IL-10-producing lung interstitial macrophages prevent neutrophilic asthma

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Abstract

Inflammatory responses contribute to host defense against harmful organisms and allergens, whereas a failure of immune tolerance can cause chronic inflammation including asthma. The lung has several innate myeloid cell subsets. Among these subsets, there are two types of macrophages: alveolar macrophages (AMs) and interstitial macrophages (IMs). However, compared with AMs, the role of IMs in lung homeostasis remains poorly understood. In this study, we characterized AMs and IMs in healthy and inflammatory conditions. Pulmonary IMs constitutively produce the anti-inflammatory cytokine IL-10 through activation of the TLR4/MyD88 pathway in a microbiota-independent manner. In addition to IMs, $Foxp3^+$ T_{reg} cells show persistent IL-10 expression in the lung, with IL-10-producing IMs more prevalent than $Foxp3^+$ T_{reg} cells. IMs, but not $Foxp3^+$ T_{reg} cells, increased IL-10 production in house dust mite-challenged mice, a model of human asthma. House dust mite-challenged *Il10*$^{-/-}$ mice exhibited severe lung pathology characterized by neutrophilia compared with that of wild-type mice. In addition, transplantation of wild-type IMs reduced neutrophilic inflammation, goblet cell mucus production, and decreased expression of lung IL-13 and Th17-related neutrophil-activating cytokines such as IL-17, GM-CSF, and TNF-α. Together, these results demonstrate that IL-10-producing IMs negatively regulate Th2- and Th17-mediated inflammatory responses, helping prevent neutrophilic asthma.

Introduction

The respiratory tract is continuously exposed to a variety of inhaled allergens. Inflammatory responses mediate clearance of harmful organisms and allergens while a failure of immune tolerance causes chronic respiratory inflammation, such as asthma. Asthma incidence has increased in developed countries over the past few decades (1). Th2-related cytokines such as IL-4, IL-5, and IL-13 mediate asthma pathogenesis (2). Recently, atypical pathologic characteristics of asthma have been identified. For instance, neutrophilic asthma, which is a severe persistent asthma, does not show basement-membrane thickening and responds poorly to glucocorticoid treatment (3). Thus, asthma is a phenotypically heterogeneous disorder, and therefore the underlying pathophysiologic mechanisms remain largely unknown.

IL-10 inhibits the inflammatory response, and defective production or action of IL-10 is linked to the development of chronic inflammatory disorders, including inflammatory bowel disease (4). Recent genome-wide association studies identified IL-10 as an important risk gene for asthma (5,6). Accordingly, lower levels of IL-10 are observed in asthma patients (7,8). In the lung, IL-10 inactivates neutrophils, eosinophils, and mast cells (9). In addition, alveolar macrophages (AMs) exhibit IL-10-dependent suppression of CD80 and CD86 expression, and thereby suppress activation of adaptive immunity (10). Moreover, transfer of dendritic cells (DCs) (11) or $CD4^+$ T cells (12) engineered to secrete IL-10 prevents experimental lung inflammation. Thus, IL-10 is critically involved in lung inflammation and asthma.

Pulmonary $Foxp3^+$ T_{reg} cells that produce IL-10 are responsible for lung homeostasis (13). In addition, several studies have shown that $Foxp3^+$ T_{reg} cell-derived IL-10 prevents experimental asthma by suppressing Th1/Th2 responses (13,14). However, other studies have demonstrated that $Foxp3^+$ T_{reg} cells inhibit airway inflammation by producing TGF-β, but not IL-10 (15). In addition, T_{reg} cell-mediated suppression of pulmonary allergic inflammation is shown to be IL-10 independent (16).

Thus, it is still controversial whether Foxp3$^+$ T_{reg} cell-derived IL-10 prevents pulmonary inflammation. In addition to Foxp3$^+$ T_{reg} cells, innate pulmonary myeloid cells play an important role in inhibiting inflammation via IL-10 production. Pulmonary DCs exhibit allergen-induced transient IL-10 production and induce IL-10 production of co-cultured T cells (17). Lung macrophages, which consist of two major subsets; AMs and interstitial macrophages (IMs), also exert tolerogenic properties. AMs inactivate T- cell responses through TGF-β and retinoic acid-dependent induction of Foxp3$^+$ T_{reg} cells (18). In addition, IMs constitutively produce IL-10, thereby preventing asthma by suppressing DC function (19). However, the precise mechanisms that regulate IL-10 production in pulmonary myeloid cells remain poorly characterized. It is also unclear which cell population, innate myeloid cells or Foxp3$^+$ T_{reg} cells, effectively suppresses asthmatic inflammation through IL-10 production.

In this study, we analyzed mechanisms underlying induction of IM IL-10 and further determined the effect of IL-10-producing IMs on HDM-induced asthma. IMs constitutively produce IL-10 via microbiota-independent activation of the TLR4/MyD88 pathway. Wild-type IM transplantation improved the severity of neutrophilic inflammation and reduced the number of mucus-loaded goblet cells in HDM-treated *Il10*$^{-/-}$ mice. This was due, in part, to downregulation of IL-13 and Th17-related neutrophil-activating mediators, leading to inhibition of neutrophilic asthma.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). *Il10*$^{-/-}$ mice and Foxp3-EGFP knock-in mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). *Myd88*$^{-/-}$, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice were generated previously and backcrossed to C57BL/6 for 8 generations (20). IL-10-Venus mice were generated as previously described (21). β-actin-eGFP mice were gifted from Dr. Okabe (Osaka University). All mice were maintained under specific pathogen- or germ-free conditions. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Osaka University.

Antibodies

Anti mouse-CD16/32 (2.4.G2), CD4-FITC (GK1.5), CD11c-FITC (N418), CD45R(B220)-FITC (RA3-6B2), CD3e-FITC (145-2C11), and MHC class II-PE (M5/114.15.2) were purchased from Tombo Biosciences. Anti mouse-CD4-Percp/Cy5.5 (GK1.5), CD45-PerCP/Cy5.5 (30-F11), CD11b-Pacific blue (M1/70), CD11c-PE-Cy7 (N418), CD8a-FITC (53-6.7), CD24-FITC (M1/169), F4/80-APC (BM8), LPAM-1-APC (DATK32), and IL-10-PE (JES5-16E3) were purchased from Biolegend. Anti mouse- Siglec-F-PE (E50-2440), Ly-6C-FITC (AL-21), Ly-6G-FITC (1A8), and Purified anti-mouse IL-10 (JES5-16E3) were purchased from BD Biosciences. Anti mouse-CD80-FITC (16-10A1), CD86-FITC (GL1) were purchased from eBioscience. Anti-GFP was purchased from Santa Cruz Biotechnology.

Flow cytometry

Flow cytometric analysis was performed using a FACS Cant II flow cytometer (BD Biosciences) with FlowJo software (Tree Star, Ashland, OR, USA). Instrumental settings were adjusted for three- or four-color stained samples.

Real-time RT-PCR

Total RNA was collected with TRIzol (Invitrogen, Carlsbad, CA, USA), and reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random primers (Toyobo, Tokyo, Japan) after treatment with RQ1 DNaseI (Promega). Quantitative real-time PCR was performed in Step one Plus (Applied Biosystems, Foster City, CA, USA) using Go Taq Q-PCR Master Mix (Promega). Amplification conditions were: 94°C (5 min), 40 cycles of 94°C (20 s), 55°C (20 s), and 72°C (50 s). All data are shown as relative mRNA levels normalized to GAPDH expression levels. The primer sequences are: *Gapdh*, 5'-CCTCGTCCCGTAGACAAAAT-3' and 5'-TCTCCACTTTGCCACTGCAA-3'; *Tlr2*, 5'-GAAACCTCAGACAAAGCGTC-3' and 5'-GCTTTTCATGGCTGCTGTGA-3'; *Tlr4*, 5'-CGCTTTCACCTCTGCCTTCACTACAG-3' and 5'-CACTACCACAATAACCTTCCGGCTC-3'; *Tlr5*, 5'-TGCTCAAACACCTGGATGCTCACTAC-3' and 5'-CAGCCGCCTGGATGTTGGAGATATG-3'; *Tlr9*, 5'-AGCTCAACCTGTCCTTCAATTACCGC-3' and 5'-ATGCCGTTCATGTTCAGCTCCTGC-3'; *Tnf*, 5'-TCCAGGCGGTGCCTATGT-3' and 5'-CACCCCGAAGTTCAGTAGACAGA-3'; *Il4*, 5'-AAGGTGCTTCGCATATTTTAT-3' and 5'-TTAGGCTTTCCAGGAAGTCTT-3'; *Il5*, 5'-AGCTCTGTTGACAAGCAATGA-3' and 5'-TTTCTTTATTAATGACAGGTT-3'; *Il6*, 5'-TGTTCTCTGGGAAATCGTGGA-3' and 5'-AAGTGCATCATCGTTGTTCATACA-3'; *Il13*, 5'-ATCTACAGGACCCAGAGGAT-3' and 5'-TAGCTGAGCAGTTTTGTTAT-3'; *Il17a*: 5'-GGACTCTCCACCGCAATGA-3' and 5'-GGCACTGAGCTTCCCAGATC-3'; *Il23p19*, 5'-CCAGCAGCTCTCTCGGAATCT-3' and 5'-GGGTCACAACCATCTTCACAC-3';

Csf2: 5'-TCGAGCAGGGTCTACGGGGC-3' and 5'-TCCGTTTCCGGAGTTGGGGG-3'. *Sfpta*, 5'-ACATCAGATTCTGCAAACAA-3' and 5'-TTGTACTTCTTTGTAATGCT-3'.

Isolation of pulmonary myeloid cells and lymphocytes

Lung tissue was cut into small pieces and incubated in PBS containing 0.5% BSA, 100 μg/ml Liberase (Roche Diagnostics Corporation, Indianapolis, IN, USA), 250 μg/mL Collagenase XI (Sigma, St Louis, MO, USA), 100 μg/mL Hyaluronidase (Sigma), and 200 μg/mL DNase I (Sigma) for 60min at 37°C. Single-cell suspensions were passed through a 40-μm cell strainer (BD Biosciences) and washed in PBS containing 2% FBS. Lung lymphocytes were isolated as previously described with modifications (22). In brief, lung tissue was cut into small pieces and incubated in PBS containing 300 U/mL Collagenase II (Worthington, Lakewood, NJ, USA), 150 μg/mL DNase I for 60 min at 37°C in a shaking water bath. Single cell suspensions were passed through 100-μm cell strainer (BD Biosciences) and washed in PBS containing 2% FBS.

Intracellular cytokine staining

$CD4^+$ T- cell intracellular IL-10 expression was analyzed using a Cytofix/Cytoperm Kit plus with Golgistop (BD Biosciences) according to the manufacturer's instructions. In brief, lung lymphocytes from $Foxp3^{GFP/+}$ mice were incubated with 50 ng/mL phorbol myristate acetate (Sigma), 5 μM calcium ionophore A23187 (Sigma), and Golgistop in complete RPMI1640 at 37°C for 4 h. Surface staining was performed with anti-CD4 for 20 min at 4°C and intracellular cytokine staining performed with anti-IL-10 for 20 min.

ELISA

Culture supernatant TNF-α, IL-6, and IL-10 levels were measured using Cytometric Bead Array (CBA) kit (BD Biosciences). Serum IgE and IgG levels were measured

using mouse IgE or IgG Ready-SET-Go (eBioscience), respectively.

Pulmonary IM transplantation and HDM challenge

IM transplantation was performed as described previously (23). In brief, 3.5×10^5 IMs from C57BL/6 mice were transferred intranasally three times into *Il10*$^{-/-}$ mice. In some experiments, IMs from β-actin-GFP mice were transferred into *Il10*$^{-/-}$ mice. Allergic inflammation was induced 2 weeks after the third transplantation, by intranasal injection of 40 μg HDM (ITEA Inc., Tokyo, Japan) dissolved in 10 μL PBS into wild-type, *Il10*$^{-/-}$, and IM-transferred *Il10*$^{-/-}$ mice on days 0–4 and 7–11. At 3 days post-challenge, lungs and bronchoalveolar lavage (BAL) were collected.

Histology

Paraffin-embedded lung samples were sectioned and stained with hematoxylin and eosin (H&E) or Alcian Blue. H&E and Alcian Blue-stained images were taken using Biozero (Keyence, Itasca, IL, USA).

Statistics

Differences between control and experimental groups were evaluated by the Student's *t* test.

Results

Characterization of pulmonary myeloid cell subsets

We analyzed lung cell surface expression of CD11c and CD11b to identify pulmonary innate myeloid subsets that constitutively produce IL-10 (Fig. 1A). Three lung subsets consisted of $CD11c^{high}$ $CD11b^{-}$, $CD11c^{mid}$ $CD11b^{+}$, and $CD11c^{-}$ $CD11b^{+}$ cells. These subsets were further subdivided based on F4/80 and MHC class II (MHCII) expression levels. In the $CD11c^{-}$ $CD11b^{+}$ and the $CD11c^{high}$ $CD11b^{-}$ cell populations, $F4/80^{+}$ $MHCII^{-}$ cells were dominant. The $CD11c^{mid}$ $CD11b^{+}$ cells were subdivided into three subsets: $F4/80^{+}$ $MHCII^{-}$, $F4/80^{+}$ $MHCII^{+}$, and $F4/80^{-}$ $MHCII^{+}$ cells. We then analyzed surface expression of several markers on the five subsets (Fig. 1B). The $CD11c^{-}$ $CD11b^{+}$ $F4/80^{+}$ $MHCII^{-}$ cells expressed Ly-6G and contained multi-segmented nuclei, indicative of pulmonary neutrophils (24). Among $CD11c^{mid}$ $CD11b^{+}$ cells, $F4/80^{+}$ $MHCII^{-}$ cell population expressed Ly-6C and therefore these cells are considered inflammatory monocytes (25), the $F4/80^{+}$ $MHCII^{+}$ cell population possessing cytoplasmic vacuoles was interstitial macrophages (IMs) with high CD86 and low CD24 expression (19,24,26), and the $F4/80^{-}$ $MHCII^{+}$ cell population was $CD11b^{+}$ dendritic cells (DCs) (24,26). The $CD11c^{high}$ $CD11b^{-}$ $F4/80^{+}$ $MHCII^{-}$ cell population with numerous cytoplasmic vacuoles was alveolar macrophages (AMs) (18). Although IMs constitutively produce IL-10 (19), it is unclear whether other innate immune subsets produce IL-10 in healthy lungs. Therefore, we examined IL-10 expression in five subsets of pulmonary innate immune cells using IL-10-venus mice (Fig. 1C). Approximately 70% of IMs and 35% of monocytes, but not neutrophils, $CD11b^{+}$ DCs, and AMs, expressed steady-state IL-10. Thus, as previously reported (19), a large fraction of IMs and a small fraction of monocytes produce IL-10.

Production of TLR-dependent IL-10 by lung IMs

Pattern recognition receptors such as Toll-like receptor (TLR) and NOD-like

receptor sense microbial components, thereby activating the innate immune system (27,28). To examine whether TLRs activate pulmonary immune cells, we analyzed *Tlr* mRNA expression in all five subsets (Fig. 2A). IMs expressed the highest levels of *Tlr2* and *Tlr4*. Neutrophils showed high expression of *Tlr4*. *Tlr5* was expressed almost equally across the five subsets. *Tlr9* was highly expressed on $CD11b^+$ DCs. We then analyzed TLR-induced production of IL-10 and pro-inflammatory cytokines by ELISA (Fig. 2B). Based on TLR expression patterns, we used TLR2 ligand FSL-1, TLR4 ligand LPS, TLR5 ligand flagellin, and TLR9 ligand CpG. In accordance with the results obtained in IL-10-venus mice (Fig. 1C), IMs constitutively produced the highest levels of IL-10. IMs also produced high amounts of IL-6 even in the absence of stimulation. Furthermore, FSL-1 and LPS induced augmented IM IL-10 and IL-6 secretion. Pulmonary monocytes increased IL-10 and TNF-α production in response to FSL-1, LPS, and flagellin. The highest TNF-α production was detected in FSL-1- and LPS-stimulated AMs. LPS-stimulated neutrophils secreted low levels of TNF-α and IL-6. In contrast, $CD11b^+$ DCs were unresponsive to TLR stimulation, whereas they constitutively produced IL-6. Thus, five subsets of pulmonary innate immune cells exhibit differential patterns of cytokine production in response to TLR stimulation, and IMs are unique in that they produce elevated levels of IL-10.

Lung microbiota-independent activation of the TLR4/MyD88 pathway mediates constitutive IL-10 production of lung IMs

To evaluate the involvement of TLR signaling in IM IL-10 production, we compared IL-10 production from wild-type and *Myd88*$^{-/-}$ mice (Fg. 3A). In *Myd88*$^{-/-}$ IMs, IL-10 production was greatly reduced compared with that in wild-type IMs. In addition, TNF-α and IL-6 production decreased in all five subsets from *Myd88*$^{-/-}$ mice, indicating TLR/MyD88 signaling-dependent production of IL-10, IL-6, and TNF-α in lung innate immune subsets. We then attempted to determine which TLR mediates IM IL-10

production. The *Tlr4* deficiency resulted in severely reduced IM IL-10 production, while a slight decline in IL-10 was observed in *Tlr2*$^{-/-}$ mice (Fig. 3B). IL-6 production was decreased in all five subsets from *Tlr2*$^{-/-}$ mice, but not *Tlr4*$^{-/-}$ mice. In *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ lungs, TNF-α production was decreased. Together, these results suggest that the TLR4/MyD88 pathway mediates IM constitutive IL-10 expression, and that the TLR2/MyD88 signaling induces pro-inflammatory cytokines such as IL-6 and TNF-α in the lung innate immune subsets.

In the intestinal lamina propria, macrophages produce IL-10 via TLR/MyD88 signaling in a commensal bacteria-dependent manner (29). The lung as well as the intestine, harbors a substantial microbial community (30). Therefore, we analyzed whether the lung microbiota mediates IM IL-10 production (Fig. 3C). In germ free (GF) mice, innate immune cells were normally present in the lung (data not shown), and there was no change in TNF-α and IL-6 production between SPF and GF mice. In addition, IL-10 production by IMs from GF mice was similar to that from SPF mice, indicating microbiota-independent induction of IM IL-10.

IL-10 suppresses TLR-induced expression of pro-inflammatory cytokines through activation of the transcription factor Stat3 in myeloid cells, leading to inhibition of Th1 and Th17 responses (9). Therefore, we investigated the effect of IL-10 on LPS-induced TNF-α and IL-6 production in pulmonary immune cell subsets (Supplementary Fig. 1). Consistent with IL-10 production patterns, LPS stimulation increased TNF-α and IL-6 secretion by IMs and monocytes from *Il10*$^{-/-}$ mice compared with that of wild-type mice. We further analyzed which cells, IL-10-producing or IL-10-nonproduing IMs, secrete pro-inflammatory cytokines. IL-10 (venus)-positive and -negative IMs were isolated from the lung of IL-10-venus mice, and stimulated with LPS or FSL-1 in the presence of neutralizing antibodies to IL-10 to exclude the effect of IL-10 (Supplementary Fig. 2). IL-10-positive IMs produced lower levels of IL-6 and TNF-α compared with those in IL-10-negative IMs, indicating that IL-10-nonproducing

IMs are major producer of IL-6 and TNF-α among IMs. These results demonstrate that IL-10 by IMs negatively regulates pro-inflammatory cytokine production by myeloid cells residing in interstitial space of the lung.

HDM challenge promotes IM IL-10 production

Development of asthma is mediated by DC-driven Th2 responses characterized by eosinophil infiltration (2). In addition to IMs, a few $CD4^+$ $Foxp3^+$ T_{reg} cells constitutively produce IL-10 in the lung (Fig. 4A) (13). Pulmonary $CD45^-$ non-haematopoetic cells did not produce IL-10 under staedy-state, whereas approximately 5% of $CD45^+$ haematopoietic cells constitutively produced IL-10 (Supplementary Fig. 3A). In addtion, approximately 55% of IL-10-producing $CD45^+$ cells was IMs, but the proportion of $CD4^+$ T cells-producing IL-10 was lower than 5%. Thus, IMs are the major subset of IL-10-producing cells in the lung (Supplementary Fig. 3B). Several studies have demonstrated IL-10- or TGF-β-dependent prevention of asthma by $Foxp3^+$ T_{reg} cells (15). Therefore, we compared IMs and $Foxp3^+$ T_{reg} cell IL-10 production during HDM-induced asthma. Mice were exposed to HDM, which induces a mouse model of asthma, and lung $Foxp3^+$ T_{reg} cells were stimulated with HDM in the presence of antigen presenting cells. IL-10 production by $Foxp3^+$ T_{reg} cells from HDM-administrated mice was similar to that in non-challenged mice (Fig. 4B). In contrast, IM IL-10 secretion was considerably enhanced following HDM exposure (Fig. 4C). We then analyzed the number of IMs and lung $Foxp3^+$ T_{reg} cells in control and HDM-challenged mice (Fig. 4D). Even in unchallenged mice, the number of IL-10-producing IMs was about 30-fold higher than that of $Foxp3^+$ T_{reg} cells. Furthermore, HDM treatment induced twice the number of IL-10-producing IMs while $Foxp3^+$ T_{reg} cells did not increase (Fig. 4 D). In the lung of HDM-challenged mice, monocytes, neutrophils, and $CD11b^+$ DCs were only marginally increased, and AMs were not increased (Supplementary Fig. 4A). Surface expression of CD80, CD86,

Ly-6C, Ly-6G, and CD24 on the five subsets from HDM-exposed mice was comparable to that in unchallenged mice (Supplementary Fig. 4 B). Thus, the number and IL-10 production was increased in IMs compared with $Foxp3^+$ T_{reg} cells during HDM-induced lung inflammation.

IMs from wild-type mice inhibit lung inflammation in HDM-challenged Il10$^{-/-}$ mice.

Because IL-10-producing IMs were predominantly present, we examined the role of IM IL-10 on HDM-induced asthma. Thus, *Il10*$^{-/-}$ mice were challenged with HDM 2 weeks after the third intranasal transfer of wild-type IMs (Fig. 5A). Transferred GFP-expressing IMs were present within the interstitial space of the lung in *Il10*$^{-/-}$ mice (Fig. 5B). In wild-type mice, HDM challenge resulted in eosinophilia with pulmonary inflammation and an increased number of mucus-laden goblet cells (Fig. 5C, D, and E). HDM-treated *Il10*$^{-/-}$ mice exhibited severe lung pathology characterized by neutrophilia and reduced eosinophil infiltration compared to HDM-treated wild-type mice (Fig. 5C) (31,32). Increased goblet cell mucus secretion was similarly observed in *Il10*$^{-/-}$ and wild-type mice. Importantly, transfer of wild-type IMs, dramatically reduced the severity of lung inflammation and neutrophil infiltration in HDM-challenged *Il10*$^{-/-}$ mice (Fig. 5C). In addition, mucus-filled goblet cells were dramatically reduced in *Il10*$^{-/-}$ mice given IMs. These findings demonstrate that IL-10-producing IMs negatively regulate neutrophil infiltration and mucus production in *Il10*$^{-/-}$ mice. We further investigated the effect of IM transplantation on IgG and IgE responses, which are mediated by IL-4 (Fig. 5F, G) (33). There was no difference in the circulating levels of HDM-specific IgG and total IgE between wild-type, *Il10*$^{-/-}$, and IM-transferred *Il10*$^{-/-}$ mice, indicating IL-10-independent regulation of IgG/IgE responses.

We then attempted to determine how IMs regulate HDM-induced pulmonary inflammation. To assess their role in Th2-mediated responses such as eosinophil infiltration and mucus production, we analyzed lung *Il5* and *Il13* expression of

HDM-exposed animals (Fig. 6A) (2,4). *Il10*$^{-/-}$ mice showed decreased *Il5* expression, which correlated with reduced eosinophil numbers (Fig. 5D) (32). Transfer of IMs led to similar *Il5* expression levels in *Il10*$^{-/-}$ mice compared with wild-type mice. *Il13* was highly expressed in *Il10*$^{-/-}$ and wild-type mice, whereas transferred IMs inhibited *Il13* expression in *Il10*$^{-/-}$ mice, coinciding with diminished eosinophil recruitment and mucus production (Fig. 5C, E). These findings suggest that IMs inhibit Th2-related inflammation through downregulation of *Il13*.

Because transfer of wild-type IMs decreased neutrophil infiltration in *Il10*$^{-/-}$ mice, we next evaluated the effect on neutrophilic inflammation. In both mice and humans, Th17 cells are implicated in neutrophil influx during asthma (34). Therefore, we investigated the expression of *Il6* and *Il23p19*, which mediate Th17 development and maintenance, in the lung from HDM-exposed mice (Fig. 6B). In *Il10*$^{-/-}$ mice, HDM challenge induced increased levels of *Il6* and *Il23p19* expression compared with wild-type mice, whereas IM transfer downregulated their expression in *Il10*$^{-/-}$ mice. We then investigated Th17-related cytokines such as IL-17A, TNF-α, and GM-CSF (CSF2) because these genes are known to activate neutrophils (34). In HDM-treated *Il10*$^{-/-}$ mice, we observed elevated *Il17a*, *Tnf*, and *Csf2* expression compared with wild-type mice (Fig. 6C). However, IM transplantation into *Il10*$^{-/-}$ mice dramatically decreased *Il17a*, *Tnf*, and *Csf2* expression. These results indicate that IL-10-producing IMs suppress Th17 responses and thereby inhibit the development of neutrophilic asthma.

Discussion

In this study, we show that pulmonary IMs constitutively produce high levels of the anti-inflammatory cytokine IL-10 via activation of the TLR4/MyD88 signaling pathway, in a microbiota-independent manner. In HDM-treated mice, the number of IL-10-producing IMs, but not $Foxp3^+$ T_{reg} cells, and IL-10 production increased compared with that of unchallenged mice. These results prompted us to analyze the role of IL-10-producing IMs in HDM-induced asthma. HDM-treated *Il10*$^{-/-}$ mice developed more severe lung inflammation accompanied with neutrophilia compared with that of wild-type mice. Furthermore, transplantation of wild-type IMs into *Il10*$^{-/-}$ mice dramatically reduced the number of infiltrating neutrophils, mucus-filled goblet cells, and the severity of HDM-induced lung inflammation. In this context, expression of IL-13 and Th17-related cytokines such as IL-17, IL-6, IL-23p19, GM-CSF, and TNF-α were downregulated. These findings demonstrate that IL-10-producing IMs prevent HDM-induced neutrophilic asthma by inhibiting Th2 and Th17 responses in *Il10*$^{-/-}$ mice.

Although neutrophil migration into inflammatory sites is essential for host defense, excessive accumulation of neutrophils damages tissue. In the lung, inappropriate neutrophil activation is implicated in inflammatory diseases such as neutrophilic asthma and chronic obstructive pulmonary disease (3,35). Thus, proper neutrophil influx is responsible for maintaining lung homeostasis. Our present study clearly demonstrates that the TLR4/MyD88 signaling is required for persistent production of IL-10 by IMs and that IL-10-producing IMs play a crucial role in preventing neutrophilic asthma by suppression of IL-13 and Th17-related cytokines. A previous study showed that *Tlr4*$^{-/-}$ IMs had lost their immunosuppressive activity to inhibit lung inflammation (19), indicating that IM-derived IL-10 via the TLR4 signaling may be required for immunosuppressive properties. Heterogeneous populations of transcription factor IRF4-expressing $CD24^+$ $CD11b^+$ cDCs promote either Th17 responses through IL-23p19 or Th2 responses in the lung (36-38). Therefore, it is

possible that IMs modulate activation of these cells through IL-10 and thereby prevent Th2- and Th17-mediated lung inflammation. In addition to the development of $CD11b^+$ cDCs, IRF4 directly induces IL-10 expression in bone marrow-derived DCs upon HDM stimulation (39). Therefore, IRF4 may augment IM IL-10 production in HDM-treated mice.

Several lung IL-10-producing myeloid cell subsets have been reported. IL-10-producing $CD11b^+$ $Gr\text{-}1^{low}$ mononuclear phagocytes suppress NK- and Th1-dependent responses in aged mice (40,41). Unlike IMs, these cells do not express CD11c and are not observed in young mice. In the intestine, macrophages and $CD103^+$ DCs contribute to the development and maintenance of IL-10-producing $Foxp3^+$ T_{reg} cells and type 1 regulatory T (Tr1) cells, respectively, by producing IL-10 (42). Pulmonary $CD11c^+$ DCs transiently produce high levels of IL-10 after antigen exposure and induce antigen-specific Tr1-like cells in draining lymph nodes (17), where IMs are not present. In our study, Tr1 cells increased in wild-type mice following HDM exposure (cell number: 1870±1127 and 4553±1951, $p < 0.0025$), albeit fewer cells compared with IL-10-producing IMs. AMs promote pulmonary tolerance by inducting $Foxp3^+$ T_{reg} cells through retinoic acid and TGF-β under steady-state conditions (18). $Foxp3^+$ T_{reg} cell-derived IL-10 is required for lung homeostasis (13). Thus, it is interesting to examine whether IL-10-producing IMs mediate induction and maintenance of lung $Foxp3^+$ T_{reg} cells and Tr1 cells. $CD11b^+$ $F4/80^+$ $Gr\text{-}1^{int}$ cells also produce high amounts of IL-10 (43,44). However, these cells are characterized by ring-shaped nuclei and prevent eosinophilic asthma, whereas IMs are mononuclear and inhibit neutrophilic asthma. Furthermore, unlike IMs, this subset develops in a TLR4/MyD88 signaling-dependent manner. Thus, the $CD11b^+$ $F4/80^+$ $Gr\text{-}1^{int}$ cells described before are distinct population from IMs characterized in this study.

In the intestine, microbiota mediates macrophage TLR/MyD88-dependent IL-10 production (29). However, pulmonary IMs produce IL-10 through the

TLR4/MyD88 pathway even in the absence of commensal bacteria. In the lung, alveolar type II cells secrete surfactants, which function in innate host defense, in response to several stimuli such as ATP, lipoproteins, and LPS (45). The surfactant protein, SP-A that was expressed in the lung independently of microbiota (Supplementary Fig. 5), was identified as an endogenous TLR4 ligand and found to induce IL-10 production in macrophages (46), although this observation remains controversial. IMs have been shown to present antigens to pulmonary DCs (47). Therefore, IMs may extend their dendrites into air space to directly sample antigens and consequently recognize SP-A through TLR4, and thereby produce IL-10.

IL-10 induces IL-5 expression in the lung during allergic inflammation (32). We also observed that transfer of IL-10-producing IMs stimulates lung IL-5 expression in HDM-challenged *Il10*$^{-/-}$ mice. Transgenic mice overexpressing lung-specific IL-10 show enhanced goblet cell mucus production, accompanied by high levels of steady-state IL-13 expression (48). Consistent with this, markedly decreased mucus production was observed in *Il10*$^{-/-}$ mice sensitized/challenged with OVA (32). In contrast, others have noted no change in goblet cell mucus secretion between wild-type and *Il10*$^{-/-}$ mice treated with aerosolized OVA (31). We also demonstrated that the number of mucus-laden goblet cells in HDM-challenged *Il10*$^{-/-}$ mice was similar to that in wild-type mice. However, transplantation of IL-10-producing IMs into *Il10*$^{-/-}$ mice led to dramatically decreased mucus production in goblet cells, as previous study has indicated (19). Mice overexpressing lung-specific IL-9 show an asthma-like phenotype (49,50). In these mice, a lack of or neutralization of IL-13, but not IL-4 and IL-5, blocked goblet cell mucus production and eosinophilic inflammation. Importantly, lung IL-13 expression decreased dramatically in IM-transferred *Il10*$^{-/-}$ mice, consistent with decreased numbers of eosinophils and mucus production. These results suggest that IMs might affect mucus production and eosinophil recruitment by suppressing IL-13 expression. IL-10 negatively regulates Th1/Th17-related genes such as IL-12, IL-1β,

IL-6, TNF-α, and GM-CSF (9). Several studies have shown that lack of IL-10 signaling induces excessive inflammatory responses against invading pathogens by promoting accumulation of neutrophils and Th17 cells in the lung (51,52). Th17-derived IL-17 activates neutrophils by inducing GM-CSF, TNF-α, and IL-8. Accordingly, administration of an anti-IL-17 monoclonal antibody prevents bronchial neutrophil influx (53). In the present study, we show that transferred IMs improve neutrophilic asthma in *Il10*$^{-/-}$ mice through downregulation of Th17-related genes such as IL-6, IL-23p19, IL-17A, GM-CSF, and TNF-α. These findings indicate that IM-derived IL-10 might modulate Th17 responses and thereby prevent neutrophilic asthma. IL-17 is also produced by various immune cells, including type 3 innate lymphoid cells (ILC3) and γδ T cells (2). Thus, IMs might modulate IL-17 production by several immune cell subsets. In addition to IMs, we observed that few lung Foxp3$^+$ T_{reg} cells persistently produced IL-10. Foxp3$^+$ T_{reg} cell-specific IL-10 deficient mice exhibit a severe lung pathology accompanied by eosinophilia following HDM exposure (13), suggesting that IL-10-producing IMs inhibit neutrophilic asthma while IL-10-producing Foxp3$^+$ T_{reg} cells help prevent eosinophilic asthma.

In present study, we have shown that IL-10-producing IMs prevent lung inflammation by regulating neutrophil infiltration and goblet cell mucus production by downregulating IL-13 and Th17-related genes. It is noteworthy that patients with lung inflammatory disorders, such as cystic fibrosis, asthma, and interstitial lung disease, show decreased IL-10 levels (7,54,55). Thus, additional studies are needed to determine whether IL-10-producing IMs are decreased in these disorders. Neutrophilic asthma is steroid-resistant and has more severe inflammation compared with that of eosinophilic asthma (3). Therefore, alternative therapeutic interventions that resolve activation of lung neutrophils are desired to improve clinical outcomes. In the future, a clinical study will be needed to characterize IM function in asthma patients as well as healthy individuals, as IMs may offer a novel therapeutic target for neutrophilic asthma.

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Competing financial interests

The authors declare no competing financial interests.

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Figure legends

Figure 1: IMs constitutively produce IL-10.

(A) Flow cytometry analysis of cells from lung of C57BL/6J mice stained for CD11b, CD11c, MHC class II, and F4/80. (B) Indicated lung myeloid cell subsets were stained with May–Grunwald Giemsa. (original magnification, 200×.) (Upper panel) Surface expression of CD80, CD86, Ly-6C, Ly-6G, and CD24 (open histogram) on indicated subsets. Filled histograms, isotype control. (Lower panel) (C) IL-10 expression on indicated lung myeloid cell subsets of IL-$10^{Venus/Venus}$ mice. All data are representative of three independent experiments.

Figure 2: TLR-dependent expression of IL-10, TNF-α, and IL-6 in pulmonary myeloid subsets. (A) Expression of *Tlr2*, *Tlr4*, *Tlr5*, and *Tlr9* in innate immune cell subsets from lung of C57BL/6J mice. All data are representative of at least two independent experiments (mean ± SD of at least triplicate PCRs on identical samples). (B) Lung immune cell subsets from C57BL/6J mice were cultured for 24 h, and the supernatants were assayed for production of IL-10, TNF-α, and IL-6 by ELISA. Graphs show mean values ± SD of duplicate well measurements. All data are representative of two independent experiments. *$P < 0.05$, **$P < 0.01$.

Figure 3: IMs produce IL-10 through microbiota-independent activation of the TLR4/MyD88 pathway. Indicated lung innate immune cell subsets from wild-type, *Myd88*$^{-/-}$ (A), *Tlr4*$^{-/-}$, *Tlr2*$^{-/-}$ (B), and germ free (GF) (C) mice were cultured for 24 h, and the supernatants were assayed for production of IL-10, TNF-α, and IL-6 by ELISA. Mean values ± SD of triplicate well measurements are shown. All data are representative of at least two independent experiments. *$P < 0.05$, **$P < 0.01$.

Figure 4: The number of IL-10-producing IMs, but not Foxp3$^+$ T_{reg} cells, increases

during HDM-induced asthma. (A) Flow cytometry analysis of lung cells from $Foxp3^{GFP/+}$ mice stained for CD4 and IL-10. (B) Lung $Foxp3^+$ T_{reg} cells from HDM-challenged $Foxp3^{GFP/+}$ mice were stimulated with HDM in the presence of antigen presenting cells for 24 h, and the supernatants were assayed for production of IL-10 by ELISA. (C) Indicated lung myeloid cell subsets from HDM-challenged mice were cultured for 24 h, and the supernatants were analyzed for the production of IL-10 by ELISA. (D) The number of IL-10-producing IMs and $Foxp3^+$ T_{reg} cells in control (n = 5) or HDM-treated mice (n = 5). All data are representative of at least two independent experiments (mean values ± SD). * not detected. $^{\#}P < 0.05$, n.s. not significant.

Figure 5: Transfer of wild-type IMs inhibits lung inflammation characterized by neutrophilia in HDM-challenged *Il10*$^{-/-}$ mice. (A) Experimental step of the HDM challenge protocol used to induce lung inflammation. (B) Lung cryosections following transfer of IMs isolated from GFP mice (original magnification, 200×.) (C) Number of eosinophils and neutrophils in BALF from wild-type, *Il10*$^{-/-}$, and wild-type IM-transferred wild-type and *Il10*$^{-/-}$ mice treated with (n = at least 6) or without (n = at least 6) HDM (mean values ± SEM). (D, E) H&E staining (D) and Alcian blue staining (E) of lung sections 2 days after the last HDM challenge (original magnification, 200×.). Data are representative of two independent experiments. (F, G) Concentration of total serum IgE (F) and HDM-specific IgG (G) in wild-type, *Il10*$^{-/-}$, and IM-transferred *Il10*$^{-/-}$ mice treated with (n = at least 7) or without (n = at least 9) HDM (mean values ± SEM). $^*P < 0.05$.

Figure 6: IL-10-producing IMs downregulate IL-13 and Th17-related cytokines in HDM-challenged mice. Expression of *Il5* and *Il13* (A), *Il6* and *Il23p19* (B), and *Il17a*, *Csf2*, and *Tnf* (C) in the lung from wild-type, *Il10*$^{-/-}$, and IM-transferred *Il10*$^{-/-}$ mice

challenged to HDM. Results are mean ± SEM from three independent experiments. *P < 0.05.

Fig. 1

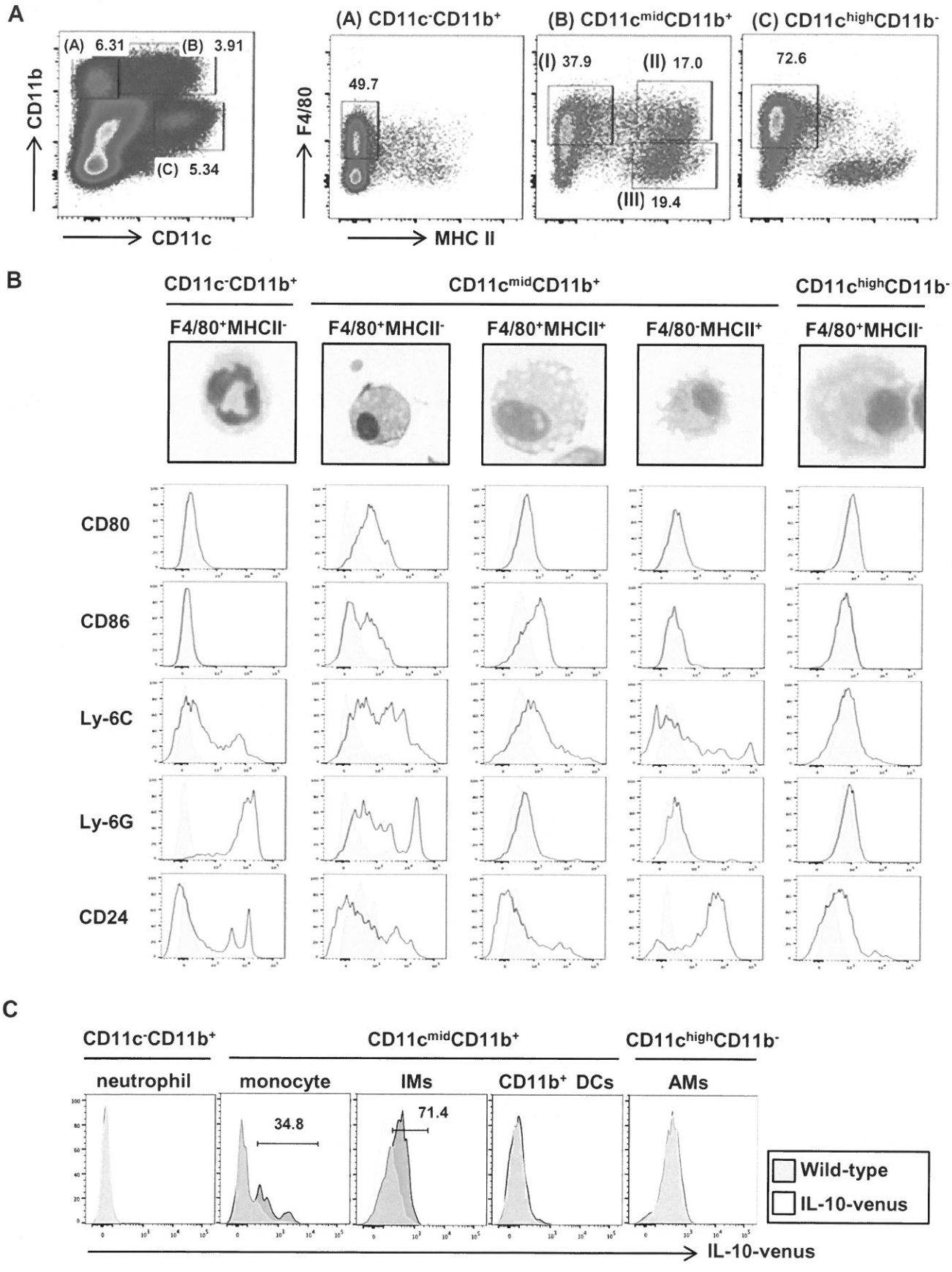

A
(A) CD11c-CD11b+
(B) CD11cmidCD11b+
(C) CD11chighCD11b-
(A) 6.31
(B) 3.91
(C) 5.34
CD11b
CD11c
49.7
F4/80
MHC II
(I) 37.9
(II) 17.0
(III) 19.4
72.6
B
CD11c-CD11b+
CD11cmidCD11b+
CD11chighCD11b-
F4/80+MHCII-
F4/80+MHCII-
F4/80+MHCII+
F4/80-MHCII+
F4/80+MHCII-
CD80
CD86
Ly-6C
Ly-6G
CD24
C
CD11c-CD11b+
CD11cmidCD11b+
CD11chighCD11b-
neutrophil
monocyte
IMs
CD11b+ DCs
AMs
34.8
71.4
Wild-type
IL-10-venus
IL-10-venus

Fig. 2

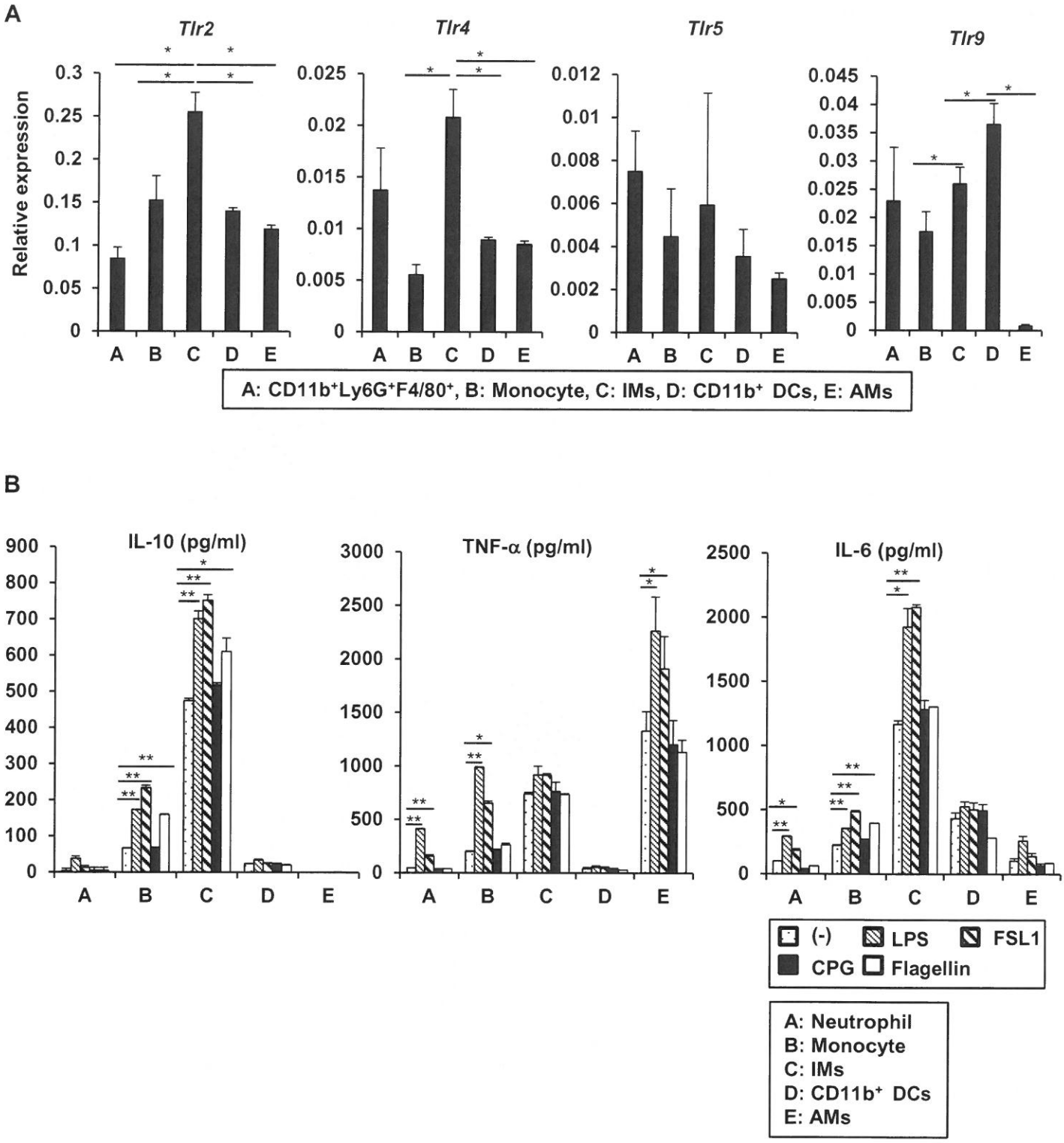

A
Tlr2
Tlr4
Tlr5
Tlr9
Relative expression
A: CD11b+Ly6G+F4/80+, B: Monocyte, C: IMs, D: CD11b+ DCs, E: AMs
B
IL-10 (pg/ml)
TNF-α (pg/ml)
IL-6 (pg/ml)
(-)
LPS
FSL1
CPG
Flagellin
A: Neutrophil
B: Monocyte
C: IMs
D: CD11b+ DCs
E: AMs

Fig. 3

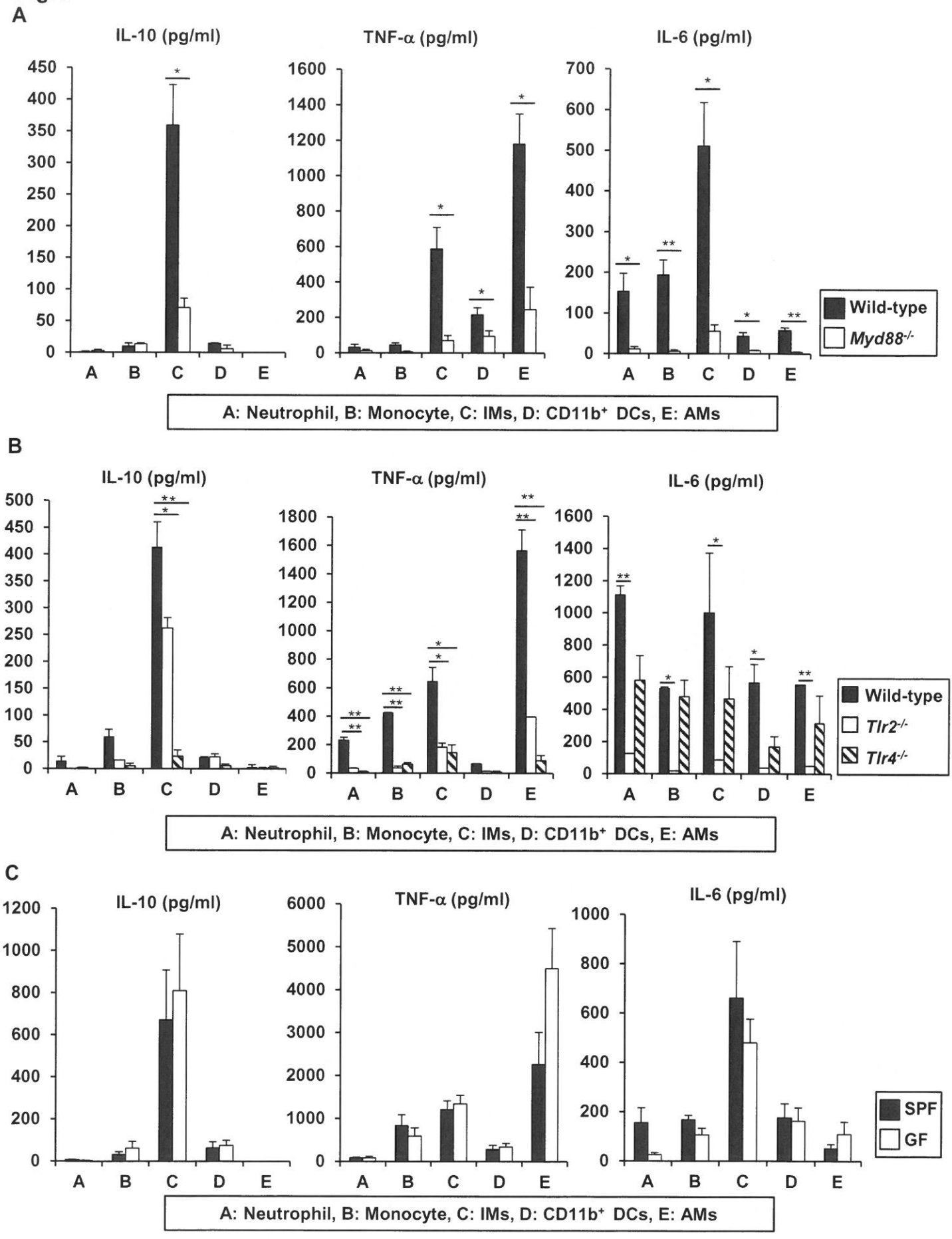

A
IL-10 (pg/ml)
TNF-α (pg/ml)
IL-6 (pg/ml)
Wild-type
Myd88-/-
A: Neutrophil, B: Monocyte, C: IMs, D: CD11b+ DCs, E: AMs
B
IL-10 (pg/ml)
TNF-α (pg/ml)
IL-6 (pg/ml)
Wild-type
Tlr2-/-
Tlr4-/-
A: Neutrophil, B: Monocyte, C: IMs, D: CD11b+ DCs, E: AMs
C
IL-10 (pg/ml)
TNF-α (pg/ml)
IL-6 (pg/ml)
SPF
GF
A: Neutrophil, B: Monocyte, C: IMs, D: CD11b+ DCs, E: AMs

Fig. 4

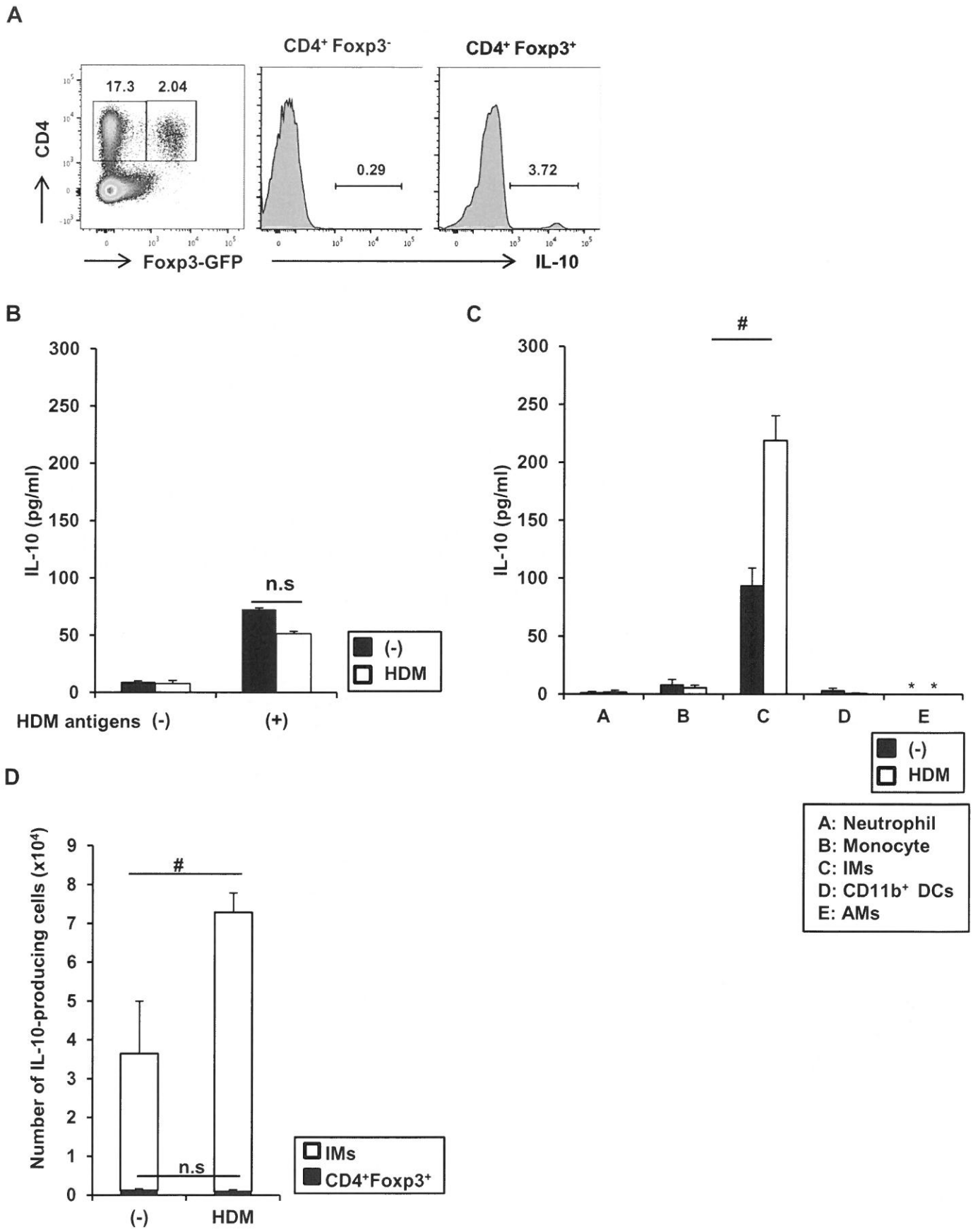

A
CD4+ Foxp3-
CD4+ Foxp3+
17.3
2.04
CD4
0.29
3.72
Foxp3-GFP
IL-10
B
IL-10 (pg/ml)
n.s
(-)
HDM
HDM antigens (-)
(+)
C
#
IL-10 (pg/ml)
A
B
C
D
E
(-)
HDM
A: Neutrophil
B: Monocyte
C: IMs
D: CD11b+ DCs
E: AMs
D
Number of IL-10-producing cells (x10⁴)
#
n.s
IMs
CD4+Foxp3+
(-)
HDM

Fig. 5

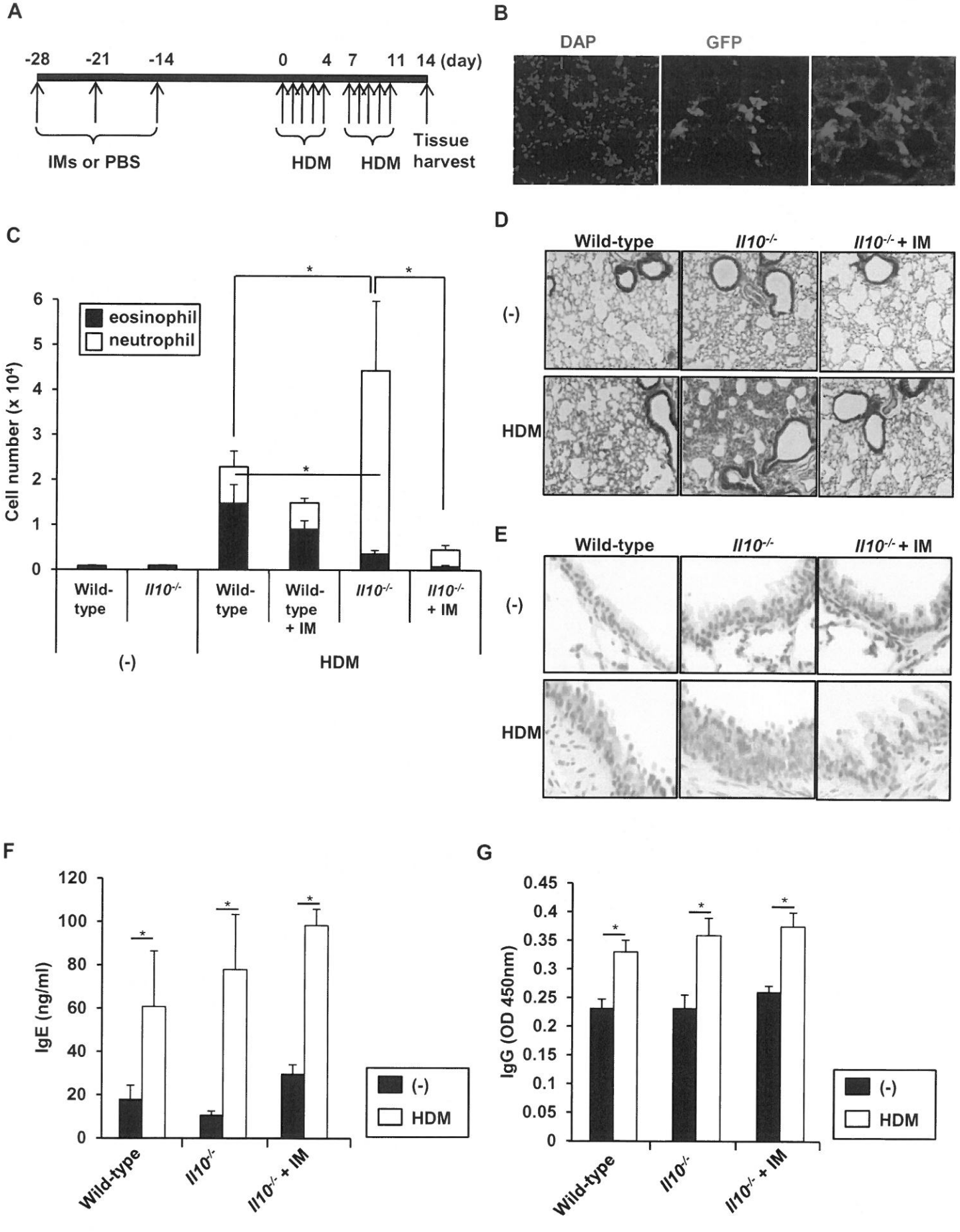
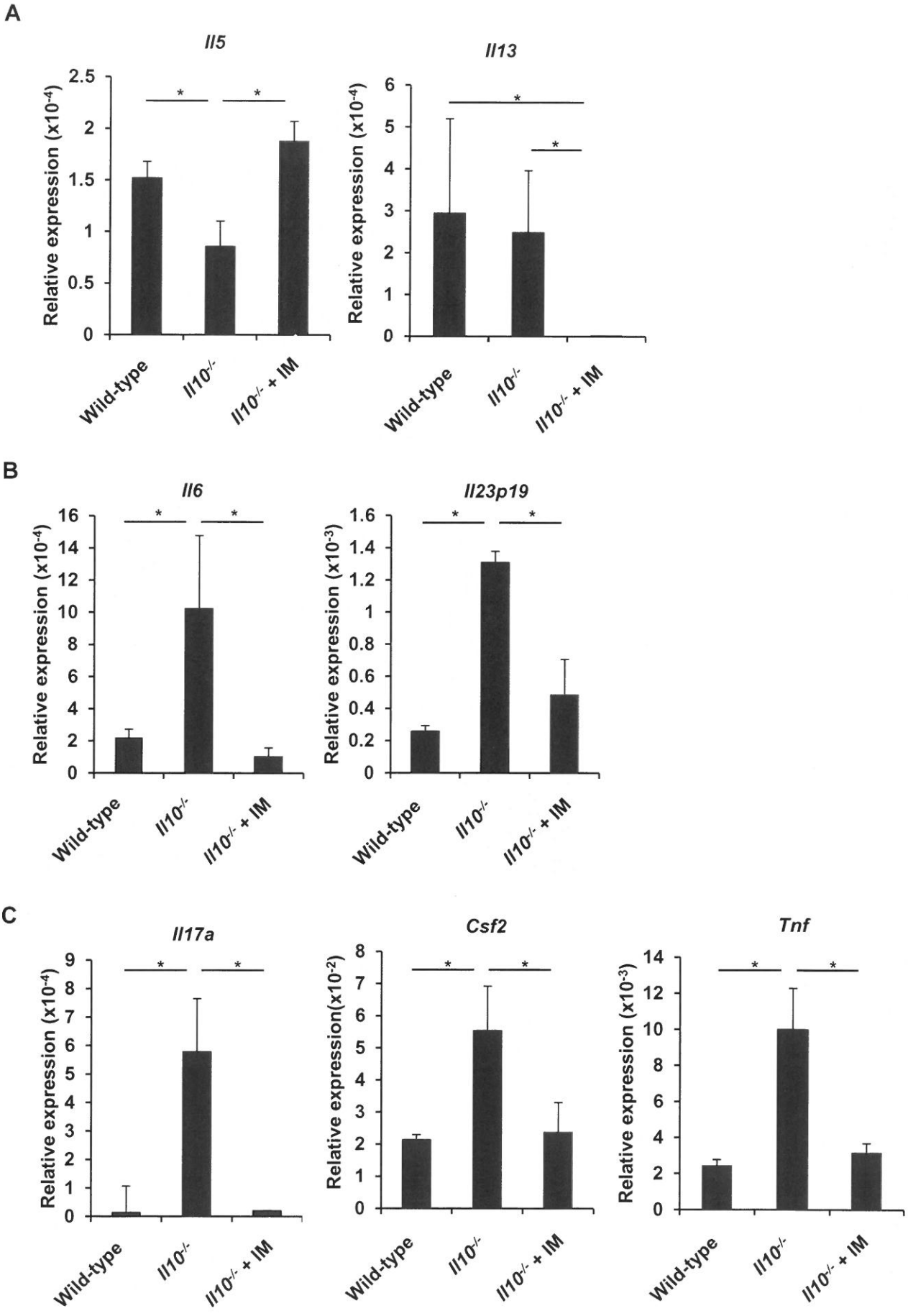
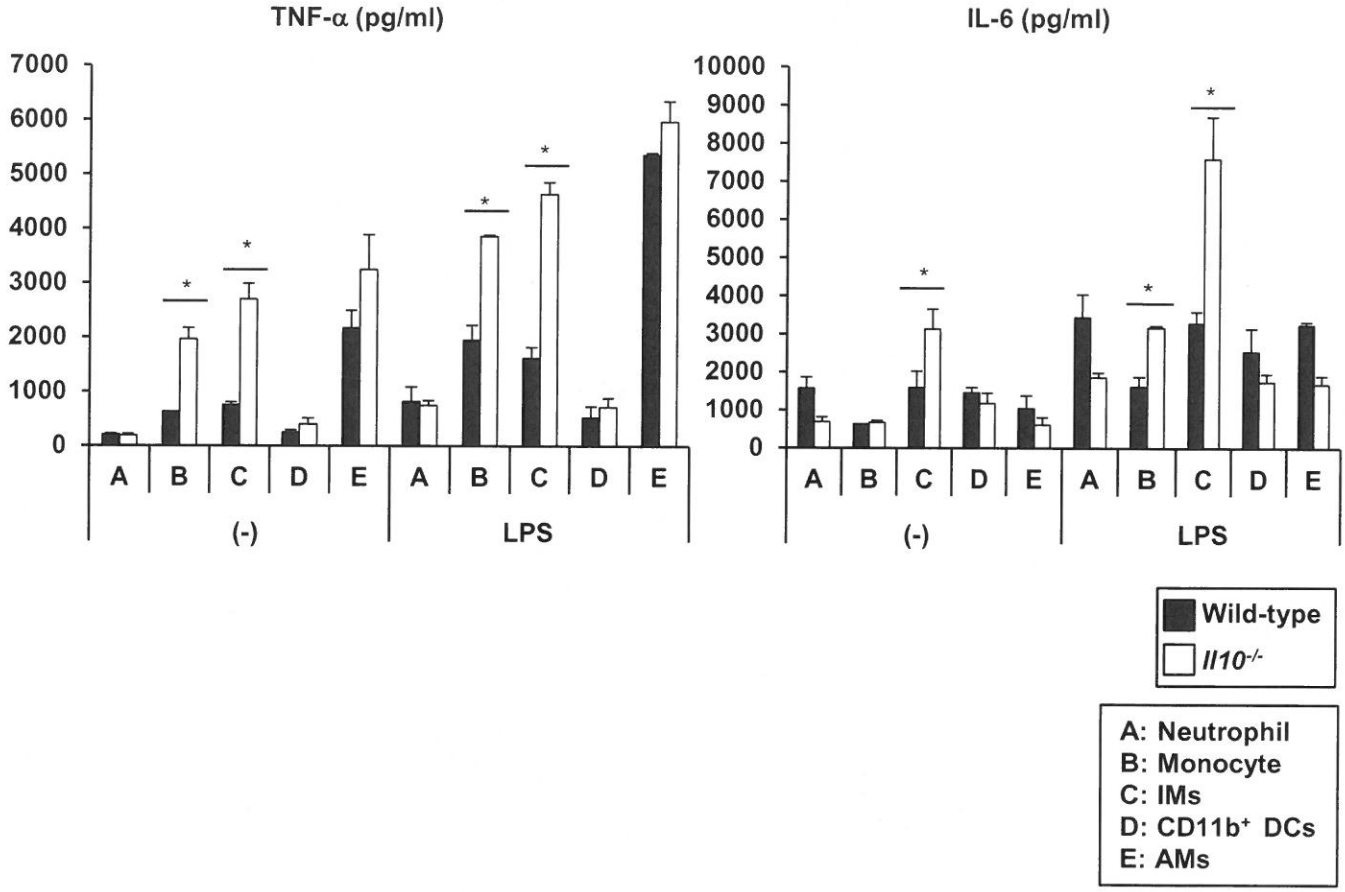

A
-28
-21
-14
0
4
7
11
14 (day)
IMs or PBS
HDM
HDM
Tissue harvest
B
DAP
GFP
C
eosinophil
neutrophil
Cell number (x 10⁴)
Wild-type
Il10-/-
Wild-type
Wild-type + IM
Il10-/-
Il10-/- + IM
(-)
HDM
D
Wild-type
Il10-/-
Il10-/- + IM
(-)
HDM
E
Wild-type
Il10-/-
Il10-/- + IM
(-)
HDM
F
IgE (ng/ml)
(-)
HDM
Wild-type
Il10-/-
Il10-/- + IM
G
IgG (OD 450nm)
(-)
HDM
Wild-type
Il10-/-
Il10-/- + IM

Fig. 6

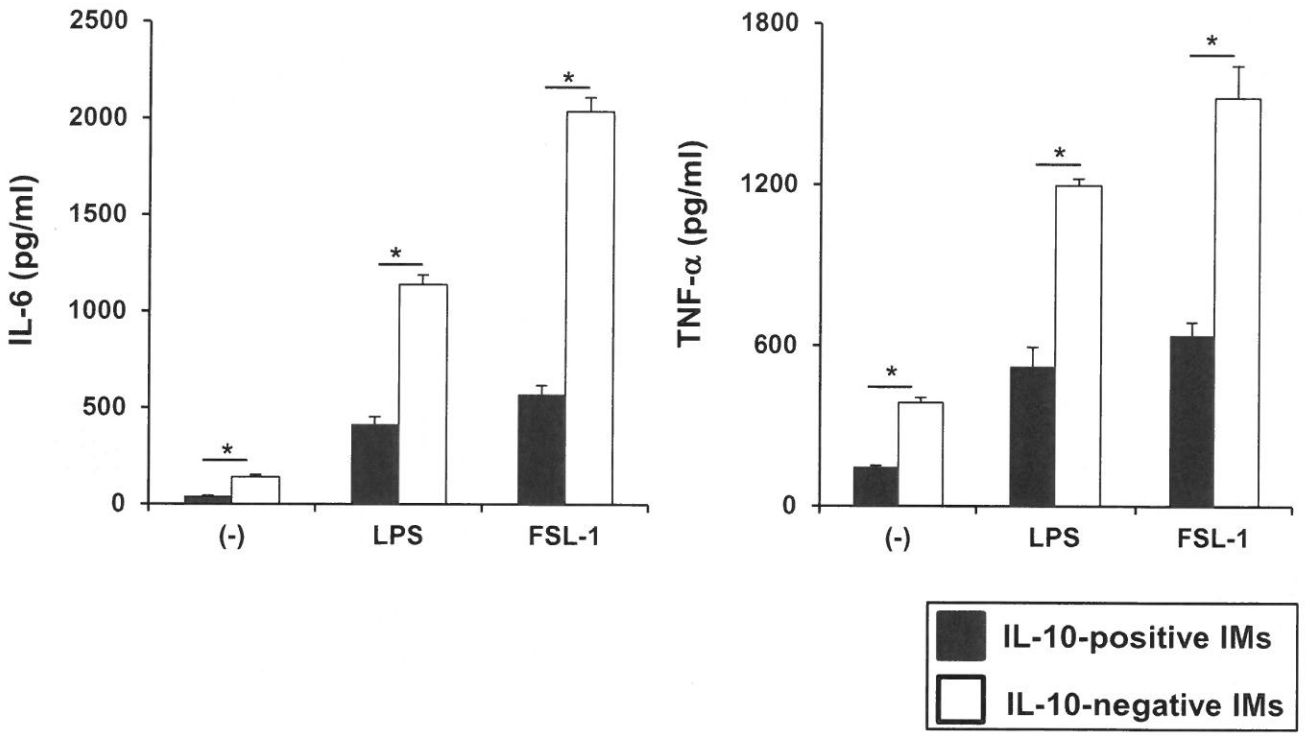

A
Il5
Relative expression (x10-4)
2.5
2
1.5
1
0.5
0
*
*
Wild-type
Il10-/-
Il10-/- + IM
Il13
Relative expression (x10-4)
6
5
4
3
2
1
0
*
*
Wild-type
Il10-/-
Il10-/- + IM
B
Il6
Relative expression (x10-4)
16
14
12
10
8
6
4
2
0
*
*
Wild-type
Il10-/-
Il10-/- + IM
Il23p19
Relative expression (x10-3)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Wild-type
Il10-/-
Il10-/- + IM
C
Il17a
Relative expression (x10-4)
9
8
7
6
5
4
3
2
1
0
*
*
Wild-type
Il10-/-
Il10-/- + IM
Csf2
Relative expression(x10-2)
8
7
6
5
4
3
2
1
0
*
*
Wild-type
Il10-/-
Il10-/- + IM
Tnf
Relative expression (x10-3)
14
12
10
8
6
4
2
0
*
*
Wild-type
Il10-/-
Il10-/- + IM

Supplementary Fig. 1



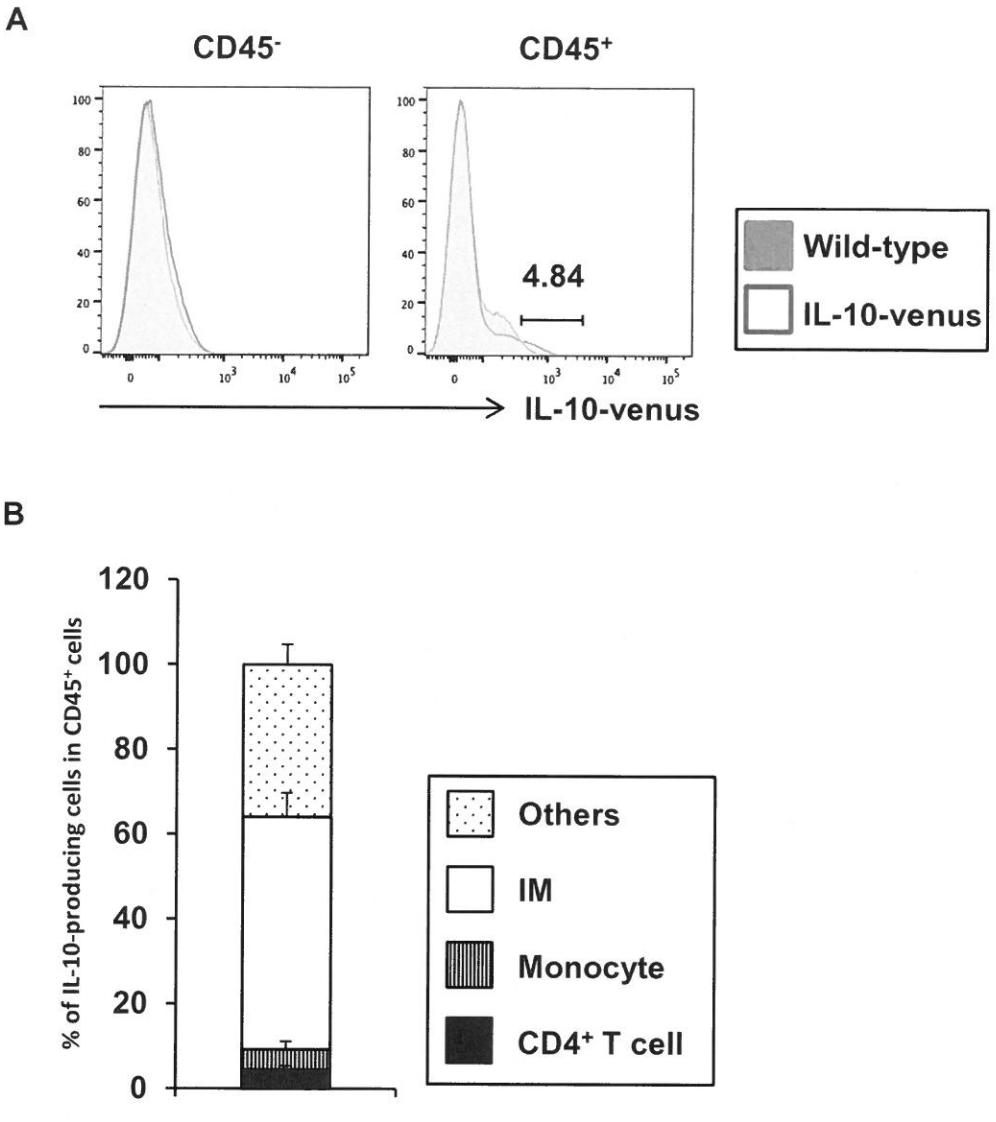
Supplementary Figure 1: IL-10-dependent suppression of pro-inflammatory cytokine production in pulmonary immune subsets. Indicated lung innate immune cell subsets from wild-type and *Il10*$^{-/-}$ mice were cultured for 24 h in the presence or absence of LPS, and the supernatants were assayed for production of TNF-α and IL-6 by ELISA. All data are representative of at least two independent experiments (mean values ± SD of duplicate well measurements). *$P < 0.05$.

Supplementary Fig. 2



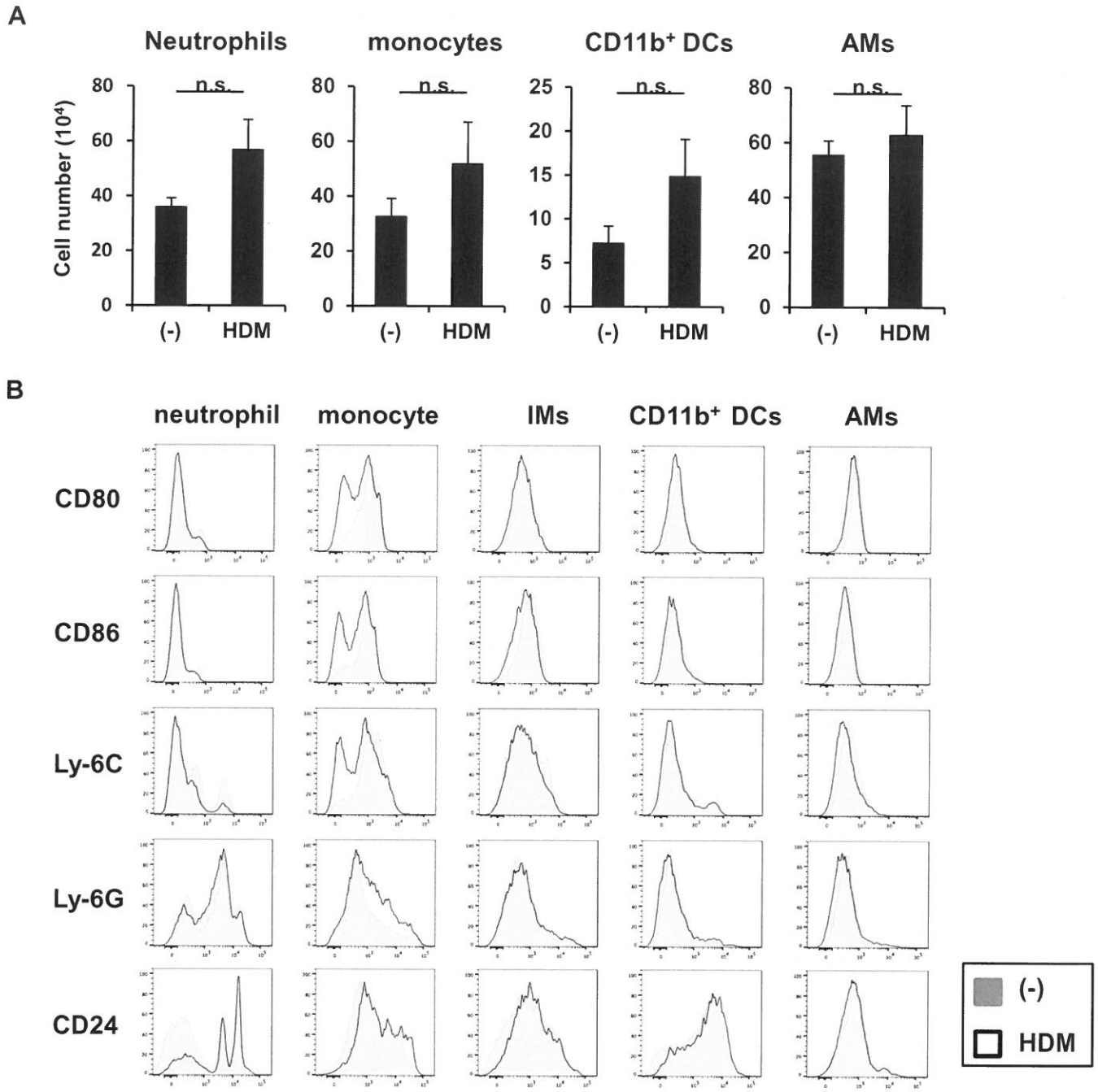
Supplementary Figure 2: IL-10-nonporducing IMs produced higher amounts of pro-inflammatory cytokines than IL-10-producing IMs did. IL-10 (venus)-positive and -negative IMs were isolated from the lung of IL-10-venus mice, and cultured in the presence of neutralizing antibodies to IL-10 for 24 h with or without LPS and FSL-1. The supernatants were assayed for production of TNF-α and IL-6 by ELISA. All data are representative of at least two independent experiments (mean values ± SD of duplicate well measurements). *P < 0.01.

Supplementary Fig. 3



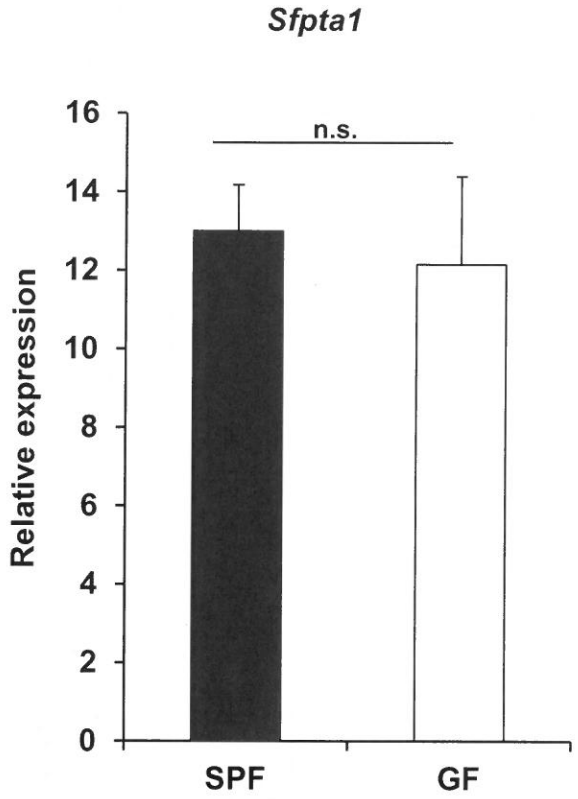
Supplementary Figure 3: Anti-inflammatory cytokine IL-10 is mainly produced by IMs in the lung under steady-state. (A) Expression of IL-10 in $CD45^+$ or $CD45^-$ cells from the lung of IL-10-venus mice. All data are representative of four independent experiments. (B) The proportion of indicated cell population among IL-10-producing $CD45^+$ lung cells in IL-10-venus mice (n = 4). Graphs show mean values ± SEM.

Supplementary Fig. 4



Supplementary Figure 4: The number of pulmonary innate immune subsets in HDM-challenged mice. (A) The number of lung innate immune cells in control (n = 3) or HDM-treated (n = 3) mice (mean values ± SEM). n.s. not significant. (B) Surface expression of CD80, CD86, Ly-6C, Ly-6G, and CD24 on indicated subsets from the lung of control (Filled histogram) and HDM-challenged (open histogram) mice.

Supplementary Fig. 5



Supplementary Figure 5: Microbiota-independent expression of SP-A in the lung. mRNA expression of *Sfpta1*, encoding surfactant associated protein A1 (SP-A) in the lung of SPF (n = 3) and germ free (GF) (n = 3) mice. Graphs show mean values ± SEM. n.s. not significant.

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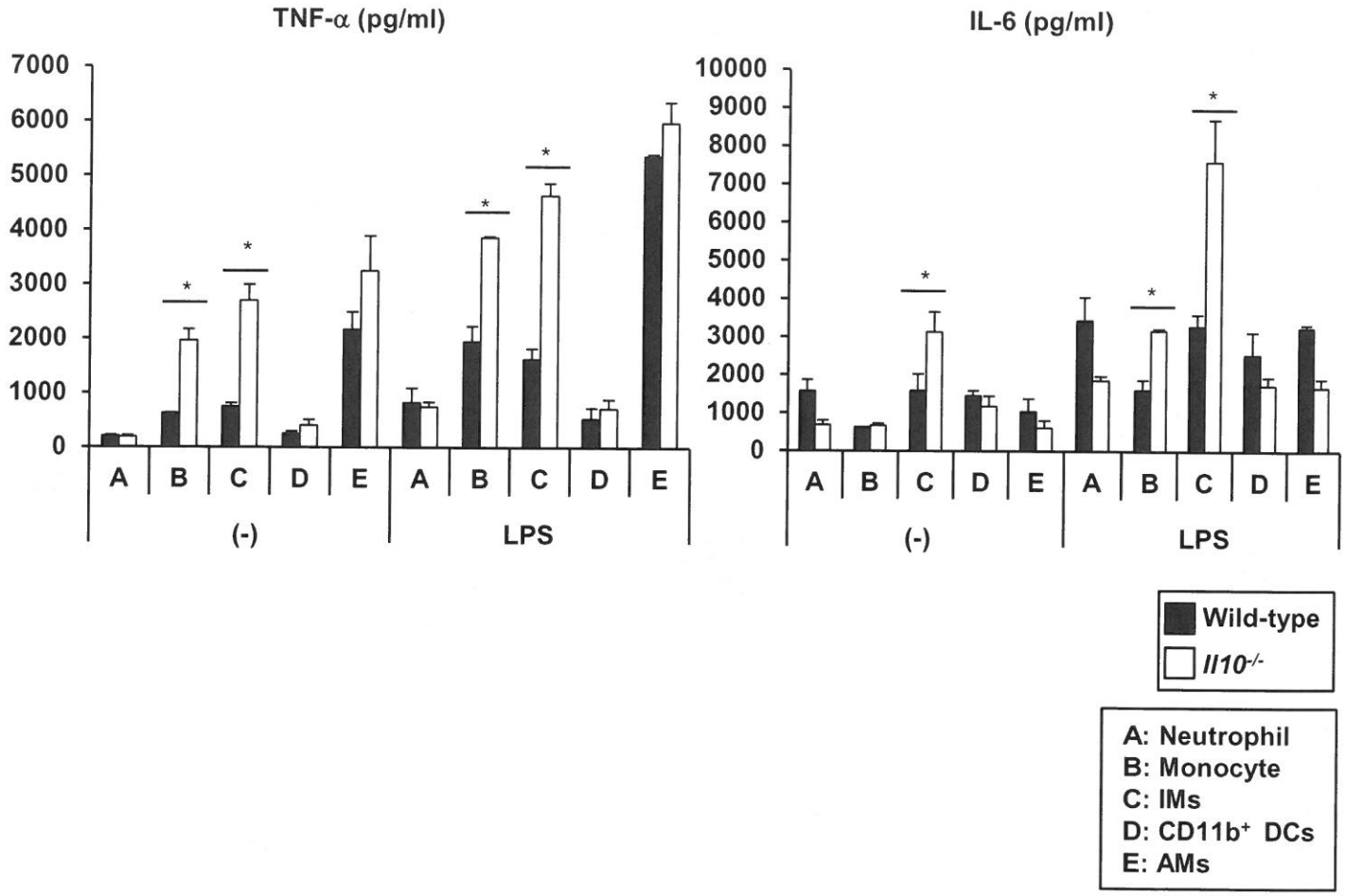

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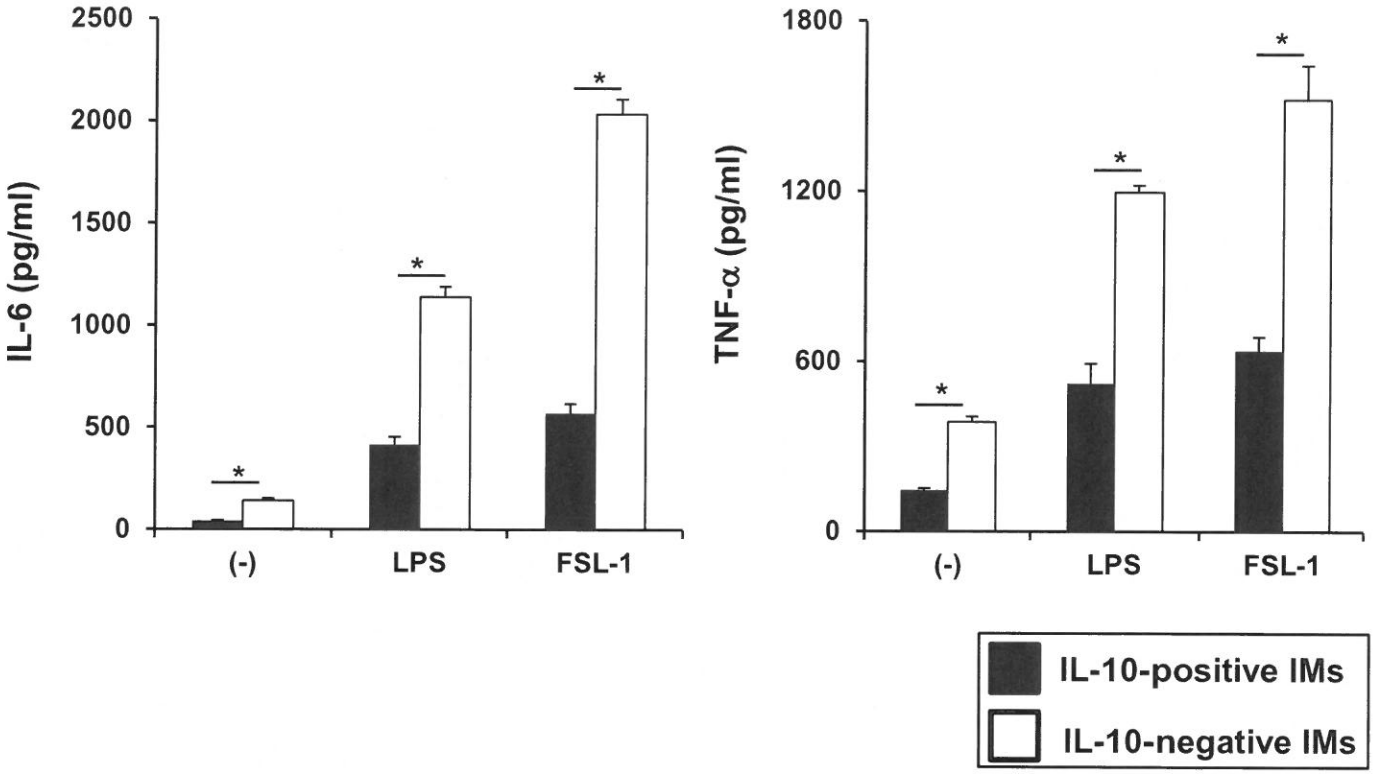

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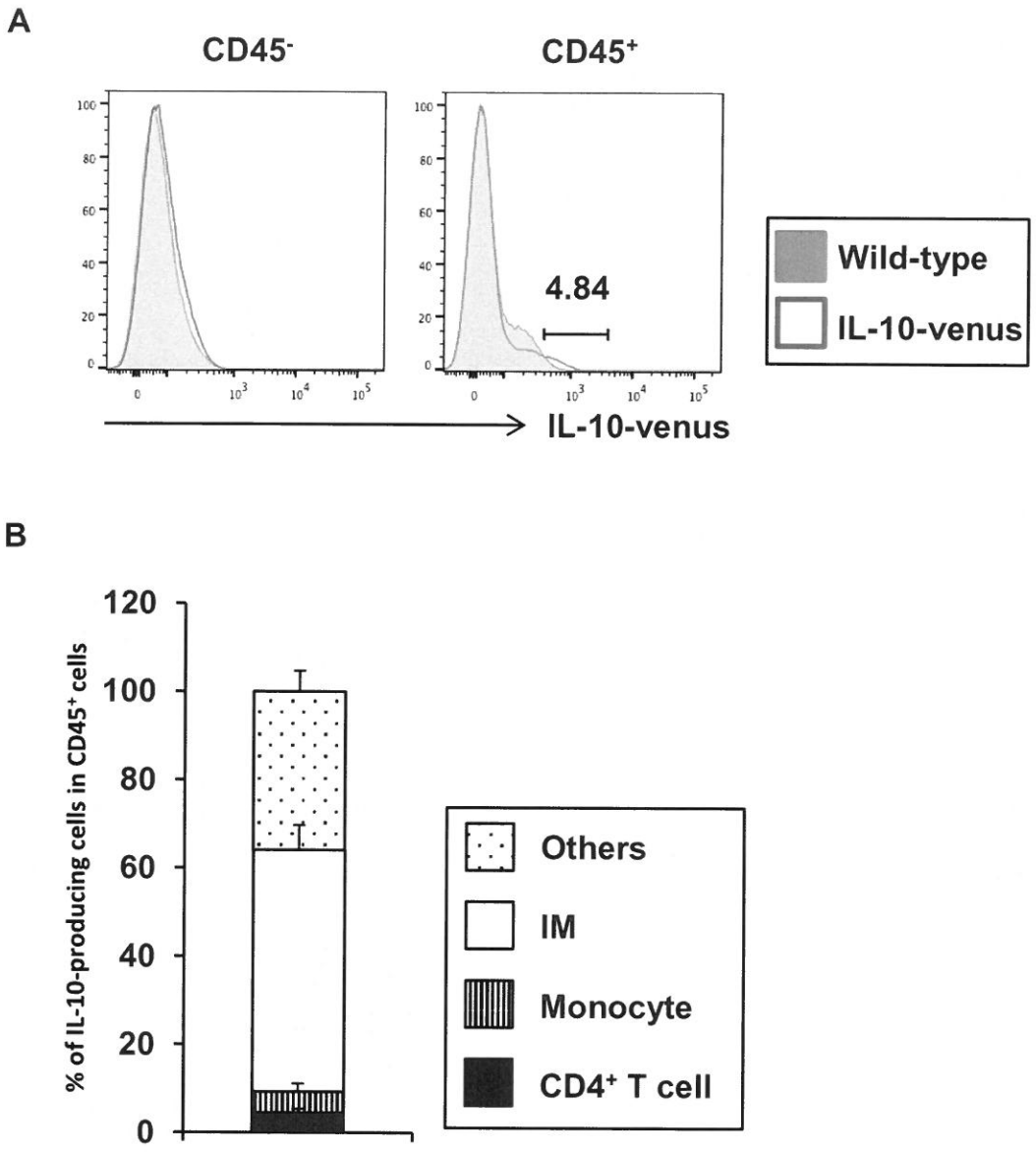

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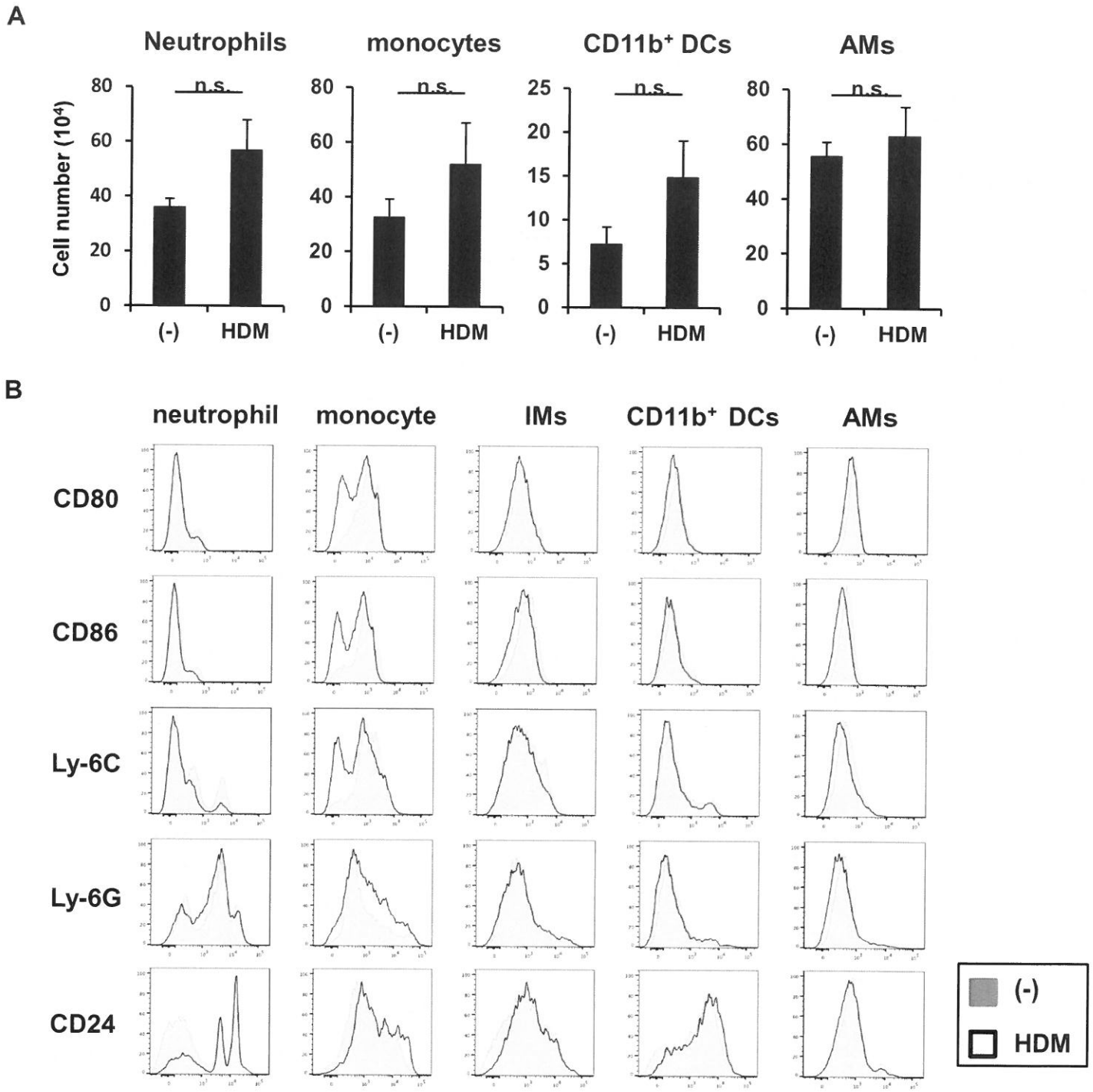

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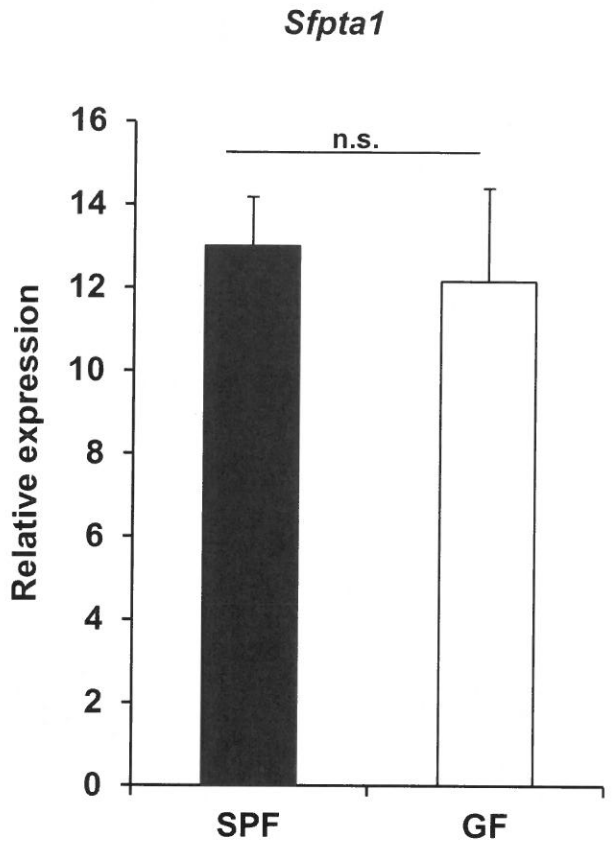

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