (Figure S2). As shown in Figure 2A, the percentage of CD4<sup>+</sup>IL-4<sup>+</sup>Th2 cells in CD3<sup>+</sup>T cells and the percentage of CD4<sup>+</sup>IL-17<sup>+</sup>Th17 cells trend to an increase in asthma patients. In contrast, as expected, the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Treg cells significantly decreased in patients when compared with controls.

Further, we compared the ratios of effector T cells to Treg cells and found that the ratios of Th2/Treg and Th17/Treg were dramatically increased in asthma patients, compared to those in controls (Figure 2B). We also found the percentages of Th2 cells were positively correlated with those of Th17 cells, and the percentages of Treg cells were positively correlated with those of Th2 cells and Th17 in asthma patients (Figure 2C).

We then analyzed the ratios of effector T cells to M-MDSC and PMN-MDSC and found that similar to Treg, the ratios of both Th2/M-MDSC and Th17/M-MDSC were increased in asthma patients, although the increase of Th17/M-MDSC was not statistically significant, perhaps due to insufficient sample number studied (Figure 2D). The percentage of Th2 was also positively correlated with that of M-MDSC in asthma patients (Figure 2D). There was no difference on the ratios of Th2/PMN-MDSC and Th17/PMN-MDSC between healthy controls and asthma patients, and no correlation was found between PMN-MDSC with Th2 or Th17 in asthma patients (data not shown).

Studies have indicated that MDSC can suppress effector T cells but promote Treg expansion.<sup>1</sup> In the present study, we have shown that in asthmatic patients the percentage of M-MDSC is decreased, and both M-MDSC and PMN-MDSC are able to suppress T cell proliferation in vitro (Figure 1). These results suggest that the reduced Treg and increased Th2/Th17 responses might be caused by the reduced M-MDSC in asthma. Therefore, strategies to increase the number or inhibitory function of MDSC may be superior to other asthma immune treatments to quell a plethora of Th2/Th17 cytokines after they have been released. We have shown that in mice adoptive transfer of murine MDSC generated in vitro could decrease intestinal inflammation and cytokine levels.<sup>9</sup> Using similar methods, we have also found that infusion of murine MDSC into asthmatic

mice could ameliorate allergic airway inflammation, as shown by reduced IgE and eosinophil infiltration (manuscript in preparation), suggesting the therapeutic potential of MDSC in asthma.

In summary, we have reported that the percentage of M-MDSC is decreased in asthma patients and that these cells can suppress T cell proliferation. These results, along with the elevated Th2 and Th17 cells and reduced Treg cells, indicate that the reduced M-MDSC may lead to abnormal T cell responses, revealing a new immune regulatory mechanism in our understanding of the pathogenesis of asthma.

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## CONFLICTS OF INTEREST

Dr Peng reports grants from Research Manitoba, grants from Children's Hospital Research Institute of Manitoba, during the conduct of the study. The other authors declare no financial conflict of interest.

Qingdong Guan<sup>1,2,3,4,5</sup> 

Bin Yang<sup>1</sup>

Richard J. Warrington<sup>2,3</sup>

Steven Mink<sup>3</sup>

Chrystyna Kalicinsky<sup>3</sup>

Allan B. Becker<sup>1,2</sup>

Elinor Simons<sup>1</sup>

Zhikang Peng<sup>1,2</sup>

<sup>1</sup>Department of Pediatrics and Child Health, University of Manitoba, Winnipeg, Manitoba, Canada

<sup>2</sup>Department of Immunology, University of Manitoba, Winnipeg, Manitoba, Canada

<sup>3</sup>Department of Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

<sup>4</sup>Cellular Therapy Laboratory, CancerCare Manitoba, Winnipeg, Manitoba, Canada

<sup>5</sup>Research Institute in Oncology and Hematology, CancerCare Manitoba, Winnipeg, Manitoba, Canada

## Correspondence

Qingdong Guan, Cellular Therapy Laboratory, CancerCare Manitoba, MS773M, 820 Sherbrook St., Winnipeg, Manitoba R3A 1R9, Canada.

Email: qdguan@gmail.com

Guan and Yang equally contributed to this work.

## ORCID

 Qingdong Guan  <https://orcid.org/0000-0003-1972-487X>

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## SUPPORTING INFORMATION

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# Cyclooxygenase inhibition in mice heightens adaptive- and innate-type responses against inhaled protease allergen and IL-33

To the Editor,

Cyclooxygenases (COXs) are responsible for the formation of prostanoids such as prostaglandins (PGs) and thromboxanes.<sup>1</sup> Nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit COX activity, provoke aspirin-exacerbated respiratory disease (AERD),<sup>2</sup> and inhibition or deficiency of COXs exacerbates allergic asthma in murine models where ovalbumin (OVA) is used for immunization,<sup>3,4</sup> although the mechanism has not been fully elucidated. Environmental allergen sources, such as house dust mites, insects, fungi, and pollen, contain proteases, which are frequently allergens. In the present study, we used the protease papain as a model allergen, which is more relevant to natural allergen exposure than OVA, and examined whether COX inhibition promotes adaptive-type (Figure 1: the adaptive papain model<sup>5</sup>) and innate-type responses (Figure 2A,B: the innate papain model;<sup>5,6</sup> Figure 2C-E: the innate IL-33 model<sup>6</sup>) in IL-33-mediated protease-dependent models (for further information, see Supporting Information).

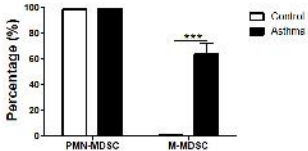
Upon inhalation of 10 µg papain, mice treated with the NSAID indomethacin (Figure 1A-D and Figure S1A-C) or aspirin (Figure S2) had severe allergic airway inflammation compared to that of the control group. Upon inhalation of 5 µg papain, which does not induce airway

inflammation without NSAID treatment, indomethacin-treated mice had increased responses including airway mucus production and airway hyperresponsiveness to methacholine (Figure 1E-I and Figure S1D,E). Genes for IL-33, a subunit of the IL-33 receptor (ST2), and thymic stromal lymphopoietin (TSLP) are susceptible loci associated with allergic diseases including asthma,<sup>7</sup> and nasal polyps from AERD patients show increased levels of IL-33 and TSLP.<sup>2</sup> Next, we evaluated whether epithelial cytokines IL-33 and TSLP were involved in the immune response upon COX inhibition. Exacerbation by COX inhibition was markedly reduced in IL-33-deficient and TSLP receptor-deficient mice (Figure 1J and Figure S3), which suggests involvement of IL-33 and TSLP.

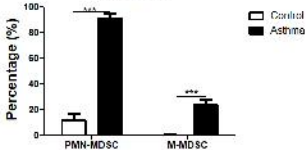
Tissue damage due to proteolytic activity of allergens induces the release and activation of IL-33.<sup>5,6,8,9</sup> Airway inflammation induced by COX inhibition in papain-inhaled mice was associated with infiltration of approximately equivalent numbers of ST2<sup>+</sup> Th cells and ST2<sup>+</sup> ILC2 and release of IL-5 and Th2-attracting chemokines into bronchial alveolar lavage fluid (BALF) (Figure 1K,L, and Figures S4 and S5). COX inhibition in papain-inhaled mice promoted differentiation of antigen-specific T cells in bronchial draining lymph nodes to produce Th2 (IL-4, IL-5, IL-13, and amphiregulin), Th9 (IL-9), Th17 (IL-17A), and Th1 cytokines (IFN-γ) on antigen restimulation; costimulation with recombinant IL-33 (rIL-33) promoted production of IL-5, IL-13, amphiregulin, IL-9, and IFN-γ, whereas stimulation with rIL-33 alone induced the production of IL-5, IL-13, IL-9, and IFN-γ (Figure 1M,N). Th cells

**Abbreviations:** AERD, aspirin-exacerbated respiratory disease; BALF, bronchial alveolar lavage fluid; COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; OVA, ovalbumin; PG, prostaglandin; rIL-33, recombinant IL-33; ST2, a subunit of the IL-33 receptor; TSLP, thymic stromal lymphopoietin.

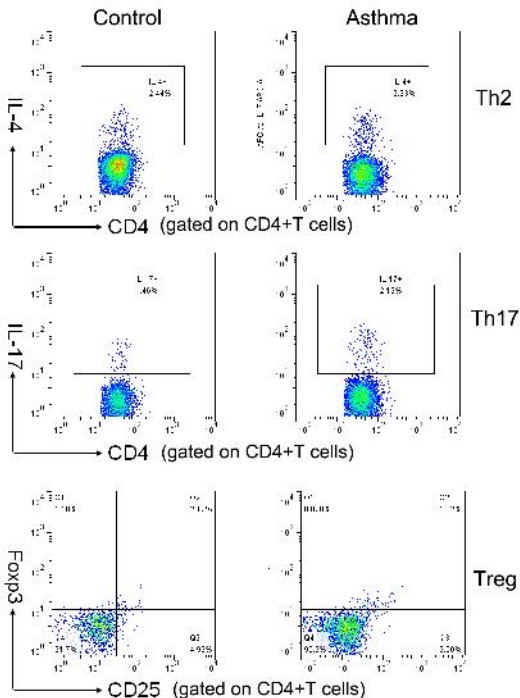
### INOS



### Arginase-1



Supplementary Figure 1



Supplementary Figure 2

**Supplementary figure legends**

Supplementary Figure 1. The expression of Arginase-1 and iNOS in MDSC subsets. The expression of Arginase-1 and iNOS in MDSC subsets were detected by intracellular staining of Arg-1 and iNOS and analyzed by flow cytometry (n=4 for asthma patients and 4 for healthy controls). \*\*\*,  $P < 0.001$

Supplementary Figure 2. The flow gating strategy for Th2, Th17 and Treg.

15 **Supplementary Table 1. Patient characteristics**

| Pts # | Sex         | Age      | Severity of asthma* |     |     |     | Allergic/ non-allergic <sup>#</sup> |            | Oral prednisone | Under treatment of inhalers | Drug name                            |
|-------|-------------|----------|---------------------|-----|-----|-----|-------------------------------------|------------|-----------------|-----------------------------|--------------------------------------|
|       |             |          | I                   | II  | III | IV  | Allergic                            | Non        |                 |                             |                                      |
| 1     | M           | 76       |                     |     |     | √   | -                                   | yes        | no              | yes                         | Advair, ventolin atrovent alvesco    |
| 2     | F           | 46       |                     | √   |     |     | yes                                 | -          | no              | yes                         | Symbicort Atrovent Ventolin          |
| 3     | F           | 43       |                     |     | √   |     | -                                   | yes        | no              | yes                         | Flovent Ventolin                     |
| 4     | F           | 49       |                     | √   |     |     | yes                                 | -          | no              | yes                         | Flovent, ventolin                    |
| 5     | F           | 43       |                     |     |     | √   | -                                   | yes        | yes 10-15mg     | yes                         | symbicort, ventolin singulair        |
| 6     | F           | 48       | √                   |     |     |     | yes                                 | -          | no              | yes                         | Advair Ventolin                      |
| 7     | F           | 78       |                     |     | √   |     | -                                   | yes        | no              | yes                         | Advair, Singulare, ventolin          |
| 8     | M           | 74       |                     |     |     | √   | yes                                 | -          | no              | yes                         | Advair, ventolin                     |
| 9     | M           | 17       |                     |     | √   |     | yes                                 | -          | no              | yes                         | ICS, LABA                            |
| 10    | M           | 58       |                     | √   |     |     | -                                   | yes        | no              | yes                         | Symbicort, ventolin                  |
| 11    | F           | 81       |                     |     |     | √   | -                                   | yes        | no              | yes                         | Advair, theophylline, ventoline      |
| 12    | F           | 56       | √                   |     |     |     | yes                                 | -          | no              | yes                         | Symbicort, Spiriva, ventolin         |
| 13    | F           | 73       |                     | √   |     |     | -                                   | yes        | no              | yes                         | Symbicort ventolin                   |
| 14    | F           | 79       |                     |     | √   |     | -                                   | yes        | no              | yes                         | Symbicort ventolin                   |
| 15    | M           | 72       |                     |     |     | √   | yes                                 | -          | no              | yes                         | Symbicort                            |
| 16    | M           | 65       |                     |     | √   |     | -                                   | yes        | no              | yes                         | Symbicort                            |
| 17    | M           | 57       |                     |     |     | √   | yes                                 | -          | no              | yes                         | Symbicort                            |
| 18    | F           | 71       | √                   |     |     |     | -                                   | yes        | no              | yes                         | Flovent                              |
| 19    | M           | 59       |                     |     |     | √   | -                                   | yes        | yes, 5mg        | yes                         | Atrovent, Symbicort, Alvesco         |
| 20    | F           | 15       |                     |     | √   |     | yes                                 | -          | no              | yes                         | ICS/LABA                             |
| 21    | F           | 47       | √                   |     |     |     | -                                   | yes        | no              | yes                         | Singular, bricanyl, symbicort        |
| 22    | F           | 58       |                     |     | √   |     | -                                   | yes        | no              | yes                         | flovent                              |
| 23    | M           | 16       | √                   |     |     |     | yes                                 | -          | no              | yes                         | Advair, Singulair, Flovent, Ventolin |
| 24    | F           | 61       |                     |     | √   |     | -                                   | yes        | yes, 10mg       | yes                         | Ventolin Singulair                   |
| Total | M=8<br>F=16 | Mean =56 | n=5                 | n=4 | n=8 | n=7 | n=10 (42%)                          | n=14 (58%) | n=3 (13%)       | n=24 (100%)                 |                                      |

16 \*Severity: According to the National Asthma Education and Prevention Program's guideline  
 17 (NIH publication no. 07-4051), patients were assessed and divided into four degrees groups  
 18 based on symptoms, pulmonary function, skin test sensitivity, adherence to prescribed drug  
 19 regimen. I, intermittent; II: mild persistent, III: moderate persistent; IV: severe persistent.

20  
 21 #, Allergic versus non-allergic based on skin testing.

## SUPPLEMENTARY MATERIALS AND METHODS

### Patients

After approval by the University of Manitoba Research Ethics Board, twenty-four asthmatic patients and eighteen age-matched healthy donors without an asthma history were recruited at Health Sciences Center, Winnipeg. According to the National Asthma Education and Prevention Program's guideline (NIH publication no. 07-4051), patients were assessed and classified as intermittent, mild, moderate and severe. Patient demographics are presented in Supplementary Table 1. After written consent, peripheral blood was taken.

### Isolation of white blood cells (WBC) and peripheral blood mononuclear cells (PBMC)

Sixteen mL of peripheral blood was drawn from each participant and placed into a heparin-treated tube. Half of the blood volume was used to isolate WBC using HetaSep (Stem Cell Technologies, Vancouver, Canada) and half was used to isolate PBMC using Ficoll as we described previously<sup>1</sup>.

### Identification of MDSC subsets, effector T cells and immune regulatory cells by flow cytometer

WBC were used to identify MDSC and Treg cells. MDSC was characterized as described previously<sup>1,2</sup>. To evaluate Treg cells, WBC were firstly stained with Percp-Cy5.5-, FITC-, PE-Cy7-, and APC- labeled antibodies against CD3, CD4, CD8 and CD25; after fixation and permeabilization, cells were intracellularly stained with PE-labeled anti-Foxp3 according to standard procedure provided by eBioscience. The WBC analysis was performed within 4-6 hours after blood draw.

Fresh PBMC were used to evaluate Th2 or Th17 cells. After incubation with a cell stimulation cocktail (PMA/inomycin/BFA) for 5-6 hours, PBMC were stained with Percp-Cy5.5- and FITC- labeled antibodies against CD3 and CD4. After fixation and permeabilization, cells were stained with PE-, and/or APC-labeled antibodies against IL-17 or IL-4 according to the manufacturer recommendations. The Th2 and Th17 analysis were performed within 12 hours after blood draw.

For intracellular staining of Arg-1 and iNOS, cells were first stained with

fluorescence-labeled antibodies against HLA-DR, CD33, CD15, and CD14 and then fixed and permeabilized using eBioscience intracellular fixation/permeabilization buffer. Cells were then stained with fluorescence labeled anti-iNOS and anti-Arg-1<sup>1</sup>.

All antibodies were purchased from eBioscience (San Diego, USA), Biolegend (San Diego, USA), Santa Cruz Biotechnology (Dallas, USA) and R&D systems (Minneapolis, USA). After staining, cells were acquired and analyzed using flow cytometry (FACS Canto II, BD Biosciences, San Jose, USA) and FlowJo software (TreeStar, San Carlos, CA, USA).

### **Inhibition by MDSC of T cell proliferation assays**

To evaluate the function of MDSC, MDSC from the peripheral blood of patients were enriched by flow sorter (BD FACSAriaIII cell sorter). The purity of MDSC cells was confirmed by flow cytometry (>90%). The suppressive effect of MDSC on anti-CD3/CD28-induced lymphocyte proliferation was then evaluated through CFSE dilution assay as described previously<sup>1</sup>. Briefly,  $1 \times 10^5$  CFSE-labeled PBMCs were stimulated with anti-CD3/CD28 microbeads (1.8  $\mu$ L/well, Life Technologies, Grand Island, NY) in the presence of M-MDSC or PMN-MDSC at the ratios of PBMC/MDSC (1:1 and 1:0.25) in 96-well plates for 5 days. The inhibition rate was calculated below:

Inhibition (%) = (Number of proliferated cells without MDSC – Number of proliferated cells with MDSC) / Number of proliferated cells without MDSC  $\times$  100%

### **Morphology of MDSC subsets in asthma patients**

$1-2 \times 10^5$  sorted M-MDSC or PMN-MDSC were used for Cytospin (Shandon, Runcorn, UK). After Cytospin, the slides were stained with HEMA 3 (Fisher Diagnostics, Pittsburg, PA) as we described previously<sup>3</sup>.

### **Statistical analysis**

Values were expressed as means  $\pm$  SD. Unpaired student t-tests were used to compare the differences. Spearman correlations were used to assess the relationship between cell values. All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Prism, USA). *P* values < 0.05 were considered statistically significant. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

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