

Myeloid-derived suppressor cell function is diminished in aspirin-triggered allergic airway hyperresponsiveness in mice

Maohua Shi, MD,^{a,b*} Guochao Shi, MD, PhD,^{c*} Juan Tang, BSc,^a Deping Kong, BSc,^a Yao Bao, MD,^c Bing Xiao, MSc,^a Caojian Zuo, MD,^a Tai Wang,^{a,‡} Qingsong Wang, PhD,^a Yujun Shen, PhD,^a Hui Wang, MD, PhD,^{a,e} Colin D. Funk, PhD,^d Jie Zhou, MD, PhD,^{b,f} and Ying Yu, MD, PhD^{a,e} *Shanghai, Guangzhou, and Beijing, China, and Kingston, Ontario, Canada*

Background: Myeloid-derived suppressor cells (MDSCs) have recently been implicated in the pathogenesis of asthma, but their regulation in patients with aspirin-intolerant asthma (AIA) remains unclear.

Objective: We sought to characterize MDSC accumulation and pathogenic functions in allergic airway inflammation mediated by COX-1 deficiency or aspirin treatment in mice.

Methods: Allergic airway inflammation was induced in mice by means of ovalbumin challenge. The distribution and function of MDSCs in mice were analyzed by using flow cytometry and pharmacologic/gene manipulation approaches.

Results: CD11b⁺Gr1^{high}Ly6G⁺Ly6C^{int} MDSCs (polymorphonuclear MDSCs [PMN-MDSCs]) recruited to the lungs are negatively correlated with airway inflammation in allergen-challenged mice. Aspirin-treated and COX-1 knockout (KO) mice showed significantly lower accumulation of PMN-MDSCs in the inflamed lung and immune organs

accompanied by increased T_H2 airway responses. The T_H2-suppressive function of PMN-MDSCs was notably impaired by COX-1 deletion or inhibition, predominantly through downregulation of arginase-1. COX-1-derived prostaglandin E₂ promoted PMN-MDSC generation in bone marrow through E prostanoid 2 and 4 receptors (EP2 and EP4), whereas the impaired arginase-1 expression in PMN-MDSCs in COX-1 KO mice was mediated by dysregulation of the prostaglandin E₂/EP4/cyclic AMP/protein kinase A pathway. EP4 agonist administration alleviated allergy-induced airway hyperresponsiveness in COX-1 KO mice. Moreover, the immunosuppressive function of PMN-MDSCs from patients with AIA was dramatically decreased compared with that from patients with aspirin-tolerant asthma.

Conclusion: The immunosuppressive activity of PMN-MDSCs was diminished in both allergen-challenged COX-1 KO mice and patients with AIA, probably through an EP4-mediated signaling pathway, indicating that activation of PMN-MDSCs might be a promising therapeutic strategy for asthma, particularly AIA. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Myeloid-derived suppressor cells, aspirin-intolerant asthma, T_H2, COX, prostaglandin, arginase

From ^athe Key Laboratory of Food Safety Research, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai; ^bthe Institute of Human Virology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou; ^cthe Department of Pulmonary Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai; ^dthe Department of Biomedical and Molecular Sciences, Queen's University, Kingston; ^ethe Key Laboratory of Food Safety Risk Assessment, Ministry of Health, Beijing; and ^fthe Key Laboratory of Tropical Disease Control (Sun Yat-sen University), Chinese Ministry of Education, Guangzhou.

*These authors contributed equally to this work.

‡Tai Wang is currently affiliated with the Perkiomen School, Pennsburg, Pa.

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Corresponding author: Ying Yu, MD, PhD, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 294 Taiyuan Rd, Shanghai, 200031, China. E-mail: yuying@sibs.ac.cn. Or: Jie Zhou, MD, PhD, Institute of Human Virology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, China. E-mail: zhouj72@mail.sysu.edu.cn.

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Aspirin-intolerant asthma (AIA) is a distinctive condition involving severe bronchospasm in an asthmatic patient caused by ingestion of aspirin or other COX-1-inhibiting nonsteroidal anti-inflammatory drug (NSAIDs).¹ Although there are 2 different COX isoforms (ie, COX-1 and COX-2), aspirin is more than 170-fold selective in inhibiting COX-1 than COX-2.² Despite the presence of COX-2, its expression is limited and enzymatic activity is diminished in nasal polyps and bronchial wall epithelial cells of patients with AIA.¹ Moreover, the majority of asthmatic patients with aspirin hypersensitivity are able to tolerate selective COX-2 inhibitors, indicating that COX-1 inhibition is implicated in the exacerbated response in patients with AIA. In addition, a positive correlation exists between prostaglandin (PG) inhibition by NSAIDs and the occurrence of AIA in asthmatic patients,³ suggesting that blockage of COX-1-derived PG biosynthesis is particularly involved in the pathogenesis of AIA.

PGE₂, a dominant COX product, mediates the proinflammatory response in many diseases, such as arthritis and cancer.⁴ However, PGE₂ displays some beneficial anti-inflammatory properties in the airways, including antifibrotic, bronchodilating, and inhibitory activities against allergic inflammation-induced pulmonary vascular smooth muscle remodeling.⁵ In asthmatic patients inhalation of PGE₂ significantly attenuates allergen-induced airway responses and pulmonary inflammation.⁶ In contrast, PGE₂

Abbreviations used

AHR:	Airway hyperresponsiveness
AIA:	Aspirin-intolerant asthma
APC:	Allophycocyanin
Arg1:	Arginase-1
ATA:	Aspirin-tolerant asthma
BALF:	Bronchoalveolar lavage fluid
BM:	Bone marrow
cAMP:	Cyclic AMP
CFSE:	5(6)-Carboxyfluorescein diacetate succinimidyl ester
CREB:	cAMP response element-binding protein
Ef-OVA:	Endotoxin-free ovalbumin
Ef-OVA _{LPS} /Ef-OVA mice:	Mice sensitized with Ef-OVA and low-dose LPS (0.1 µg) and challenged with Ef-OVA
EP:	E prostanoïd receptor
KO:	Knockout
L-NMMA:	L-N ^G -monomethyl arginine
MDSC:	Myeloid-derived suppressor cell
Mo-MDSC:	Monocytic MDSC
NO:	Nitric oxide
NSAID:	Nonsteroidal anti-inflammatory drug
OVA:	Ovalbumin
PE:	Phycocerythrin
PG:	Prostaglandin
PKA:	Protein kinase A
PMN-MDSC:	Polymorphonuclear MDSC
ROS:	Reactive oxygen species
TX:	Thromboxane
WT:	Wild-type

generation is markedly reduced in pulmonary tissues from patients with AIA compared with those from control subjects,⁷ whereas treatment with PGE₂ administered by means of inhalation can prevent aspirin-triggered bronchoconstriction in patients with AIA.¹ These findings indicate that PGE₂ produced primarily from COX-1 plays a crucial role in the pathogenesis of AIA; however, its exact mechanism in patients with AIA has yet to be fully elucidated.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that consist of 2 distinct subsets: Ly6G⁺Ly6C^{int} polymorphonuclear MDSCs (PMN-MDSCs) and Ly6G[−]Ly6C^{high} monocytic MDSCs (Mo-MDSCs).⁸ In general, MDSCs can differentiate into mature granulocytes, dendritic cells, and macrophages in the bone marrow (BM), and their expansion was discovered in a range of pathologic conditions, including tumors, infections, trauma, and chronic inflammatory diseases, such as diabetes and inflammatory bowel disease.^{8,9} Recently, LPS was shown to induce the expansion of CD11b⁺Gr1^{int}F4/80⁺ immature myeloid cells in patients with allergic airway inflammation.¹⁰ These cells alleviated asthma by suppressing the dendritic cell-mediated reaction of primed T_H2 cells,^{10,11} suggesting a potential protective effect of MDSCs in the development of asthma. Both clinical observations and laboratory experiments demonstrated that COX and its product, PGE₂, were involved in the regulation of activation and accumulation of MDSCs.¹² We hypothesized that the suppressive function of MDSCs might be impaired by COX-1 inhibition in an allergen-induced airway hyperresponsiveness (AHR) model with increased T_H2 activation.

METHODS

See the **Methods** section in this article's Online Repository at www.jacionline.org for a detailed explanation of the methods and materials used in this study.

RESULTS**PMN-MDSCs are negatively correlated with allergen-induced AHR phenotype in mice with COX-1 inhibition**

Allergic asthma is an inflammatory disease initiated and driven by T_H2 cytokines. LPS is often present with ovalbumin (OVA) allergen during sensitization in mouse models and is responsible for CD4⁺ T-cell differentiation. Low-dose LPS induces T_H2 responses, whereas high-dose LPS induces T_H1 responses.^{13,14} Indeed, a considerable amount of endotoxin (49.7 ± 2.0 EU/mg, data not shown) could be detected in the OVA preparation, as previously reported.¹⁵ Both endotoxin-free ovalbumin (Ef-OVA) plus low-dose LPS (Ef-OVA_{LPS}) and traditional OVA sensitization protocols were used to induce airway inflammation in mice to explore whether MDSCs are involved in COX-1 inhibition-mediated AHR. As shown in Fig 1, A, and Fig E1, A, in this article's Online Repository at www.jacionline.org, a population of CD11b⁺Gr1^{high} cells was significantly increased in lungs from OVA-challenged mice when compared with PBS-challenged mice (1.40- to 1.65-fold, *P* < .05). Strikingly, the CD11b⁺Gr1^{high} population was much lower in aspirin-treated or COX-1 knockout (KO) mice than in wild-type (WT) mice both before and after OVA challenge (Fig 1, B, and see Fig E1, B), suggesting that the reduction in CD11b⁺Gr1^{high} cell counts might contribute to COX-1 inhibition-mediated AHR. Immunofluorescence staining further confirmed that CD11b⁺Gr1⁺ MDSCs accumulated in the peribronchial areas of the lungs of these mice (Fig 1, C, and see Fig E1, C). A reduction in MDSC counts was also observed in other immune tissues and organs, such as the BM and spleen (see Fig E2, A, and Fig E3, A, in this article's Online Repository at www.jacionline.org). As expected, COX-1 deficiency or aspirin administration aggravated airway inflammatory responses to OVA in mice, as evidenced by increased bronchoalveolar lavage fluid (BALF) protein levels, increased eosinophil counts in BALF, more inflammatory cells surrounding bronchioles and blood vessels, and increased T_H2 cytokine levels and airway resistance (see Fig E2, B-F, and Fig E3, B-G). Flow cytometric analysis revealed that the CD11b⁺Gr1^{high} cells were predominantly Ly6G⁺Ly6C^{int} (>92%) in airway inflammation status and thus were named PMN-MDSCs, whereas Ly6G[−]Ly6C⁺ cells/Mo-MDSCs (Fig 1, D, and see Fig E1, D) could be clearly separated as gated in CD11b⁺Gr1^{int}, as previously described.¹⁶ Again, the number of PMN-MDSCs, but not Mo-MDSCs, was significantly decreased in the BM, spleens, and lungs of WT/aspirin and COX-1 KO AHR mice compared with that seen in control animals (Fig 1, E, and see Fig E1, E; Fig E2, A; and Fig E3, A). Moreover, the PMN-MDSC population was negatively correlated with protein secretion in BALF in OVA-treated mice (Fig 1, F, and see Fig E1, F), indicating that PMN-MDSCs alleviate the airway inflammation induced by OVA.

We next investigated whether infusion of additional PMN-MDSCs could inhibit the airway response to OVA (Fig 2, A, and see Fig E4, A, in this article's Online Repository at www.jacionline.org). PMN-MDSC infusion in WT mice resulted in

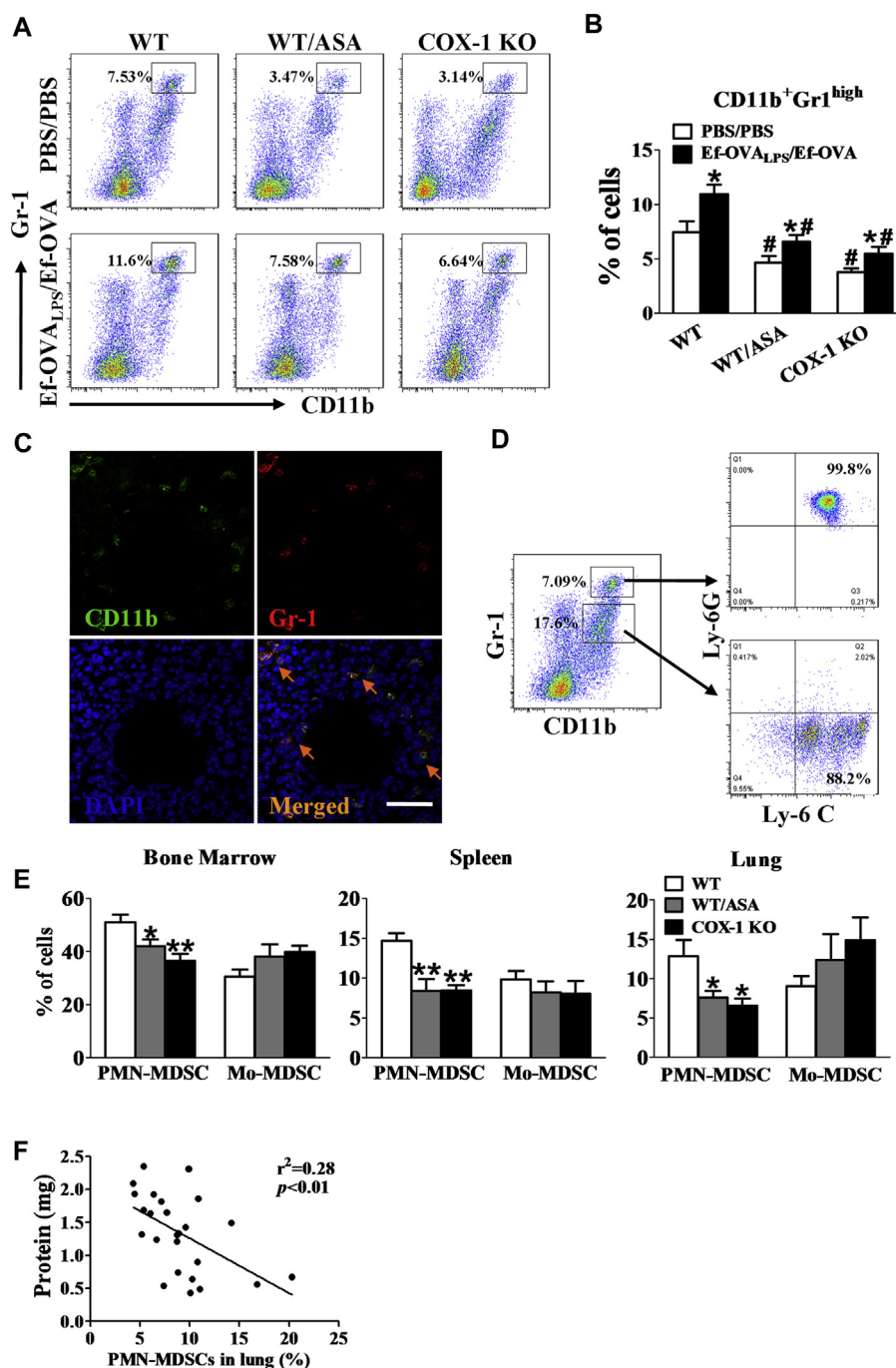


FIG 1. Characterization of MDSCs accumulated in Ef-OVA_{LPS}/Ef-OVA mice: effects of aspirin (ASA) treatment and COX-1 deficiency. **A** and **B**, Representative flow charts (Fig 1, A) and quantitation of CD11b⁺Gr1^{high} cells (Fig 1, B) in the lung are shown. * $P < .05$ versus the PBS group and # $P < .05$ versus the WT group ($n = 8-10$). **C**, Representative immunofluorescent micrographs of MDSCs in lung sections from allergic mice. Scale bar = 50 μm. **D**, Characteristic analysis of CD11b⁺Gr1^{high} MDSCs. **E**, MDSC subsets in the BM, spleens, and lungs of Ef-OVA-challenged mice. * $P < .05$ and ** $P < .01$ versus the WT group ($n = 8-10$). **F**, Correlation analysis of infiltrated PMN-MDSCs in the lung with BALF total protein from WT, WT/ASA, and COX-1 KO mice challenged by Ef-OVA.

a mild decrease in airway inflammation with no statistical difference (Fig 2, B and C, and see Fig E4, B and C). PMN-MDSC transfer to COX-1 KO mice significantly attenuated the airway inflammatory response to OVA; this included reduced inflammatory cell infiltration in both the peribronchial

and perivascular regions (Fig 2, B and C, and see Fig E4, B and C), reduced protein levels (Fig 2, D, and see Fig E4, D) and infiltrated inflammatory cell counts (Fig 2, E, and Fig E4, E), and decreased TH2 cytokine secretion in BALF (Fig 2, F, and see Fig E4, F).

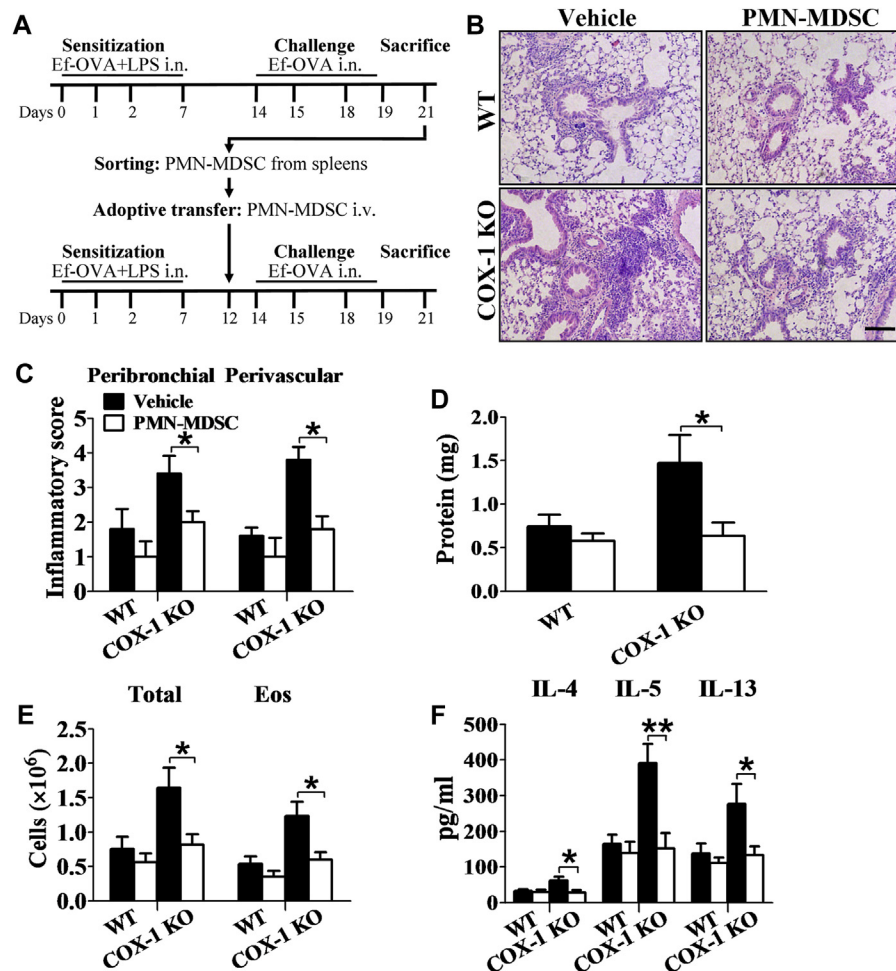


FIG 2. Adoptive transfer of PMN-MDSCs alleviates COX-1 deficiency-mediated AHR in Ef-OVA_{LPS}/Ef-OVA mice. **A**, Schematic for protocol for administration of PMN-MDSCs to Ef-OVA-challenged mice. **B** and **C**, Representative hematoxylin and eosin staining (Fig 2, B) and quantification of inflammatory scores (Fig 2, C) of lung sections from mice undergoing PMN-MDSC adoptive transfer and challenged with Ef-OVA. Scale bar = 50 μ m. **D-F**, Protein secretion (Fig 2, D), inflammatory cell recruitment (Fig 2, E), and cytokines secreted in BALF (Fig 2, F) from mice undergoing adoptive transfer. * $P < .05$ and ** $P < .01$ ($n = 6-8$).

Impaired immunosuppressive function of PMN-MDSCs contributes to OVA-induced AHR in COX-1-deficient mice

The suppressive function of the expanded PMN-MDSCs was examined in aspirin-treated and COX-1 KO mice. As anticipated, PMN-MDSCs from all 3 groups displayed significant immunosuppressive function, which was measured as a decrease in T-cell proliferation. However, the functional activity of PMN-MDSCs from both aspirin-treated and COX-1-deficient mice was markedly reduced compared with that from WT control animals (Fig 3, A and B). Consistently, the levels of cytokines secreted by activated T_H2 cells in MDSC/T-cell cocultured medium, including IL-4, IL-5, and IL-13, were also greater in the aspirin-treated and COX-1-deficient PMN-MDSCs (Fig 3, C).

Because the immunosuppressive function of PMN-MDSCs was impaired by COX-1 deficiency or inhibition in the OVA-induced airway inflammation model, we envisioned that BM from WT donors might rescue the defective MDSC phenotype in COX-1-deficient mice and thus mitigate pulmonary airway responses. Genotyping of both tail biopsy specimens and blood samples

confirmed successful BM reconstitution (Fig 3, D, and see Fig E5, A, in this article's Online Repository at www.jacionline.org). The reduced PMN-MDSC counts in BM from OVA-challenged COX-1 KO mice were completely recovered by WT BM transplantation (WT $\rightarrow$ KO), whereas MDSC generation was decreased in WT recipients reconstituted by COX-1 KO (KO $\rightarrow$ WT; Fig 3, E, and see Fig E5, B and C, and Fig E6, A, in this article's Online Repository at www.jacionline.org), suggesting that COX-1 in BM-derived cells played an important role in the generation of PMN-MDSCs. Interestingly, the pulmonary infiltration of PMN-MDSCs in KO $\rightarrow$ WT chimeras was comparable with that in WT $\rightarrow$ WT controls (Fig 3, E, and see Fig E5, C). Although the generation of PMN-MDSCs in WT $\rightarrow$ KO BM was normal, the pulmonary PMN-MDSC expansion in OVA-treated WT $\rightarrow$ KO mice was significantly lower than that in WT $\rightarrow$ WT control mice (40% to 46% decrease), although notably increased compared with that in KO $\rightarrow$ KO experimental mice (Fig 3, E, and see Fig E5, C). In line with the increase in PMN-MDSC counts in the lungs, OVA-induced airway inflammation was significantly alleviated in WT $\rightarrow$ KO mice (Fig 3, F and G, and see Fig E5, D and E), as evidenced by reduced

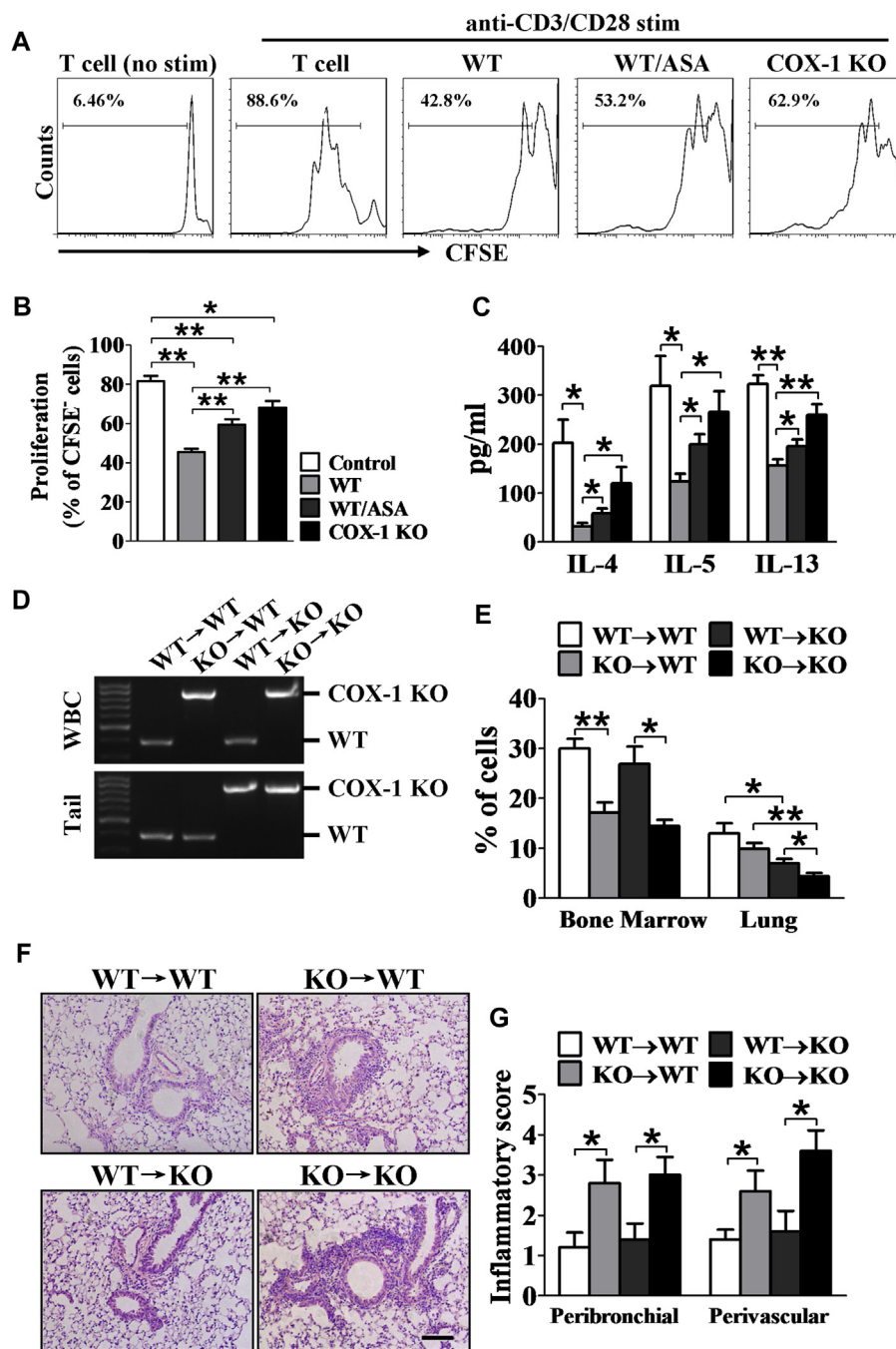


FIG 3. Impaired immunosuppressive function of PMN-MDSCs contributes to COX-1 inhibition/deletion-mediated AHR in Ef-OVA_{LP}/Ef-OVA mice. **A**, Representative flow charts for proliferation of T cells cocultured with PMN-MDSCs from WT, WT/aspirin, and COX-1 KO mice. **B**, Quantitation of proliferation of T cells shown in Fig 3, A. **C**, T_H2 cytokine production from coculture medium. **D**, BM transplantation confirmation by means of genotyping. **E**, PMN-MDSC accumulation in BM and lungs of BM-transplanted mice challenged with Ef-OVA. **F** and **G**, Representative hematoxylin and eosin staining (Fig 3, F) and inflammatory scores (Fig 3, G) of lung sections for mice undergoing BM transplantation. Scale bar = 50 μ m. * P < .05 and ** P < .01, as indicated (n = 6-8).

inflammatory cell infiltration and T_H2 cytokine secretion (see Fig E5, F-H, and Fig E6, B-D), whereas KO→WT BM transplantation led to dramatic deterioration of OVA-induced airway inflammation. These observations indicate that COX-1 activity in the lung is essential for the pulmonary recruitment of PMN-MDSCs from BM in OVA-challenged mice.

Next, we examined the COX-derived PG production from lung tissues in response to OVA challenge. PGE₂ and PGI₂ (6-keto-PGF_{1 α} , a stable hydrolysis product) are the major PGs in inflamed lung tissue, levels of which are decreased by aspirin treatment (WT/aspirin) or COX-1 deficiency (see Fig E7, A, in this article's Online Repository at www.jacionline.org), as reported

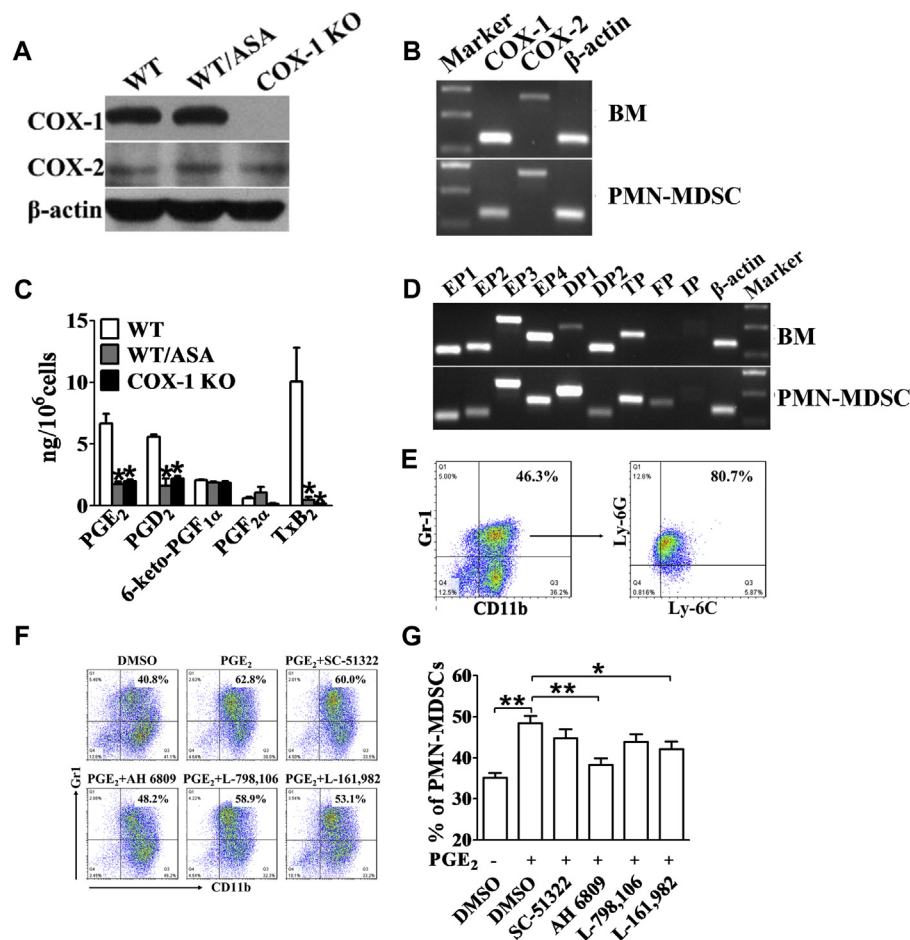


FIG 4. COX-1-mediated BM-derived MDSC expansion through PGE₂-EP2/EP4 signaling *in vitro*. **A**, Western blot analysis of COX-1 and COX-2 in BM cells. **B**, COX-1 and COX-2 expression in BM and BM-derived PMN-MDSCs. **C**, PG profiles in BM cells. **D**, Expression of PG receptors in BM cells and BM-derived PMN-MDSCs. **E**, Characteristic analysis of BM-derived PMN-MDSCs. **F**, Effect of EP antagonists on PMN-MDSC generation from cultured BM. **G**, Quantitative data for the effect of PGE₂ and EP antagonists on the generation of BM-derived PMN-MDSCs. **P* < .05 and ***P* < .01 (*n* = 4). ASA, Aspirin; DMSO, dimethyl sulfoxide.

previously.^{17,18} We did not detect any differences in leukotriene B₄ production among the 3 groups (data not shown). As previously determined,^{19,20} PMN-MDSC infiltration and migration is mediated by the chemokine receptor CXCR2, whereas Mo-MDSC recruitment occurs through CCR2. We found that gene expression of CCR1, CCR2, CXCR2, and CXCR3 in PMN-MDSCs from OVA-treated mice was not influenced by COX-1 inhibition or genetic deficiency (see Fig E7, B and C). Gene expression of the CXCR2 ligands CXCL2 and CXCL3 and the CXCR3 ligand CXCL4 was suppressed by more than 60% in OVA-challenged lungs after aspirin treatment or COX-1 disruption in mice (see Fig E7, D), which might lead to reduced expansion of PMN-MDSCs in COX-1 KO recipient mice (Fig 3, E, and see Fig E5, C).

PMN-MDSC generation in BM is mediated by COX-1-derived PGE₂ through EP2/EP4 receptors

Expression of COX-1 was abundant in primary BM cells, whereas expression of COX-2 was minimal (Fig 4, A and B), indicating that COX-1 is the dominant isoform for PG synthesis in

BM. These most abundant PGs in BM, including PGE₂, PGD₂, and thromboxane (TX) B₂ (TXA₂ hydrolysis metabolite), were suppressed by aspirin treatment or COX-1 deficiency (Fig 4, C), and all PG receptors except I prostanoid receptor (IP) were expressed (Fig 4, D) in BM cells and PMN-MDSCs.

BM cells were treated with different PG receptor antagonists during MDSC generation induced by IL-4/GM-CSF to examine which PG receptors mediate MDSC expansion. Numbers of Ly-6C⁺Ly-6G⁺ PMN-MDSCs, the major subtype of MDSCs in our experimental system (Fig 4, E), increased by more than 50% in cultures supplemented with PGE₂ (Fig 4, F and G). Notably, the effect of PGE₂ on PMN-MDSC generation was nearly abolished by AH 6809, a selective EP2 antagonist. In addition, L-161,982, a selective EP4 antagonist, had a modest but significant effect on PGE₂-mediated PMN-MDSC expansion (Fig 4, F and G). There were no detectable effects of PGD₂ or TX on PMN-MDSC generation through their corresponding receptors (see Fig E8 in this article's Online Repository at www.jacionline.org), although their production was also impaired in BM cells from COX-1 KO mice (Fig 4, C).

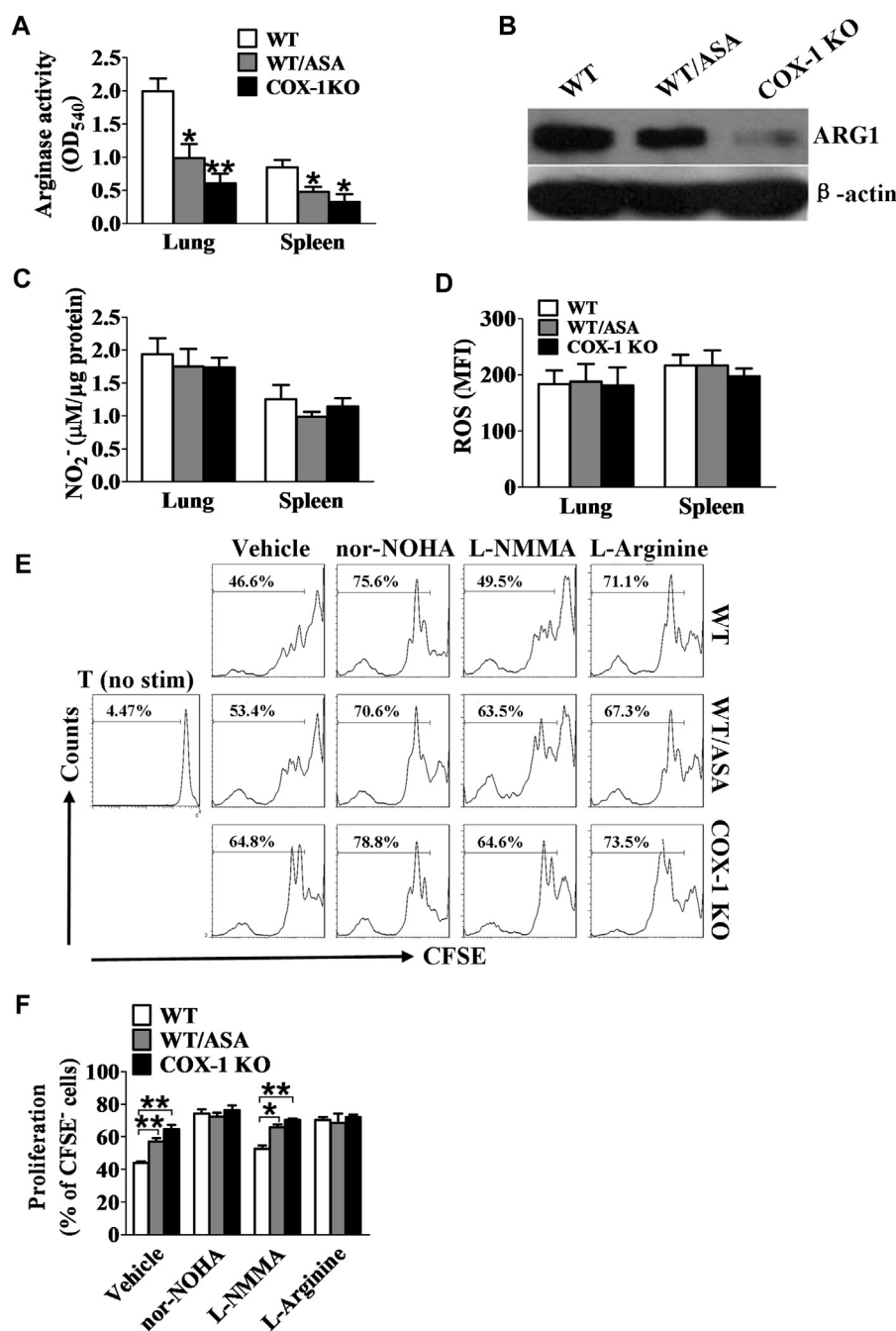


FIG 5. Arg1 activity of PMN-MDSCs is inhibited by aspirin (ASA) blockade of COX-1 or COX-1 gene disruption in OVA-challenged mice. **A**, Arginase activity in PMN-MDSCs in the lungs and spleens of OVA-challenged mice. **B**, Arg1 protein levels in spleen PMN-MDSCs from WT, WT/aspirin, and COX-1 KO mice. **C** and **D**, NO metabolites and ROS products of PMN-MDSCs from lungs and spleens of OVA-challenged mice (n = 4). **E**, Representative flow charts for proliferation of CD4⁺ T cells cocultured with spleen PMN-MDSCs from WT, WT/aspirin, and COX-1 KO mice. **F**, Quantitation data for Fig 5, E. *P < .05 and **P < .01, as indicated (n = 4).

PMN-MDSC function is dependent on arginase activity in aspirin-treated or COX-1-deficient mice

L-Arginine metabolism is associated with the suppressive activity of PMN-MDSCs under some pathologic conditions.²¹ Therefore we analyzed products of L-arginine metabolism, including arginase activity and nitric oxide (NO) and reactive oxygen species (ROS) production, in PMN-MDSCs isolated from

the lung and spleen. The results showed that arginase activity was dramatically decreased in tissues from both WT/aspirin and COX-1 KO mice when compared with that from WT mice (Fig 5, A). In support of the changes in arginase activity, expression of arginase-1 (Arg1) also displayed similar reductions in both protein and mRNA levels (Fig 5, B, and see Fig E9, A, in this article's Online Repository at www.jacionline.org). Inducible nitric oxide

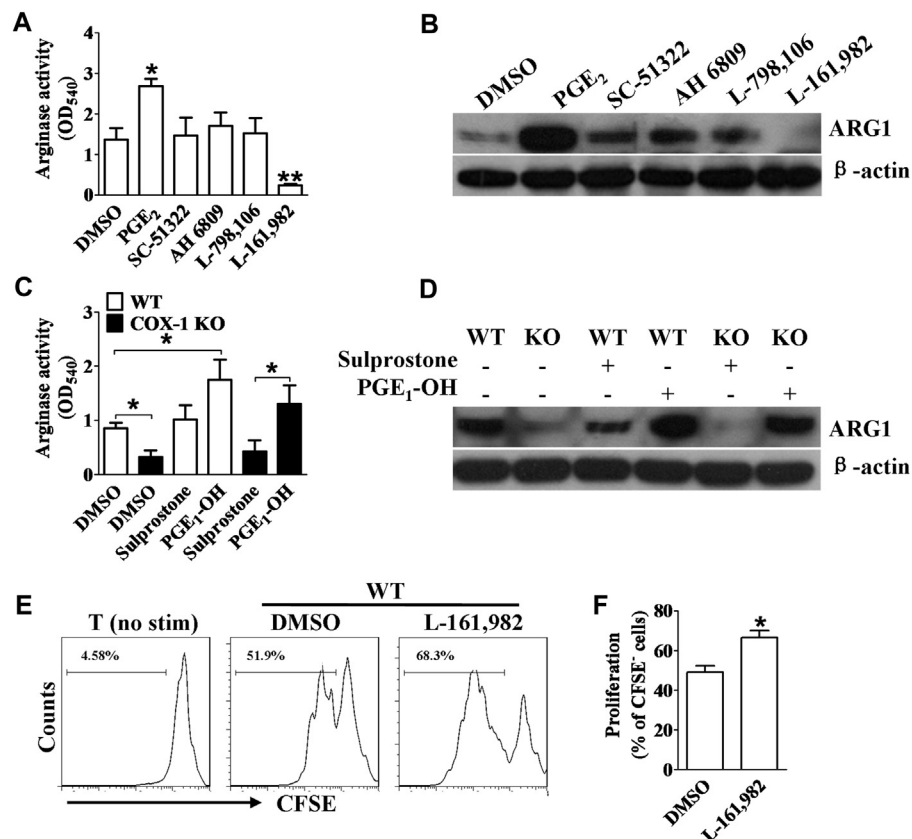


FIG 6. PGE₂ regulates Arg1 expression through EP4 in BM-derived PMN-MDSCs. **A**, Effect of EP antagonists on arginase activity in MDSCs. **B**, Effect of EP antagonists on Arg1 expression in MDSCs. **C**, Effect of PGE₁-OH on arginase activity in MDSCs. **D**, Effect of PGE₁-OH on Arg1 expression in MDSCs. **E**, Effect of the EP4 selective antagonist L-161,982 on suppressive activity of PMN-MDSCs. **F**, Quantitation data for Fig 6, E. **P* < .05 and ***P* < .01 (*n* = 4). DMSO, Dimethyl sulfoxide.

synthase expression in PMN-MDSCs was reduced by COX-1 deletion (see Fig E9, B), but NO metabolites in the lung tissue were not altered among the 3 groups (Fig 5, C), probably because of weak expression of inducible nitric oxide synthase. The levels of ROS (Fig 5, D) and p47^{phox}, which is responsible for their production (see Fig E9, C), did not display any changes in PMN-MDSC counts among the WT/aspirin, COX-1 KO, and WT groups. Administration of L-arginine or an arginase inhibitor (N ω -hydroxy-L-arginine [nor-NOHA]) completely abolished the differences in suppressive activity of PMN-MDSCs among the 3 groups (ie, WT, WT/aspirin, and COX-1 KO), whereas L-N^G-monomethyl arginine (L-NMMA), a specific inhibitor of nitric oxide synthase, did not display any significant effects compared with the dimethyl sulfoxide control group (Fig 5, E and F).

COX-1-mediated Arg1 expression depends on PGE₂-EP4/cyclic AMP/protein kinase A signaling in PMN-MDSCs

PGE₂ is one of the major PG products in PMN-MDSCs, as in BM cells (data not shown). As shown in Fig 6, A and B, arginase activity and Arg1 expression were markedly increased in PGE₂-treated cells compared with levels in the control group. In contrast to other EP antagonists, treatment with the selective EP4 antagonist L-161,982 caused significant suppression of arginase activity

and resulted in minimal Arg1 protein expression. The EP4 agonist PGE₁-OH, but not the EP1/EP3 agonist sulprostone, completely restored arginase activity and Arg1 expression of BM-derived PMN-MDSCs from COX-1 KO mice to levels comparable with those from WT mice (Fig 6, C and D). PGD₂ and TXA₂, the products of which were also generated by COX-1 during PMN-MDSC generation in BM, did not display any effect on arginase activity through their receptors (see Fig E10 in this article's Online Repository at www.jacionline.org). In support of the biological effects of PGE₂-EP4 signaling on MDSC function, administration of the EP4 antagonist L-161,982 clearly suppressed the function of PMN-MDSCs from WT mice, as evidenced by increased T-cell proliferation in an MDSC/T-cell coculture system (Fig 6, E and F).

Because the EP4 receptor has been reported to couple to Gs/cyclic AMP (cAMP)/protein kinase A (PKA) signaling,²² we next explored whether this signaling pathway was involved in COX-1-mediated Arg1 regulation. Ectopic expression of COX-1 in COX-1 KO BM-derived PMN-MDSCs remarkably increased both arginase activity and Arg1 expression, whereas administration of the PKA inhibitor H-89 or L-161,982 compromised these effects (Fig 7, A and B). The change in Arg1 expression was in concert with alterations in cAMP response element-binding protein (CREB) phosphorylation and intracellular cAMP levels (Fig 7, B-D) in PMN-MDSCs, supporting the notion that the EP4/cAMP/PKA/CREB signaling cascade participated in

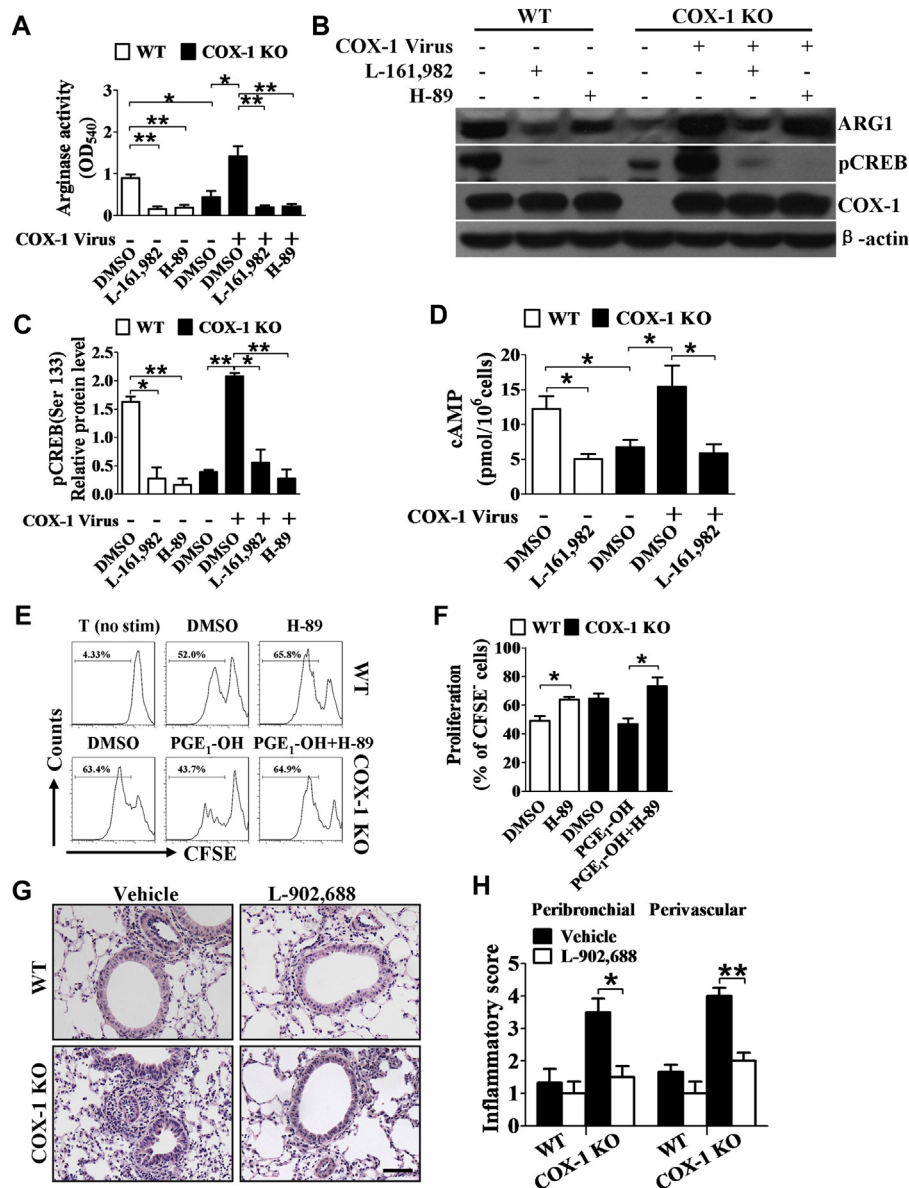


FIG 7. COX-1 contributes to the regulation of Arg1 expression through EP4/PKA/CREB signaling in PMN-MDSCs. **A** and **B**, Effect of L-161,982 and H-89 on arginase activity (Fig 7, **A**) and Arg1 expression (Fig 7, **B**) in BM-derived PMN-MDSCs ($n = 4$). **C**, Quantitative analysis of phospho-CREB in Fig 7, **B** ($n = 4$). **D**, Effect of L-161,982 on cellular cAMP levels in PMN-MDSCs from COX-1 and WT mice ($n = 6$). **E**, Effects of H-89 and PGE₁-OH on the immunosuppressive activity of PMN-MDSCs from OVA-challenged COX-1 KO and WT mice. **F**, Quantitative analysis of T-cell proliferation, as shown in Fig 7, **E** ($n = 4$). **G**, Effects of L-161,982 on OVA-induced AHR in COX-1 KO mice. **H**, Quantification of inflammatory scores of lung sections for Fig 7, **G**. * $P < .05$ and ** $P < .01$, as indicated ($n = 8-10$). Scale bar = 50 μ m.

COX-1-mediated Arg1 regulation. Accordingly, H-89 treatment restrained the suppressive activity of MDSCs from WT mice, as evidenced by increased CD4⁺ T-cell proliferation, whereas the effect of PGE₁-OH on the function of MDSCs from COX-1 KO mice was blocked by H-89 (Fig 7, **E** and **F**).

To further elucidate the role of PGE₂-EP4 signaling on PMN-MDSCs *in vivo* and in the pathogenesis of allergen-induced AHR in COX-1 KO mice, the EP4 selective agonist L-902,688 was administered to OVA-challenged mice. We observed that L-902,688 moderately ameliorated airway inflammation induced by OVA challenge in WT mice (Fig 7, **G** and **H**, and see Fig E11 in

this article's Online Repository at www.jacionline.org). Strikingly, L-902,688 administration significantly attenuated airway hyperreaction in COX-1 KO mice but not in WT mice, as evidenced by reduced inflammatory cell infiltration and T_H2 cytokine secretion in lungs (Fig 7, **G** and **H**, and see Fig E11).

Immunosuppressive function of PMN-MDSCs is impaired in patients with AIA

Next, we examined whether the immunosuppressive function of PMN-MDSCs is impaired in patients with AIA. PBMCs from

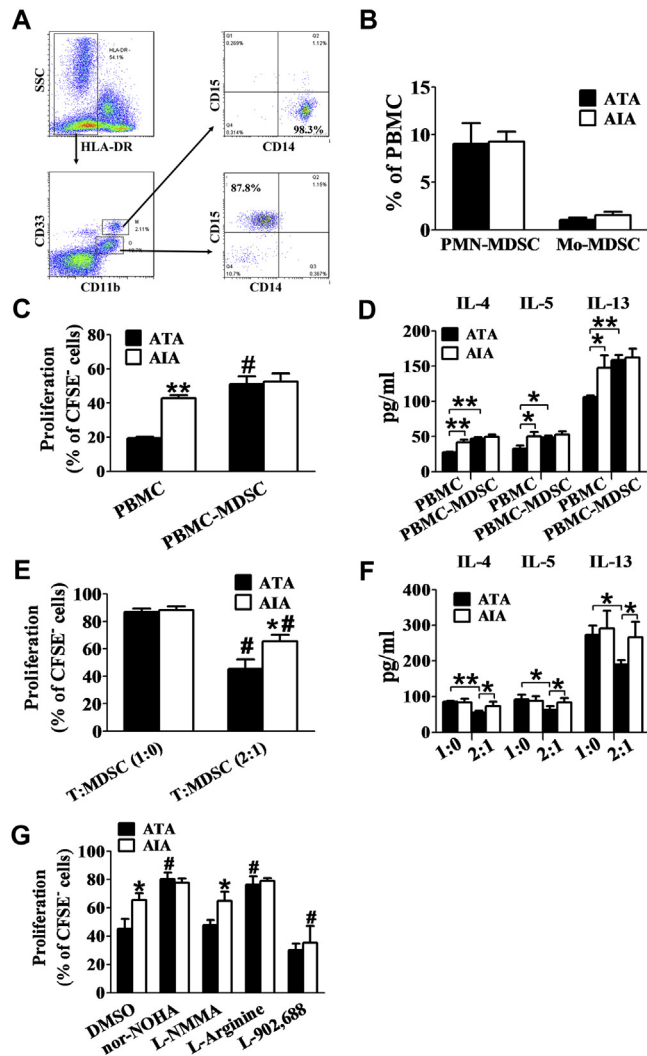


FIG 8. Immunosuppressive function of PMN-MDSCs is impaired in patients with AIA. **A**, Flow cytometric analysis of MDSCs from asthmatic patients. **B**, Frequency of PMN-MDSCs and Mo-MDSCs in PBMCs from patients with ATA and those with AIA. PBMCs or PBMCs with MDSC depletion (PBMC-MDSCs) were stimulated with PHA ($n = 6-11$). **C**, Proliferation of CD4⁺ T cells was examined by using CFSE dilution. ** $P < .01$ versus patients with ATA and # $P < .01$ versus PBMCs ($n = 6$). **D**, Cytokine production in culture from Fig 8, C. * $P < .05$ and ** $P < .01$ ($n = 6$). **E**, Suppressive effects of PMN-MDSCs from patients with ATA and those with AIA. * $P < .05$ versus patients with ATA and # $P < .01$ versus the T:MDSC (T cell/PMN-MDSC ratio [T:MDSC] 1:0) group ($n = 5$). **F**, T_H2 cytokine secretion from the above coculture. * $P < .05$ and ** $P < .01$ ($n = 5$). **G**, Effect of nor-NOHA, L-arginine supplementation, L-NMMA, and L-902,688 on suppressive function of PMN-MDSCs from both patients with ATA and those with AIA. * $P < .05$ versus patients with ATA and # $P < .05$ versus dimethyl sulfoxide (DMSO) control ($n = 5$).

patients with AIA and age-matched patients with aspirin-tolerant asthma (ATA; see Table E1 in this article's Online Repository at www.jacionline.org) were collected, and the subpopulations and frequencies of MDSCs were determined by using flow cytometry. Both HLA-DR⁺CD11b⁺CD33^{high}CD14⁺CD15⁺ (Mo-MDSC) and HLA-DR⁺CD11b⁺CD33^{int}CD14⁺CD15⁺ (PMN-MDSC) cells^{8,23} were clearly gated among PBMCs (Fig 8, A). PMN-MDSCs are the major population of human MDSCs in peripheral blood (9-fold greater than Mo-MDSCs). We did not observe differences in the proportions of PMN-MDSC and Mo-MDSC in

PBMCs between patients with AIA and those with ATA (Fig 8, B). Interestingly, CD4⁺ T-cell proliferation and T_H2 cytokine secretion after PHA stimulation in peripheral blood from patients with AIA was significantly increased compared with that seen in patients with ATA. This difference was completely abrogated by MDSC depletion (PBMC-MDSC; Fig 8, C and D, and see Fig E12, A, in this article's Online Repository at www.jacionline.org), suggesting the immunoregulatory function of MDSC in patients with AIA was impaired. These observations were further confirmed in an MDSC/T-cell coculture system, in which the suppressive effect of PMN-MDSCs was less pronounced in patients with AIA than that in patients with ATA, despite PMN-MDSCs from all patients efficiently suppressing the CD4⁺ T-cell responses after anti-CD3/CD28 stimulation (Fig 8, E and F, and see Fig E12, B). Consistent with the observations from mice that the suppressive function of PMN-MDSCs is dependent on arginase activity, the dissimilarity of PMN-MDSC function between patients with AIA and those with ATA vanished after supplementation with L-arginine or the arginase inhibitor nor-NOHA in MDSC/T-cell coculture experiments, whereas the nitric oxide synthase blocker L-NMMA displayed no effect (Fig 8, G). Moreover, administration of the EP4 agonist L-902,688 augmented the inhibitory capacity of PMN-MDSCs on T-cell proliferation and diminished the difference between patients with AIA and those with ATA (Fig 8, G).

DISCUSSION

Accumulation of MDSCs was discovered in the circulation, lymph nodes, and BM and at tumor tissues in most patients with cancer.²⁴ They inhibit both adaptive and innate immunity and subsequently promote tumor escape from immune surveillance and facilitate tumor growth.²⁵ In allergy challenge models robust expansion of MDSCs was observed in immune organs and BM despite 30% to 40% reduction through COX-1 inhibition or genetic deletion. Consistent with other studies,^{26,27} adoptive transfer of MDSC subsets clearly suppressed AHR to OVA allergen, and replacement of defective MDSCs in COX-1 null mice by BM transplantation ameliorated pulmonary symptoms and blocked IL-13 and IL-4 secretion in BALF, supporting the notion that MDSCs can suppress T_H2 responses in asthmatic patients. Strikingly, a PMN-MDSC subset that infiltrated the lungs was associated negatively with pulmonary inflammation in this OVA challenge model. Thus the differential functional manifestations of MDSCs in asthmatic and tumorigenic environments might explain partially the epidemiologic findings that the risk of most common cancers, such as stomach and brain cancers, is reduced in asthmatic patients with increased T_H2 immunity.²⁸⁻³⁰

Despite the complexity of AIA pathogenesis, resultant symptoms appear to be caused by the aberrant expansion of T_H2 cells and increased secretion of T_H2 cytokines (eg, IL-13) in the airways.³¹ In patients with AIA, PGE₂ generation in response to proinflammatory stimuli by fibroblasts from both bronchioles³² and nasal mucosal tissue^{17,33} was markedly suppressed. In rodents with allergic airway inflammation, blockage of PGE₂ production by genetic deletion of the COX-1 enzyme^{17,18} or pharmacologic inhibition by NSAIDs led to an increase in AHR, which depends on T_H2 cytokines. Interestingly, decreased EP2 receptor expression was reported in immune cells from patients with AIA,³⁴ and polymorphisms in EP2 and other EP receptor genes are associated with AIA.³⁵⁻³⁷ Consistent with these findings, we found PGE₂ derived from COX-1 plays an important

role in MDSC expansion in BM through EP2 and EP4 receptors. Activation of both EP2 and EP4 heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors could liberate G protein $\beta\gamma$ subunits and activate the phosphoinositide 3-kinase pathway, which is likely to be a vital signaling event in generation of MDSCs in BM.³⁸ Moreover, the immunosuppressive function of PMN-MDSCs is dramatically reduced in aspirin-treated/COX-1-deficient mice and in patients with AIA, which is also correlated with arginase activity in PMN-MDSCs. Moreover, COX-1 expression was downregulated in nasal/bronchial epithelial cells and fibroblasts from patients with AIA.^{32,39} We further demonstrated that COX-1-derived PGE₂ governs Arg1 expression predominantly through an EP4/cAMP/PKA/CREB pathway in BM-derived PMN-MDSCs, which are analogous to BM-derived macrophages⁴⁰ and MDSCs in the tumor microenvironment.⁴¹ An EP4 agonist upregulates Arg1 expression in PMN-MDSCs and enhances the effect of PMN-MDSCs on T-cell suppression but also ameliorates allergen-induced AHR by COX-1 disruption in mice, further supporting that EP4 might be a therapeutic target for asthma, particularly AIA. However, we did not observe the decreased population of PMN-MDSCs in patients with AIA, as seen in aspirin-treated/COX-1-deficient mice, probably because of lack of ingestion of aspirin or other NSAIDs by the patients at the time of sampling.

In summary, we demonstrated that the immunosuppressive function of PMN-MDSCs was impaired in allergy-induced airway inflammation evoked by aspirin treatment or COX-1 gene disruption in mice and in patients with AIA through downregulation of EP4-mediated phosphorylation of CREB and Arg1 expression, which subsequently led to increased T_H2 cell activation. Infusion of normal PMN-MDSCs, BM transplantation, and EP4 agonist treatment could alleviate the allergy-induced AHR evoked by COX-1 deletion in mice. These findings might open new approaches and enhance therapeutic options in asthmatic patients, particularly patients with AIA.

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Key messages

- The immunosuppressive function of PMN-MDSCs was impaired in an allergic airway inflammation mouse model by COX-1 inhibition, and in AIA patients.
- This likely occurs through downregulation of Arg1 expression by suppressing PGE₂/EP4/cAMP/PKA/CREB signaling.

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METHODS

Mice

Six- to 8-week-old female mice were used in all experiments in the study. All mice were maintained on a mixed C57BL/6×Sv129 genetic background for more than 20 generations in pathogen-free conditions. All procedures were approved by the Institutional Animal Care and Use Committee of the Institution for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China.

Study population

Adult asthmatic patients were recruited from outpatients in the Department of Pulmonary Medicine of Shanghai Ruijin Hospital from August 2012 to May 2013 (Table E1). All patients were given diagnoses based on the criteria for asthma according to the Global Initiative for Asthma guidelines. All patients with AIA had a clinical record of recurrent attacks induced by the ingestion of aspirin or other NSAIDs, which was confirmed based on the results of a modified oral aspirin challenge test performed before this study.^{E1} Briefly, doubling doses (40, 80, 160, and 320 mg) of aspirin were administered every 3 hours. FEV₁ was recorded every 10 minutes after administration. AIA was defined as when FEV₁ decreased 20% or more from the baseline. All subjects recruited had a more than 5-year history of diagnosed asthma. Subjects with respiratory tract infection within 4 weeks, ischemic cardiovascular disease, renal/liver dysfunction, tumor, or autoimmune disease were excluded from the study. This study was approved by the Ethics Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and the Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. All subjects provided written consent.

Allergy-induced airway inflammation models in mice

Mice were sensitized intranasally 4 times with Ef-OVA (InvivoGen, San Diego, Calif) and low-dose LPS (Sigma-Aldrich, St Louis, Mo; Ef-OVA_{LPS}; 50 µg of OVA combined with 0.1 µg of LPS in 25 µL of PBS) on days 0, 1, 2, and 7 and then challenged 4 times intranasally with Ef-OVA (50 µg in 25 µL of PBS) on days 14, 15, 18, and 19. For the traditional allergy-induced airway inflammation model (mice sensitized and challenged with OVA [OVA/OVA; Sigma]), mice were intraperitoneally sensitized with 50 µg of OVA (Grade V, Sigma-Aldrich) emulsified in 10 mg of aluminum hydroxide (200-µL total volume, Sigma-Aldrich) on days 0 and 7, followed by intranasal challenge with OVA (50 µg in 50 µL of PBS) once on days 14, 15, and 16. Two days after the last challenge, mice were killed for further analysis. Aspirin was administered to mice in drinking water (100 mg/L) in the indicated group.

Flow cytometric analysis

Cells were stained with fluorochrome-conjugated antibodies, according to the manufacturer's protocols. Flow cytometry was performed with a BD FACSAria flow cytometry system (BD Biosciences, San Jose, Calif), and data were analyzed with FlowJo software (Tree Star, Ashland, Ore). The fluorescent-conjugated anti-mouse antibodies fluorescein isothiocyanate-CD11b (M1/70), allophycocyanin (APC)-CD11b (M1/70), phycoerythrin (PE)-Gr1 (RB6-8C5), APC-Gr1 (RB6-8C5), PE-Ly6C (HK1.4), and PE-Cy5-CD4 (GK1.5) and their corresponding isotype controls were purchased from eBioscience (San Diego, Calif). APC-Ly6G (1A8) and PE-Cy7-Ly6G (1A8) were purchased from BD Biosciences. The following fluorescent-conjugated anti-human antibodies were purchased from eBioscience: APC-CD11b (ICRF44), PE-HLA-DR (L243), fluorescein isothiocyanate-CD33 (WM53), PE-Cy7-CD14 (61D3), eFluor450-CD15 (HI98), and APC-CD4 (OKT4) and their corresponding isotype controls.

BM transplantation

Allogeneic BM transplantation was conducted, as previously described.^{E2} Briefly, donor WT and COX-1 KO mice were killed, and BM cells were

collected from femurs and tibias by means of flushing. Recipient mice were lethally irradiated with a total of 9.5 Gy of total body irradiation administered in 3 bursts (one 3.5-Gy dose and two 3-Gy doses administered 1.5 hours apart) from a ¹³⁷Cs source (MDS Nordion, Ottawa, Ontario, Canada), and then 5 × 10⁶ BM cells from donor mice in 200 µL of sterilized PBS were injected through the tail vein to reconstitute the hematopoietic system.

Isolation of MDSCs from lungs

Lungs of allergic mice were separated and minced on ice and then incubated with 0.5 mg/mL collagenase type 4 (Worthington Biochemical, Lakewood, NJ) in RPMI 1640 medium (Life Technologies, Grand Island, NY) in a volume of 15 mL per lung for 2 hours at 37°C with continuous agitation in an incubator. The crude suspensions were further filtered through 70-µm cell strainers to obtain single-cell suspensions. Single-cell suspensions from lungs were fractionated by means of Percoll (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. CD11b⁺Gr1^{high} cells were subsequently isolated through flow cytometric sorting.

Adoptive transfer of PMN-MDSCs

CD11b⁺Gr1^{high} cells from the spleens of allergic mice were purified by means of flow cytometric cell sorting. For adoptive transfer, 2 × 10⁶ isolated cells were injected through the tail vein 2 days before OVA challenge (Fig 2, A, and see Fig E4, A).

Airway resistance analysis in mice

Airway responsiveness to acetylcholine chloride was evaluated with an AniRes 2005 mouse lung function analysis system (SYNOL High-Tech, Beijing, China) 24 hours after the last OVA challenge. Mice were anesthetized with chloral hydrate (400 mg/kg, Sigma-Aldrich) 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 at baseline and after intravenous administration of different doses of acetylcholine chloride (200 or 400 µg/kg, Sigma-Aldrich) through tail vein injection. The signals of lung resistance were collected continuously, and the maximal values for airway resistance were expressed as the fold change relative to baseline.

In vitro generation of MDSCs from BM cells

BM cells were obtained from the femurs and tibias of mice and cultured in 24-well plates in RPMI 1640 medium containing 10% FBS (HyClone Laboratories, Logan, Utah). The medium was supplemented with 50 µmol/L 2-mercaptoethanol (Life Technologies), 10 ng/mL IL-4 (PeproTech, Rocky Hill, NJ), and 20 ng/mL GM-CSF (PeproTech). After 5 days of culture, cells were harvested, and the proportion of MDSCs was analyzed by using flow cytometry. PGE₂ (2 µmol/L), PGD₂ (2 µmol/L), and different selective antagonists for PGE₂ receptors (SC-51322, 5 µmol/L; AH 6809, 2 µmol/L; L-798,106, 10 µmol/L; and L-161,982, 1 µmol/L) were added in the culture, as indicated, to test the role of different PGs on MDSC generation.

Analysis of *in vitro* immunosuppressive function of murine PMN-MDSCs

CD4⁺ T cells were isolated from naive BALB/c mouse splenocytes by using a T-cell enrichment column (R&D Systems, Minneapolis, Minn) and then stained with 1 µmol/L 5(6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience), according to the manufacturer's instructions. Ly-6G⁺ PMN-MDSCs were purified from spleens of allergic mice by using the mouse the MDSC MicroBeads isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CFSE-labeled purified CD4⁺ T cells were seeded in 96-well plates at 2 × 10⁵ cells per well and cocultured with PMN-MDSCs (1:1 ratio) in the presence of 1 µg/mL each of plate-bound anti-CD3e (17A2, eBioscience) and soluble anti-CD28 (37.51, eBioscience) mAbs for 72 hours. Cells were pre-treated with 10 µmol/L nor-NOHA (Cayman Chemical, Ann Arbor, Mich), 10 µmol/L L-NMMA (Cayman Chemical), or 1 mmol/L L-arginine (Sigma-Aldrich), as indicated, and then stained with PE-Cy5-conjugated CD4

antibody and analyzed by using flow cytometry for CFSE dilution of CD4⁺ T cells.

Human PBMC isolation, T-cell proliferation assay, and measurement of ROS, NO production, and arginase activity

Preparation of PBMCs, T-cell proliferation assay, and measurement of ROS, NO production, and arginase activity were described previously.^{E3} Different selective antagonists for PGE₂ receptors (SC-51322, 5 μmol/L; AH 6809, 2 μmol/L; L-798,106, 10 μmol/L; and L-161,982, 1 μmol/L), the EP1/3 agonist sulprostone (10 μmol/L), the EP4 agonist PGE₁-OH (10 μmol/L), and the PKA inhibitor H-89 (10 μmol/L) were applied in the culture for 24 hours, as indicated, to examine whether PGE₂ regulates arginase activity in BM-derived MDSCs.

Lung histology and immunofluorescence microscopy

Lungs were fixed in 4% neutral paraformaldehyde (Sigma-Aldrich), 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 assessed, as previously described^{E4}: 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 magnification by examining at least 40 consecutive fields.

Both CD11b and Gr1 antibodies (diluted 1:100) were used for immunofluorescence staining to determine MDSC counts in inflamed lungs. Images were captured with a Zeiss LSM510 META Laser Scanning Confocal Microscope (Carl Zeiss, Oberkochen, Germany).

Preparation of lentivirus expressing COX-1

A COX-1 expression cassette was cloned into FG12 between the *Xba*I and *Xho*I sites to construct the COX-1 overexpression lentiviral vectors. The correct construct was confirmed by means of DNA sequencing. HEK-293 T cells were cotransfected with appropriate amounts of vector plasmid, the lentiviral packaging constructs pRSVREV and pMDLg/pRRE, and the VSV-G expression plasmid pHCMVG. The viral supernatants were harvested on days 2 and 3 after transfection and then filtered with 0.4-μm filters and stored at −80°C. For COX-1 overexpression, BM cells (2 × 10⁶ cells/well) were infected with COX-1 or green fluorescent protein viral preparations in the presence of 8 μg/mL Polybrene (Santa Cruz Biotechnology, Dallas, Tex) overnight. After viral incubation, medium was replaced with 2 mL of fresh RPMI 1640 supplemented with 10% FBS, 50 μmol/L 2-mercaptoethanol, 10 ng/mL IL-4, and 20 ng/mL GM-CSF. The efficiency of infection was determined by means of Western blotting for COX-1 expression.

Endotoxin measurement

Endotoxin levels in OVA samples were detected by using the Limulus amoebocyte lysate assay with the Chromogenic LAL Endotoxin Assay Kit (GenScript USA, Piscataway, NJ), according to the manufacturer's instructions.

ELISA

Cytokine levels in BALF supernatants and cell-culture media were assayed by using ELISA, according to the manufacturer's instructions (R&D Systems).

LTB₄ measurement in lungs of allergic mice

Lungs of allergic mice were homogenized in ice-cold PBS by using steel beads. Residual tissue was separated by using centrifugation, and the supernatant was collected. LTB₄ levels in supernatants were measured with a Leukotriene B₄ EIA Kit (Cayman Chemical) and normalized to the concentration of soluble protein.

Routine laboratory experiments

Detection of cellular cAMP, RNA preparation, quantitative RT-PCR, Western blot analysis, and PG profiling were described previously.^{E5} The primers for quantitative RT-PCR are summarized in Table E2.

Statistical analysis

Data analysis was performed with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, Calif). Results were expressed as means ± SEMs 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 .05 were considered statistically significant.

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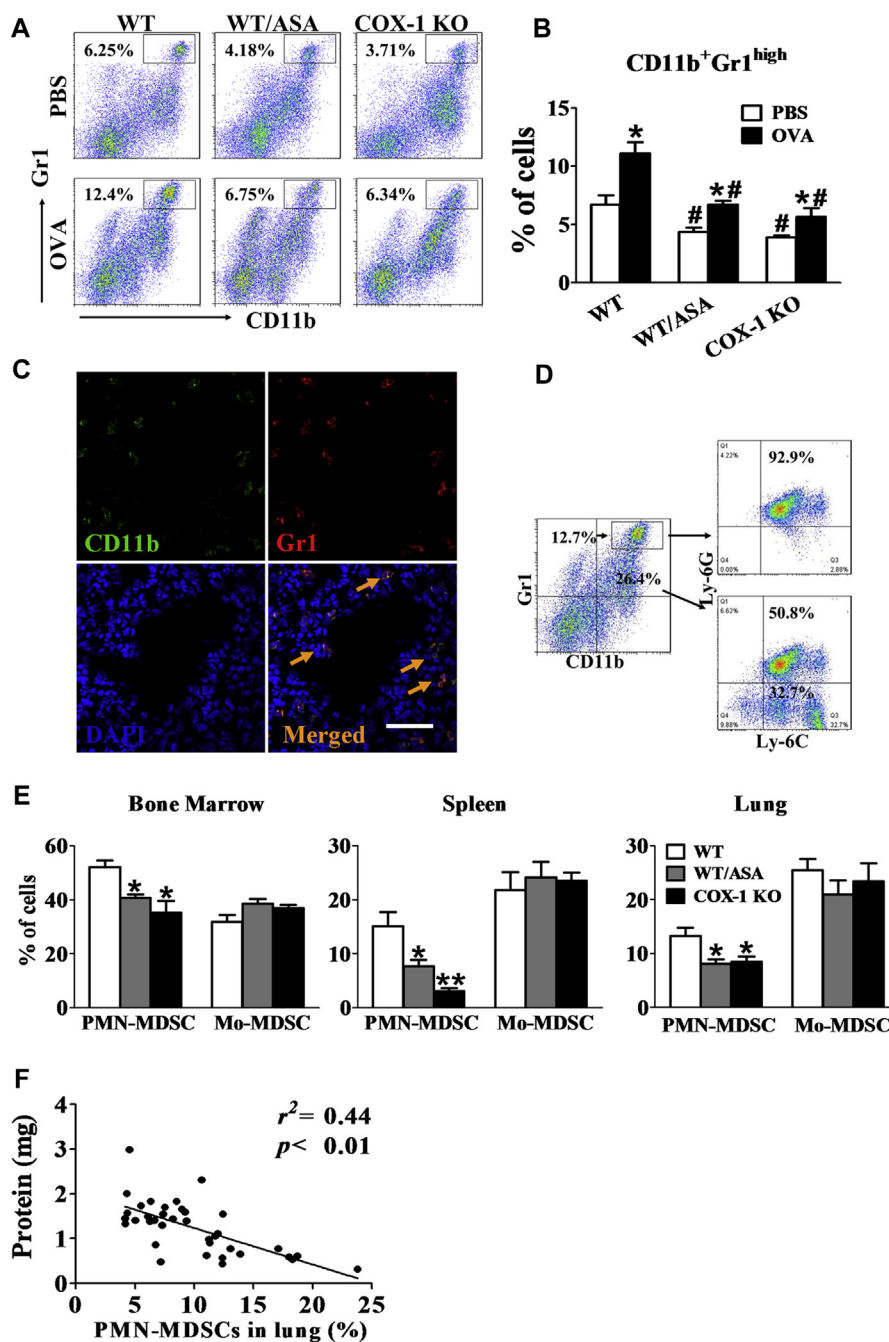


FIG E1. Characterization of MDSCs accumulated in OVA/OVA mice: effects of aspirin (ASA) treatment and COX-1 deficiency. **A** and **B**, Representative flow charts (Fig E1, A) and quantitation of CD11b⁺Gr1^{high} cells (Fig E1, B) in the lung are shown. * $P < .05$ versus the PBS-challenged group and # $P < .05$ versus the WT group ($n = 10-15$). **C**, Representative immunofluorescent micrographs of MDSCs in lung sections from allergic mice. Scale bar = 50 μ m. **D**, Characteristic analysis of CD11b⁺Gr1^{high} MDSCs. **E**, MDSC subsets in the BM, spleens, and lungs of OVA-challenged mice. * $P < .05$ and ** $P < .01$ versus WT ($n = 10-15$). **F**, Correlation analysis of infiltrated PMN-MDSCs in the lung with BALF total protein from OVA/OVA mice.

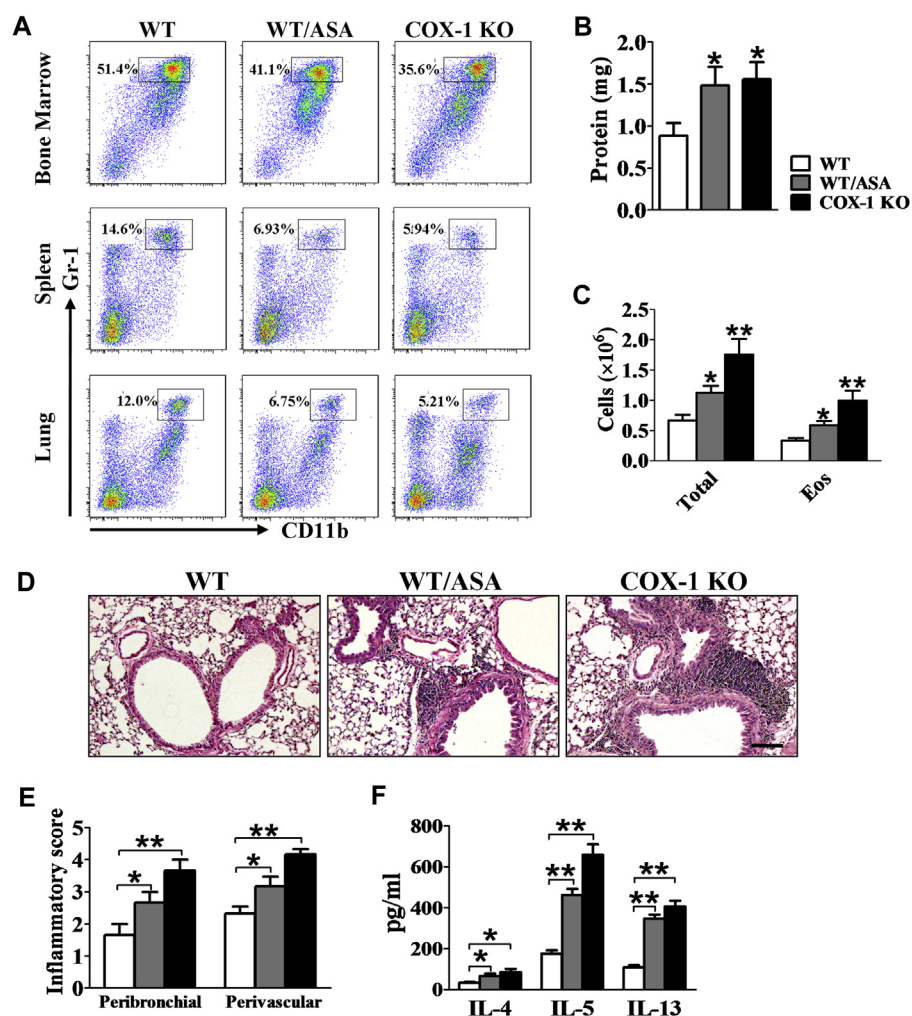


FIG E2. Aspirin (ASA) treatment or COX-1 deletion aggravated airway inflammation in Ef-OVA_{LPS}/Ef-OVA mice. **A**, Representative flow charts for CD11b⁺Gr1^{high} cells in BM, spleens, and lungs of allergic mice. **B** and **C**, Protein secretion (Fig E2, B) and inflammatory cell recruitment (Fig E2, C) in BALF of Ef-OVA_{LPS}/Ef-OVA mice. **P* < .05 and ***P* < .01. *Eos*, Eosinophils. **D**, Representative hematoxylin and eosin staining of lung sections from Ef-OVA-challenged WT, WT/aspirin, and COX-1 KO mice. Scale bar = 50 μm. **E**, Quantitation of infiltrated inflammatory cells in peribronchial and perivascular areas in Fig E2, D. **P* < .05 and ***P* < .01. **F**, T_H2 cytokines in BALF from Ef-OVA_{LPS}/Ef-OVA mice. **P* < .05 and ***P* < .01 (n = 8-10).

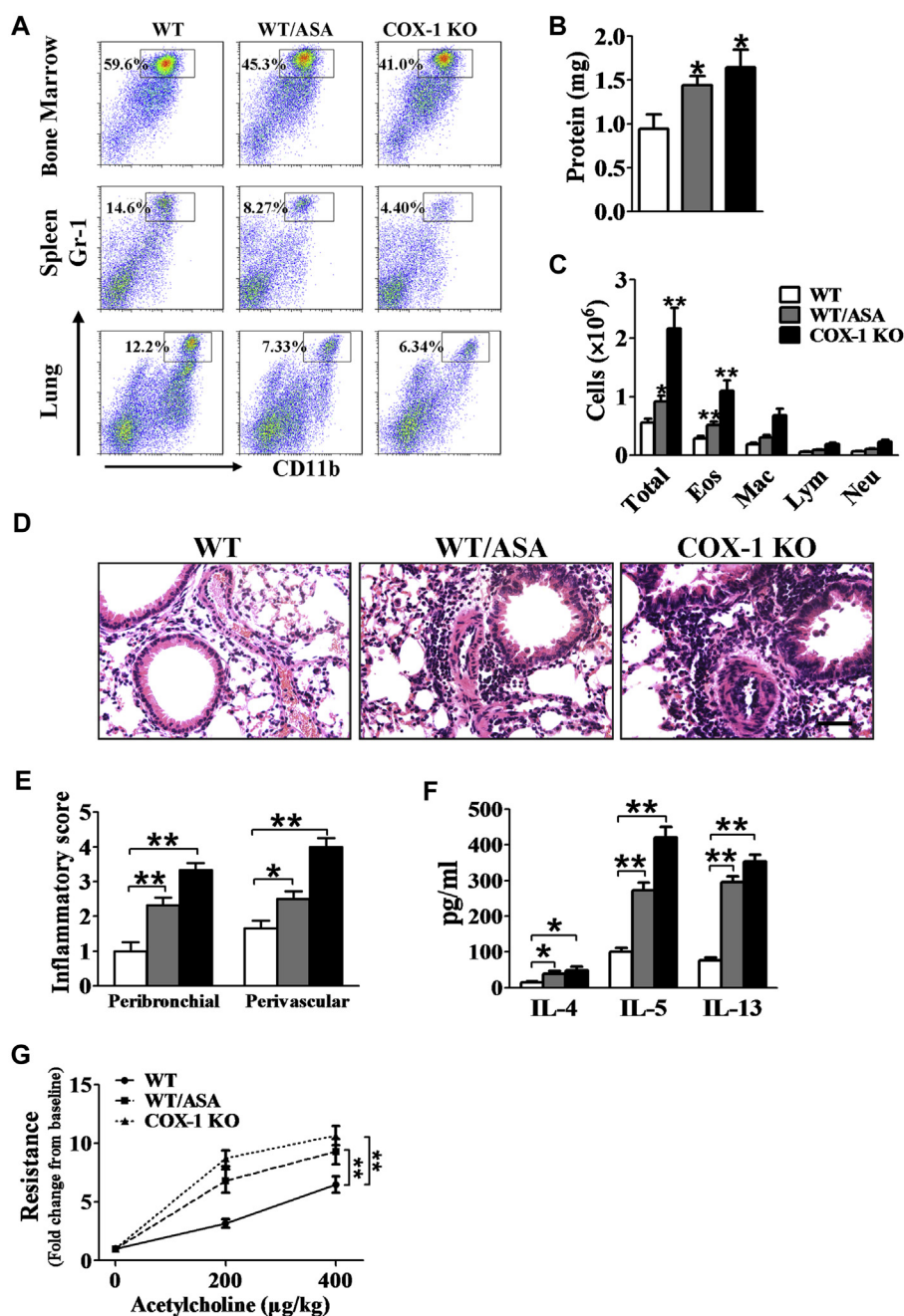


FIG E3. Aspirin (ASA) treatment or COX-1 deletion aggravated airway inflammation in OVA/OVA mice. **A**, Representative flow charts for PMN-MDSCs in BM, spleens, and lungs of allergic mice. **B** and **C**, Protein secretion (Fig E3, **B**) and inflammatory cell recruitment (Fig E3, **C**) in BALF of OVA/OVA mice. $*P < .05$ and $**P < .01$ ($n = 10-15$). *Eos*, Eosinophils; *Lym*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils. **D**, Representative hematoxylin and eosin staining of lung sections from OVA-challenged WT, WT/aspirin, and COX-1 KO mice. Scale bar = 50 μ m. **E**, Quantitation of infiltrated inflammatory cells in peribronchial and perivascular areas in Fig E3, **D**. $*P < .05$ and $**P < .01$ ($n = 10-15$). **F**, Secretion of T_H2 cytokines in BALF in OVA/OVA mice. $*P < .05$ and $**P < .01$ ($n = 10-15$). **G**, Airway resistance was monitored in response to acetylcholine stimulation in OVA-challenged WT, WT/aspirin, and COX-1 KO mice. $**P < .01$ versus the WT group ($n = 10-15$).

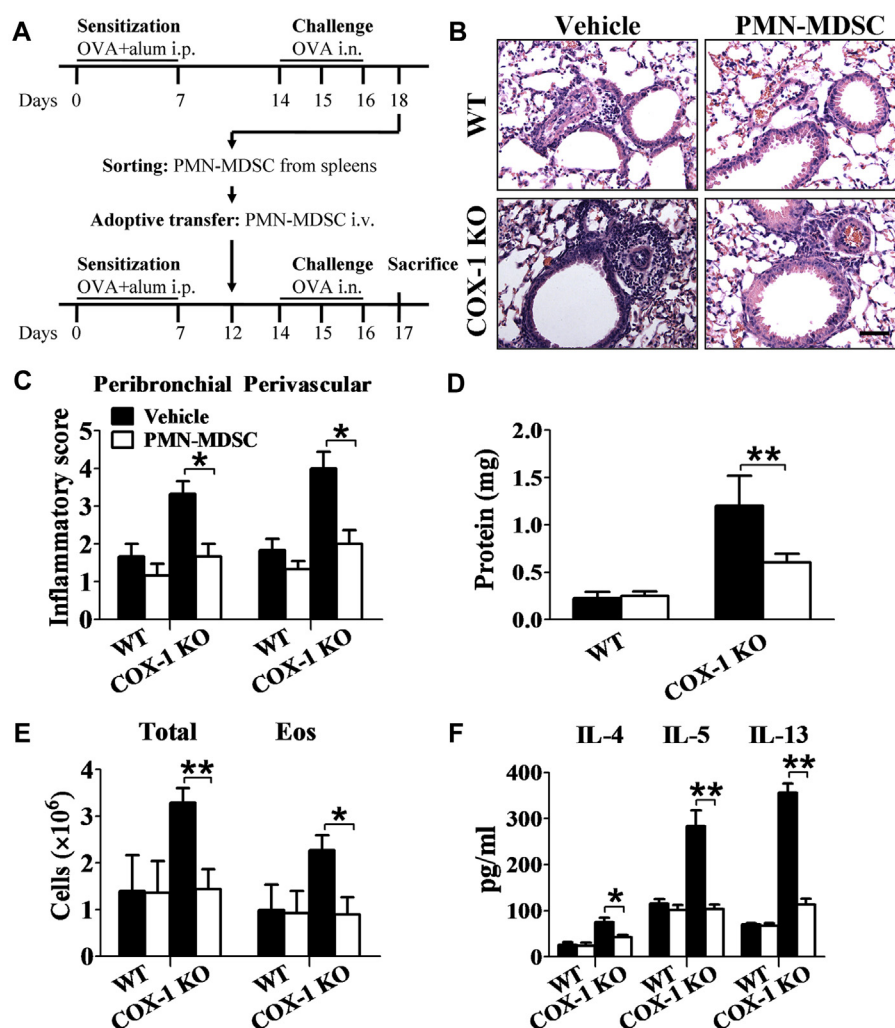


FIG E4. Adoptive transfer of PMN-MDSCs alleviates COX-1 deficiency-mediated AHR in OVA/OVA mice. **A**, Schematic for protocol of administration of PMN-MDSCs to OVA-challenged mice. *i.n.*, Intranasal; *i.p.*, intraperitoneal. **B** and **C**, Representative hematoxylin and eosin staining (Fig E4, **B**) and quantification of inflammatory scores (Fig E4, **C**) of lung sections from mice undergoing adoptive transfer with PMN-MDSCs and challenged with OVA. Scale bar = 50 μ m. **D** and **E**, Protein secretion (Fig E4, **D**) and inflammatory cell recruitment (Fig E4, **E**) in BALF from mice undergoing adoptive transfer. **F**, Cytokines secreted in BALF from mice undergoing adoptive transfer. * $P < .05$ and ** $P < .01$ ($n = 8-10$).

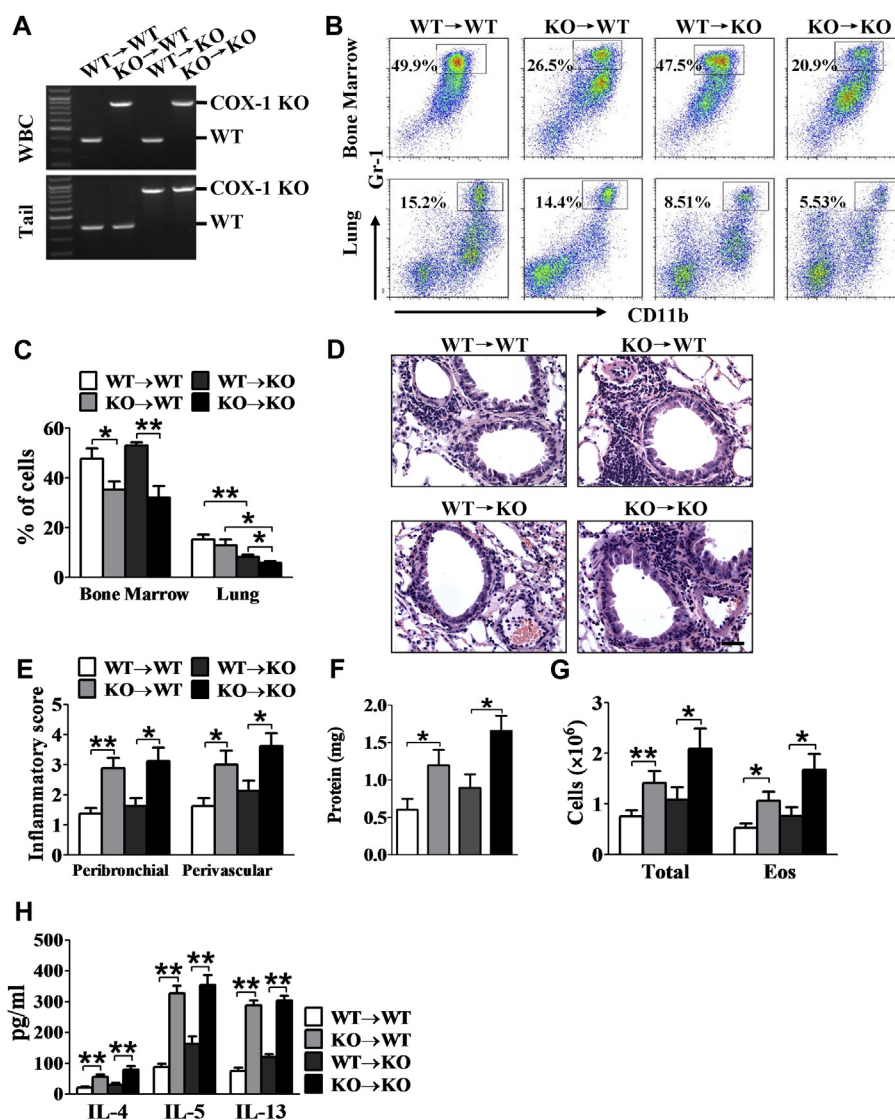


FIG E5. BM transplantation alleviates COX-1 deficiency-mediated AHR in OVA/OVA mice. **A**, BM transplantation between WT and COX-1 KO mice was confirmed by using genotyping. **B** and **C**, Representative flow charts (Fig E5, **B**) and quantitation of PMN-MDSCs (Fig E5, **C**) in BM and lungs of allergic mice. **D**, Representative hematoxylin and eosin staining of lung sections. Scale bar = 50 μm. **E**, Infiltrated inflammatory cells in peribronchial and perivascular areas. **F-H**, Protein secretion (Fig E5, **F**), inflammatory cell recruitment (Fig E5, **G**), and T_H2 cytokine secretion (Fig E5, **H**) in BALF. * $P < .05$ and ** $P < .01$ ($n = 8-10$).

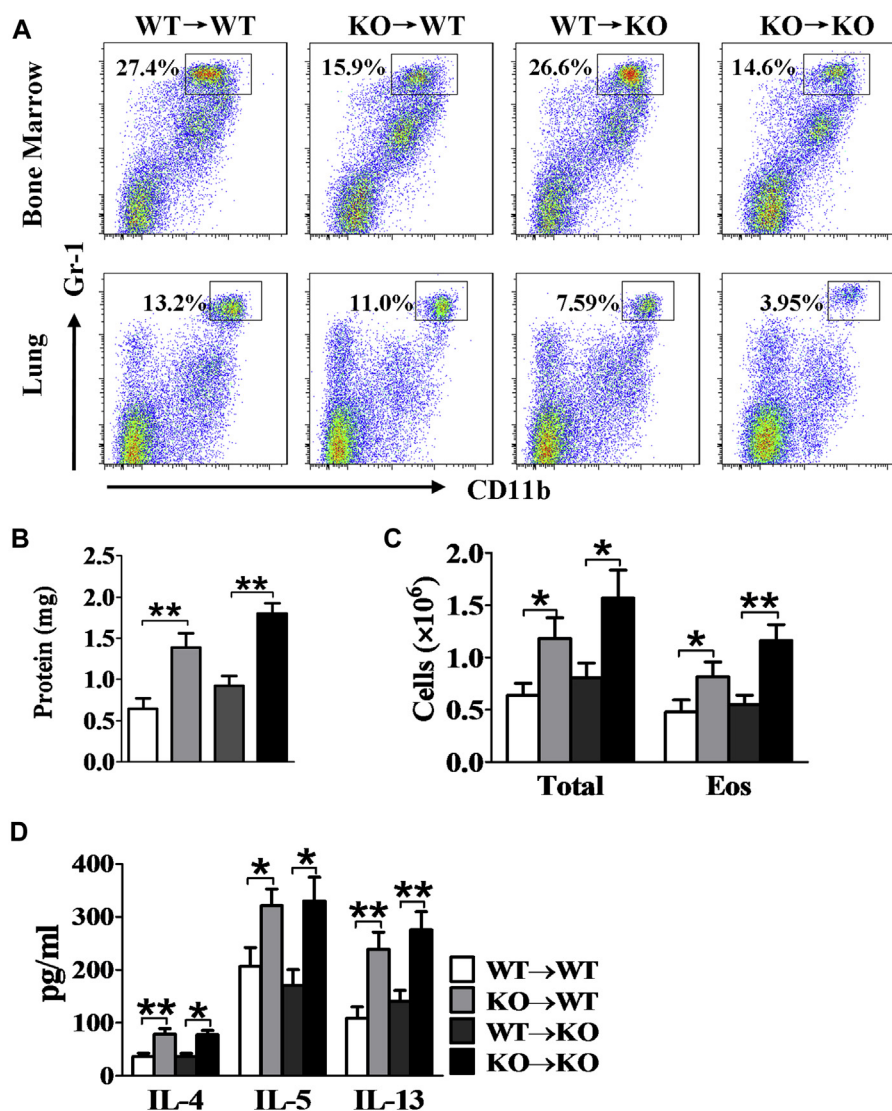


FIG E6. BM transplantation alleviates COX-1 deficiency-mediated AHR in Ef-OVA_{LPS}/Ef-OVA mice. **A**, Representative flow charts for PMN-MDSCs in BM and lungs of BM-transplanted Ef-OVA_{LPS}/Ef-OVA mice. **B** and **C**, Total protein (Fig E6, **B**) and total cells and eosinophil infiltration (Fig E6, **C**) in BALF of BM-transplanted Ef-OVA_{LPS}/Ef-OVA mice. * $P < .05$ and ** $P < .01$, as indicated. **D**, Cytokine (IL-4, IL-5, and IL-13) production in BALF from BM-transplanted mice was measured by using ELISA. * $P < .05$ and ** $P < .01$ ($n = 6-8$).

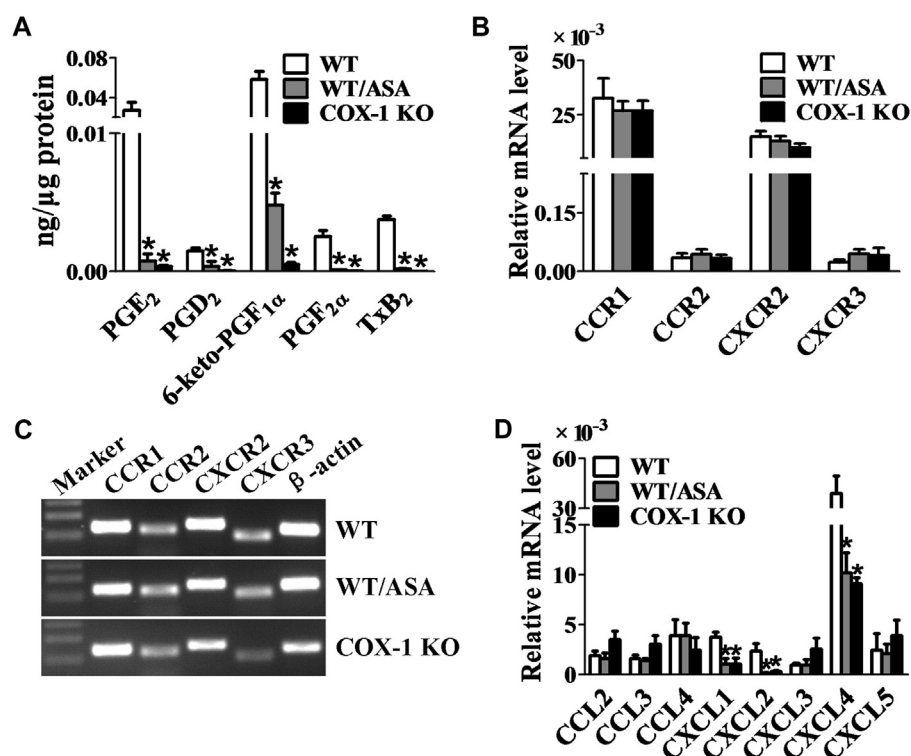


FIG E7. Diminished PMN-MDSC-associated chemokine expression in lungs from aspirin (ASA)-treated and COX-1-deficient mice. **A**, PG profile in lungs from OVA-challenged WT, WT/aspirin, and COX-1 KO mice. * $P < .01$ versus WT ($n = 8010$). **B-D**, Gene expression for chemokine receptors of spleen PMN-MDSCs (Fig E7, *B* and *C*) and lungs (Fig E7, *D*) from OVA-challenged WT, WT/aspirin, and COX-1 KO mice were analyzed by using quantitative RT-PCR and normalized to β -actin. Fig E7, *C*, Representative image of agarose gel chemokine receptor quantitative RT-PCR products. * $P < .05$ versus WT ($n = 6$).

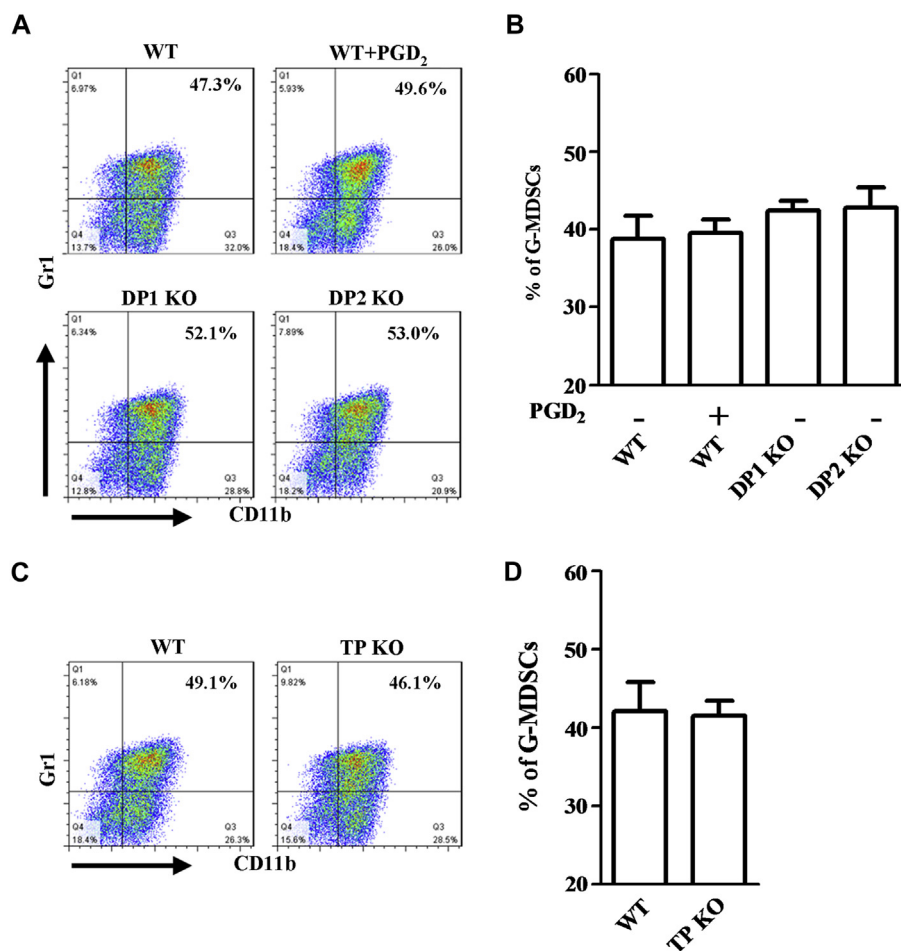


FIG E8. PGD₂/DP receptor signaling and TXA₂/TP receptor signaling are not involved in the regulation of PMN-MDSC expansion in BM. **A** and **B**, Representative flow charts for CD11b⁺Gr1⁺ cells (Fig E8, A) and quantitative data (Fig E8, B) for CD11b⁺Gr1⁺Ly6G⁺Ly6C⁺ PMN-MDSCs from GM-CSF- and IL-4-stimulated BM cells isolated from DP1 KO, DP2 KO, and WT mice. **C** and **D**, Representative flow charts for CD11b⁺Gr1⁺ cells (Fig E8, C) and quantitative data for CD11b⁺Gr1⁺Ly6G⁺Ly6C⁺ PMN-MDSCs (Fig E8, D) from GM-CSF- and IL-4-stimulated BM cells isolated from TP KO and WT mice (n = 4).

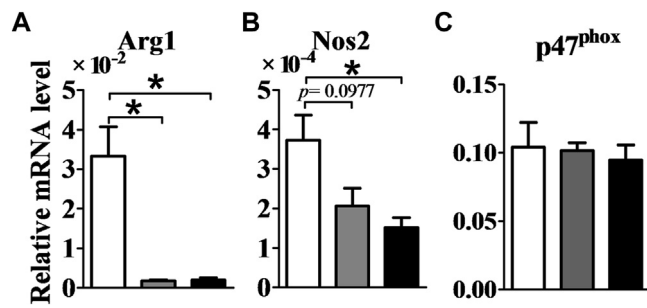


FIG E9. Expression of immunosuppressive factors of PMN-MDSCs in OVA-challenged WT, WT/aspirin, and COX-1 KO mice. Expression of Arg1 (**A**), inducible nitric oxide synthase (*Nos2*; **B**), and p47^{phox} (**C**) in spleen PMN-MDSCs from OVA-challenged WT, WT/aspirin, and COX-1 KO mice analyzed by using quantitative RT-PCR. Cycle threshold values were normalized to β-actin. **P* < .05 versus the WT group (n = 4).

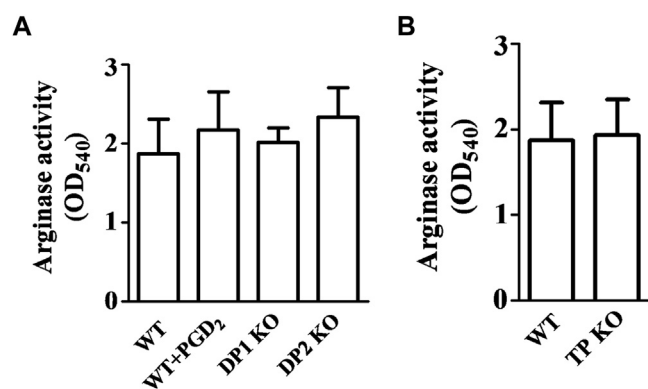


FIG E10. DP1, DP2, and TP receptors are not involved in regulation of Arg1 activity in PMN-MDSCs. BM cells from WT, DP1 KO, DP2 KO, and TP KO mice were cultured in 24-well plates in complete medium supplemented with GM-CSF and IL-4 for 5 days. WT cells were treated with 2 μ mol/L PGD₂ or left untreated. The cells were lysed, and arginase activity was measured, as described in the Methods section (n = 4).

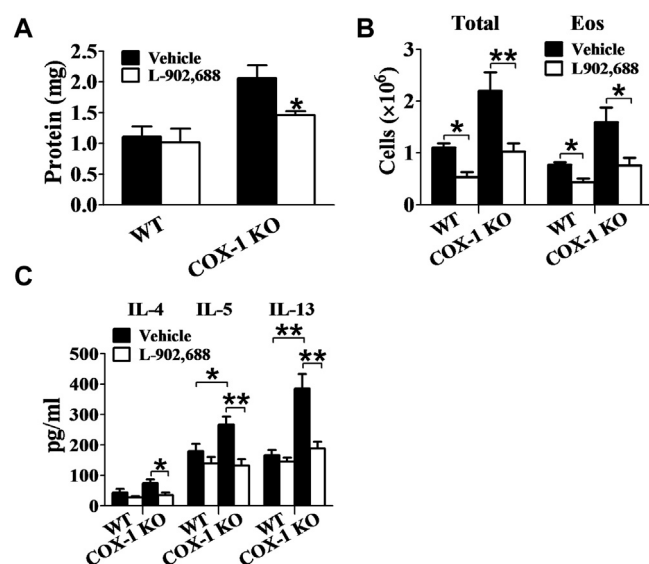


FIG E11. EP4 agonist L-902,688 administration alleviates OVA-induced AHR in COX-1 KO mice. OVA-challenged COX-1 KO and WT control animals were administered a selective EP4 agonist L-902,688, as described in the Methods section. **A** and **B**, Protein (Fig E11, **A**) and total cells and eosinophil (*Eos*) infiltration (Fig E11, **B**) in BALF from L-902,688-treated COX-1 KO and WT mice were quantitated. * $P < .05$ and ** $P < .01$, as indicated ($n = 8-10$). **C**, Cytokine (IL-4, IL-5, and IL-13) production in BALF from L-902,688-treated COX-1 KO and WT mice. * $P < .05$ and ** $P < .01$ ($n = 8-10$).

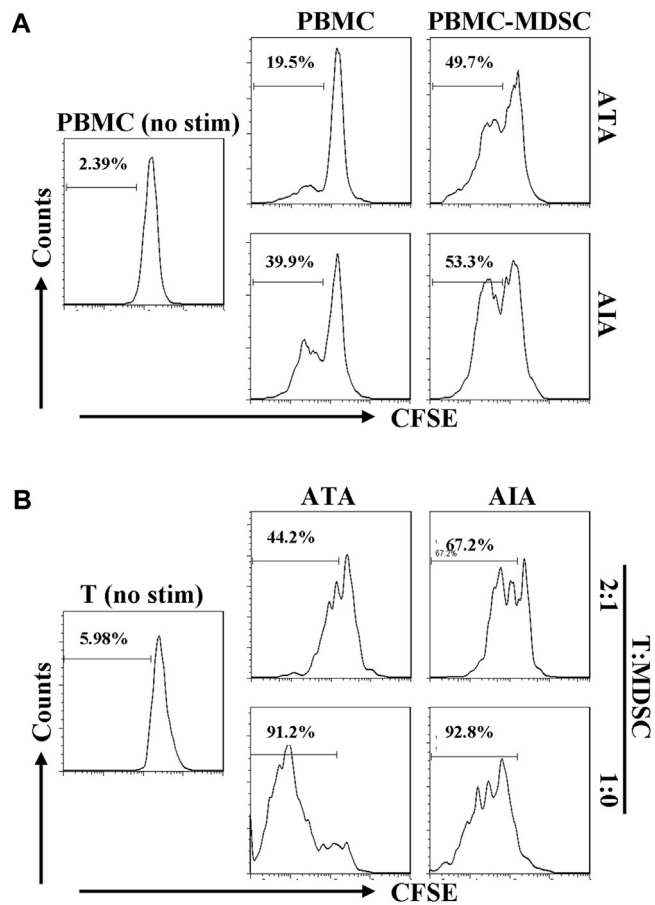


FIG E12. Immunosuppressive function of PMN-MDSCs is impaired in patients with AIA. **A**, PBMCs or PBMC-MDSCs from donors were stimulated with PHA. Representative flow charts for proliferation of CD4⁺ T cells are shown. **B**, CD4⁺ T cells were stimulated with anti-CD3/CD28 antibodies and then cocultured with PMN-MDSCs from the same donors for 3 days, and T-cell proliferation was evaluated by means of CFSE labeling. Representative flow charts for proliferation of CD4⁺ T cells are shown.

TABLE E1. Clinical characteristics of asthmatic patients

	Patients with ATA	Patients with AIA	<i>P</i> value
Male/female sex (no.)	4/7	2/4	.90
Age (y)	52.3 ± 4.6	60.7 ± 3.6	.24
Duration of asthma (y)	11.6 ± 2.0	15.8 ± 3.2	.26
FEV ₁ (% predicted)	73.1 ± 3.4	59.6 ± 7.5	.08
PEF (L/s)	4.0 ± 0.3	3.0 ± 0.4	.07

Values are presented as means ± SEMs.

FEV₁, Forced expiratory volume in 1 second; PEF, peak expiratory flow.

TABLE E2. Sequences of primers used in this study

COX-1 forward	ATGAGTCGAAGGAGTCTCTCG	CCL2 forward	TTAAAAACCTGGATCGGAACCAA
COX-1 reverse	GCACGGATAGTAACAACAGGGA	CCL2 reverse	GCATTAGCTTCAGATTTACGGGT
COX-2 forward	TTCAACACACTCTATCACTGGC	CCL3 forward	TGTACCATGACACTCTGCAAC
COX-2 reverse	AGAAGCGTTTGCGGTACTCAT	CCL3 reverse	CAACGATGAATTGGCGTGAA
EP1 forward	GGGCTTAACCTGAGCCTAGC	CCL4 forward	TTCCTGCTGTTTCTCTTACACCT
EP1 reverse	GTGATGTGCCATTATCGCCTG	CCL4 reverse	CTGCTGCGCTCTTTTGGTCAG
EP2 forward	TCCCTAAAGGAAAAGTGGGACC	CXCL1 forward	ACTGCACCCAAACCGAAGTC
EP2 reverse	GAGCGCATTAAACCTCAGGACC	CXCL1 reverse	TGGGGACACCTTTTAGCATCTT
EP3 forward	CCGGAGCACTCTGCTGAAG	CXCL2 forward	AAGTTTGCCTTGACCCTGAA
EP3 reverse	CCCCACTAAGTCGGTGAGC	CXCL2 reverse	AGGCACATCAGGTACGATCC
EP4 forward	ACCATTCCTAGATCGAACCGT	CXCL3 forward	AGTGCCTGAACACCCTACCA
EP4 reverse	CACCACCCCGAAGATGAACAT	CXCL3 reverse	GGCAAACCTCTTGACCATCC
DP1 forward	ATGAACGAGTCTATCGCTGT	CXCL4 forward	CTTCTGGGCCTGTTGTTTCT
DP1 reverse	ACACGAGCACATAAAAGACCG	CXCL4 reverse	CATTCTTCAGGGTGGCTATG
DP2 forward	AGATGGTCCAGCTTCCAAACC	CXCL5 forward	GTTCCATCTCGCCATTCTATGC
DP2 reverse	ACAGGATGAGTCCGTTTTCCA	CXCL5 reverse	GCGGCTATGACTGAGGAAGG
TP forward	GTGGTCTTCGGGCTCATATTC	CCR1 forward	CTCATGCAGCATAGGAGGCTT
TP reverse	CCCACGAGCTGAACCATCAT	CCR1 reverse	ACATGGCATCACCAAAAATCCA
FP forward	CTGGACTCATCGAAACACAA	CCR2 forward	TGTGATTGACAAGCACTTAGACC
FP reverse	AGGAAGCCTTTGACTTCTGTCTA	CCR2 reverse	TGGAGAGATACCTTCGGAACCT
IP forward	GTTCAAGACTCGGTAGGACCT	CXCR2 forward	ATGCCCTCTATTCTGCCAGAT
IP reverse	GAGAGCTGTTTCGAGCATAGG	CXCR2 reverse	GGTGCTCCGGTTGTATAAGATGA
Arg1 forward	CTCCAAGCCAAAGTCCTTAGAG	CXCR3 forward	TACCTTGAGGTTAGTGAACGTCA
Arg1 reverse	AGGAGCTGTCATTAGGGACATC	CXCR3 reverse	CGCTCTCGTTTTCCCATAATC
Nos2 forward	GTTCTCAGCCCAACAATACAAGA	β-actin forward	CGTGCGTGACATCAAAGAGAAG
Nos2 reverse	GTGGACGGGTCGATGTCAC	β-actin reverse	CGTTGCCAATAGTGATGACCTG
p47 ^{phox} forward	AGAGCATCCACCAGCGTTCT		
p47 ^{phox} reverse	GATTGTCTTTGTGCCATCC		

DP, D prostanoid receptor; *FP*, F prostanoid receptor; *IP*, I prostanoid receptor; *Nos2*, inducible nitric oxide synthase; *TP*, thromboxane A2 receptor.