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Complement Receptor C5aR1/CD88 and Dipeptidyl Peptidase-4/CD26 Define Distinct Hematopoietic Lineages of Dendritic Cells

Hideki Nakano,* Timothy P. Moran,*+,† Keiko Nakano,* Kevin E. Gerrish,‡ Carl D. Bortner,§ and Donald N. Cook*

Dendritic cells (DCs) induce adaptive immunity by acquiring Ags and presenting peptides derived from them to naïve T cells (1). In nonlymphoid tissues such as the lung, two major CD11c+ DC subsets can be identified based on their display of the integrins αE (CD103) and αM (CD11b) (2, 3). Lung CD103+ DCs are a homogeneous population and are similar to CD8α+ DCs, which are primarily found in lymphoid tissues. Both of these DC types are derived exclusively from FLT3-dependent DC precursors (preDCs) and are therefore termed conventional DCs (cDCs), including CD103+ and CD11bhi cDCs, express dipeptidyl peptidase-4/CD26. Flow cytometric analysis of multiple organs, including the kidney, liver, lung, lymph nodes, small intestine, and spleen, confirmed that reciprocal display of CD88 and CD26 can reliably distinguish FLT3-independent moDCs from FLT3-dependent cDCs in C57BL/6 mice. Similar results were obtained when DCs from BALB/c mice were analyzed. Using this novel approach to study DCs in mediastinal lymph nodes, we observed that most blood-derived lymph node–resident DCs, as well as tissue-derived migratory DCs, are cDCs. Furthermore, cDCs, but not moDCs, stimulated naïve T cell proliferation. We anticipate that the use of Abs against CD88 and CD26 to distinguish moDCs and cDCs in multiple organs and mouse strains will facilitate studies aimed at assigning specific functions to distinct DC lineages in immune responses. The Journal of Immunology, 2015, 194: 3808–3819.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; BMDC, BM-derived DC; C5aR1, complement 5a receptor 1; cDC, conventional DC; DC, dendritic cell; DPP4, dipeptidyl peptidase-4; FLT3, FLT3 ligand; HDE, house dust extract; LN, lymph node; MHC-H, MHC class II; mLN, mediastinal lymph node; moDC, monocyte-derived DC; preDC, DC precursor; qPCR, quantitative PCR.

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progress has also been made in this area. Abs against CD14 and CD64 are widely used to identify macrophages, and recent studies have shown that these molecules are also displayed by some CD11c⁺MHC class II (MHC-II)⁺CD11b⁺ DCs, presumably CD11b⁺ moDCs (15). Accordingly, display of CD14 and CD64 has been used to identify moDCs, together with the marker CD244, which is displayed on some CD11b⁺ cDCs (16, 17). This is a useful strategy for discriminating between cDCs and moDC in the lung and small intestine, but it is of limited utility for distinguishing DC lineages in other organs (15, 17). For example, in skin-draining lymph nodes (LNs), cDCs identified by their high expression of Zbi/b46 have relatively high levels of CD14 (12), which is associated with moDCs in the lung (11, 15). It is therefore important to develop novel strategies that can reliably discriminate between cDCs and moDCs in multiple organs and in multiple mouse strains. In the present study, we analyzed gene expression of moDCs and cDCs prepared from the lung to identify novel markers of these two lineages. We found that complement C5a receptor 1 (C5aR1) and C5b8 are selectively expressed on moDCs, including Ly-6C⁺–resident moDCs and Ly-6C⁰ inflammatory moDCs. Conversely, CD103⁺ cDCs and CD11b⁺ cDCs display high cell surface levels of dipeptidyl peptidase-4 (DPP4)/CD26. Abs against these molecules can reliably identify the major lineages of cDCs and moDCs in multiple peripheral and lymphoid tissues, including kidney, liver, lung, small intestine, spleen, and lung-draining LNs.

Materials and Methods

**Mice**

BALB/cJ, C57BL/6J, CD45.1 (B6.SJL-Ptprc<sup>+</sup> Pepck<sup>−/−</sup>BoyJ), and OT-II (B6.Cg-Tg(TcraTcrb)24Cbn/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Flt3L<sup>+/−</sup> (C57BL/6-Flt3L<sup>+/−</sup>) mice and control C57BL/6N mice were from Taconic (Germantown, NY). Mice were housed in specific pathogen-free conditions at the National Institute on Environmental Health Sciences and used between 6 and 12 wk of age in accordance with guidelines provided by the Institutional Animal Care and Use Committees.

**Allergic sensitization**

In some experiments, mice were anesthetized by isoflurane inhalation and given 50 μl PBS containing 0.1 μg LPS (Sigma-Aldrich, St. Louis, MO) together with 100 μg endotoxin-free OVA (Profs, Regensburg, Germany) (LPS/OVA) or 10 μl mouse dust extract (HDE) together with OVA (HDE/OVA) by oropharyngeal aspiration as described previously (18, 19).

**Preparation and ex vivo analysis of DCs**

DCs were prepared from mouse lung, mediastinal LNs (mLNs), spleen, kidney, liver, and from the small intestine. After removal of Peyer’s patches, small intestines were washed extensively in PBS containing 5 mM EDTA, 145 μg DTT, and 10% FBS as previously reported (20). Minced tissues were digested as described previously (21), with minor modifications in incubation times as follows: 10 min for liver, 30 min for mLN, spleen, kidney, and small intestine; and 60 min for lung. Low-density cells, collected from gradient centrifugation using 16% Nycodenz (Accurate Chemical, Westbury, NY), were diluted to 1–2 × 10⁶ cells/ml. High-density cells, collected from gradient centrifugation using 16% Nycodenz (Accurate Chemical, Westbury, NY), were diluted to 1–2 × 10⁷ cells/ml. mDCs were digested as previously (22), with minor modifications in incubation times as follows: 10 min for liver, 30 min for mLN, spleen, kidney, and small intestine; and 60 min for lung. Low-density cells, collected from gradient centrifugation using 16% Nycodenz (Accurate Chemical, Westbury, NY), were diluted to 1–2 × 10⁷ cells/ml. mDCs were digested as previously (22), with minor modifications in incubation times as follows: 10 min for liver, 30 min for mLN, spleen, kidney, and small intestine; and 60 min for lung.

**Calcium flux**

Intracellular calcium was measured using Fluo-4 (Life Technologies, Grand Island, NY) as described previously (22). Briefly, DCs in HBSS containing 1 mM Ca<sup>2+</sup>, 1 mM Mg<sup>2+</sup>, and 0.5% BSA were labeled with 4 μM Fluo-4 for 30 min at 37°C in the presence of 4 mM probenecid. After washing, cells were resuspended in 500 μl buffer and allowed to rest for at least 15 min prior to analysis. The Fluo-4 signals were recorded by flow cytometry for 30 s prior to adding 10 μM Ca<sup>2+</sup> agonistic peptide (FKP-D(Cha)-Cha-R) (AnaSpec, Fremont, CA) and then monitored for 12 min. Ionomycin (2 μl 1 mg/ml, EMD Millipore, Billerica, MA) was added at the end to ensure the presence of the calcium indicator.

**Gene expression analysis**

Microarray experiments were done in triplicate with DCs purified from pooled lungs of 20 mice (total of 60 mice). The ranges of analyzed DC numbers were as follows: CD103⁺ DCs, 1.98–2.53 × 10⁶ cells; CD11b⁺ CD14<sup>+</sup>Ly-6C<sup>+</sup> DCs, 3.04–3.54 × 10⁵ cells; CD11b⁺CD14<sup>+</sup>Ly-6C<sup>−</sup> DCs, 1.24–1.37 × 10⁵ cells; CD11b⁺Ly-6C<sup>−</sup> DCs, 3.35–8.11 × 10⁴ cells. Total RNA was isolated from each subset using RNeasy kit (Qiagen, Valencia, CA) and comprehensive gene expression analysis was conducted using Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix). After scanning arrays, data were obtained using the GeneChip Command Console software using the MS5 algorithm (Affymetrix). Microarray data are available at the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo/info/linking.html; accession no. GSE46980). To identify subset-specific genes, data were further analyzed using IPA software (Ingenuity). For quantitative PCR (qPCR) analysis, total RNA from purified lung DC subsets prepared independently from microarray was isolated using TRIzol (Life Technologies). The total RNA was converted to cDNA.
FIGURE 1. Display of CD88/C5aR1 on lung moDCs. (A) Pre- and postpurification of CD11bhiCD14hiLy-6Clo moDCs and CD11bhiCD14hiLy-6Clo cDCs from C57BL/6 mouse lungs following inhalation of LPS/OVA. Data shown are from a single experiment, representative of three. (B–D) Flow cytometric analyses of CD88 display on lung DC subsets from C57BL/6 mice. Similar results were obtained in three independent experiments. (B) CD88 display on CD11bhiCD14hi, CD11bhiCD14lo, and CD103+ lung DCs at steady-state. Mean fluorescence intensity (MFI) is indicated. (C) CD88 display on CD103+ DCs, CD11bhiCD14hi, CD11bhiCD14lo, and CD11bhiLy-6Chi DCs after instillation of LPS/OVA. (D) MFI of CD88 display on lung DC subsets. Mean values ± SEM from three mice are shown. *p, 0.01, **p, 0.001, ***p, 0.0001. (E) Co-display of CD88 and CD64 on CD11bhi lung DCs. (F) C5a-mediated Ca2+ influx in the indicated lung DCs purified from lungs of LPS/OVA-treated C57BL/6 mice. DCs were loaded with Fluo-4, and fluorescence was measured by flow cytometry before and after treatment with C5a peptide and ionomycin. Eight to 14 × 10⁴ cells per sample were acquired, and 909 ± 295 cells were analyzed for each time point. A representative result from three independent experiments is shown. *p < 0.05, Fluo-4 signals in C5a-treated cells compared with mock-treated cells at each time point.
with oligodeoxythymidine primers and a SuperScript III First-Strand kit (Life Technologies). PCR amplification was performed with SYBR Green Master Mix (Applied Biosystems) and an Mx3000P qPCR system (Agilent Technologies, Santa Clara, CA) using the following primers:

- **Gapdh**, forward, 5′-AACTTTGGCATTGTGGAAGG-3′, reverse, 5′-AACTTTGGCATTGTGGAAGG-3′;
- **Il12a**, forward, 5′-CTAGACAAGGGCATGCTGGT-3′, reverse, 5′-GCTTCTCCCACAGGAGGTTT-3′;
- **Irf4**, forward, 5′-AATCCCCATTGAGCCAAGCA-3′, reverse, 5′-TCGTCGTGGTCAGCTTTTC-3′;
- **Irf8**, forward, 5′-ACAATCAGGAGGTGGATGCTT-3′, reverse, 5′-CGTGGCTGGTTCAGCCTTTGT-3′;
- **Nos2**, forward, 5′-CCCCGCTACTTACTCCATCAG-3′, reverse, 5′-GGCTTCAGGTTCCTGATCCAA-3′;
- **Tnf**, forward, 5′-GATCGGTCCCCAAAGGGATGA-3′, reverse, 5′-TGCTCCTCCACTTGGTGGTTT-3′;
- **Zbtb46**, forward, 5′-ATCACTTCTCACTACGGCAT-3′, reverse, 5′-AAGACGTTCTTATGTGCCTTGAA-3′.

The relative expression of each gene was normalized to *Gapdh* expression.

### Statistical analysis

Data are presented as means ± SEM. Statistical differences between groups were calculated using a two-tailed Student *t* test, unless indicated otherwise. A *p* value < 0.05 was considered significant.

## Results

**Monocyte-derived pulmonary CD11b<sup>hi</sup> DCs selectively express C5aR1/CD88**

Reciprocal display of CD64 and CD24 has been reported to define moDCs and cDCs, respectively, at least for DCs prepared from the lung or small intestine (15–17, 23). Using C57BL/6 mice, we confirmed that lung CD11b<sup>hi</sup> DCs, defined as CD11b<sup>hi</sup>CD11c<sup>+</sup>MHC-II<sup>+</sup>autofluorescence<sup>−</sup> cells (Supplemental Fig. 1A) can be clearly resolved into CD14<sup>hi</sup>(CD64<sup>hi</sup>)CD24<sup>lo</sup> moDCs and CD14<sup>lo</sup>(CD64<sup>lo</sup>)CD24<sup>hi</sup> cDCs (Supplemental Fig. 1B). Because BALB/c mice are frequently used in animal models of allergic diseases such as asthma, we also tested whether these same markers can similarly distinguish CD11b<sup>hi</sup> cDCs and moDCs in that strain. Unexpectedly, Abs against CD14, CD64, and CD24 failed to resolve CD11b<sup>hi</sup> lung DCs from BALB/c mice into the distinct moDC and cDC populations. This was because a relatively small number of BALB/c DCs stained for CD24, and because many

![CD26/DPP4 display on lung cDCs.](http://www.jimmunol.org/)

(A) Venn diagram indicating the cellular location of proteins encoded by genes expressed ~2-fold higher in CD103<sup>+</sup> cDCs and in CD11b<sup>hi</sup> cDCs than in either moDC subset. (B) Display of CD26 on CD103<sup>+</sup> and CD11b<sup>hi</sup> lung DCs during steady-state conditions and after instillation of LPS/OVA. (C) Mean fluorescence intensity (MFI) ± SEM of CD26 display on lung DC subsets. Data are from two mice. *p < 0.01, **p < 0.001.
CD24lo DCs were also CD14lo or CD64lo (Supplemental Fig. 1B). We next analyzed DCs from lung-draining mLNs of C57BL/6 and BALB/c mice and found that these Abs also failed to clearly resolve CD11bhi DCs into their cDC and moDC components. Unlike CD11bhi DCs from C57BL/6 mouse lungs, CD24 display on LN DCs was not inversely correlated with display of either CD14 or CD64 (Supplemental Fig. 1C, 1D). The latter findings are consistent with the previously reported high levels of CD14 on CD11bhi cDCs, and of ZBTB46-GFP on CD24lo DCs in skin-draining LNs (11–13). Thus, neither CD14 nor CD64 can identify moDCs in all organs and mouse strains, underscoring the need for more widely applicable cell surface markers.

We reasoned that comprehensive gene expression analysis of well-defined populations of CD11bhi moDC and cDC populations might reveal genes that are reciprocally expressed in these DC lineages, regardless of their anatomical location. Because display levels of CD14 are sufficient to identify CD11bhi moDCs and cDCs in lungs of C57BL/6 mice, we purified CD11bhiCD14hi Ly-6Clo-resident moDCs and CD11bhiCD14hiLy-6Chi cDCs from lungs of these animals using flow cytometry–based cell sorting (Fig. 1A). RNA was separately prepared from these two populations, and microarray-based gene expression analysis was performed. As expected, expression of CD14 was several-fold higher in moDCs than in cDCs, confirming effective separation of these two populations. We also found that three other cell membrane protein-encoding genes, C5ar1, C3ar1, and Ptgser2, were expressed at much higher levels in moDCs than in cDCs (Table I). We focused on C5ar1, because it had the greatest difference in expression between moDCs and cDCs. This gene encodes the well-characterized complement receptor C5ar1 (CD88), and Abs that recognize it are readily available. Flow cytometric analysis confirmed that CD88 is displayed at higher levels on CD11bhiCD14hi DCs than on CD11bhiLy-6Chi DCs (Fig. 1A, Supplemental Fig. 2C, Table II). The gene encoding DPP4/CD26 had the highest expression in CD11bhiCD14lo cDCs (Table II). Flow cytometric analyses confirmed that CD103+ cDCs uniformly display high levels of CD26 at the protein level, whereas CD11bhi DCs in the lung comprise two major populations that can be distinguished by their relative display of CD26. These two populations were observed at steady-state, as well as during LPS/OVA-induced inflammation (Fig. 2B). Display of CD26 inversely correlated with that of CD88, suggesting that CD88 is highly displayed by cDCs, but not by moDCs.

**Table II.** Intensities of genes encoding plasma membrane proteins selectively expressed by cDCs

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<th>Gene Symbol</th>
<th>CD11bhiCD14hi</th>
<th>CD103+</th>
<th>CD11bhiCD14lo</th>
<th>CD11bhiLy-6Cin</th>
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<td>1709</td>
<td>468</td>
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<td>2076</td>
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<td>493</td>
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<tr>
<td>Prnp</td>
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Mean intensity in microarray analysis from three replicates (20 mice each) is shown.

**Conventional pulmonary CD11bhi DCs selectively express DPP4/CD26**

We next sought to identify a novel cell surface marker that is specific for cDCs. We compared gene expression in two cDC subsets (CD103+ DCs and CD11bhiCD14lo DCs) with that of two moDC subsets (CD11bhiCD14hi DCs and CD11bhiLy-6Cin DCs) (Supplemental Fig. 2A, 2B). As expected from previous studies, many genes (including Xcr1) were differentially expressed between CD103+ and CD11bhi cDCs (25–27). Of greater relevance to the goal at hand, we also identified 52 genes that were expressed at least 2-fold higher in cDC subsets than in moDC subsets, and 18 of those genes encoded membrane proteins (Fig. 2A, Supplemental Fig. 2C, Table II). The gene encoding DPP4/CD26 had the highest expression in CD11bhiCD14lo cDCs (Table II). Flow cytometric analyses confirmed that CD103+ cDCs uniformly display high levels of CD26 at the protein level, whereas CD11bhi DCs in the lung comprise two major populations that can be distinguished by their relative display of CD26. These two populations were observed at steady-state, as well as during LPS/OVA-induced inflammation (Fig. 2B). Display of CD26 inversely correlated with that of CD88, suggesting that CD26 is highly displayed by cDCs, but not by moDCs.

**CD11bhiCD88hi DCs are monocyte-derived**

To confirm that CD11bhiCD88hiCD26lo DCs are indeed moDCs, we purified Ly-6Cin monocytes from CD45.1 mouse BM (Supplemental Fig. 3A, 3B), adoptively transferred them into CD45.2 mouse, and instilled LPS/OVA into the airways of recipient mice to induce inflammation. One day after this treatment, many CD45.1+ donor-
derived monocytes had differentiated into inflammatory DCs, as evidenced by their acquisition of CD11c and MHC-II. Freshly isolated monocytes did not display CD88 (Fig. 3A), but they acquired its expression upon their differentiation to DCs (Fig. 3B). By day 3 posttransfer, most donor-derived cells no longer displayed high levels of Ly-6C, but maintained display of CD88 (Fig. 3B). Very few CD88lo donor cells were seen at either time point, confirming that monocytes give rise almost exclusively to CD88hi moDCs.

To confirm that CD88hiCD26hi cells are cDCs, we studied mice lacking FLT3L, a cytokine that promotes cDC development. In agreement with previous reports (8, 11), Flt3L−/− mice were essentially devoid of CD103+ CDCs and had severe reductions in CD11bhiCD88loCD26hi DCs in the lung (Fig. 3C, 3D). In contrast, CD11bhiCD88hi DCs were abundant in Flt3L−/− mice (Fig. 3C, 3E), as would be expected for cells derived from monocytes. Thus, multiple independent experiments confirmed that at least for lungs of C57BL/6 mice, CD88 and CD26 are reliable markers to identify moDCs and cDCs, respectively.

Our finding that CD11bhi DCs of BALB/c mice are not effectively resolved into distinct populations by Abs against CD64 and CD14 (Supplemental Fig. 1B, 1D) prompted us to test whether

**FIGURE 3.** Confirmation of developmental lineages of CD88hi and CD26hi lung DCs. (A) CD88 display on monocytes freshly isolated from BM. (B) Differentiation of CD45.1 monocytes following their adoptive transfer into CD45.2 recipient mice. Shown are the gating strategy to identify CD45.1+ donor cells as well as their display of CD88 and Ly-6C 1 or 3 d after instillation of LPS/OVA into recipient mice. (C–E) Flow cytometric analysis of lung DCs of wild-type and Flt3L−/− mice at steady-state and 16 h after instillation of LPS/OVA. (C) Cytograms and percentages of CD11bhiCD88loCD26hi and CD11bhiCD88hiCD26hi lung DCs. (D) Percentages of CD103+ lung DCs. (E) Cytograms and percentages of CD11bhiCD88loLy-6Clo, CD11bhiCD88hiLy-6Clo, and CD11bhiCD88hiLy-6Chi lung DCs (n = 3). Results shown are from one of three experiments yielding similar results. *p < 0.05.
CD26 and CD88 are more effective in this regard. We harvested DCs from the lung of BALB/c mice and found that the latter two molecules could indeed resolve CD11b+ DCs into two distinct populations: CD88hiCD26lo DCs and CD26hiCD88lo DCs (Supplemental Fig. 4A). Using this approach to evaluate DC changes in the lung following LPS/OVA instillation, we observed that cell numbers for all types of CD11b+ DCs increased, with the largest increase seen for CD88hiCD26lo inflammatory DCs.

Analysis of moDCs and cDCs in the lung

To further characterize moDCs and cDCs in the lung, we used flow cytometry–based cell sorting to purify CD103+ cDCs, CD11bhiCD88hiCD26lo moDCs, and CD11bhiCD88hiCD26lo moDCs from untreated mice and from mice that had inhaled LPS/OVA. Analysis of gene expression by qPCR revealed that CD11bhiCD88hi CD26lo moDCs highly expressed Il12a, Tnf, and Nos2, which are highly expressed in monocyte-derived cells, such as inflammatory DCs and inflammatory macrophages (28, 29). These genes were not highly expressed in CD103+ cDCs or in CD11bhiCD88hi CD26lo cDCs (Fig. 4). Conversely, CD103+ cDCs and CD11bhi CD88loCD26hi cDCs exclusively expressed Zbtb46, which is expressed by all cDCs (12, 13). As expected, Irf8 was expressed in CD103+ cDCs, whereas Irf4 was expressed in CD11bhiCD88lo CD26hi cDCs (Fig. 4).

Analysis of moDCs and cDCs in lung-draining LNs

During steady-state, most DCs in skin-draining and mesenteric LNs are tissue-derived 11c+HiMHC-II+ migratory DCs and blood-derived CD11b+HiMHC-II+–resident DCs. Both of these DC populations express Zbtb46, suggesting they are cDCs (12, 13). However, during inflammation, many blood-derived moDCs are recruited to the LN (4, 30, 31), and these cells also display high levels of MHC-II (32). Thus, when analyzing cells from inflamed LNs, high levels of MHC-II cannot distinguish migratory cDCs from moDCs. We therefore tested whether these two DC populations can be distinguished using Abs against CD88 and CD26. During steady-state, CD11bhiCD88loCD26hi were the major DC population, followed by CD103hi cDCs, and relatively few CD11bhiCD88hiCD26hi DCs were seen (Fig. 5). A marked reduction of CD11bhiCD88hiCD26hi DCs in Flt3L−/− mouse LNs confirmed that these cells are indeed cDCs (Fig. 5B, 5C). Following instillation of LPS/OVA to induce mild inflammation in the lung, the number of CD103+ DCs in mLNs increased dramatically and these cells became the major DC population. Modest increases were seen for CD11bhiCD88loLy-6Clo(CD26lo) cDCs, CD11bhiCD88hiLy-6Clo(CD26hi) moDCs and CD11bhiCD88hiLy-6Clo(CD26hi) inflammatory moDCs in both C57BL/6 and BALB/c mice (Fig. 5C, Supplemental Fig. 4B), but the two moDC subsets remained minor populations. Importantly, the strict dependence of CD11bhiCD88hiCD26lo DCs on the presence of Flt3L regardless of immunological status indicates that CD88 and CD26 can reliably resolve two different lineages of DCs in LNs.

T cell activation by moDCs and cDCs

An important characteristic of DCs is their ability to stimulate proliferation of naive T cells. To evaluate this property in moDCs and cDCs prepared from the lung, we cultured naive CD4+ T cells from OVA–specific TCR (OT-II) transgenic mice together with various lung DC subsets. Because HDEs represent an environmentally relevant source of adjuvants promoting allergic airway inflammation (19, 22), HDE/OVA was administered to the airways of mice prior to preparing DCs from their lungs. Both cDC subsets (CD103+ cDCs and CD11bhiCD88hi cDCs) induced robust naive CD4 T cell proliferation as measured by dilution of CFSE, and each purified subset was more efficient in this regard than were total DCs (Fig. 6A, 6B). In contrast, neither moDC subset (CD11bhiCD88hi lung–resident moDCs or CD11bhiLy-6Clo inflammatory moDCs) induced proliferation of CD4+ T cells. T cell counts following the cocultures confirmed that both cDC subsets stimulated T cell proliferation much better than did moDCs (Fig. 6C). These results demonstrate that, at least in the lung, moDCs do not efficiently stimulate naive T cell proliferation, suggesting they fulfill a different function, such as production of proinflammatory cytokines. In support of this, moDCs produced much larger amounts of IL-6 and TNF-α than did cDCs (Fig. 6D).

LNs that drain peripheral tissue contain both resident DCs and migratory DCs, the former develop from precursors arriving to LNs directly from the blood, whereas the latter migrate from peripheral tissue to LNs through the lymphatics (4, 31). Compared to resident DCs, migratory DCs display higher amounts of MHC-II I-A, but lower levels of CD11c (20). We found that both types of DCs were markedly reduced in mLNs of Flt3L−/− mice (Supplemental Fig. 3C), in agreement with our previous results (Fig. 5) and indicating that most resident DCs, as well as migratory DCs, are cDCs. Although resident DCs in skin-draining LNs acquire soluble Ags that are passively carried to LNs through the lymphatics and stimulate T cells (31, 33, 34), it has been unclear whether resident DCs in lung-draining mLNs are also capable of stimulating naive
T cells, and whether this is dependent on their developmental lineage. To address this issue, we prepared total mLN DCs and separately purified CD103⁺ migratory cDCs, CD11b⁺CD88⁻I-A⁺CD11c⁻migratory cDCs, CD11b⁺CD88⁻I-A⁻CD11c⁺ resident cDCs, and CD11b⁺CD88⁺Ly-6C⁺ inflammatory moDCs from mLNs of mice 1 d after instillation of HDE/OVA. Total mLN DCs, as well as both types of migratory cDCs, induced robust proliferation of naive CD4⁺ T cells as measured by CFSE dilution assay and by cell count, whereas mLN-resident cDCs and inflammatory moDCs did not (Fig. 6E–G). To test the possibility that a longer time is required for resident DCs to acquire T cell–stimulating activity, we cultured naive CD4⁺ T cells with DCs prepared from mLNs 3 d after allergic sensitization. Although CD103⁺ DCs harvested at this time point displayed a reduced ability to stimulate naive CD4⁺ T cell proliferation compared with their counterparts harvested at 1 d after sensitization, both migratory cDC subsets, CD103⁺ DCs and CD11b⁺CD88⁻I-A⁺, still robustly stimulated T cell proliferation, whereas resident cDCs and moDCs did not (Fig. 6H, 6I). These results suggest that at least in the allergic sensitization model tested in the present study, the ability to stimulate naive CD4⁺ T cell proliferation is restricted to the lung-derived migratory cDCs.

Use of CD88 to identify in vitro–generated moDCs

BM is a rich source of DC progenitors and is therefore widely used to generate large numbers of DCs for study. Typically, BM cells are cultured in media containing either FLT3L or GM-CSF. We found that after culture in FLT3L-containing media, ∼90% of BM-derived DCs (BMDCs) were CD88⁺, suggesting that these conditions primarily give rise to cDCs. In contrast, BM cultured with GM-CSF gave rise to both CD88⁻ and CD88⁺ (Supplemental Fig. 3D). Including IL-4 in the media together with GM-CSF, a procedure widely used for generation of BMDCs, failed to yield large numbers of CD88⁻ cells (data not shown), likely because IL-4 suppresses Cd88 mRNA expression in these moDCs (35). To determine whether precursor cell identity determines...
CD88 display on their DC progeny, we separately purified monocytes and preDCs from BM and cultured them with GM-CSF without IL-4. Freshly isolated monocytes did not display CD88 (Fig. 3A), but most monocyte-derived CD11c+MHC-II+ cells after culture were CD88hi (Supplemental Fig. 3E). In contrast, CD11c+MHC-II+ cells derived from preDCs were primarily CD88lo. These data provide additional evidence that CD88 display on DCs is determined by their developmental lineage, and further suggest that CD88 display can be used to resolve BMDCs into their moDC and cDC components.

CD88 and CD26 distinguish moDCs and cDCs in multiple organs

Previous studies have shown that although CD24 and CD64 can be used to distinguish CD11bhi moDCs from CD11bhi cDCs in the lung and small intestine, these markers do not discriminate be-
tween lineages of DCs prepared from other organs (17). We confirmed that splenic CD8α+ DCs, as well as CD103+ DCs in kidney, liver, and small intestine, display a CD64loCD24hi phenotype, but that CD11bhi DCs in the spleen, kidney, and liver are not resolved into their moDC and cDC components (Supplemental Fig. 4C). We therefore tested the ability of Abs to CD88 and CD26 to distinguish cDCs from moDCs in various tissues. CD8α+ DCs and CD11bhi DCs prepared from the spleen all displayed a CD88hi CD26hi cDC phenotype (Fig. 7A), consistent with previous reports that there are very few moDCs in the spleen (9, 12). This was confirmed by marked reductions of CD11bhiCD26hi DCs and CD8α+ DCs in spleens of Flt3L−/− mice (Fig. 7B). In the kidney, liver, and small intestine, CD103+ DCs were uniformly CD88hi CD26hi. Importantly, CD11bhi DCs were effectively resolved into CD88hiCD26hi DCs and CD88hiCD26hi DCs (Fig. 7A). The former were Flt3L-dependent, whereas the latter were Flt3L-independent, confirming their identities as cDCs and moDCs, respectively (Fig. 7B). These results demonstrate that reciprocal display of CD88 and CD26 is widely useful to resolve two distinct lineages of DCs in many different organs.

Discussion
Because of their ability to link the innate and adaptive arms of the immune response, DCs are currently the focus of intense investigation. In recent years, it has become clear that there are many types of DCs, and that they can be subdivided based on their developmental lineage or display of different cell surface markers. However, assignment of specific functions to these different DC subsets has been difficult, in part because cell surface markers that can unequivocally resolve CD11bhi moDCs from their CD11bhi cDC counterparts in multiple organs and multiple strains have been lacking. In the present study, we found that CD88 is highly displayed on the surface of moDCs and that this display can distinguish them from cDCs. Although we initially identified CD88 as a marker of moDCs prepared from lungs, subsequent experiments revealed that it is also expressed in moDCs from several other organs, including the kidney, liver, and small intestine. For these organs, CD88hi DCs were present at normal numbers in Flt3L−/− mice, which display marked reductions in cDCs, but not moDCs. Additionally, adoptive transfer of monocytes gave rise almost exclusively to CD88hi cells, and CD88 was highly displayed on moDCs in multiple organs of two different mouse strains. These observations are in agreement with a study of C5aR-GFP reporter mice, which showed that C5aR-GFP fluorescence associates with monocyte/macrophage markers such as CD11b, F4/80, or Ly-6C/G, but not the cDC marker CD8α or the plasmacytoid DC marker PDCA-1 (36). We did not conduct an exhaustive study of all organs, however, and it is possible that some of them contain DC populations whose lineages cannot be identified solely by display of CD26 and CD88.

Although our present work focused on reciprocal display of CD88 and CD26 as a means to define moDC and cDC populations, respectively, it is possible that these molecules also affect DC function. C5a elicited Ca2+ influx in moDCs, indicating that CD88-mediated signaling is functional in these cells. Previous studies have shown that CD88−/− mice have exacerbated allergic inflammation upon allergen inhalation (37–39), and that Cd88−/− pulmonary DCs promote increased production of chemokines by T cells (37). However, in vitro–generated Cd88−/− BMDCs produce lower levels of Th17-inducing cytokines, such as IL-1β, IL-23, and TGF-β, than do wild-type BMDCs, and they have a diminished capability to induce allergic inflammation upon adoptive transfer (40). Thus, the role of CD88 in DC function remains to be solved.

The role of CD26 in DC function is also poorly understood. This transmembrane glycoprotein has many functions, including peptidase activity, T cell costimulatory activity, and cell adhesion properties (41, 42). Although Cd26−/− mice display enhanced...
allergic airway inflammation (43), it is unclear whether this is due to altered function of DCs or lymphocytes, as CD26 is also expressed by T and B cells (42). Our observation that CD26 is highly expressed by cDCs is consistent with some previous reports (17, 23), and the ability to selectively delete CD26 as well as CD88 on DCs should be helpful to understand the role of these molecules in DC function.

We found that in the spleen, most CD11b<sup>hi</sup> DCs (as well as CD80<sup>+</sup> DCs) are CD88<sup>−</sup>CD26<sup>−</sup>. This suggests that most splenic DCs are cDCs, in agreement with previous reports (9, 12). However, the relative abundance of moDCs and cDCs in most tissue-draining LNs is still unclear, although skin-draining LNs contain both Z<sup>b</sup>b4<sup>+</sup>-dependent and -independent cells (44). We previously found that although cDCs in the lung can migrate to regional mLNs, moDCs do not (11). Consequently, lung-derived DCs in regional LNs are almost exclusively cDCs. In addition to these migratory DCs, LNs also contain nonmigratory (LN-resident) DCs that probably arise from precursors that have direct access to LNs from the blood. The developmental lineage of these LN-resident DCs has been controversial (4, 12, 30), despite the fact that they are the major population of DCs in LNs, at least during steady-state conditions. Our ability to discriminate between these lineages by their reciprocal display of CD88 and CD26 prompted us to study their abundance in mLNs. We observed that most mLN-resident DCs (CD11b<sup>hi</sup>-I<sup>A</sup>-CD11c<sup>−</sup>), as well as most migratory DCs (CD103<sup>+</sup> and CD11b<sup>−</sup>I<sup>A</sup>-CD11c<sup>−</sup>), are cDCs. This is consistent with the finding that although blood-derived monocytes can accumulate in LN in the setting of severe inflammation (11, 31), this does not happen to an appreciable extent during steady-state conditions, or even following allergic sensitization (11). It seems likely, therefore, that most LN-resident DCs develop in situ from preDCs, which have been previously shown to reside in lymphoid organs, including LNs (45).

An important characteristic of DCs is their ability to stimulate naive T cells. We found that lung-derived migratory CD103<sup>+</sup> cDCs and migratory CD11b<sup>−</sup> cDCs induced robust proliferation of naive CD4<sup>+</sup> T cells, whereas moDCs did not. This finding is in agreement with a recent study showing that CD64<sup>+</sup> moDCs poorly stimulate naive T cells (17), and consistent with a role of moDCs as suppressive cells. However, some groups have reported that moDCs can promote T cell proliferation (32, 46). It remains to be seen whether some of the apparently discordant results of previous studies are due to differences in DCs from different LNs, or from incomplete separation of moDCs from cDCs. We anticipate that the strategy we have developed to clearly resolve moDCs from cDCs in multiple tissues will be helpful in this regard.

Our finding that mLN-resident CD11b<sup>−</sup> cDCs poorly stimulated T cell proliferation was somewhat unexpected because in skin-draining LNs, resident cDCs are reported to activate T cells and induce their proliferation in vivo (33, 47). It is possible that Ags drain more efficiently to skin LNs than to mLNs because although Ag was readily detected in resident DCs of skin-draining LNs after immunization with OVA or MHC class I-E<sup>α</sup> protein (31, 33), we were unable to detect fluorescent OVA in mLN-resident cDCs even after instilling high amounts of OVA (H. Nakano and D. Cook, unpublished observations). Alternatively, LN-resident DCs in skin-draining LNs might possess different activities than their counterparts in mLNs, possibly because they are exposed to different environmental factors. It is also conceivable that migratory DCs and resident DCs cooperate to stimulate T cells in mLNs, as they are reported to do in skin-draining LNs (34). Regardless of the correct explanation, the ability to discriminate each LN-resident DC subset using reliable markers should facilitate future studies of their development and functions in immune responses.

The definition of DCs based on their morphology, surface molecule expression, and function has recently been called into question because macrophages and DCs can share some of these features (48). CD11c<sup>+</sup>MHC-II<sup>−</sup> cells are widely regarded to be DCs, but some macrophages can also display high amounts of these cell surface proteins (11, 49, 50). Similarly, some activated macrophages exhibit dendrite-like pseudopodia usually associated with DCs (51). Even the ability to activate naïve T cells, one of the functional definitions of DCs, can be observed for some macrophages (50). It has therefore been proposed that classification of DCs should be based on their hematopoietic lineage with the term “DC” being reserved for cDCs (17, 52, 53). According to this proposed definition, neither inflammatory moDCs nor tissue-resident moDCs would be classified as DCs. The most widely used approach to generate human DCs is to culture blood-derived monocytes with appropriate stimuli, such as GM-CSF and IL-4. Similar approaches are used to generate mouse DCs, which possess all known features of DCs (32), but these cells would no longer be called DCs according to the proposed definition. It remains to be seen whether this new definition will be widely adopted, but it might help to standardize nomenclature and therefore communication of findings. Regardless of how moDCs are defined, it had been difficult to reliably determine their ontogeny in animals that have not been genetically altered. Our present finding that reciprocal display of CD88 and CD26 can distinguish cDCs (or DCs) from moDCs (or monocyte-derived cells) in multiple organs and in different mouse strains should facilitate studies of how these different cell types initiate, propagate, and regulate immune responses.

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**Disclosures**

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**References**


