

T_H2-type inflammation instructs inflammatory dendritic cells to induce airway hyperreactivity

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Abstract

Dendritic cells (DCs) play critical roles in determining the fate of CD4⁺ T cells. Among DC subpopulations, monocyte-derived inflammatory DCs (iDCs) have been shown to play an important role in the induction of adaptive immune responses under inflammatory conditions. Although previous studies have shown that DCs have an indispensable role in the induction of allergic airway inflammation and airway hyperreactivity (AHR) in murine asthma models, the precise roles of iDCs in the asthmatic responses remain largely unknown. We show here that T_H2 cell-mediated inflammation in murine asthma models induces the expression of some markers of alternatively activated macrophage such as arginase 1 and resistin-like molecule- α in iDCs by a mechanism depending on the intrinsic expression of STAT6. In contrast, T_H1 cell-mediated inflammation induces iDCs to express TNF- α and inducible nitric oxide synthase (iNOS), markers of TNF- α - and iNOS-producing DCs. Moreover, we show that iDCs under a T_H2 environment play an important role in the induction of AHR, independently of allergic airway inflammation. Our results thus indicate the importance of iDCs in the induction of AHR as downstream effector cells in T_H2 cell-mediated asthmatic responses.

Keywords: arginase 1, asthma, inflammatory DC, STAT6

Introduction

Dendritic cells (DCs) have a strong ability to induce primary immune responses in naive T cells, thereby playing a critical role in bridging between innate and adaptive immune responses (1). Following antigen uptake, conventional DCs (cDCs) migrate to the draining lymph nodes and present antigens to antigen-specific naive T cells, leading to diverse immune responses (1). Meanwhile, under inflammatory conditions, monocytes (Mo) are recruited to the inflamed tissues where they develop to inflammatory DCs (iDCs) (1–4). iDCs also induce adaptive immune responses by activating antigen-specific CD4⁺ and CD8⁺ T cells (1, 3, 5, 6). In addition, recent studies have suggested that TNF- α - and inducible nitric oxide synthase (iNOS)-producing DCs (Tip-DCs), which are developed from Ly6C^{high} Mo, play an important role in the pathogen clearance as effector cells, although the relationship among Tip-DCs, iDCs, inflammatory Mo and classically activated macrophages (M ϕ) is still controversial (3, 7, 8).

Asthma is a complex disease characterized by allergic airway inflammation and airway hyperreactivity (AHR) (9–11). The roles of DCs in initiating T_H2 cell differentiation and inducing allergic airway inflammation have been established in

murine models of asthma (1, 10, 12). In addition, it has been shown that the depletion of CD11c⁺ cells during the elicitation phase of a murine asthma model prevents allergen-induced eosinophilic inflammation and AHR (13). More recently, it has been shown that CD11b⁺ DCs amplify T_H2 cell-mediated airway inflammation by recruiting T_H2 cells into the lungs during the elicitation phase of a murine asthma model (6). These findings suggest that in addition to their roles in the initiation of T-cell responses, DCs also play an important role in amplifying T_H2 cell-mediated responses at the elicitation phase of allergic airway inflammation. However, the precise role of iDCs in inducing allergic airway inflammation and AHR still remains largely unknown.

In this study, we show that T_H2 cell-mediated inflammation induces Mo-derived iDCs to express some markers of alternatively activated M ϕ (aaM ϕ), arginase 1 and resistin-like molecule- α (RELM- α), by a mechanism depending on the intrinsic expression of STAT6. In contrast, T_H1 cell-mediated inflammation induces iDCs to express Tip-DC markers. We also show that iDCs under a T_H2 environment play a critical role in the induction of AHR, independently of allergic airway

inflammation. Our results thus indicate the importance of iDCs in the induction of AHR as effector cells in T_H2 cell-mediated asthmatic responses.

Methods

Mice

BALB/c mice were purchased from Charles River Laboratories (Kanagawa, Japan). CCR2^{-/-} mice (14) on a C57BL/6 background were obtained from Dr M. Takeya (Kumamoto University). STAT6^{-/-} mice (15) were obtained from Dr K. Takeda (Osaka University). CD45.1 C57BL/6 mice were provided by RIKEN BioResource Center (RIKEN BRC) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. Enhanced green fluorescent protein transgenic (eGFP-Tg) mice on a C57BL/6 background were described previously (16). DO11.10 mice on a BALB/c background and OT-II mice on a C57BL/6 background were housed in microisolator cages under pathogen-free conditions. The Chiba University Animal Care and Use Committee approved the animal procedures used in this study.

Ovalbumin-induced allergic airway inflammation

Mice (6–8 weeks old) were immunized intra-peritoneally twice with 10 µg of ovalbumin (OVA; Sigma-Aldrich, St Louis, MO, USA) in 4 mg of aluminum hydroxide at a 2-week interval, and 2 weeks after the second immunization, the sensitized mice were challenged with aerosolized OVA (50 mg ml⁻¹) dissolved in PBS for 20 min through a DeVilbiss 646 nebulizer (DeVilbiss Corp, Somerset, PA, USA) (17). As a control, PBS alone was administered through the nebulizer.

Lung cell preparation

Mice were anesthetized with intra-peritoneal injection of Somnopentyl (1.5 mg per mouse; Kyoritsu, Tokyo, Japan). Lungs were removed from the mice after perfusion through right ventricle with PBS, cut into small fragments and digested with collagenase A solution (1 mg ml⁻¹; Roche, Indianapolis, IN, USA) in RPMI1640 medium containing 10% fetal calf serum for 40 min at 37°C with continuous agitation. After filtering through a 0.07-mm nylon mesh to remove aggregates, viable cells were enriched by 30% Percoll (GE Healthcare, Uppsala, Sweden) gradient centrifugation according to the manufacturer's instruction. Non-hematopoietic cells including epithelial cells and parenchymal cells were removed by this procedure. Lung cell suspensions were obtained after red blood cells (RBCs) were lysed with ACK lysis buffer.

FACS analysis

Lung cell suspensions were incubated with anti-CD16/32 mAb (BD Biosciences, San Diego, CA, USA) to block Fc-mediated binding, stained with the indicated antibodies and analyzed on a FACSCalibur (BD Biosciences) using CellQuestPro software (BD Biosciences) and FlowJo software (Tree Star Inc., San Carlos, CA, USA). The following antibodies were purchased from BD Biosciences: FITC-conjugated anti-CD8α (53-6.7), FITC-conjugated anti-CD11c (HL3), FITC-conjugated

anti-TCR-Vβ5.1/5.2 (MR9-4), PE-conjugated anti-CD19 (1D3), PE-conjugated anti-CD31 (MEC13.3), PE-conjugated anti-CD80 (16-10A1), PE-conjugated anti-CD86 (GL1), PE-conjugated anti-Siglec-F (E50-2440), PE-conjugated anti-Ly6C (AL-21), Biotin-conjugated anti-CD40 (3/23) and Biotin-conjugated anti-Ly6C (AL-21). The following antibodies were purchased from BioLegend (San Diego, CA, USA): FITC-conjugated anti-CD103 (2E7), PE-conjugated anti-TCR-Vα2 (B20.1), PerCP/Cy5.5-conjugated anti-I-A/I-E (M5/115.15.2), PerCP/Cy5.5-conjugated anti-CD11b (M1/70), allophycocyanin-conjugated anti-CD4 (RM4-5), allophycocyanin-conjugated anti-Ly6G (1A8), allophycocyanin-conjugated anti-EpCAM (G8.8), allophycocyanin-conjugated anti-CD45.1 (A20), Alexa Fluor 647-conjugated anti-CD11b (M1/70) and PE/Cy7-conjugated anti-CD11b. The following antibodies were purchased from eBioscience (San Diego, CA, USA): FITC-conjugated anti-CD45.2 (104) and FITC-conjugated anti-F4/80 (BM8).

FACS sorting

Lung cell suspensions were pooled from 9 to 14 mice and each myeloid cell population and epithelial cells (CD45.2⁻ CD31⁻ EpCAM⁺ cells in the upper layer of Percoll gradient centrifugation) were sorted by a FACS Aria cell sorter (BD Biosciences). The resultant cells were at least >88% pure for each cell population by FACS analysis.

Naive CD4⁺ T-cell isolation

Naive CD4⁺ T cells were collected from lymph nodes and spleens using a CD4⁺ CD62⁺ T-cell isolation kit II according to the manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). The resultant cells were >98% pure CD4⁺ CD62L⁺ T cells by FACS analysis.

CFSE labeling of CD4⁺ T cells and cell proliferation assay

OVA-specific naive CD4⁺ T cells from DO11.10 mice were labeled with CFSE (1 µM; Invitrogen, Grand Island, NY, USA) for 10 min at 37°C according to the manufacturer's instruction. CFSE-labeled CD4⁺ T cells (1 × 10⁵ cells) were stimulated with OVA323-339 peptide (200 ng ml⁻¹) in the presence of lungs myeloid cell populations isolated from the lungs of OVA-challenged BALB/c mice at the indicated ratios on a 96-well flat bottom plate. Forty-eight hours later, the frequency of dividing CD4⁺ T cells was analyzed by flow cytometry.

Transfer of bone marrow Mo

A single cell suspension of bone marrow (BM) cells was obtained from 8-week-old eGFP-Tg mice. Neutrophils (Neut) and RBCs were removed from BM cells by density centrifugation using Ficoll-Paque™ PREMIUM 1.084 (GE Healthcare). BM Mo were enriched by removing CD11c⁺, Ly6G⁺ and c-Kit⁺ cells by magnetic cell sorting according to the manufacturer's instruction (Miltenyi Biotec). Subsequently, BM Mo were positively collected by magnetic cell sorting with anti-CD115 biotin (AFS98; BioLegend) and anti-biotin microbeads (Miltenyi Biotec). The resultant cells were >95% pure CD115⁺ Ly6C⁺ BM Mo.

OVA-sensitized C57BL/6 mice were challenged with OVA inhalation as described above, and 2 h later, the mice were

injected intravenously with eGFP-Tg BM Mo (1×10^6 cells per mouse). Forty-eight hours after OVA inhalation, GFP⁺ cells in the lung and the spleen were analyzed by flow cytometry.

Adoptive transfer experiments of antigen-induced airway inflammation

OVA-specific naive CD4⁺ T cells (1×10^6 ml⁻¹) from OT-II mice were stimulated with plate-bound anti-CD3 mAb ($1 \mu\text{g ml}^{-1}$, 145-2C11; BD Biosciences) in the presence of anti-CD28 mAb ($1 \mu\text{g ml}^{-1}$, 37.51; BD Biosciences) for 3 days in T_H1-polarizing conditions [IL-12 (10 ng ml^{-1} ; Peprotech Inc. Rocky Hill, NJ, USA) and anti-IL-4 mAb ($5 \mu\text{g ml}^{-1}$, 11B11; BD Biosciences)] or in T_H2-polarizing conditions [IL-4 (10 ng ml^{-1}) and anti-IFN- γ mAb ($5 \mu\text{g ml}^{-1}$, XMG1.2; BD Biosciences)]. After cells were cultured for another 2 days in the presence of IL-2 (10 ng ml^{-1} ; Peprotech Inc.), cells were washed twice with PBS and transferred intravenously into the recipient mice (1×10^7 cells per mouse). Twenty-four hours later, these mice were challenged with OVA inhalation once or three times at a 24-h interval.

Quantitative real-time PCR analysis

Total cellular RNA was extracted with PureLink™ RNA Mini kit (Invitrogen). Reverse transcription was performed using an iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA, USA). Quantitative PCR (qPCR) was performed with an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). The levels of arginase 1, RELM- α , Ym1, macrophage mannose receptor (MMR), DC-SIGN, iNOS or TNF- α mRNA were normalized to the levels of β -actin mRNA. The sequences of PCR primers are as follows: arginase 1, forward primer: 5'-CTCCAAGCCAAAGTCCTT AGAG-3' and reverse primer: 5'-AGGAGCTGTCATTAGGGA CATC-3'; RELM- α , forward primer: 5'-TTGCAACTGCCTGTG CTTAC-3' and reverse primer: 5'-CTGGGTTCTCCACCTCT TCA-3'; Ym1, forward primer: 5'-CAGGTCTGGCAATTCTTC TGAA-3' and reverse primer: 5'-GTCTTGCTCATGTGTGTAA GTGA-3'; MMR, forward primer: 5'-CTCTGTTCAGCTATTGG ACGC-3' and reverse primer: 5'-CGGAATTCTGGGATTCA GCTTC-3'; DC-SIGN, forward primer: 5'-CCTTGTCAAAGATC GACTGTGC-3' and reverse primer: 5'-CCCATCCAAGTGTA GCCTCTC-3'; iNOS, forward primer: 5'-GTTCTCAGCCCAAC AATACAAGA-3' and reverse primer: 5'-GTGGACGGGTGCA TGTCAC-3'; TNF- α , forward primer: 5'-ATGGCCTCCCTCTCA TCAGT-3' and reverse primer: 5'-GCTCCTCCACTTGGTGGT TT-3'; β -actin, forward primer: 5'-GGCTGTATCCCTCCAT CG-3' and reverse primer: 5'-CCAGTTGGTAACAATGCCA TGT-3'.

Cytokine assay

The amounts of IL-4, IL-5 and IL-13 in the bronchoalveolar lavage fluid (BALF) were measured by ELISA kits [IL-4 (BD Biosciences), IL-5 and IL-13 (R&D Systems, Minneapolis, MN, USA)] according to the manufacturer's instruction.

Immunoblotting

Immunoblotting was performed as described previously (18) by using anti-arginase 1 antibody (sc-20150; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HSP 90 α / β

antibody (sc-7947; Santa Cruz Biotechnology) and anti-Rabbit IgG (H+L) HRP Conjugate (81-6120; Zymed, San Francisco, CA, USA).

Histological analysis

After lungs were perfused with PBS, the left lobes of the lungs were fixed in 10% formaldehyde for 48 h, embedded in paraffin, sectioned (4- μm thick) and stained with hematoxylin and eosin according to standard protocols.

Measurement of airway reactivity

Airway reactivity to aerosolized acetylcholine was assessed by using a computer-controlled small animal ventilator system (flexiVent; SCIREQ Inc., Montreal, Quebec, Canada), as described elsewhere (19).

Generation of mixed BM chimeric mice

CD45.1 mice (6-week-old) on a C57BL/6 background were irradiated (9.5 Gy) and injected with BM cells (2×10^6 cells per mouse) from CD45.1 wild-type (WT) mice and CD45.2 STAT6^{-/-} mice on a C57BL/6 background at a 2:1 ratio. Eight weeks later, OT-II CD4⁺ T cells that had been stimulated under T_H2-polarizing conditions were transferred intravenously to the chimeric mice. These mice were then challenged with OVA inhalation three times at a 24-h interval. Twenty-four hours after the final inhalation, lung cells were isolated from the mice ($n = 10$) and pooled cells were stained with anti-CD45.2 FITC, anti-CD11c PE, anti-I/A-I/E PerCP/Cy5.5, anti-CD11b PE/Cy7 and anti-CD45.1 allophycocyanin. WT iDCs (CD11b⁺ CD11c⁺ I-A/I-E⁺ CD45.1⁺ CD45.2⁻ cells) and STAT6^{-/-} iDCs (CD11b⁺ CD11c⁺ I-A/I-E⁺ CD45.1⁻ CD45.2⁺ cells) were isolated by a FACSaria cell sorter.

Data analysis

Data are summarized as means $\pm$ SD. The statistical analysis of the results was performed by the unpaired *t*-test. *P* values <0.05 were considered significant.

Results

CD11b^{high} CD11c^{int} MHC II^{high} iDCs accumulate in the lung during allergic airway inflammation

Although the roles of DCs in the sensitization to allergens are well characterized, their roles in the effector phase of allergic airway inflammation are still poorly understood. To address the roles of DCs during the effector phase of allergic airway inflammation, we first reevaluated DCs at the site of allergic airway inflammation by flow cytometric and morphological analysis. As shown in Fig. 1A, based on the expression of CD11b, CD11c, Siglec-F, MHC II, Ly6C and Ly6G, we defined six myeloid populations in the lung of OVA-sensitized and -challenged BALB/c mice: eosinophils (Eo; CD11b^{high} CD11c^{low} Siglec-F^{high}), iDCs (CD11b^{high} CD11c^{int} MHC II^{high}), cDCs (CD11b^{low} CD11c^{int} MHC II^{high}), alveolar macrophages (AMs; CD11b^{int} CD11c^{high} Siglec-F^{high}), Mo (CD11b^{high} CD11c^{low} Ly6c^{high} Ly6g^{low}) and Neut (CD11b^{high} CD11c^{low} Ly6c^{high} Ly6g^{high}). The morphological analyses of isolated cell populations showed that iDCs

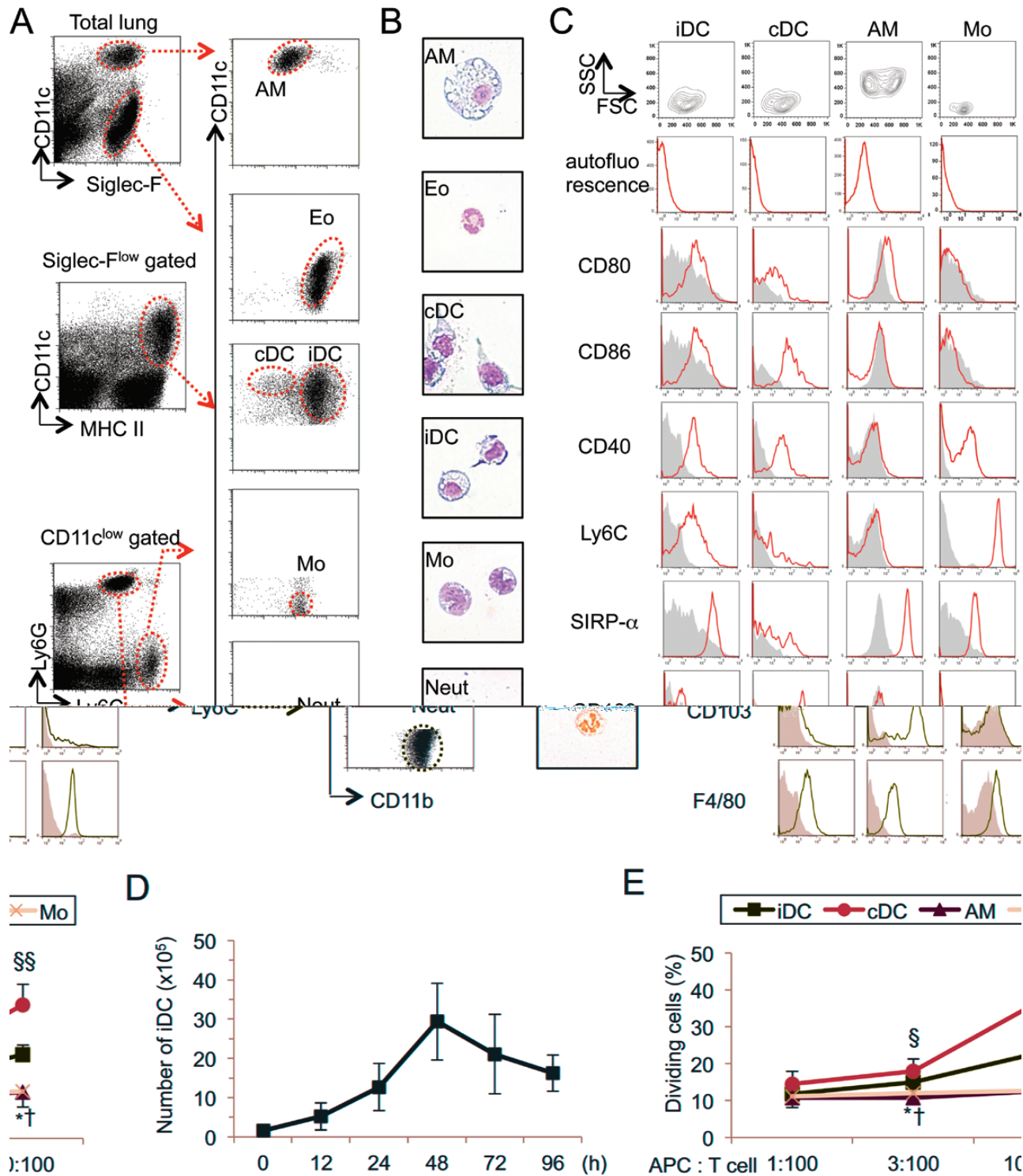


Fig. 1. CD11b^{high} CD11c^{int} MHC II^{high} cells are increased in the lung during allergic airway inflammation. (A and B) OVA-sensitized BALB/c mice were challenged with inhaled OVA and 48h later, cells were harvested from the lung and stained with a mixture of antibodies against CD11c, Siglec-F, MHC II and CD11b or a mixture of antibodies against CD11c, Ly6C, Ly6G and CD11b. (A) Flow cytometric analyses identified six types of myeloid cells: Eo, iDCs, cDCs, AMs, Mo and Neut. (B) Each myeloid population was sorted by flow cytometry and cytopsin cell preparations were stained with Wright-Giemsa solution. Data are representative of four independent experiments. (C) OVA-sensitized BALB/c mice were challenged with inhaled OVA and 48h later, forward and side scatter, autofluorescence and the expression levels of CD80, CD86, CD40, Ly6C, SIRP-α, CD103 and F4/80 of iDCs (CD11b^{high} CD11c^{int} MHC II^{high} cells), cDCs (CD11b^{low} CD11c^{int} MHC II^{high} cells), AMs (CD11b^{int} CD11c^{high} MHC II^{low} cells) and Mo (CD11b^{high} CD11c^{low} Ly6C^{high} cells) were examined by flow cytometry. Filled histograms show staining controls with isotype-matched control antibody. Data are representative of three independent experiments. (D) OVA-sensitized BALB/c mice were challenged once with inhaled OVA. At the indicated times after the inhaled OVA challenge, the number of iDCs (CD11b^{high} CD11c^{int} MHC II^{high} cells) in the lung

had small dendrites and shared common features with cDCs (Fig. 1B). As shown in Fig. 1C, iDCs expressed co-stimulatory molecules such as CD80, CD86 and CD40. iDCs also expressed Ly6C and SIRP- α (Fig. 1C) as previously described (1). On the other hand, iDCs expressed lower levels of CD103 as compared with those in cDCs (Fig. 1C). Furthermore, iDCs lacked autofluorescence and showed distinct forward and side scatter characteristics from AMs (Fig. 1C). The number of iDCs in the lung begun to increase at 12 h after OVA inhalation in OVA-sensitized BALB/c mice and reached a peak at 48 h after OVA inhalation (Fig. 1D).

To evaluate the ability of iDCs in the lung as antigen-presenting cells, CFSE-labeled OVA-specific naive CD4⁺ T cells were stimulated with OVA323-339 peptide in the presence of isolated iDCs, cDCs, AMs or Mo for 48 h, and the frequency of dividing T cells (CFSE^{low} cells) was evaluated by flow cytometry. As shown in Fig. 1E, iDCs and cDCs but not AMs or Mo induced the proliferation of naive CD4⁺ T cells, although iDCs were less effective than cDCs. Taken together, these results suggest that iDCs at the site of allergic airway inflammation have distinct properties from cDCs and AMs.

iDCs accumulate in the site of allergic airway inflammation in a CCR2-dependent manner

Previous studies have suggested that iDCs are derived from Mo (2, 3). To examine whether iDCs at the site of allergic airway inflammation are derived from Mo, CD115^{high} Ly6C^{high} BM Mo were isolated from eGFP-Tg mice and transferred to OVA-sensitized mice at 2 h after the inhaled OVA challenge. As shown in Fig. 2A, transferred Mo developed into CD11b^{high} CD11c^{int} MHC II^{high} iDCs at the site of allergic airway inflammation, whereas in the spleen where no inflammation was induced, the majority of transferred Mo was still negative for CD11c expression (the frequency of CD11b^{high} CD11c^{int} MHC II^{high} cells in GFP⁺ cells in lung $47.3 \pm 4.9\%$ versus the frequency of CD11b^{high} CD11c^{int} MHC II^{high} cells in GFP⁺ cells in spleen $5.3 \pm 2.6\%$, means $\pm$ SD, $n = 3$).

Given that the recruitment of Ly6C^{high} Mo into inflammatory sites has been shown to depend on CCR2 (3, 8), we next examined whether the accumulation of iDCs in the lung depends on CCR2. Antigen-induced accumulation of iDCs in the lung was significantly decreased in T_H2 cell-transferred and OVA-challenged CCR2^{-/-} mice as compared with that in T_H2 cell-transferred and OVA-challenged WT mice (Fig. 2B and C). Taken together, these results suggest that iDCs at the site of allergic airway inflammation are derived from Mo, which are recruited into the lung in a CCR2-dependent manner.

iDCs share some features with aaM ϕ

To further examine the features of iDCs, we then examined the expression of DC- and macrophage-related genes in iDCs by qPCR analysis. Importantly, iDCs isolated from the site of allergic airway inflammation expressed mRNA for several markers of aaM ϕ (20), such as arginase 1 and RELM- α as compared with those in cDCs, AMs or Mo (Fig. 3A). iDCs in the lung also expressed a DC marker DC-SIGN as cDCs did (Fig. 3A). On the other hand, iDCs in the lung did not express mRNA for other markers of aaM ϕ , Ym1 or MMR (Fig. 3A). Moreover, mRNA levels of arginase 1 in iDCs in the lung were significantly higher than those in lung epithelial cells (Fig. 3B), whereas mRNA levels of RELM- α in iDCs in the lung were lower than those in lung epithelial cells (Fig. 3B). The strong expression of arginase 1 in iDCs at the site of allergic airway inflammation was confirmed at protein levels by immunoblotting (Fig. 3C). In addition, consistent with the reduced numbers of iDCs in the lungs of T_H2 cell-transferred and OVA-challenged CCR2^{-/-} mice (Fig. 2B and C), arginase 1 levels in the lung in T_H2 cell-transferred and OVA-challenged CCR2^{-/-} mice were significantly reduced as compared with those in T_H2 cell-transferred and OVA-challenged WT mice (Fig. 3D). Taken together, these results suggest that iDCs are the main producer of arginase 1 at the site of allergic airway inflammation.

A T_H2 environment induces aaM ϕ markers in iDCs in a STAT6-dependent manner

We next examined the role of T_H2 cells in the induction of aaM ϕ markers in iDCs. As shown in Fig. 4A, the expression of arginase 1 and RELM- α was induced in iDCs in the lungs of T_H2 cell-transferred and OVA-challenged mice but not in T_H1 cell-transferred and OVA-challenged mice, although the numbers of iDCs in the lungs of T_H1 cell-transferred and OVA-challenged mice were similar to those in T_H2 cell-transferred and OVA-challenged mice (T_H1 cell-injected mice 25.2 ± 11.4 versus T_H2 cell-injected mice 28.5 ± 6.6 , $\times 10^5$ cells per mouse, $n = 4$). In contrast, in the lungs of T_H1 cell-transferred and OVA-challenged mice, iDCs expressed TNF- α and iNOS (Fig. 4A), which are makers for Tip-DCs (7). These results indicate that a T_H2 environment is important for the induction of aaM ϕ markers in iDCs.

We next examined the role of IL-4/IL-13-STAT6 pathways in the induction of aaM ϕ markers in iDCs in a T_H2 environment by using mixed BM chimeric mice. BM cells from CD45.1 WT mice and CD45.2 STAT6^{-/-} mice were mixed and transferred to irradiated CD45.1 WT mice and 2 months later, OVA-specific T_H2 cells were transferred to the mice. The chimeric mice were then challenged with OVA inhalation three times and 24 h after the last OVA inhalation, STAT6^{-/-} iDCs and WT iDCs were isolated from the lung by flow cytometry. As shown in Fig. 4B, the induction of aaM ϕ markers was reduced in

was evaluated by flow cytometry. Data are means $\pm$ SD of the number of iDCs, $n = 4-6$ mice for each time point. (E) OVA-sensitized BALB/c mice were challenged with inhaled OVA and 48 h later, iDCs, cDCs, AMs and Mo were isolated from the lung by flow cytometry. CFSE-labeled OVA-specific naive CD4⁺ T cells from DO11.10 mice (1×10^5 cells) were stimulated with OVA323-339 peptide (200 ng ml^{-1}) in the presence of isolated iDCs, cDCs, AMs or Mo for 48 h at the indicated ratios. The frequency of dividing CD4⁺ T cells was determined by flow cytometry. Data are means $\pm$ SD of the frequency of dividing CD4⁺ T cells from four experiments. [§]Significantly different between iDCs and cDCs, [§] $P < 0.05$, ^{§§} $P < 0.01$. *Significantly different between iDCs and AMs, * $P < 0.05$. [†]Significantly different between iDCs and Mo, [†] $P < 0.05$.

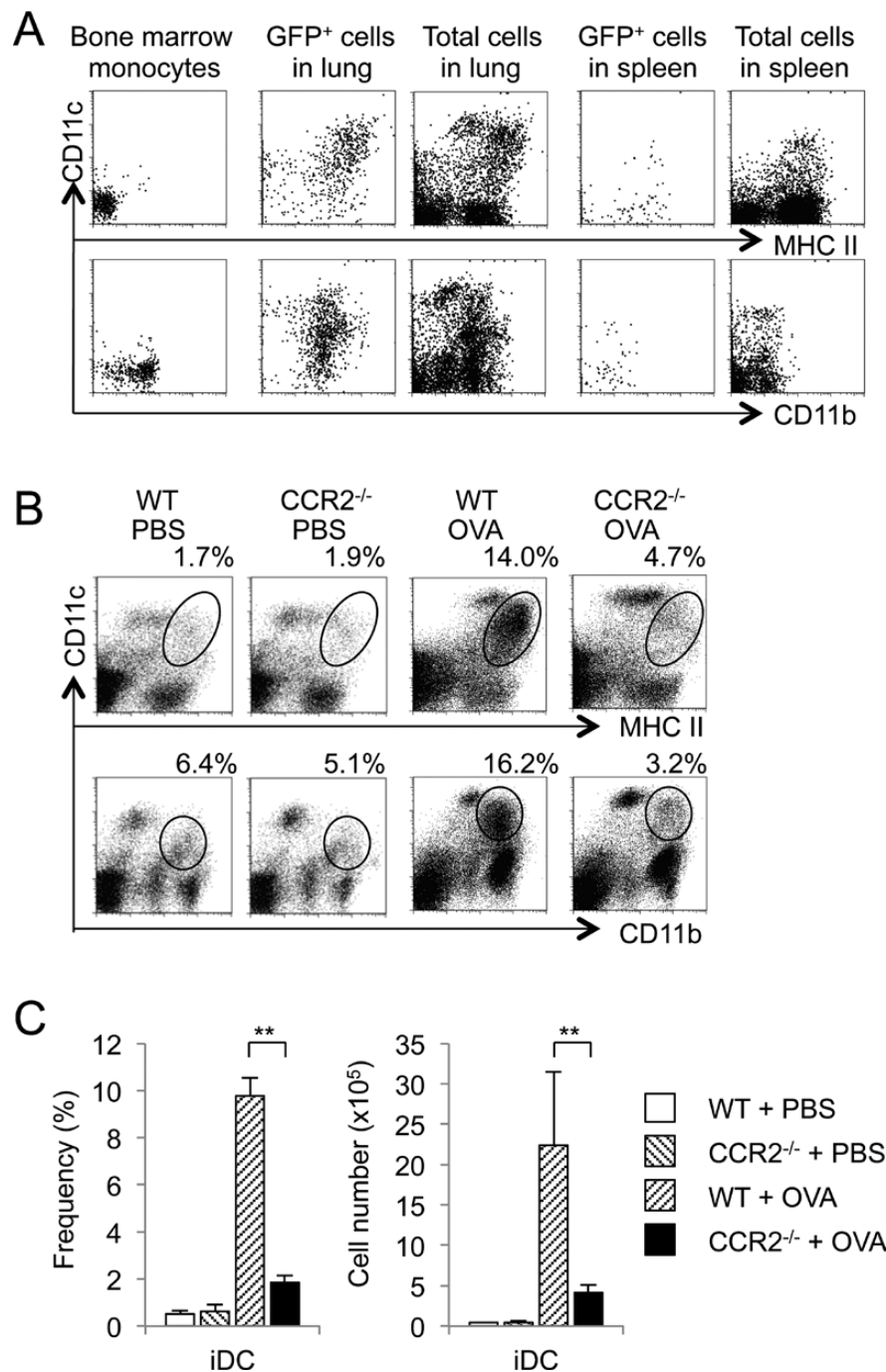


Fig. 2. Mo-derived iDCs accumulate in the site of allergic airway inflammation in a CCR2-dependent manner. (A) OVA-sensitized C57BL/6 mice were challenged with inhaled OVA and 2 h later, the mice were injected intravenously with BM Mo (1×10^6 cells) from eGFP-Tg mice. Forty-eight hours after the inhalation, cells in the lung and spleen were analyzed by flow cytometry. Freshly isolated BM Mo were also analyzed by flow cytometry. Data are representative of three independent experiments. (B and C) OVA-specific T_H2 cells were transferred intravenously to CCR2^{-/-} mice or littermate WT mice. Twenty-four hours later, these mice were challenged with inhaled OVA or PBS. Forty-eight hours after the inhalation, cells harvested from the lung were analyzed by flow cytometry. Shown are representative FACS profiles of cells in the lung (B) and the frequency and number of iDCs (CD11b^{high} CD11c^{int} MHC II^{high} cells) in the lung (C). Circles indicate the gating area. Data are means $\pm$ SD, $n = 5$ mice in each group, ** $P < 0.01$.

STAT6^{-/-} iDCs as compared with that in WT iDCs isolated from the same chimeric mice. In contrast, the expression of iNOS was rather enhanced in STAT6^{-/-} iDCs (Fig. 4B). These results indicate that intrinsic expression of STAT6 in iDCs is required for the induction of aaMq markers.

iDCs play a critical role in the induction of AHR, independently of the induction of T_H2 cell-mediated allergic airway inflammation

We next evaluated the role of iDCs in the induction of allergic airway inflammation and AHR by analyzing T_H2 cell-transferred

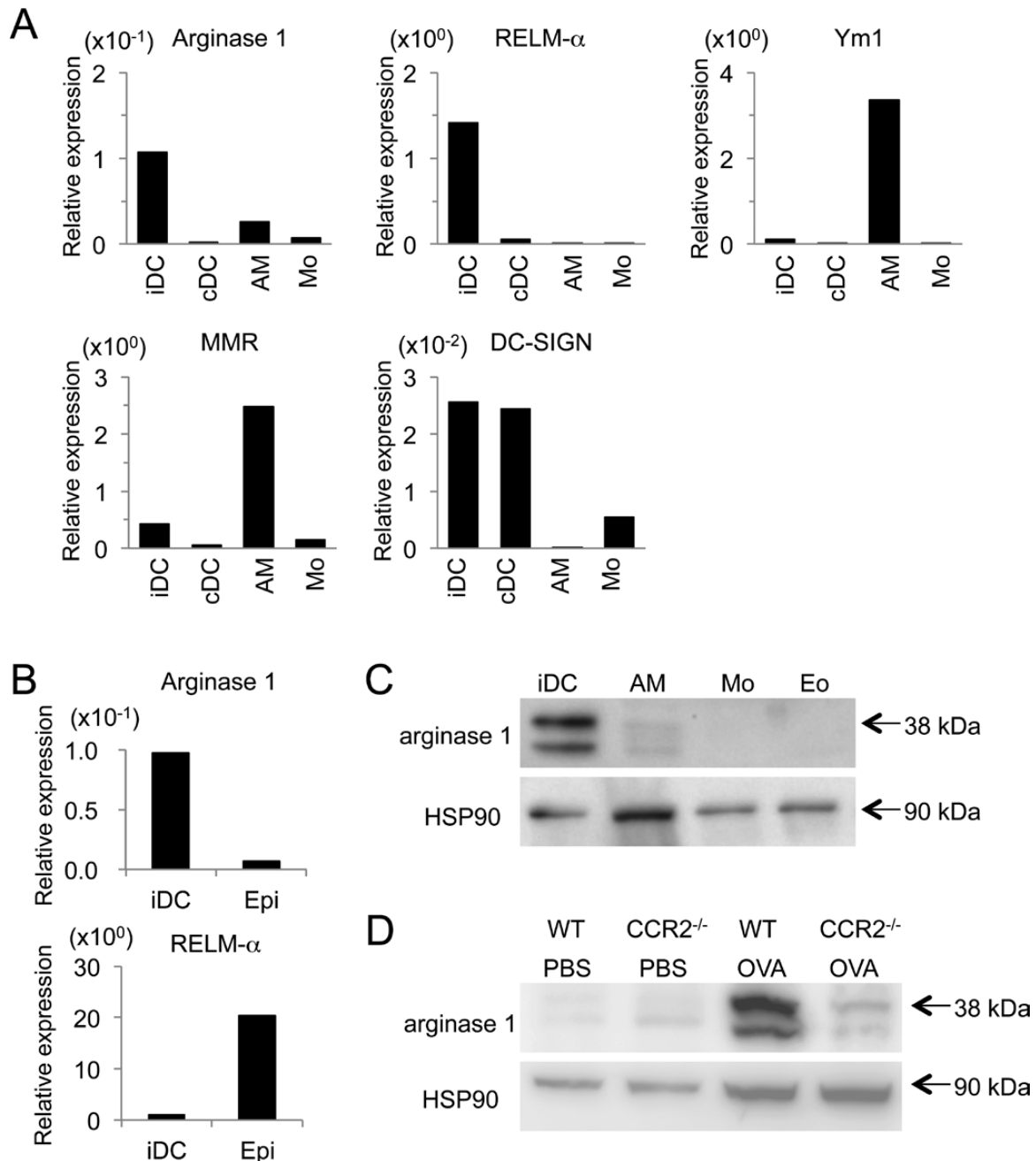


Fig. 3. iDCs share some features with aaM ϕ . (A) OVA-sensitized BALB/c mice were challenged with inhaled OVA and 48 h later, iDCs (CD11b^{high} CD11c^{int} MHC II^{high} cells), cDCs (CD11b^{low} CD11c^{int} MHC II^{high} cells), AMs (CD11b^{int} CD11c^{high} MHC II^{low} cells) and Mo (CD11b^{high} CD11c^{low} Ly6C^{high} cells) were isolated by flow cytometry. The expression levels of the indicated genes (arginase 1, RELM- α , Ym1, MMR and DC-SIGN) were evaluated by qPCR. Shown are representatives of relative expression from four independent experiments. (B) OVA-sensitized BALB/c mice were challenged with inhaled OVA and 48 h later, iDCs and epithelial cells (Epi) were isolated by flow cytometry. The expression levels of arginase 1 and RELM- α in iDCs and epithelial cells were examined by qPCR. Shown are representatives of relative expression from three independent experiments. (C) OVA-sensitized BALB/c mice were challenged with inhaled OVA and 48 h later, iDCs, AMs, Mo and Eo were isolated by flow cytometry and the lysates of each cell population were subjected to immunoblotting with anti-arginase 1 antibody and anti-HSP90 α/β antibody. Shown are representative of three independent experiments. (D) OVA-specific T_H2 cells were transferred intravenously to CCR2^{-/-} mice or littermate WT mice and 24 h later, these mice were challenged with OVA or PBS inhalation. Forty-eight hours after the inhalation, lung cell suspensions were harvested and cell lysates were subjected to immunoblotting with antibodies against arginase 1 and HSP90 α/β . The results shown are representative of three independent experiments.

and OVA-challenged CCR2^{-/-} mice. As shown in Fig. 5, antigen-induced infiltration of transferred OVA-specific T_H2 cells in the lung (Fig. 5A) and the levels of T_H2 cytokines (IL-4, IL-5 and

IL-13) in the BALF (Fig. 5B) were indistinguishable between T_H2 cell-transferred CCR2^{-/-} mice and T_H2 cell-transferred WT mice. On the other hand, consistent with the data shown in

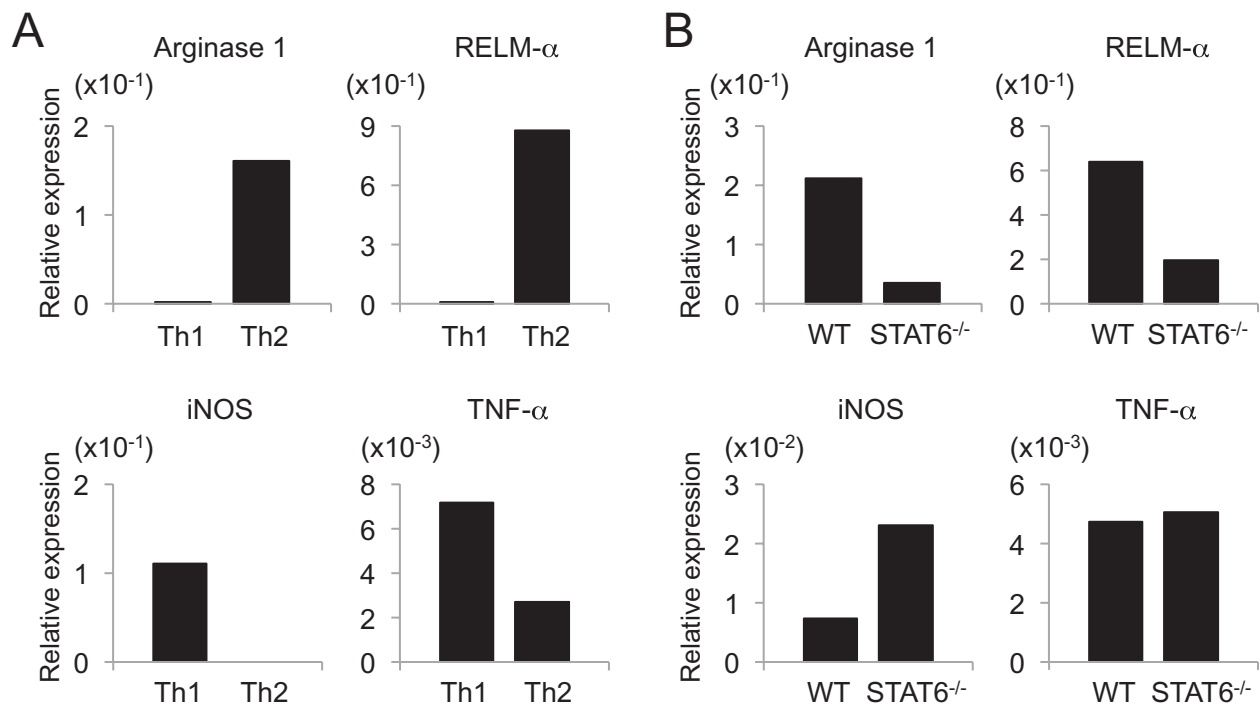


Fig. 4. T_H2-type airway inflammation induces the expression of aaMφ markers in iDCs in a STAT6-dependent manner. (A) OVA-specific T_H1 or T_H2 cells were transferred intravenously to WT mice and 24 h later, these mice were challenged with OVA inhalation three times at a 24-h interval. Twenty-four hours after the last OVA inhalation, iDCs were isolated by flow cytometry and the expression levels of the indicated genes were examined by qPCR. Shown are representatives of relative expression from four independent experiments. (B) BM cells from CD45.1 WT mice and CD45.2 STAT6^{-/-} mice were mixed and transferred intravenously to irradiated CD45.1 WT recipient mice as described in the Methods. Eight weeks later, OVA-specific T_H2 cells were transferred intravenously to the chimeric mice and the mice were challenged with OVA inhalation three times at a 24-h interval. CD45.1⁺ WT iDCs and CD45.2⁺ STAT6^{-/-} iDCs were isolated by flow cytometry and the expression levels of the indicated genes were examined by qPCR. Shown are representatives of relative expression from four independent experiments.

Fig. 2B, antigen-induced infiltration of iDCs and Mo into the lung was significantly reduced in T_H2 cell-transferred and OVA-challenged CCR2^{-/-} mice as compared with that in T_H2 cell-transferred and OVA-challenged WT mice (Fig. 5C). However, antigen-induced infiltration of Eo, Neut, T cells and B cells was indistinguishable between T_H2 cell-transferred and OVA-challenged CCR2^{-/-} mice and T_H2 cell-transferred and OVA-challenged WT mice (Fig. 5C). Furthermore, T_H2 cell-transferred and OVA-challenged CCR2^{-/-} mice had similar levels of antigen-induced peribronchial and perivascular inflammation of the lung to T_H2 cell-transferred and OVA-challenged WT mice (Fig. 5D). These results suggest that iDCs are not required for the induction of T_H2 cell-mediated allergic airway inflammation if antigen-specific T_H2 cells were adoptively transferred.

Importantly, however, AHR, which is an important characteristic of asthma, was significantly reduced in T_H2 cell-transferred and OVA-challenged CCR2^{-/-} mice as compared with that in T_H2 cell-transferred and OVA-challenged WT mice (Fig. 6A). In addition, adoptive transfer of WT iDCs isolated from the site of allergic airway inflammation restored AHR in T_H2 cell-transferred and OVA-challenged CCR2^{-/-} mice (Fig. 6B). Taken together, these results suggest that iDCs at the site of allergic airway inflammation play a critical role in the induction of AHR by a mechanism independent of T_H2 cell-mediated allergic airway inflammation.

Discussion

In this study, we show that T_H2 cell-mediated airway inflammation instructs Mo-derived iDCs to express some aaMφ markers by a mechanism depending on the intrinsic expression of STAT6. We also show that iDCs under a T_H2 environment play an indispensable role in the development of AHR, independently of allergic airway inflammation.

We show that iDCs accumulated at the site of allergic airway inflammation express some aaMφ markers. We identified iDCs as a distinct population from cDCs or AMs at the site of allergic airway inflammation not only by flow cytometric and morphological analyses but also by antigen-presenting capacity (Fig. 1). We found that iDCs isolated from the site of allergic airway inflammation expressed aaMφ markers, arginase 1 and RELM-α, but did not express other aaMφ markers, Ym1 and MMR (Fig. 3A). In contrast, AMs at the site of allergic airway inflammation expressed aaMφ markers, MMR and Ym1, but small amounts of arginase 1 and RELM-α (Fig. 3A). We thus demonstrate that iDCs and AMs express different aaMφ markers even under the same allergic inflammatory condition. It might be related to the functional roles of those cells, such as a strong phagocytic activity of AMs.

We also show that T_H2 cell-mediated airway inflammation induces iDCs to express aaMφ markers through a mechanism depending on the intrinsic expression of STAT6. We found that the expression of aaMφ markers was induced in iDCs in the

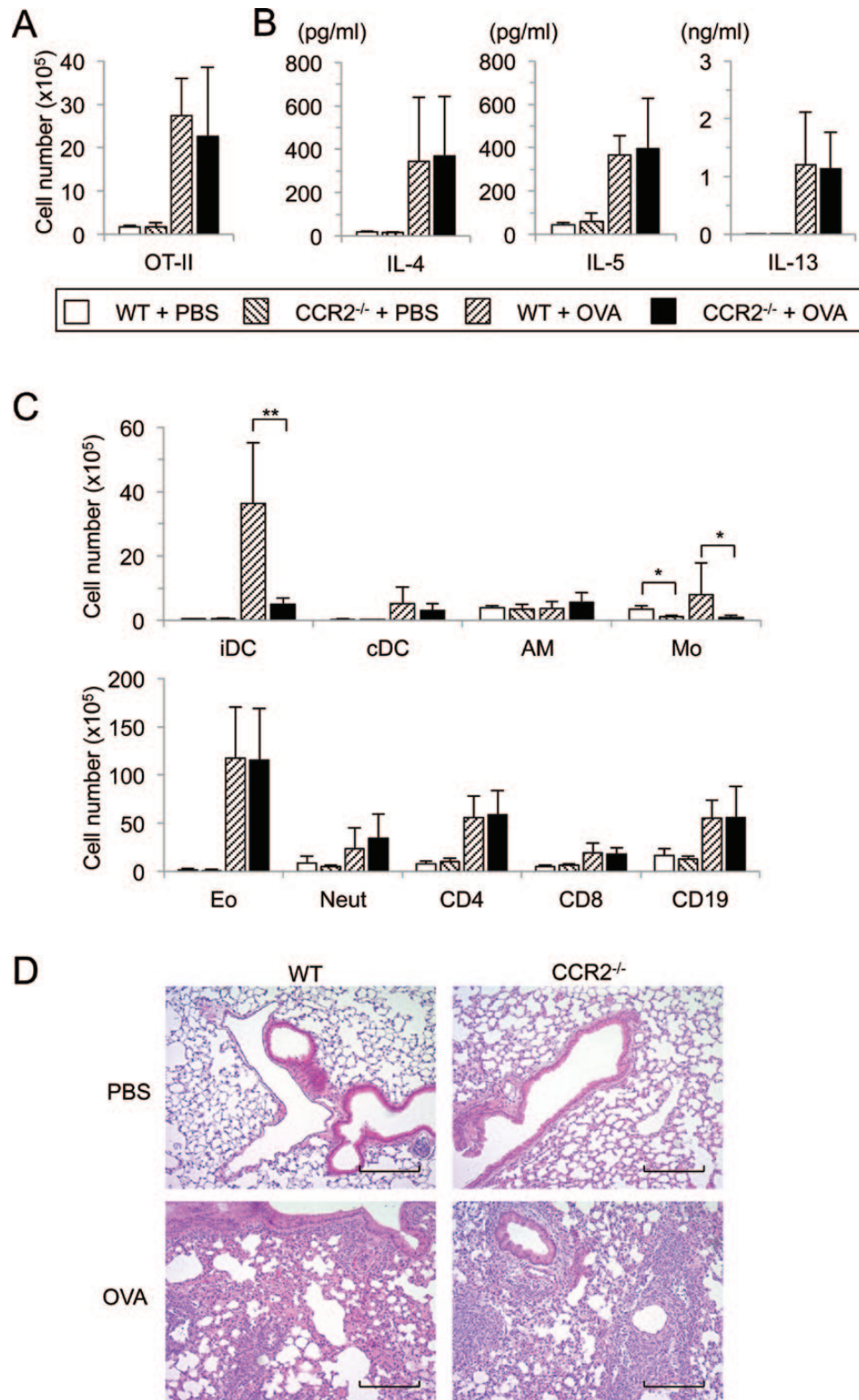


Fig. 5. T_H2 cell-mediated allergic airway inflammation is indistinguishable between T_H2 cell-transferred and antigen-challenged CCR2^{-/-} mice and WT mice. OVA-specific T_H2 cells were transferred intravenously to CCR2^{-/-} mice or littermate WT mice and 24 h later, these mice were challenged with OVA inhalation three times at a 24-h interval. (A–C) Twenty-four hours after the last OVA inhalation, the numbers of the transferred T cells (CD4⁺ Vα2⁺ Vβ5.1/5.2⁺ cells) (A) and indicated cell populations (C) harvested from the lung and IL-4, IL-5 and IL-13 levels in the bronchoalveolar lavage fluid (B) were evaluated. Data are means $\pm$ SD, $n = 5$ mice in each group. * $P < 0.05$, ** $P < 0.01$. (D) Twenty-four hours after the last OVA inhalation, histological analysis of the lung was performed. Representative photomicrographs of lung sections with hematoxylin and eosin staining are shown, $n = 4$ mice in each group. Scale bars: 200 μ m.

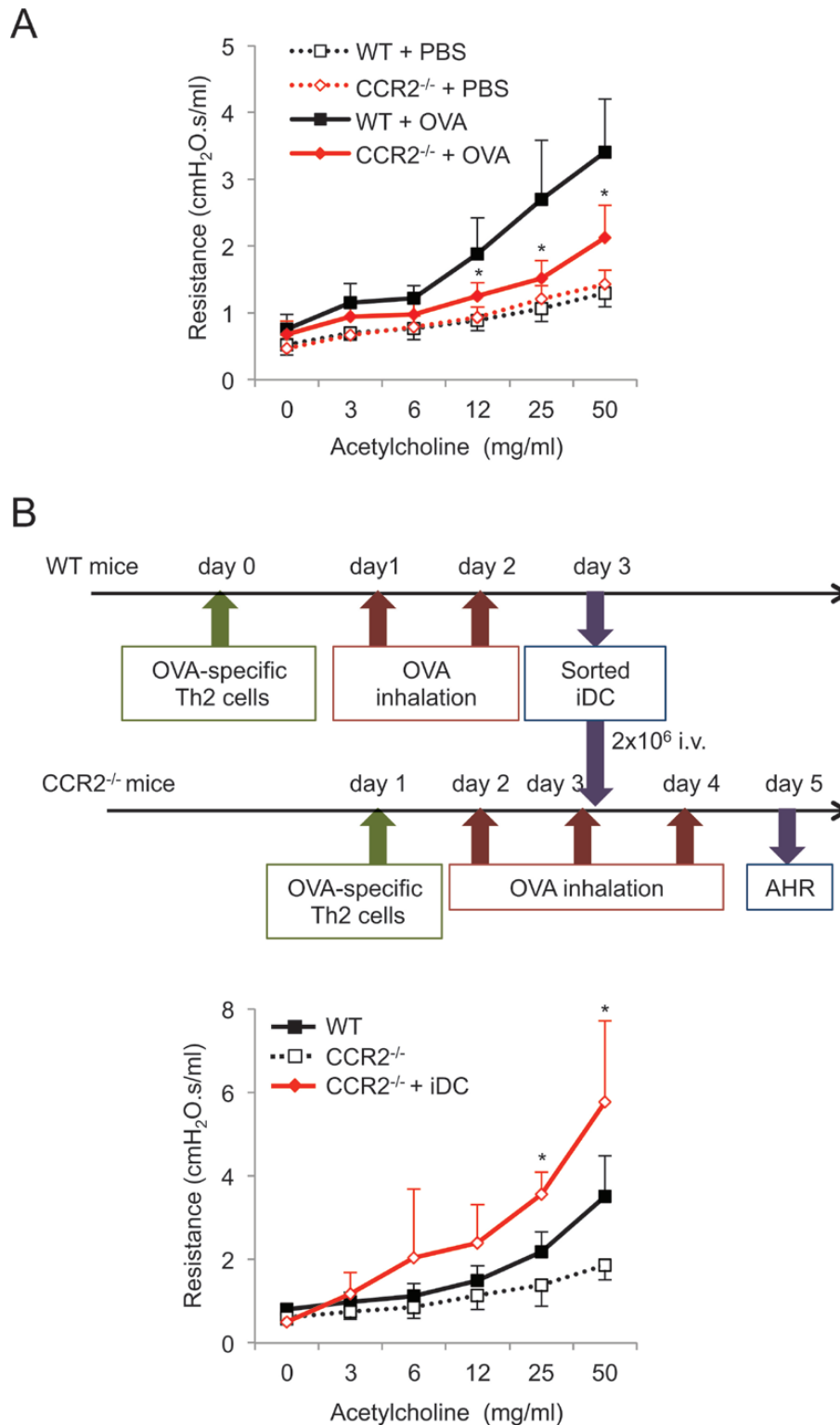


Fig. 6. iDCs play a critical role in the induction of AHR. (A) OVA-specific T_H2 cells were transferred intravenously to CCR2^{-/-} mice or littermate WT mice and 24 h later, these mice were challenged with OVA or PBS inhalation three times at a 24-h interval. Airway resistance was measured at 24 h after the last OVA inhalation by the flexiVent system. Data are means $\pm$ SD for 4–7 mice in each group. *Significantly different from the mean value of OVA-inhaled WT mice, * $P < 0.05$. (B) Similar to A, OVA-specific T_H2 cells were transferred intravenously to CCR2^{-/-} mice or littermate WT mice and 24 h later, these mice were challenged with OVA inhalation three times at a 24-h interval. Where indicated, iDCs (2×10^6 cells per mouse) isolated from T_H2 cell-transferred and OVA-challenged WT mice were transferred to CCR2^{-/-} mice just after the second OVA inhalation. Airway resistance was measured at 24 h after the final OVA inhalation. Data are means $\pm$ SD for three mice in each group. *Significantly different from the mean value of the response of OVA-inhaled CCR2^{-/-} mice without cell transfer. * $P < 0.05$.

lung during T_H2 cell-mediated inflammation but not T_H1 cell-mediated inflammation (Fig. 4A). By using mixed BM chimeric mice, we also showed that the induction of aaMφ markers was reduced in STAT6^{-/-} iDCs as compared with that in WT iDCs even though these cells were isolated from the same recipient mice (Fig. 4B). These results indicate that not only a T_H2 environment at the site of airway inflammation but also intrinsic expression of STAT6 in iDCs is required for the induction of aaMφ markers in iDCs, consistent with a previous finding on Mφs (21). On the other hand, we found that iDCs expressed Tip-DC makers in the lung of T_H1 cell-mediated inflammation (Fig. 4A). Therefore, our findings indicate that an inflammatory environment produced by CD4⁺ T cells influences the gene expression profile of iDCs, suggesting that in addition to the instruction from DCs to T cells to initiate and amplify the responses of effector T cells (1, 22), there is a reverse instruction from T cells to iDCs to provoke the effector function in iDCs in the effector phase of adaptive immune responses.

Moreover, we show that iDCs at the site of allergic airway inflammation play an indispensable role in the induction of AHR, independently of allergic airway inflammation. We found that T_H2 cell-mediated allergic airway inflammation was similarly induced in CCR2^{-/-} mice and WT mice in an adoptive T_H2 cell transfer model of asthma (Fig. 5), although antigen-induced infiltration of iDCs into the lung was significantly reduced in T_H2 cell-transferred CCR2^{-/-} mice as compared with that in T_H2 cell-transferred WT mice (Fig. 5C). These results suggest that the CCR2-iDCs axis is not required for the recruitment and cytokine production of activated T_H2 cells and subsequent T_H2 cytokine-mediated allergic airway inflammation. In contrast, importantly, AHR was significantly reduced in T_H2 cell-transferred CCR2^{-/-} mice as compared with that in T_H2 cell-transferred WT mice (Fig. 6A). We also showed that the adoptive transfer of WT iDCs isolated from the site of allergic airway inflammation restored AHR in T_H2 cell-transferred CCR2^{-/-} mice (Fig. 6B). Taken together, these results suggest that the CCR2-iDCs axis plays a critical role in the induction of AHR by a mechanism independent of T_H2 cell-mediated allergic airway inflammation. On the other hand, a previous study has shown that the depletion of cells expressing CD11c, which include iDCs, cDCs and AMs, during allergen challenge abrogates both allergic airway inflammation and AHR (13). Therefore, it is possible that other populations of CD11c⁺ cells including cDCs might be more importantly involved in the induction of T_H2 cell-mediated allergic airway inflammation than iDCs.

Previous studies have shown that IL-4-stimulated BM-derived DCs (BMDCs) express some aaMφ markers. Munder *et al.* (23) have shown that IL-4 induces the expression of arginase 1 in BMDCs, whereas Cook *et al.* (24) have recently shown that IL-4 induces the expression of RELM-α and Ym1 but not arginase 1 in BMDCs. On the other hand, we found that iDCs at the site of allergic airway inflammation express higher levels of arginase 1 and RELM-α but not Ym1 (Fig. 3A), indicating that the expression pattern of aaMφ markers is different between IL-4-stimulated BMDCs and iDCs at the site of airway allergic inflammation. In addition, we found that the administration of IL-4-stimulated BMDCs could not restore the reduced AHR in CCR2^{-/-} mice (A. Iwata and H. Nakajima, unpublished observation). These findings suggest that IL-4 signaling itself is not sufficient but a T_H2

environment at the site of allergic airway inflammation is required for DCs to induce AHR.

Regarding the mechanisms underlying iDC-mediated AHR, we show that iDCs at the site of allergic airway inflammation are the main producers of arginase 1 (Fig. 3). We found that iDCs isolated from the site of allergic airway inflammation expressed higher levels of arginase 1 as compared with those in cDCs, AMs, Mo or lung epithelial cells (Fig. 3A and B). Recent evidence has indicated that arginase, which converts L-arginine into L-ornithine and urea, plays a key role in the induction of AHR in asthma. It has been shown that arginase activity in the lung is significantly increased not only in murine models of asthma (25) but also in patients with asthma (25–27) and that the inhibition of arginase activity results in reduced AHR in some asthma models (26, 28, 29). The enhanced arginase activity in the lung is considered to result in reduced bioavailability of L-arginine to NOS, thereby causing a deficiency of bronchodilating NO (30). The fact that the administration of L-arginine reduces AHR (31) supports this notion. Taken together, these results suggest that iDC-derived arginase 1 might be involved in the iDC-mediated induction of AHR. Further studies using iDC-specific conditional knockout mice for arginase 1 are needed to test the hypothesis. The studies, which determine the roles of STAT6 expressed in iDCs in causing AHR may also provide important information on the mechanism underlying iDC-mediated AHR.

Recently, Egawa *et al.* (32) have shown that inflammatory Mo recruited to allergic skin in a CCR2-dependent manner acquire an aaMφ-like phenotype in response to basophil-derived IL-4 and exert an anti-inflammatory function in IgE-mediated chronic allergic inflammation, a model where basophils rather than T cells play a critical role for the elicitation of allergic responses (33). On the other hand, we found here that T_H2 cell-mediated airway inflammation instructs Mo-derived iDCs to express some aaMφ markers and that iDCs under a T_H2 environment play a critical role in the development of AHR, independently of allergic airway inflammation. The relationship between inflammatory Mo in IgE-mediated chronic skin inflammation and iDCs in T_H2 cell-dependent asthma models is unclear at present. A direct comparison between these two populations may reveal the mechanism of the difference in allergic responses between the skin and the airways.

In conclusion, our findings suggest that T_H2 cell-mediated airway inflammation induces AHR by modifying the function of iDCs at the site of allergic inflammation. Our results should add a new insight into the pathogenesis of AHR and raise the possibility that iDCs could be a novel therapeutic target for asthma.

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