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Conventional and Monocyte-Derived CD11b$^+$ Dendritic Cells Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen

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SUMMARY

Dendritic cells (DCs) are crucial for mounting allergic airway inflammation, but it is unclear which subset of DCs performs this task. By using CD64 and MAR-1 staining, we reliably separated CD11b$^+$ monocyte-derived DCs (moDCs) from conventional DCs (cDCs) and studied antigen uptake, migration, and presentation assays of lung and lymph node (LN) DCs in response to inhaled house dust mite (HDM). Mainly CD11b$^+$ cDCs but not CD103$^+$ cDCs induced T helper 2 (Th2) cell immunity in HDM-specific T cells in vitro and asthma in vivo. Studies in *Flt3l*$^{-/-}$ mice, lacking all cDCs, revealed that moDCs were also sufficient to induce Th2 cell-mediated immunity but only when high-dose HDM was given. The main function of moDCs was the production of proinflammatory chemokines and allergen presentation in the lung during challenge. Thus, we have identified migratory CD11b$^+$ cDCs as the principal subset inducing Th2 cell-mediated immunity in the LN, whereas moDCs orchestrate allergic inflammation in the lung.

INTRODUCTION

Allergic asthma is controlled by CD4$^+$ T helper (Th) lymphocytes that cause eosinophilic airway inflammation, mucus overproduction, and airway constriction. Dendritic cells (DCs) are the most proficient antigen-presenting cells of the lung and play a crucial role in the immune response to inhaled allergens like house dust mite (HDM) by taking up the antigen and bringing it to the draining mediastinal (M) LNs of the lung, where they polarize the Th cell immune response (reviewed in Lambrecht and Hammad, 2012). Dendritic cells are strategically located in close vicinity to airway epithelial cells that secrete cytokines as well as endogenous danger signals that activate the local network of DCs of the lung and promote Th2 cell-mediated immunity to the allergen (Kool et al., 2011). The crucial role of DCs in mediating Th2 cell-mediated immunity to inhaled antigens was revealed in CD11c-DTR transgenic mice in which conditional depletion of all CD11c^{hi} lung DCs abolished development of Th2 cell-mediated immunity to HDM allergen (Hammad et al., 2010). Dendritic cells also play a crucial role during the recall phase of the allergic response, when primed Th2 cells return to the effector site of the lung and control aspects of allergic inflammation. During this stage, phenotypically mature DCs are always seen in close proximity to effector Th cells inside areas of peribronchial inflammation (Huh et al., 2003). Conditional depletion of lung DCs during the recall phase of the allergic response abolishes the salient features of asthma (van Rijt et al., 2005, 2011).

In recent years, researchers have defined distinct DC subsets differing in surface marker expression and development (Geissmann et al., 2010). Common DC progenitors (CDPs) expressing the hematopoietic cytokine receptor Flt3 generate preconventional DCs (pre-cDCs) and plasmacytoid DCs (pDCs) (Onai et al., 2007). Pre-cDCs develop further in the lung into two subsets of cDCs, expressing either CD103 or CD11b integrins and displaying unique functions (Sung et al., 2006). CD103$^+$ DCs are specialized in cross-presentation of exogenous viral antigen to CD8$^+$ T cells (Desch et al., 2011; GeurtsvanKessel et al., 2008). Conversely, CD11b$^+$ DCs seem specialized in presenting soluble antigen to CD4$^+$ T cells and produce large amounts of proinflammatory chemokines (Beaty et al., 2007; del Rio et al., 2007). Meanwhile, plasmacytoid DCs are important for controlling overt airway inflammation through induction of regulatory T cells (de Heer et al., 2004).

It is assumed that lung cDCs derive from the circulating pre-cDC progenitor and are closely related to lymphoid tissue cDCs (Ginhoux et al., 2009). Some studies, however, have also shown that circulating monocytes can give rise to lung DCs in steady state (Jakubzick et al., 2008b; Varol et al., 2007). The

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contribution of monocytes to the DC pool is by far more important under inflammatory conditions. At times of infection or inhalation of allergens, barrier epithelial cells of the lung produce proinflammatory chemokines like CCL2 and CCL7 that act on the bone marrow to increase the release of CCR2^{hi} monocytes (Hammad et al., 2009; León et al., 2007; Serbina and Pamer, 2006). When these monocytes reach the airway mucosa as part of an inflammatory response, they can upregulate major histocompatibility complex class II (MHCII) glycoproteins and CD11c and give rise to monocyte-derived DCs (moDCs) that also express CD11b integrin and therefore easily contaminate the CD11b^{+} cDC population (Robays et al., 2007). The precise function of moDCs in lung immunity is unclear and most probably underestimated, because reliable identification of moDCs is problematic. In other parts of the body, moDCs can be fully capable of presenting antigen and priming immunity to the same extent as cDCs, favoring Th1 cell immunity (Hohl et al., 2009; Nakano et al., 2009; Wüthrich et al., 2012).

Here we have sought to clarify which subset of lung DCs is important for presenting the clinically relevant HDM allergen at times of first and repeated allergen inhalation. Unraveling this division of labor between DC subsets in allergy identified CD11b^{+} cDCs and moDCs as important players in the allergic cascade that could be inhibited for therapeutic intervention.

RESULTS

Ly6C Is a Specific but Insensitive Marker to Discriminate moDCs from CD11b^{+} cDCs in the Lung

Numerous methods have been used to identify the different CD11c^{+} DC subsets of the lungs, but like in many other organs, these fail to separate CD11b^{+} cDCs from CD11b^{+} moDCs. In defining a gating strategy, we first gated out CD11c^{+}MHCII^{int} alveolar macrophages based on their strong autofluorescent signal, SSC^{hi} granularity, and positive staining for the lectin Siglec-F (Figure 1A). The remaining MHCII^{+}CD11c^{+} lung DCs were then further separated into CD103^{+} cDCs and CD11b^{+} cells (Figure 1B). Lung pDCs were identified as CD11c^{+} MHCII^{lo}CD11b^{-}Ly6C^{+}mPDCA-1^{+} cells (Figure S1A available online). To identify CD11b^{+} moDCs among the CD11b^{+} cDCs, many investigators have used the monocytic Ly6C marker (León et al., 2007), but Ly6C is expressed only temporarily when monocytes differentiate into DCs. In our hands Ly6C expression on lung DCs represented a continuum within CD11b^{+} lung DCs, making it virtually impossible to place a cut-off marker between positive and negative cells. We verified whether Ly6C^{hi} cells and Ly6C^{lo} cells represented extremes of the spectrum and identified moDCs and cDCs, respectively. We used a competitive lineage tracing strategy to identify both subsets, based on differential CCR2-dependent egress from the bone marrow (Figure 1C). CD11b^{+} cDCs are derived from pre-cDCs that leave the bone marrow in a CCR2-independent manner. moDCs are derived from monocytes that require CCR2 for egress from the bone marrow. We therefore generated competitive mixed CD45.1 WT and CD45.2 *Ccr2*$^{-/-}$ BM chimeric mice. In reconstituted mice, monocytes had a high CD45.1 WT:CD45.2 *Ccr2*$^{-/-}$ ratio strongly in favor of the CD45.1 WT origin, whereas T cells, B cells, and neutrophils in the blood had a much lower ratio of CD45.1:CD45.2. In the lung, CD103^{+} cDCs as well as pDCs had a low CD45.1:CD45.2 ratio similar to neutrophils and lymphocytes, unequivocally indicating they were of nonmonocytic origin. However, Ly6C^{hi}CD11b^{+} DCs had a CD45.1:CD45.2 ratio similar to blood monocytes, reliably identifying these cells as monocyte-derived cells and validating the usefulness of Ly6C as a specific moDC marker. Ly6C^{lo} CD11b^{+} DCs were found to have an intermediate CD45.1: CD45.2 ratio, signifying contamination of the Ly6C^{lo}CD11b^{+} gate with monocyte-derived cells. Therefore, Ly6C staining underestimates the amount of monocyte-derived cells within the CD11b^{+} DC population and is a poorly sensitive method to identify moDCs.

CD64 and MAR-1 Staining Discriminates All Monocyte-Derived DCs from CD11b^{+} Conventional DCs

We next sought to find a more complete separation strategy of CD11b^{+} DCs. A subset of lung DCs found in inflammatory lung lesions after HDM exposure or viral infection can be stained with the MAR-1 antibody, directed against the high-affinity IgE receptor FcεRIα chain, although it was not shown whether MAR-1 staining was able to identify moDCs exclusively or was also upregulated on cDCs in inflammatory sites (Grayson et al., 2007). MAR-1^{+}CD11b^{+} DCs had a CD45.1 WT:CD45.2 *Ccr2*$^{-/-}$ ratio comparable to that of blood monocytes, validating the use of MAR-1 as a specific moDC marker among CD11b^{+} DCs. Very similar to the use of Ly6C, however, MAR-1^{lo}CD11b^{+} DCs displayed an intermediate mixed chimeric ratio, again indicative of contamination with moDCs. moDCs recovered from the muscle upon intramuscular vaccination or from the colitic intestine express high amounts of the high-affinity IgG receptor FcγR1(CD64) (Langlet et al., 2012; Tamoutounour et al., 2012). With a combined staining protocol for MAR-1 and CD64, double-positive CD11b^{+} DCs displayed a chimeric ratio comparable to blood monocytes. Strikingly, lung MAR-1 CD64 double-negative CD11b^{+} DCs cells had a chimeric ratio comparable to T cells and B cells, illustrating the absence of ‘‘false negative’’ moDC and indicating a proper identification of CD11b^{+} cDCs with this gating strategy. With CD64 and MAR1 markers as the gold standard to identify moDCs, we found that the use of Ly6C or MAR1 as binary markers had a sensitivity of only 57% and 51%, respectively (Figure 1C).

Ly6C^{+} Monocytes Develop into CD64^{+}MAR-1^{+} DCs in the Lungs of HDM-Exposed Mice

To valididate our gating strategy in the lung of inflamed mice, we transferred 2×10^6 carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled Ly6C^{hi} monocytes into monopenic *Ccr2*$^{-/-}$ mice exposed to HDM allergen. CFSE^{+} cells could be readily identified within the lung CD11c^{+}MHCII^{+} DC gate and these cells displayed a CD11b^{+}MAR-1^{+}CD64^{+} phenotype almost exclusively, validating the sensitivity and specificity of our gating approach (Figure 1D). The CFSE^{+} DCs expressed an intermediate amount of Ly6C, indicative of downregulation of Ly6C as monocytes become moDCs. This downregulation of Ly6C explains the high rate of false-negative moDCs when Ly6C is used as the discriminator for moDCs.

As shown in Figure 1E, steady-state lungs contained only a small population of moDCs within the CD11b^{+}MHCII^{+}CD11c^{+} gate. However, intratracheal (i.t.) administration of a high dose

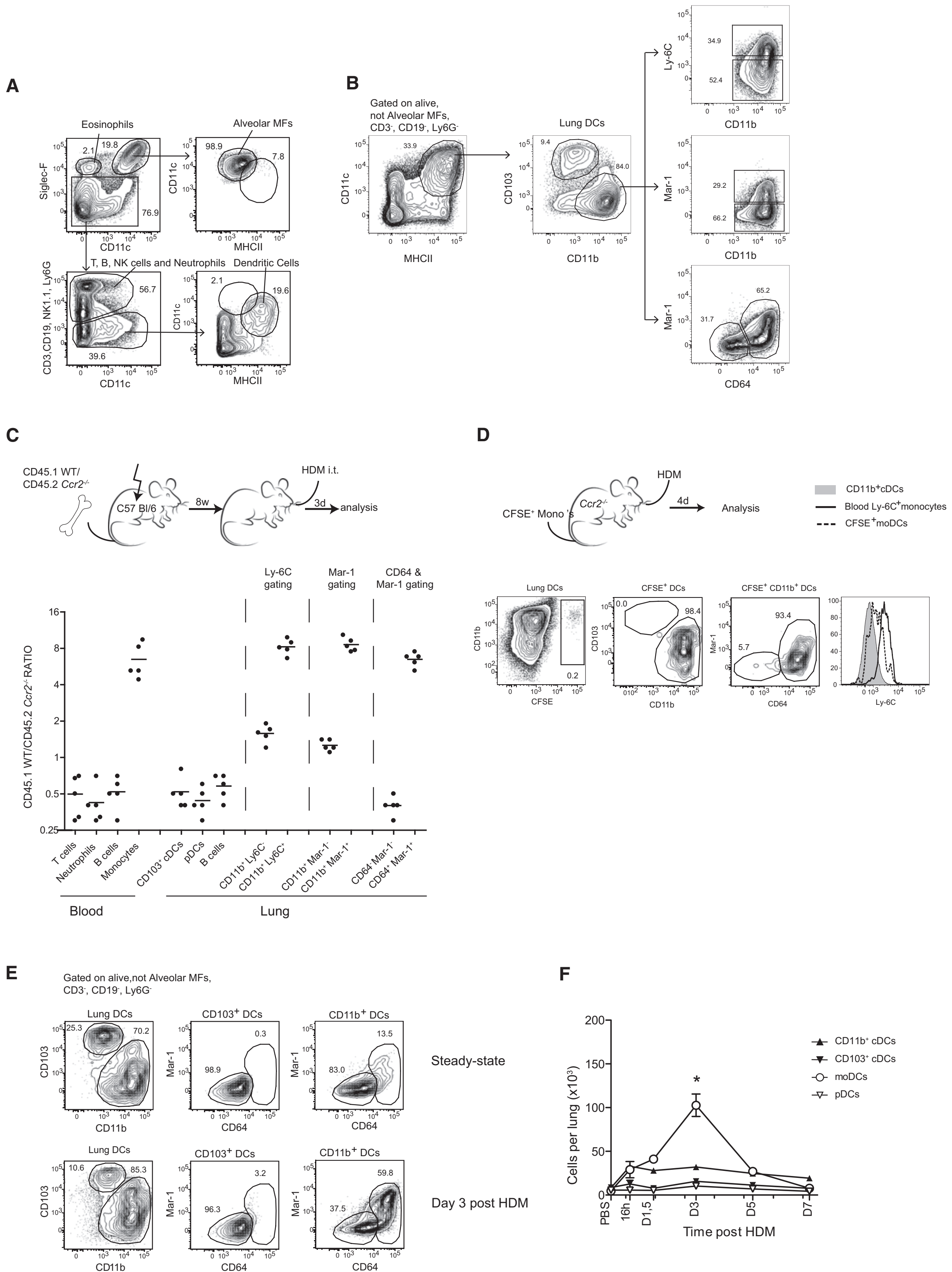

Figure 1. A Combination of MAR-1 and CD64 Expression Allows the Proper Identification of cDCs and moDCs in HDM-Exposed Lungs
(A) Gating strategy for lung cell populations in mice administered 100 μg HDM i.t.

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of HDM (i.e., 100 μg) induced a massive increase in this moDC population (Figure 1E). Tracking over time of the distinct DC subsets upon exposure to a high dose of HDM revealed a massive but temporary expansion of moDCs in the lungs, with a peak occurring 3 days after exposure (Figure 1F). pDCs and CD103^{+} cDCs were hardly affected by HDM exposure.

Accumulation of DC Subsets in the Mediastinal LN upon HDM Exposure

In the MLN, lung-derived migratory DCs can be distinguished from LN-resident DCs because of their high expression of MHCII and CCR7 (Figure 2A). Migratory DCs were divided into CD103^{+} and CD11b^{+} cells and analyzed for MAR-1 and CD64 expression (Figure 2B). While in the steady-state, only CD103^{+} cDCs and CD11b^{+} cDCs were found in the migratory DC gate of the MLN, HDM exposure of naive mice resulted in the accumulation of MAR-1^{+} DCs in the MLN. These MAR-1^{+} DCs in the MLN had slightly lower expression of CD64 compared with lung moDCs, yet still expressed the highest amounts of CD64 among MLN DCs. In contrast to resident DCs, all the migratory DC subsets expressed CCR7, although at varying amounts (Figure 2C). Overall, cDCs had higher expression of CCR7 compared with moDCs. As expected from their nonmigratory nature, pDCs did not express CCR7 (Figure 2C) and as such pDCs present in the MLN probably represent LN-resident pDCs. Analysis of the CD45.1 WT:CD45.2 CCR2 ratio on different DC subsets in the MLN showed CCR2 dependency of only the MAR1^{+}CD64^{+} DCs and showed no increase in the amount of false-negative moDCs in the CD11b^{+} cDC gate (Figure 2D). A peak accumulation of DCs in the MLN was observed already 1.5 days after HDM exposure, consisting mainly of CD103^{+} and CD11b^{+} cDCs. There was a transient and minor accumulation of moDCs in the MLN at day 3 after HDM (Figure 2E). Overall, the area under the curve, representing the total amount of DCs accumulating over time, was highest for CD11b^{+} cDCs, followed by CD103^{+} cDCs, moDCs, and pDCs.

HDM Uptake and Transport by Lung DC Subsets

Because most groups that have used HDM allergen in mouse models of asthma have used doses ranging from 1 to 100 μg per administration for sensitization, we chose three doses within this range to be administered i.t. and studied the antigen distribution 3 days later. Most of the fluorescent HDM was taken up by lung CD11c^{+} cells, including alveolar macrophages (MFs) and DCs (Figure 3A). In the MLN, fluorescent HDM was found almost exclusively in MHCII^{hi}CD11c^{+} cells, representing migratory DCs (Figure 3B). The transport of HDM to the MLN occurred through active cellular transport and not via passive lymphatic drainage through a disrupted epithelial barrier as indicated by the fact that all accumulation of fluorescent HDM in the MLN was abolished in *Ccr7*$^{-/-}$ mice (Figure 3C). The total amount of DC subsets as well as the total amount of HDM^{+} DC subsets in the lungs (Figure 3D) and MLNs (Figure 3F) of naive mice exposed to different doses of HDM is graphically represented as pie charts. The percentage of DC subsets or macrophages taking up HDM allergen in the lung and MLN is represented in Figures 3E and 3G, respectively. Overall, CD11b^{+} cDCs and moDCs took up HDM most efficiently whereas the uptake of HDM allergen by lung CD103^{+} cDCs occurred only with the highest dose of allergen. The recruitment of moDCs was strongly driven by allergen dose, and the few moDCs that were recruited in response to low and intermediate doses of HDM already took up the antigen quite efficiently. Increasing the amount of allergen also led to progressively more moDCs taking up HDM in the lung. Among other APCs, macrophages became almost universally antigen laden irrespective of dose, whereas pDCs and B cells poorly took up HDM. In the MLN, the migratory HDM^{+} cells were highly enriched for CD11b^{+} cDCs. Only with intermediate and high doses of HDM did we observe a marked population of CD103^{+} cDCs and moDCs that had taken up HDM. Little or no fluorescent signal was found in LN-resident cDCs, pDCs, and B cells (Figures 3B and 3G). Although moDCs represented the major DC subset in the lungs upon exposure to an intermediate or high HDM dose, they remained the smallest DC subset among migratory DCs within the MLN in all conditions, indicating that moDCs are poorly migratory.

Th Cell Polarization in HDM-Specific T Cells Induced by DC Subsets

To detect the induction of primary immune responses to HDM ex vivo, we generated T cell receptor (TCR) transgenic (Tg) mice (dubbed 1-DERβ mice) recognizing an immunodominant peptide derived from Der p 1 allergen. Naive CD62L^{+}CD4^{+} cells obtained from 1-DERβ proliferated vigorously when exposed to HDM-pulsed or Der p 1 peptide-pulsed bone marrow DCs, and dividing T cells upregulated CD44 as a sign of effector generation (Figure 4A). The precursor frequency of HDM-reactive CD4^{+} T cells was calculated from CFSE profiles to vary between 3% and 5% depending on donor, whereas in non-Tg mice this frequency was always below 1% (data not shown). We next studied the ex vivo potential of different APCs sorted from the MLN or lung of HDM-exposed mice. Proliferation in 1-DERβ T cells was observed only when MHCII^{hi}CD11c^{hi} migratory DCs were used as APCs; no proliferation was seen with MLN B cells or lung alveolar MFs as APCs (Figure 4B). When migratory DCs were further subdivided, moDCs and CD11b^{+} cDCs were best at inducing T cell proliferation (Figure 4C). We then measured the production of prototypic Th1 cell (IFN-γ), Th17 cell (IL-17), and Th2 cell (IL-13) and regulatory

(B) Lung MHCII^{+}CD11c^{+} cells are subdivided in CD103^{+} and CD11b^{+} DCs. The CD11b^{+} DCs can be subdivided further on the basis of Ly6C expression, MAR-1 expression, or the combined MAR-1 and CD64 expression.

(C) Scatter plot graph of cell populations in the blood and lungs of chimeric mice reconstituted with equal amounts of CD45.1^{+} WT and CD45.2^{+}*Ccr2*$^{-/-}$ bone marrow cells. The origin of the cells was determined with the CD45.1 WT/CD45.2 *Ccr2*$^{-/-}$ ratio.

(D) Phenotypic analysis of CFSE^{+}Ly6C^{hi} monocytes transferred into *Ccr2*$^{-/-}$ mice.

(E) Gating strategy for pulmonary CD103^{+} cDCs, CD11b^{+}CD64^{-}Mar-1^{-} cDCs and CD11b^{+}CD64^{+}Mar-1^{+} moDCs 3 days after PBS or HDM exposure.

(F) Kinetics of CD103^{+} cDCs, CD11b^{+} cDCs, moDCs, and pDC recruitment to the lungs of HDM-exposed mice.

Data represent at least two (B) and three (A, C–F) independent experiments with three to six mice per group. *$p < 0.05$. See also Figure S1.

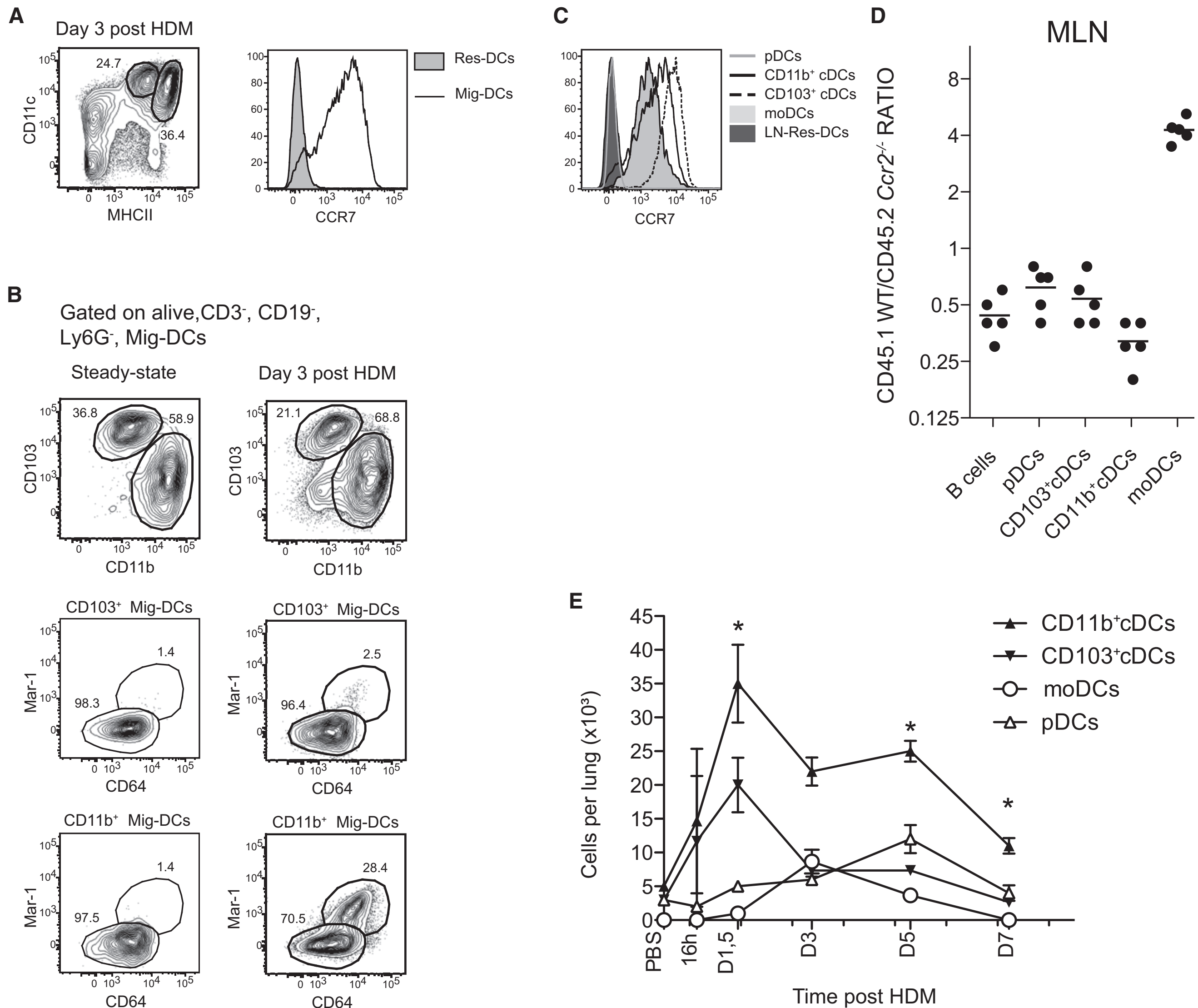


Figure 2. A Combination of MAR-1 and CD64 Expression Discriminates between cDCs and moDCs in the MLN

(A) CCR7 expression by MHCII^{hi}CD11c^{+} migratory DCs and MHCII^{int}CD11c^{+} resident DCs in the MLNs of mice exposed to HDM.

(B) Gating strategy for CD103^{+} cDC, CD11b^{+} cDC, and CD11b^{+}CD64^{+}Mar-1^{+} mo-DC populations in the MLN of PBS- or HDM-exposed mice.

(C) CCR7 expression by different DC subsets. Mig-DCs were subdivided into CD103^{+} cDCs (CD103^{+}CD11b^{-}MAR-1^{-}CD64^{-}), CD11b^{+} cDCs (CD103^{-}CD11b^{+}MAR-1^{-}CD64^{-}), and moDCs (CD103^{-}CD11b^{+}MAR-1^{+}CD64^{+}) and their CCR7 expression was compared to pDCs (CD103^{-}CD11b^{-}Ly-6C^{+} mPDCA-1^{+}) and Res-DCs.

(D) Scatter plot graph showing the CD45.1 WT/CD45.2 *Ccr2*$^{-/-}$ ratio for MLN populations of chimeric mice reconstituted with equal amounts of CD45.1^{+} WT and CD45.2^{+}*Ccr2*$^{-/-}$ bone marrow cells.

(E) Kinetics of recruitment of DC subsets to the MLNs of HDM-exposed mice.

Data represent at least two (D) or three (A–C, E) independent experiments with three to six mice per group. *$p < 0.05$.

(IL-10) cytokines in the supernatant of these cultures (Figure 4D). CD11b^{+} cDCs and moDCs were most proficient in inducing all these cytokines, with a slight bias for Th1 cell induction by moDCs, whereas CD103^{+} cDCs induced no IFN-γ and low amounts of IL-17, IL-13, and IL-10. Because lung macrophages did not induce T cell proliferation, and because pDCs took up very little HDM allergen, we did not perform these Th cell polarization assays with these cells as APCs. Compared with CD103^{+} cDCs, CD11b^{+} cDCs expressed considerably higher amounts of cathepsin S, lysosomal-associated membrane protein-1 (Lamp1), and the peptide exchanger H2DMb1, all involved in the MHCII-processing pathway. Meanwhile, moDCs expressed intermediate amounts of processing machinery (Figure 4E). We did not obtain sufficient pDCs to obtain cDNA from this fraction. Thus, CD11b^{+} cDCs and moDCs induced the highest amount of effector cytokines in a Th cell polarization assay, related to higher antigen-processing capacity, in these cells.

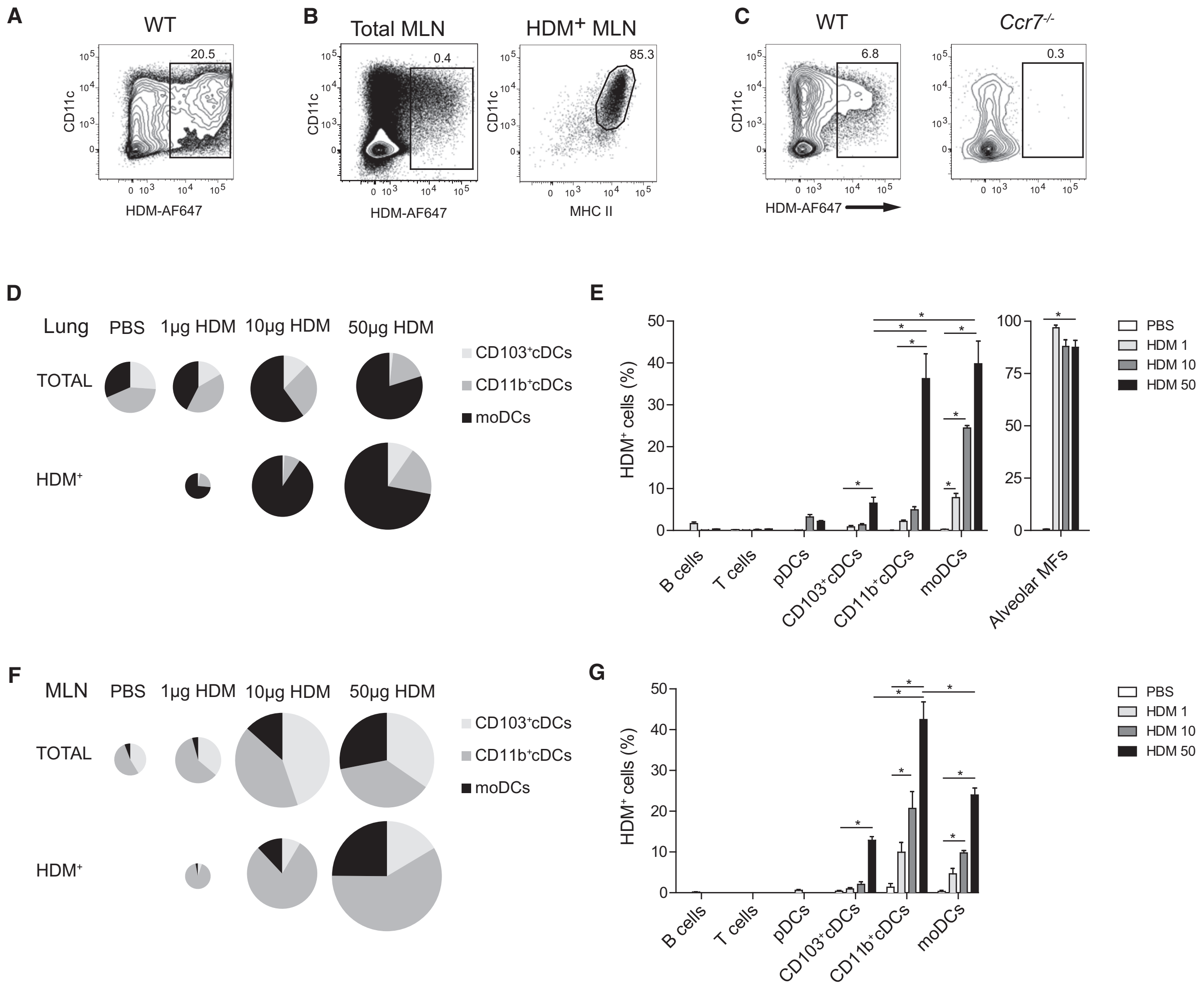


Figure 3. HDM Uptake and Presentation by Distinct DC Subsets during the Sensitization Phase of Allergic Airway Inflammation

(A–C) Uptake of Alexa Fluor 647 (AF647)-labeled HDM by CD11c$^+$ cells in the lung (A) and MLNs of WT (B, C) and *Ccr7*$^{-/-}$ (C) mice administered with the allergen 72 hr earlier.

(D) Pie charts depicting the relative proportion of each DC subset within the total or the AF647-HDM$^+$MHCII$^+$CD11c$^+$ population in the lung of mice administered with increasing doses of allergen.

(E) Proportion of AF647-HDM$^+$ lung populations 72 hr after the allergen administration.

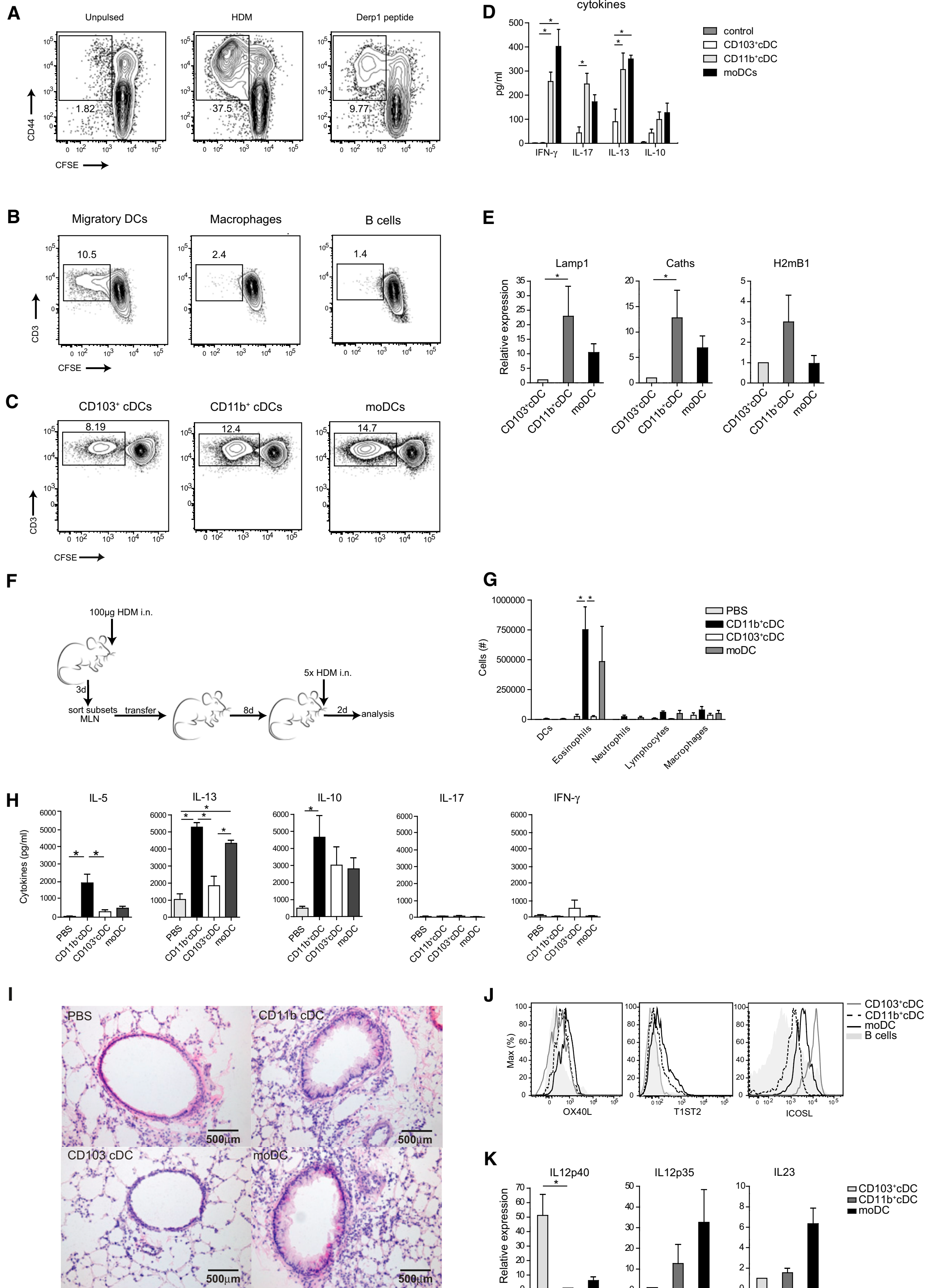
(F) Pie charts depicting the relative proportion of each DC subset within the total or the AF647-HDM$^+$MHCII$^+$CD11c$^+$ population in the MLNs of mice administered with increasing doses of allergen.

(G) Proportion of AF647-HDM$^+$ MLN populations 72 hr after the allergen administration.

Data represent three independent experiments with three to six mice per group. Error bars represent the SEM. *$p < 0.05$.

CD11b$^+$ cDCs and moDCs but Not CD103$^+$ cDCs Are Sufficient to Induce HDM Sensitization

To evaluate whether the HDM presentation by the distinct lung DC subsets is sufficient to prime Th2 cell-mediated immunity to HDM in vivo, we developed an intratracheal DC-transfer protocol to sensitize mice to HDM (Figure 4F). The different DC subsets were sorted ex vivo from the MLN of mice that received 100 µg of HDM allergen 3 days earlier. The high dose of HDM ensured that all lung DC subsets were loaded with HDM and migrated efficiently to the MLN (Figure 3F). Mice receiving CD11b$^+$ cDCs or moDCs developed increased BAL fluid cellularity and eosinophilic infiltration upon HDM aerosol rechallenge. In contrast, negative control mice that received PBS-sensitized CD11b$^+$ DCs and mice that received CD103$^+$ cDCs isolated from the MLN of HDM-sensitized mice did not develop eosinophilic infiltration (Figures 4G and 4I). In addition, HDM restimulation of MLN cells isolated from mice sensitized by transfer of CD11b$^+$ cDCs or moDCs but not CD103$^+$ cDCs induced the production of the Th2 cell-associated cytokine IL-13, indicating that both CD11b$^+$ cDCs and moDCs but not CD103$^+$ DCs are



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capable of inducing a proper Th2 cell-mediated effector response to HDM in vivo (Figure 4H). IL-5 and IL-10 were induced mainly by the CD11b^{+} cDCs, whereas none of the subsets induced measurable IL-17 in vivo.

We next questioned how CD11b^{+} cDCs and moDCs promoted Th2 cell-mediated immunity and addressed the expression of known Th2 cell-associated skewing factors by flow cytometry (Figure 4J). OX40L on DCs has been shown to promote Th2 cell-mediated immunity in the lung (Jember et al., 2001), and we have observed reduced Th2 cell-mediated immunity to HDM when mice received a blocking Ab to OX40L at the time of priming (data not shown). Although in general the expression of OX40L were low, these were highest on moDCs and CD11b^{+} cDCs compared with CD103^{+} cDCs or reference B cells.

OX40L expression of pulmonary DCs is controlled by IL-33 (Besnard et al., 2011), and development of Th2 cell-mediated immunity to HDM critically depends on IL-33 released from airway epithelial cells (Willart et al., 2012). We therefore measured the expression of T1/ST2, the receptor for IL-33, and found that mainly CD11b^{+} cDCs and moDCs expressed it, whereas CD103^{+} cDCs did not. ICOSL has been shown to be involved in DC-driven Th2 cell-mediated immunity to allergens (Lambrecht et al., 2000) but can also control respiratory tolerance by DCs. ICOSL was mainly expressed by CD103^{+} cDCs. Although these findings offer some explanation for the potential Th2 cell bias of ex vivo sorted HDM-exposed CD11b^{+} cDCs and moDCs, they do not explain why moDCs have a slightly higher Th1 cell-inducing bias in vitro. The predominant cytokine driving Th1 cell development is IL-12p70. We therefore measured the mRNA expression of IL-12p40 and the rate limiting IL-12p35 subunits of bioactive IL-12 (Figure 4K). Upon HDM exposure in vivo, moDCs expressed the highest amounts of IL-12p35 and intermediate amounts of IL-12p40, whereas CD11b^{+} cDCs expressed intermediate amounts of IL-12p35. moDCs also expressed the highest amounts of IL-23p19.

CD11b^{+} cDCs but Not CD103^{+} cDCs Are Necessary for Th2 Cell-Mediated Immune Response Induction to Low-Dose HDM Allergen

Having established that adoptive transfer of ex vivo purified CD11b^{+} cDCs and moDCs was *sufficient* to prime Th2 cell-mediated immunity to HDM upon adoptive transfer, we next wanted to study which of these would be *necessary* to do so in vivo, especially because functions of moDCs and CD11b^{+} cDCs were largely overlapping. To deplete cDCs while leaving moDCs unaffected, we reasoned that *Flt3l*-deficient mice might be a good model. Both CD103^{+} and CD11b^{+} cDCs derive from a *Flt3*-expressing pre-cDC progenitor, whereas moDCs derive from monocytes. In the lungs of HDM-exposed *Flt3l*-deficient mice, there was indeed a strong reduction in CD103^{+} cDCs (Figure 5A). There were many residual CD11b^{+} DCs in the lungs of allergen-exposed *Flt3l*$^{-/-}$ mice, yet these universally expressed CD64 and MAR-1, identifying them as moDCs (Figure 5B). *Flt3l*$^{-/-}$ mice were sensitized and challenged with HDM allergen (1 μg and 5 times 10 μg, respectively), as previously reported (Willart et al., 2012). In this low-dose allergen-exposure protocol, *Flt3l*$^{-/-}$ mice displayed significantly less eosinophil, neutrophil, and lymphocytic infiltration as compared to WT mice exposed to the same protocol (Figure 5C). The size of the LNs in *Flt3l*$^{-/-}$ mice was so small that we were unable to measure MLN Th cell effector cytokine production in this experiment. Because *Flt3l*$^{-/-}$ mice lack both CD11b^{+} and CD103^{+} cDCs, we also set up an additional experiment to deplete endogenous CD103^{+} cDCs of the lungs during HDM sensitization, employing langerin-DTR mice (Kissenpfennig et al., 2005). Lung CD103^{+} cDCs are universally and exclusively langerin positive, demonstrating langerin promotor activity and leading to selective depletion of this subset upon DT treatment (Figure 5D). As shown in Figure 5E, depletion of CD103^{+} cDCs at the time of HDM sensitization did not lead to reduction in Th2 cell-dependent eosinophilic influx or mediastinal LN Th2 cell-associated cytokine production (Figure 5F).

Contribution of Monocyte-Derived DCs to Th2 Cell-Mediated Immunity to HDM

The experiments in *Flt3l*$^{-/-}$ mice suggest that CD11b^{+} cDCs are required for Th2 cell-mediated immunity to HDM, but adoptive transfer studies (Figures 4G–4I) demonstrate that moDCs can be sufficient to induce Th2 cell priming to HDM and others have shown that *Flt3l*$^{-/-}$ mice normally develop asthma to high-dose cockroach allergen (Walzer et al., 2005). One possibility would be that in the low-dose HDM-sensitization model, moDCs do not get properly triggered to migrate to the MLN. We therefore performed experiments in which *Flt3l*$^{-/-}$ mice

Figure 4. CD11b^{+} cDCs and moDCs Induce HDM-Specific Sensitization

(A) Proliferation of CFSE-labeled 1-DERβ T cells cocultured for 4 days with unpulsed, HDM extracts or Der p 1 peptide.

(B) Proliferation of CFSE-labeled 1-DERβ T cells cocultured for 4 days with migratory DCs and B cells sorted from the MLN of HDM-exposed mice, or with macrophages sorted from the lungs of HDM-exposed mice.

(C) Proliferation of CFSE-labeled 1-DERβ T cells cocultured for 4 days with DC subsets sorted from the MLN of HDM-exposed mice.

(D) Cytokine production in supernatants of CFSE-labeled 1-DERβ T cells cocultured for 4 days with different MLN-derived DC subsets.

(E) mRNA expression of Lamp1, CathS, and H2mB1 in CD103^{+} cDCs, CD11b^{+} cDCs, and moDCs. mRNA expression was normalized against housekeeping genes and represented as relative expression compared to CD103^{+} cDCs (i.e., the lowest-expressing DC subset).

(F) Protocol of allergic airway induction by adoptively transferred DCs.

(G) Differential cell counts in the bronchoalveolar lavage of mice adoptively transferred with the different DC subsets on day 0 and rechallenged with HDM.

(H) Cytokine production by MLN cells restimulated ex vivo for 4 days with HDM extracts.

(I) Hematoxylin-eosin staining of lung sections from mice injected with the different DC subsets on day 0 and rechallenged with HDM.

(J) Expression of ICOSL, OX40L, and T1/ST2 (IL-33R) on CD103^{+} cDCs, CD11b^{+} cDCs, moDCs, and B cells from the lungs at day 3 after HDM sensitization.

(K) mRNA expression of IL-12p40, IL-12p35, and IL-23p19 in CD103^{+} cDCs, CD11b^{+} cDCs, and moDCs. mRNA expression was first normalized against housekeeping genes and then represented as relative expression as compared to CD103^{+} cDCs (i.e., the lowest-expressing DC subset).

Data are representative of at least three (A–D, F–K) or two (E) independent experiments with three to six mice. PCR data are from three independent experiments in which each time cells from ten mice were pooled and separated into the distinct DC subsets. Error bars represent the SEM. *$p < 0.05$. See also Figure S2.

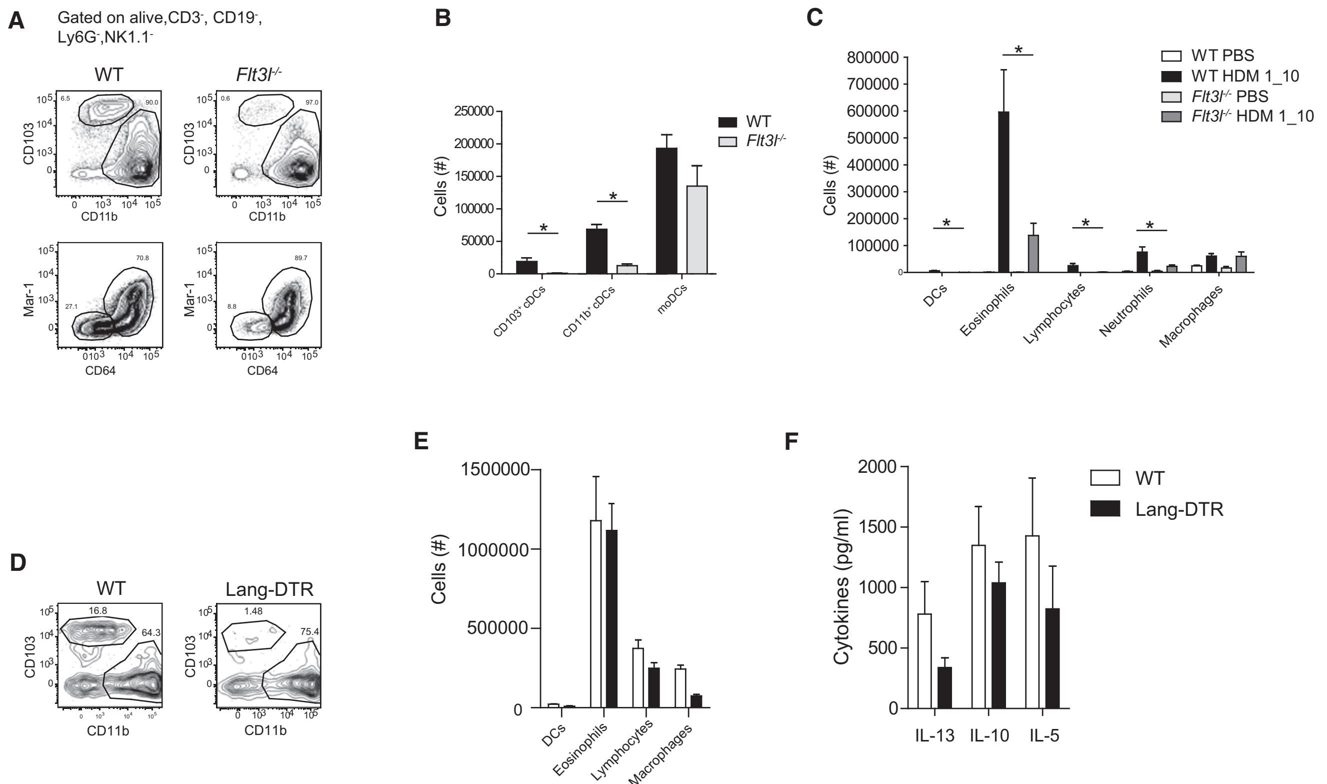


Figure 5. CD11b+ cDCs but Not CD103+ cDCs Are Necessary during the Sensitization and the Challenge Phase of HDM-Induced Allergic Airway Inflammation

(A) Analysis of the different DC subsets in the lungs of WT and *Flt3l*$^{-/-}$ mice 3 days after HDM administration.

(B) Number of CD103+ cDCs, CD11b+ cDCs, and moDCs in the lungs of *Flt3l*$^{-/-}$ and WT mice 3 days after HDM sensitization.

(C) *Flt3l*$^{-/-}$ mice or WT were sensitized with 1 μg HDM and challenged with 10 μg HDM. BAL-differential cell counts were obtained 72 hr after the last HDM challenge i.t.

(D) Langerin-DTR mice or WT mice were injected i.p. with 500 ng DT. The presence of CD103+ cDCs was evaluated in the lungs 24 hr later.

(E) Langerin-DTR or WT mice were sensitized and challenged with HDM as described in Figure 4C. BAL-differential cell counts were obtained 72 hr after the last allergen challenge.

(F) Cytokine production by MLN cells restimulated ex vivo for 4 days with HDM.

Data are representative of at least three independent experiments with four to six mice per group. Error bars represent the SEM. *p < 0.05.

were exposed to a very high dose of 100 μg of HDM during both sensitization and challenge, a dose shown to lead to significant accumulation of moDCs in the MLN (Figure 3E). In this protocol, Flt3L-deficient mice developed airway inflammation (Figure 6A) and Th2 cell-associated cytokine production (Figure 6B) that was comparable with WT mice, showing that moDCs are sufficient to induce Th2 cell-mediated immunity to HDM even in the absence of CD11b+ cDCs, when high dose of allergen is given. As such, we conclude that when the allergen dose is sufficiently high to induce migration of moDCs to the MLN, these moDCs can effectively replace the CD11b+ cDCs for the induction of Th2 cell responses. Conversely, this suggests that moDCs may play a redundant role during airway inflammation when CD11b+ cDCs are present. Unfortunately, no mice strains are available in which moDCs can be selectively depleted with antibodies or genetic targeting techniques. To address the functional capacities of moDCs in HDM-driven asthma in vivo, we therefore resorted to the use of *Ccr2*$^{-/-}$ mice, in which monocytes fail to exit the bone marrow. Previous studies have shown that CCR2 is a predominant chemokine receptor that is responsible for recruitment of pulmonary CD11b+ DCs to the allergen- or pathogen-exposed lung. When exposed to a large dose of HDM allergen, *Ccr2*$^{-/-}$ mice had normal numbers of CD103+ cDCs and CD11b+ cDCs, whereas the number of CD64+MAR1+ moDCs was reduced by at least 50% (Figure 6C). When exposed to a high-dose allergen challenge protocol, *Ccr2*$^{-/-}$ mice developed less eosinophilic airway inflammation compared with WT littermates, whereas the number of lymphocytes were identical between groups (Figure 6D), suggesting an important role for monocyte-derived cells in allergic airway inflammation. Indeed, the numbers of Th2 cell-priming CD11b+ cDCs were identical in these animals (Figure 6C), so the most likely explanation would be that the effects of CCR2 deficiency were mainly due to effects of moDC deficiency at the challenge phase of eosinophilic airway inflammation.

We therefore set up experiments to look at the recruitment and APC function of cDCs and moDCs during the allergen challenge phase. Mice were first sensitized to HDM and challenged with fluorescent HDM 10 days later. As shown in Figures 6E and 6F, allergen challenge with 10 or 50 μg of HDM in already sensitized

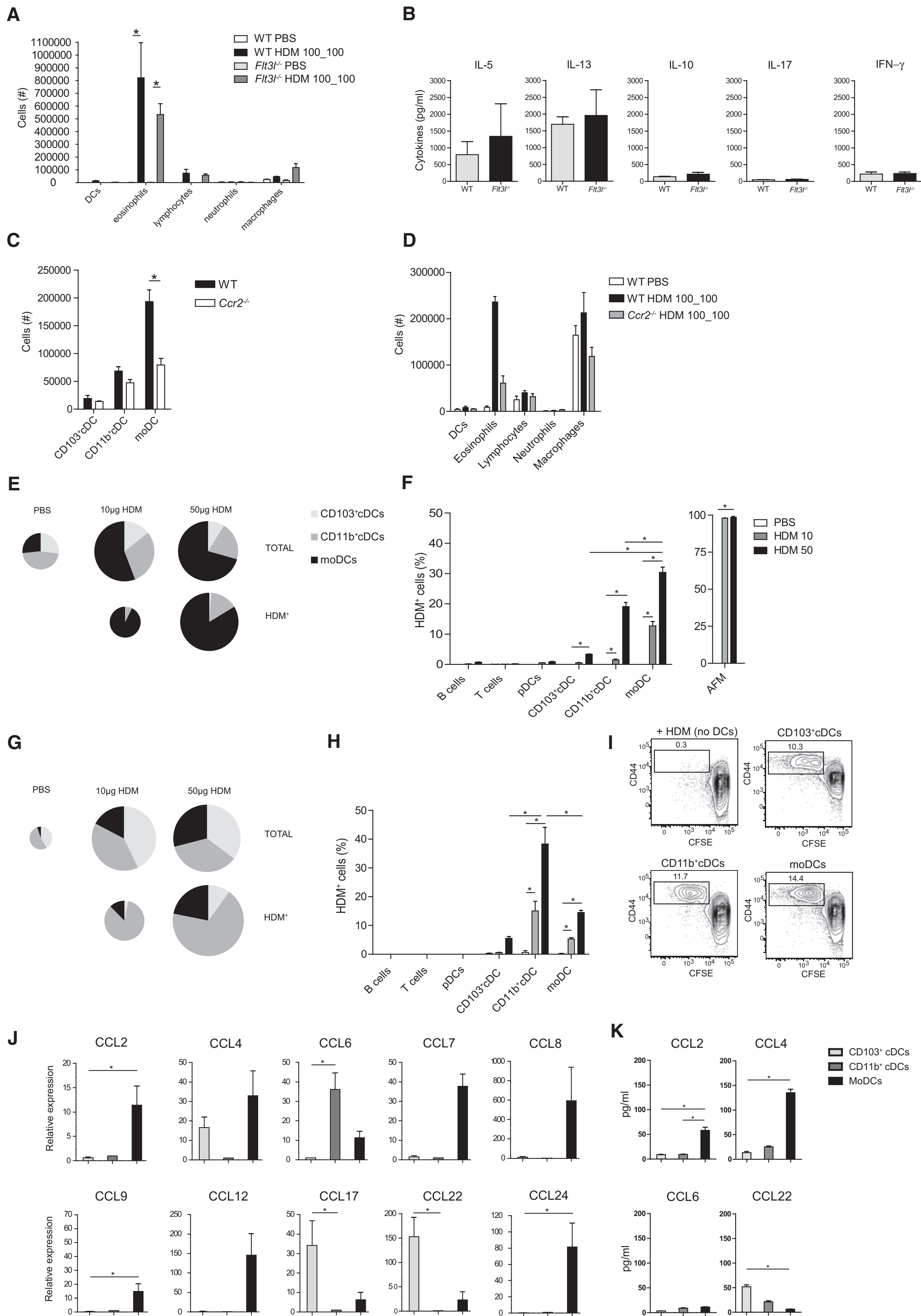
mice led to a strong increase in HDM$^+$ moDCs in the lungs, and globally the distribution pie charts of DCs taking up Ag in the lung were very comparable to the pie charts seen in naive mice exposed to the same dose of HDM during the sensitization phase (compare Figures 6E and 6F with Figures 3D and 3E). Note that the moDCs clearly represented the DC subset with the highest HDM content within the lungs during the challenge phase. In the MLN of sensitized and challenged mice, moDCs and both subsets of cDCs were seen to carry HDM antigen, but as during the sensitization phase moDCs represented a minor DC population within the MLN (Figures 6G and 6H). As during the sensitization phase, virtually all alveolar macrophages were HDM positive (Figure 6F). MHCII antigen-processing machinery was identically expressed in DCs sorted from the challenge phase compared with the sensitization phase (data not shown). When DC subsets were sorted from the lung during the challenge phase and used to stimulate 1-DERβ CD4$^+$ T cells, all three subsets were able to induce T cell division, as seen by their CFSE dilution, indicative of antigen processing of the HDM to immunogenic peptides (Figure 6I), yet moDCs are slightly more proficient in doing so, most probably as a result of the higher percentage of Ag uptake by these cells. Because *Ccr2*$^{-/-}$ mice had reduced and *Flt3l*$^{-/-}$ mice had intact eosinophilic airway inflammation in the high-dose allergen exposure model, we finally addressed whether any of the DC subsets would have a direct contribution by producing proinflammatory mediators. We therefore measured the mRNA expression of various chemokines known to influence allergic airway inflammation by direct effects on Th effector cells, mononuclear cells, or eosinophils. As shown in Figure 6J, moDCs strongly expressed various chemokines with activity on eosinophils and monocytes (CCL24, eotaxin2; CCL2, MCP1; CCL7, MCP3; CCL12, MCP5) as well as CCL4 and CCL9. Strikingly, CD11b$^+$ cDCs produced hardly any chemokines. As reported before, CD103$^+$ cDCs were the predominant source of CCL17 (TARC) and CCL22 (MDC) that have a potential to attract Th2 cells and regulatory T (Treg) cells expressing CCR4. Amounts of CXCL1, CXCL10, CCL3, CCL5, and CCL8 were all very low. moDCs not only strongly expressed various chemokine mRNAs, they also produced more CCL2 and CCL4 protein compared to cDCs. At protein level, CCL22 was produced mainly by CD103$^+$ cDCs. The production of CCL6 protein could not be confirmed in any DC subset (Figure 6K). Therefore, the known bias for chemokine production within the CD11b$^+$ lung DCs seems to reside within the moDC fraction.

DISCUSSION

Dendritic cells of the lung are a heterogeneous population of hematopoietic cells that can be broadly split in CD11b$^+$ and CD103$^+$ subsets and derive from a common DC progenitor. Adoptive transfer studies as well as developmental genetic tagging studies with LysMCre reporter systems have revealed that lung DCs in steady state can also originate from monocytes, and this pathway could be predominant when inflammation is induced (Jakubzick et al., 2008a). Although there is no problem in reliably identifying CD103$^+$ cDCs, many groups have searched for markers that could separate moDCs from cDCs within the CD11b$^+$ gate. We found a combination of two specific markers, CD64 and MAR-1, that unequivocally separate CCR2-dependent moDCs from CCR2-independent CD11b$^+$ cDCs and that performs much better than the traditionally used Ly6C marker in identifying all moDCs. We propose that this gating strategy can be used also in studies addressing the developmental origin of lung DC subsets. In support, we readdressed the presence of cDCs in *Flt3l*$^{-/-}$ mice and found that both CD103$^+$ cDCs and CD11b$^+$ cDCs were totally absent from the lungs, whereas residual CD11b$^+$ DCs were universally CD64$^+$MAR1$^+$ moDCs. One can argue that CD64 by itself might suffice to separate both subsets of CD11b$^+$ lung DCs. However, in moDCs migrating to the MLNs and carrying HDM allergen, we noticed some reduction in the intensity of CD64 staining. MAR1 staining, however, compensated for this loss, because moDCs became uniformly MAR1$^+$ upon migration to the MLN. CD64 is emerging as a universal discriminating marker for moDCs and macrophages, as we also described its usefulness in DC phenotyping in the muscle, intestine, and skin and others found it to be a marker for tissue-resident macrophages (Gautier et al., 2012).

With this gating strategy and a series of depletion and adoptive transfer studies, we addressed which of the migratory DCs was necessary and sufficient for inducing Th2 cell-mediated immunity to HDM allergen. We did not address the involvement of resident cDCs of the MLN, because all allergen accumulation in the MLN was eliminated in CCR7-deficient mice and all antigen-carrying DCs were CCR7 positive. Under conditions of physiological low allergen exposure (1 μg dose), induction of Th2 cell-mediated immunity to HDM was the exclusive function of CD11b$^+$ cDCs, as indicated by the fact that *Flt3l*$^{-/-}$ DCs no longer mounted Th2 cell-dependent eosinophilic airway inflammation. Because *Flt3l*$^{-/-}$ mice lack migratory and resident cDCs, this conclusion could be reached only by comparison with langerin-DTR mice, in which the subset of CD103$^+$ cDCs is exclusively depleted, showing no reduction in the strength of the Th2 cell-mediated immune response. Upon adoptive transfer to naive mice, only CD11b$^+$ cDCs and not CD103$^+$ cDCs were able to induce Th2 cell-dependent eosinophilic airway inflammation. The fact that CD103$^+$ cDCs do not induce Th2 cell-mediated immunity to HDM is at odds with a recent paper reaching the opposite conclusion (Nakano et al., 2012). In this work, BHX2 mice were analyzed carrying a mutation in the *Irf8* gene that leads to aberrant development of CD103$^+$ cDCs. These mice failed to develop asthma, leading the authors to conclude that CD103$^+$ cDCs were essential. However, BHX2 mice also have a myeloproliferative syndrome that could suppress development of allergy and have abnormal IL-12p40 production (Turcotte et al., 2005). Also, induction of Th2 cell-mediated immunity by purified CD103$^+$ cDCs was read out in vitro through polyclonal activation of T cells, which might have unraveled a weak Th2 cell response induced by CD103$^+$ cDCs. Finally, differences in the dose and batch of HDM used might have led to opposing effects compared with our study.

When we increased the dose of HDM allergen and concomitantly induced a stronger TLR4-dependent innate immune response accompanied by production of the CCR2 ligand CCL2, more moDCs were attracted to the lungs, and some of these moDCs were able to migrate to the MLN, where they were sufficient to prime for Th2 cell immunity. Also, when the dose of HDM was increased in *Flt3l*$^{-/-}$ mice, there was no longer



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any necessity for cDCs, because all features of airway inflammation were induced by residual and recruited moDCs. This dose dependency of allergen on the relative contribution of moDCs versus cDCs might also explain why Walzer et al. (2005) have previously noted that *Flt3l*$^{-/-}$ mice had intact features of cockroach-driven asthma, because they used a similarly high dose of 100 μg of cockroach extract to elicit asthma (Walzer et al., 2005). The finding that moDCs are able to induce Th2 cell-mediated immunity when high-dose allergens are given is surprising but not totally unexpected. Most of the papers that have addressed the Th cell-polarizing capacity of moDCs have used either Th1 cell-associated adjuvants (complete Freund's adjuvant) or strong bacterial (*Listeria*), parasitic (*Leishmania*), viral (influenza), or fungal (*Aspergillus, Histoplasma, Blastomyces*) infection models and have reached the conclusion that moDCs induce Th1 cell-mediated immunity (Hohl et al., 2009; León et al., 2007; Nakano et al., 2009; Wüthrich et al., 2012). We also noticed that moDCs were slightly biased for higher IFN-γ induction compared with cDCs, accompanied by higher mRNA expression of IL-12p35 and IL-12p40. However, there was a concomitant induction of Th2 cell-associated cytokines in vitro and in vivo. One of the arguments for Th1 cell induction by moDCs is that *plt* mice, lacking CCL19 and the lymphoid form of CCL21, are biased for Th1 cell-mediated immunity when immunized with OVA and CFA. These mice have intact moDCs in the nodes yet lack all CCR7-dependent migratory cDCs. However, *plt* mice have also been shown to have enhanced features of Th2 cell-mediated immunity in models of OVA-alum driven asthma (Grinnan et al., 2006). Comparing these studies shows that the Th cell bias of moDCs really depends on the type of inciting pathogen and/or nature of the adjuvant, most probably determining whether high IL-12 production is induced or not. In support, we have shown that the lack of Th2 cell-mediated immunity induced by OVA-alum in mice depleted of all DCs can be reverted by adoptive transfer of monocytes, developing into moDCs (Kool et al., 2008). In more Th2 cell-biased infection models like *Schistosoma mansoni* infection, CCR2-deficient animals, lacking monocyte egress and most probably therefore also moDCs, have reduced features of Th2 cell-mediated immunity (Lu et al., 1998).

Upon allergen challenge of the lung in sensitized mice, all subsets of moDCs and cDCs were recruited to the lung and mainly CD11b^{+} cDCs and moDCs had captured the HDM allergen in the lung. The increase in CD11b^{+} lung DCs has previously been reported in OVA-driven models of asthma (Robays et al., 2007), and we now show that this is mainly due to an increase in moDCs. CD11b^{+} lung DCs are a known source of proinflammatory chemokines that could directly influence inflammation at the target site, whereas CD103^{+} cDCs were shown to be important for recruitment of Th2 cells via production of Th2 cell-selective chemokines CCL17 and CCL22 (Ortiz-Stern et al., 2011). By subdividing CD11b^{+} DCs, we found that proinflammatory chemokine production is almost the exclusive function of moDCs; CD11b^{+} cDCs hardly produce them. Many of the chemokines produced have direct chemotactic activity for eosinophils and effector T cells in the airways (Gonzalo et al., 1998), which might explain why depletion of all DCs or CD11b^{+} myeloid cells at times of allergen challenge (Medoff et al., 2009) and absence of only 50% of moDCs in *Ccr2*$^{-/-}$ mice has such dramatic effects on airway eosinophilia. We also found the Th2 and Treg cell-tropic chemokines CCL17 and CCL22 to be selectively produced by CD103^{+} cDCs, but we do not understand the full impact of this in our model system, because depletion of CD103^{+} cDCs during challenge did not reduce features of asthma (data not shown). Because CD103^{+} cDCs represent only a minor fraction of total lung DCs during allergen challenge, it is most likely that the majority of the production of CCL17 and CCL22 still derives from moDCs, which also produce CCL17 and CCL22 albeit in lower amounts. In conclusion we unraveled an important division of labor between DC subsets of the lung: CD11b^{+} cDCs are necessary for priming Th2 cell immunity to physiological low doses of HDM allergen by acting as APCs, whereas moDCs play a predominant proinflammatory role in asthma, via production of chemokines.

EXPERIMENTAL PROCEDURES

Mice

Langerin-DTR, *CD45.1*, *CD45.2*, *Ccr7*$^{-/-}$, and *Ccr2*$^{-/-}$ mice were bred at the animal facility of the University of Ghent. *Flt3l*$^{-/-}$ mice were provided by M. Manz. β chain TCR Tg mice (1-DERβ) specific for Der p 1 peptide were generated as described in Supplemental Experimental Procedures. Experiments were approved by the animal ethical committee of the University of Ghent.

Figure 6. Function of moDCs in Th2 Cell Immunity and Allergen Challenge Phase

(A) *Flt3l*$^{-/-}$ or WT mice were sensitized with a high HDM dose (i.e., 100 μg of HDM), followed by five challenges of 100 μg HDM i.n. on consecutive days. 72 hr after the last HDM challenge, the BALf differential cell counts were determined.

(B) The MLNs were restimulated with HDM and cytokine production was measured in the culture supernatants.

(C) Number of CD103^{+} cDCs, CD11b^{+} cDCs, and moDCs in the lungs of *Ccr2*$^{-/-}$ and WT mice 3 days after administration of 100 μg of HDM.

(D) *Ccr2*$^{-/-}$ mice or WT were sensitized and challenged as in (A). Differential cell counts were analyzed 72 hr after the last HDM challenge.

(E) Pie charts depicting the relative proportion of each DC subset within the total or the AF647-HDM^{+}MHCII^{+}CD11c^{+} population in the lung of mice administered with increasing doses of allergen during the challenge phase.

(F) Proportion of AF647-HDM^{+} lung populations 72 hr after the allergen administration.

(G) Pie charts depicting the relative proportion of each DC subset within the total or the AF647-HDM^{+}MHCII^{+}CD11c^{+} population in the MLNs of mice administered with increasing doses of allergen during the challenge phase.

(H) Proportion of AF647-HDM^{+} MLN populations 72 hr after the allergen administration.

(I) Proliferation of CFSE-labeled 1-DERβ T cells cocultured for 4 days with DC subsets sorted from the lungs of mice during the challenge phase.

(J) mRNA expression of chemokines by lung DC subsets sorted 3 days after the last HDM challenge. mRNA expression was first normalized against housekeeping genes and then represented as relative expression to the lowest-expressing DC subset.

(K) Chemokine production measured in supernatants of DC subsets sorted from the lungs 3 days after the last HDM challenge and plated overnight.

Data represent three (A, B, E–J) and two (C, D, K) independent experiments with three to seven mice per group. Error bars represent the SEM. *$p < 0.05$. See also Table S1.

HDM Labeling, Immunization, and Depletion

HDM (*Dermatophagoidespteronyssinus* extracts, Greer Laboratories) was labeled with a AF647 labeling kit (Invitrogen) and injected i.t. dissolved in 80 μl of PBS. Analysis was performed on enzyme-digested lung and MLN cells. To induce allergic airway inflammation, mice were sensitized i.n. with 1 μg HDM extracts on day 1 and were subsequently challenged with 10 μg HDM i.n. on days 7–11. 3 days after the last challenge, lungs, BAL, and MLNs were collected. *Flt3l*$^{-/-}$ and WT control mice were also sensitized and challenged with 100 μg HDM in a high-dose model. To study the challenge phase, mice were injected with 1 μg HDM i.n. followed after 1 week by challenge with PBS or 10 or 50 μg HDM-AF647 i.n. The CD103^{+} cells were depleted with langerin-DTR C57BL/6 mice by i.p. injection of 500 ng diphtheria toxin (Sigma-Aldrich) at day −1 and −2.

Chimeric Mice

CD45.2 C57BL/6 mice were irradiated with an 8 Gy dose. After 5 hr, 2 × 10^{6} mixed bone marrow cells of CD45.1 C57BL/6 and CD45.2 *Ccr2*$^{-/-}$ mice (ratio 50/50) were injected i.v. After 8 weeks, mice were injected with 100 μg HDM i.t. After 3 days, blood, lung, and MLN were obtained and stained as described above.

Monocyte Transfer

Monocytes were sorted as Ly6C^{hi}CD11b^{+} cells from spleen and bone marrow from *Rag*$^{-/-}$ mice. After sorting, monocytes were CFSE labeled, according to the protocol from the manufacturer (Invitrogen). In *Ccr2*$^{-/-}$ mice, 2 × 10^{6} cells were injected i.v., together with 100 μg HDM i.t. After 4 days, lungs were obtained and analyzed.

T Cell Proliferation and Cytokine Production

To study proliferation of 1-DERβ T cells, CD4^{+} T cells were cocultured with CD103 cDCs, CD11b cDCs, or moDCs from the MLNs (5 T cells:1 DC ratio), 3 days after i.t. administration of HMD. During challenge, the 1-DERβ T cells were cocultured with CD103 cDCs, CD11b cDCs, or moDCs from the lung, 3 days after one challenge, 1 week after sensitization (5 T cells:1 DC ratio). For experiments with HDM-specific T cells, CD4^{+} L-selectin^{hi} cells were MACS purified from 1-DERβ TCR transgenic mice according to the protocol of the manufacturer and labeled with CFSE, prior to coculture with ex vivo sorted DC subsets. To study T cell proliferation, cells are stained after 3 days of coculture with combinations of CD3 (145-2C11), CD4 (RM4-5), CD69 (H1,2F3), and CD44 (IM7) and CFSE profiles determined. Cytokines were measured with commercially available ELISA kits (BD).

Adoptive Transfer

To test the capacities of CD103^{+} and CD11b^{+} DCs to induce Th2 cell sensitization, CD103^{+} cDCs, CD11b^{+} cDCs, or MAR1^{+}CD64^{+} moDCs were sorted from MLNs 3 days after the i.t. administration of 100 μg HDM, with a FACS ARIA II flow cytometer. 30,000 cells of these PBS- or HDM-sensitized subsets were administered i.t. into C57B/6 mice. 1 week later, mice were challenged five consecutive days with i.n. injection of HDM (10 μg HMD dissolved in 40 μl PBS, Greer Laboratories), as described above.

Statistics

For all experiments, the difference between two groups was calculated with the Mann-Whitney U test for unpaired data. If two or more groups were compared, Kruskal-Wallis test was used (GraphPad Prism version 5.0; GraphPad, San Diego, CA). Differences were considered significant when $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.10.016.

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