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Dendritic cell-associated lectin 2 (DCAL2) defines a distinct CD8α^- dendritic cell subset

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ABSTRACT

CLRs on DCs play important roles in immunity and are expressed selectively on certain DC subsets. Murine DCAL2 (myeloid inhibitory C-type lectin/Clec12a) is a type-II CLR with an ITIM. Using a mouse DCAL2-specific mAb, we found that DCAL2 is expressed at relatively high levels on APCs and that DCAL2 expression can be used to divide CD8α^- DCs into DCAL2$^+$DCIR2$^-$ and DCAL2$^-$DCIR2$^+$ subpopulations. CD8α^-DCAL2$^+$ DC, CD8α^-DCIR2$^+$ DC, and CD8α^+DCAL2$^+$ DC subsets each express different levels of TLRs and respond to unique classes of TLR ligands by producing distinct sets of cytokines. Whereas CD8α^-DCAL2$^+$ DCs robustly produce cytokines, including IL-12, in response to CpG, CD8α^-DCIR2$^+$ DCs produce only TNF-α and IL-10 in modest amounts when stimulated with zymosan. However, CD8α^-DCIR2$^+$ DCs, unlike the other DC subsets, strongly up-regulate OX40L when stimulated with bacterial flagellin. As predicted from their cytokine expression, CD8α^-DCAL2$^+$ DCs efficiently induced Th1 responses in the presence of CpG in vitro and in vivo, whereas CD8α^-DCIR2$^+$ DCs induced Th2 cells in response to flagellin. Thus, CD8α^-DCAL2$^+$ DCs comprise a distinct CD8α^- DC subset capable of supporting Th1 responses. DCAL2 is a useful marker to identify a Th1-inducing CD8α^- DC population. *J. Leukoc. Biol.* 91: 437–448; 2012.

Introduction

DCs are a family of APCs that bridge innate and adaptive immune responses and induce several distinct pathways of T cell differentiation [1–3]. In response to certain pathogenic stimuli, some DCs produce IL-12 and induce Th1 cell expansion [2]. Th1 cells predominantly produce IFN-γ and play a major role in protection against intracellular pathogens and tumors, whereas Th2 cells produce IL-4, IL-5, and IL-13 and promote resistance against helminth infections and mediate allergic responses (reviewed in refs. [4–6]). One common way to distinguish mouse DC subsets is by their expression of CD4 and CD8α [7, 8]. CD8α^+ DCs produce a large amount of IL-12p70 [9, 10], whereas CD8α^- DCs produce little if any IL-12 but are able to secrete IL-10, TNF-α, and TGF-β [11–13]. These and other observations have led to the paradigm that CD8α^+ DCs, rather than CD8α^- DCs, induce Th1 responses [6, 14, 15], and until recently, using CD4 and CD8 as markers has been a standard way to subset DCs.

A number of CLR family members are expressed on DCs, and distinct DC subsets have been identified based on their expression of CLRs as well as TLRs [16–18]. For example, BDCA-2 is a specific marker for human pDCs [19], Langerin is only expressed on Langerhans cells in the skin [20], and Clec9A is selectively expressed by CD8α^+ DCs in mice and on the putative BDCA-3$^+$ human DC counterpart [21–23]. The CLR, DEC205, is found mainly on CD8α^+ DCs located in the T cell zones of peripheral lymphoid tissues, whereas DCIR2 is relatively restricted to CD8α^- DCs located in splenic MZs and bridging channels [7, 24]. When antigens are targeted to DEC205$^+$ cells using antigen anti-DEC205 conjugates, they primarily trigger T cells to produce IFN-γ, whereas antigen anti-DCIR2 conjugates preferentially induce T cells to make IL-4 [14, 15, 25]. Thus, the expression pattern of CLRs on DCs has been useful to define different DC subsets that regulate qualitatively different immune responses.

Previously, we and others [26–31] characterized the CLR, DCAL2 (myeloid inhibitory C-type lectin/C-type lectin-like molecule-1/Clec12a). DCAL2 shares homology with CLR-like receptors on NK cells [32] and is closely related to Dectin-1 and LOX-1 [29]. One study has suggested that DCAL2 may bind an endogenous ligand(s) [30]. Human DCAL2 is expressed on monocytes and on blood and monocyte-derived DCs [26, 27, 29, 33]. The cytoplasmic tail of DCAL2 contains

Abbreviations: BDCA-2=blood DC antigen-2, BM=bone marrow, BMDC=bone morrow-derived DC, CLR=C-type lectin receptor, DCAL=DC-associated lectin, DCIR=DC immunoreceptor, DN=double-negative, GATA-3=GATA-binding protein 3, L=ligand, MZ=marginal zone, Nod1=nucleotide-binding oligomerization-1, OVAp=OVA peptide, pDC=plasmacytoid DC, PDCA-1=plasmacytoid DC antigen-1, PDL=programmed death ligand, SHP=Src homology-2-containing tyrosine phosphatase, SP=single-positive, SSC=side-scatter

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an ITIM that can bind tyrosine phosphatases, suggesting DCAL2 may mediate some inhibitory signals [29]; indeed, an anti-DCAL2 mAb suppressed LPS-induced IL-12p40 production by human DCs, but it also enhanced CD40-driven IL-12p40 levels [27].

In this study, we developed a mAb to further characterize mouse DCAL2. We detected DCAL2 on pDCs, CD8α^+ DCs, and to a lesser degree, on B cells, but DCAL2 was not expressed on peripheral T cells or NK cells. Notably, we also found that DCAL2 expression was useful for subdividing CD8α^- DCs into DCAL2$^+$DCIR2$^-$ and DCAL2$^-$DCIR2$^+$ subsets. Although CD8α^+DCAL2$^+$ DCs have been thought to be the major subset that induces Th1 responses [14, 15], CD8α^- DCAL2$^+$ DCs also produced significant amounts of IL-12 and supported Th1 responses. In contrast, CD8α^-DCIR2$^+$ DCs induced IL-4 responses. Unlike the method using CD4 expression to divide DCs [34], subsetting CD8α^- DCs based on DCAL2 and DCIR2 expression can be useful to identify Th1- or Th2-inducing DC subsets.

MATERIALS AND METHODS

Mice

Male 7- to 9-week-old C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME, USA) were used in this study. For some experiments (Supplemental Fig. 1), Fms-like tyrosine kinase-3 ligand-secreting B16 melanoma cells (FL-B16) were injected to increase the yield of DCs [35]. All mice were maintained in our specific pathogen-free facility. The Institutional Animal Care and Use Committee of the University of Washington (Seattle, WA, USA) approved all animal work.

Primary cell culture and cell lines

CD11c$^+$ DCs were purified from the spleens of C57BL/6J mice. Briefly, spleens were digested with Liberase TL and DNAse (Roche, South San Francisco, CA, USA) at 37°C for 45 min with mechanical stirring. CD11c$^+$ cells were enriched by positive selection using CD11c microbeads, following the manufacturer's protocol (Miltenyi Biotec, Sacramento, CA, USA). Enriched cells were then sorted using a FACSAria (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were gated through SSC and forward-scatter for the appropriate size and morphology for DCs, gated on CD11c$^+$ CD3$^-$B220$^-$NK1.1$^-$ populations to exclude T cells, B cells, NK cells, and pDCs, and then sorted into CD8α^+DCAL2$^+$, CD8α^-DCAL2$^+$, or CD8α^-DCAL2$^-$ populations. The DCAL2 mAb did not alter DC maturation and cytokine production (Supplemental Fig. 2).

Our flow cytometry staining schemes for staining and sorting are as follows: CD4 T cells, CD3$^+$CD4$^+$; CD8 T cells, CD3$^+$CD8$^+$; B cells, B220$^+$CD19$^+$; NK cells, DX5$^+$NK1.1$^+$; macrophages, CD107b$^+$; pDCs, CD11c^{int}PDCA-1$^+$B220$^+$; CD8α^+DCs, CD11c$^+$CD8α^+CD3$^-$B220$^-$NK1.1$^-$; CD8α^-DCs, CD11c$^+$CD8α^-CD3$^-$B220$^-$NK1.1$^-$; monocytes (CD11b$^+$F480$^+$); neutrophils (7/4^{high}Gr-1^{high}); basophils (SSC^{high}IgE$^+$); eosinophils (7/4^{low}F4/80$^+$SSC^{high}).

The YAC-1, A20, WHI231, JAWS II, RAW264.7, and NIH3T3 cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI or DMEM (Thermo Scientific, Waltham, MA, USA) following the manufacturer's recommended conditions. BMDCs were obtained by culturing BM cells from C57BL/6J mice for 7 days in the presence of 30 ng/ml GM-CSF and 10 ng/ml IL-4 (Fitzgerald, North Acton, MA, USA).

Real-time PCR

Panels of mouse tissue cDNA were purchased from Becton Dickinson. Total RNA from cell lines and primary sorted cells were extracted as recommended by the manufacturer or directly isolated using the Qiagen RNeasy kit (Qiagen, Chatsworth, CA, USA). First-Strand cDNA synthesis was performed using oligodTs or random hexamers and AMV RT (Invitrogen, Carlsbad, CA, USA) in standard reverse-transcription reactions. DCAL2 expression was analyzed by RT-PCR using SYBR Green (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). We used the following specific primers for DCAL2: forward 5′-cctgctctgctcggaat-3′ and reverse 5′-ttctgggcaacaatgcaa-3′. PCR reaction was performed at 95°C for 10 min and 40 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min. These data were standardized to β-actin expression.

Sorted DC subsets were also analyzed for their expression of PRRs. First-Strand cDNA was obtained similarly, and real-time PCR was performed using TaqMan gene-expression assays and probes for TLRs, Nod1, Ipaf/NLRC4, and RIG-I, as described by the manufacturer (Applied Biosystems, Life Technologies). These expression levels were standardized by the expression of 18S ribosomal RNA as an internal control.

Development of DCAL2 fusion proteins and a stable transfectant expressing DCAL2

The predicted extracellular region of DCAL2 was cloned using the following primers: forward 5′-gctccatggttggcaacagaaatgataaaatcg-3′ and reverse 5′-gtagcggccgcctgctatcctctggg-3′. These primers added *NcoI* and *NotI* sites to the PCR product, which was cloned further into pMT/Bip/V5-His (Invitrogen). The obtained plasmids, together with pCoHygro, hygromycin-resistent plasmids (Invitrogen), were transfected into S2 cells (Invitrogen). Selected transfectants were expanded and induced by adding copper sulfate in the culture following the vector manufacturer's protocol. DCAL2-V5-His was purified using the Ni_2 matrix column (Qiagen).

The entire coding region of DCAL2 was also cloned using the following primers: forward 5′-gccggtacctattcatcaatgtctgaagaaattgtt-3′ and reverse 5′-gccgaattcctaagcgtaatctggaacatcgtatgggtacctgctatcctctgg-3′. The forward primer adds *KpnI* and a Kozak sequence, and the reverse primer adds *EcoRI*, a stop codon, and a HA tag. The full-length DCAL2-HA was then cloned into pcDNA3.1 (Invitrogen). The obtained vectors or control vectors were nucleofected into NIH3T3 cells using nucleofector kits for NIT3T3 cells (Lonza, Walkersville, MD, USA) based on the manufacturer's protocol. Cells were selected in G418 (Roche). DCAL2 expression was detected by a mAb against DCAL2. and DCAL2-bright cells were selected by sorting them on a flow cytometer. Neither empty vector-nucleofected cells nor untreated cells expressed DCAL2.

Development of mAb specific for mouse DCAL2

mAb to DCAL2 were developed as described previously [36]. Briefly, Lou/W rats were injected i.p. with a purified DCAL2-His-V5 fusion protein with CFA as an adjuvant. Rats were boosted at weeks 3 and 4. Ten days after the second boost, polyclonal antisera were examined by cell-based ELISA. The final injection was performed, spleens were harvested after 3 days, and hybridomas were made by fusion with rat Y3-D10 cells and selected in hypoxanthine-aminopterin-thymidine-containing media. ELISAs using the DCAL2-His-V5 and a control protein were performed to determine positive clones. Supernatants from the positive clones were tested further by flow cytometry using DCAL2-transfected NIH3T3 cells. We selected one mAb, P4G2 (rat IgG2a), for further studies and used a nonbinding rat IgG2a (eBioscience, San Diego, CA, USA) as a negative control for our experiments.

Cytokine analyses

Sorted DCs were cultured in complete RPMI media with 30 ng/ml GM-CSF (Fitzgerald) and stimulated for 24 h with CpG-ODN2216 at 1–20

μg/ml, flagellin from *Bacillus subtilis* (InvivoGen, San Diego, CA, USA) at 1 ng/ml–1 μg/ml, or zymosan (Sigma-Aldrich, St. Louis, MO, USA) at 10–100 μg/ml. Cultured supernatants were analyzed for the amounts of TNF-α, IL-6, IL-10, IL-12p40, and IL-12p70 using ELISA kits (R&D Systems, Minneapolis, MN, USA), as described in the manufacturer's protocol.

ELISPOT assays were performed to analyze the frequency of CD4 T cells producing IFN-γ and IL-4. Mice were injected i.v. with 1×10^5 cells of an OVA-pulsed DC subset, and 8 days later, splenocytes were obtained and cultured for 24 h in the presence of different doses (10 nM–1 μM) of CD4-specific OVAp (323–339). Splenocytes were plated at 5×10^5–1×10^6 cells/well on MultiScreen HTS-HA filter plates (Millipore, Billerica, MA, USA), and after 24 h, cells were removed, and ELISPOT was performed using ELISPOT antibodies for IFN-γ and IL-4 (Becton Dickinson), following the manufacturer's protocol. The number of spots was enumerated using an ELISPOT reader.

T cell differentiation

Th cell differentiation by DC subsets was examined in vitro and in vivo. For in vitro analyses, we performed DC–T cell coculture. DC subsets were sorted as described above. CD4 T cells from WT or OT-II mice were purified using EasySep negative selection kit (Stemcell Technologies, Vancouver, BC, Canada), following the manufacturer's protocol. Sorted DCs (5×10^4) and WT CD4 T cells (1×10^5) were cocultured in 96-well round-bottom plates in the presence of CpG (10 μg/ml) or flagellin (1 ng/ml) with soluble anti-CD3 (10–50 ng/ml, clone 17A2). Similarly, sorted DCs and OT-II CD4 T cells were cocultured in the presence of CpG (10 μg/ml) or flagellin (1 ng/ml) with 2.5 μM OVAp (323–339). After 3–4 days of culture, supernatants were collected and analyzed for IFN-γ and IL-4 by ELISA. CD4 T cells from WT mice were also examined for the expression of GATA-3 after cocultured with sorted DC subsets in the presence of anti-CD3 mAb (100 ng/ml) and CpG (10 μg/ml) for 3 days. Cells were restimulated with ionomycin (1 μM) and PMA (50 ng) in the presence of GolgiStop for 4 h, and GATA-3 was stained for flow cytometric analysis.

For in vivo studies, we modified a similar approach as described earlier [14]. FACS-sorted DCs were pulsed 18 h with OVA (100 μg/ml) in the presence of CpG (10 μg/ml) or flagellin (100 ng/ml). This process was performed in the presence of 20 ng/ml GM-CSF. DCs were washed with PBS and injected into naive mice i.v. at 1×10^5 DCs/mouse. At Days 8 and 14, splenocytes were harvested and restimulated with 10 nM–1 μM OVAp (323–339) for 24 h, followed by ELISPOT assays.

RESULTS

DCAL2 is expressed at highest levels on APCs

We first measured mRNA levels of DCAL2 in mouse tissues, cell lines, and primary immune cells (**Fig. 1A–C**). DCAL2 mRNA expression was highest in spleen (Fig. 1A); it was also expressed at moderate levels in heart, skeletal muscle, and lung tissues. The B cell (A20) and myeloid lines (JAWS II, Raw264.7) expressed DCAL2 mRNA (Fig. 1B). Among splenic cell populations (Fig. 1C), DCAL2 mRNA expression was highest in pDCs (tenfold higher than unstimulated BMDCs), CD8α⁺ DCs (sixfold), and CD8α⁻ DCs (twofold).

To investigate DCAL2 protein expression, we generated a rat mAb specific for mouse DCAL2 and analyzed its distribution on cell lines and primary cells by flow cytometry (Fig. 1D–G). The DCAL2 mAb detected DCAL2 on NIH3T3 transfected with a DCAL2-containing plasmid but not with control vectors, demonstrating that it was specific for mouse DCAL2 (Fig. 1D). Furthermore, DCAL2 mAb did not bind to Dectin-1; costaining of Dectin-1 and DCAL2 revealed a large Dectin-1⁻DCAL2⁺ population in the spleen (mainly B cells), as well as smaller Dectin-1⁺DCAL2⁻ and Dectin-1⁺DCAL2⁺ populations (Fig. 1D). DCAL2 was expressed at high levels on B cell lines, moderate levels on myeloid cell lines, and not on NIH3T3 cells (Fig. 1D and E). In the spleen, CD8α⁺ DCs and pDCs expressed high levels of DCAL2, whereas CD8α⁻ DCs showed two distinct populations: DCAL2-high and DCAL2-low/negative. Unlike human DCAL2 [27, 29, 33], mouse DCAL2 was detected on primary B cells. Splenic NK cells and T cells did not express DCAL2, which is expressed on thymocytes at different points in T cell development (Fig. 1F). CD4⁻CD8⁻ cells and CD8 SP cells expressed high levels of DCAL2, whereas CD4⁺CD8⁺ and CD4 SP cells expressed low levels. As reported previously [30], DCAL2 was also found on blood monocytes, neutrophils, basophils, eosinophils, and B cells but not on blood CD4 and CD8 T cells (Fig. 1G). Although blood NK cells are DCAL2⁻, BM NK cells are DCAL2⁺, as shown before by Pyz et al. [30] (Fig. 1G). In summary, in peripheral lymphoid tissues, DCAL2 is mainly expressed on APCs but also present in other tissues.

DCAL2 expression is down-regulated in some cells after TLR signaling [30]. However, DCAL2 expression on primary splenic DCs was unchanged or only slightly down-regulated after CpG stimulation (data not shown). Ligating CLRs can modulate cytokine expression and maturation of DCs (reviewed in ref. [37]), and ligating human DCAL2 upregulates DC-lysosome-associated membrane protein in DCs treated with zymozan [27]. Cross-linking human DCAL2 also affected DC responses to LPS or anti-CD40 stimulation [27]. Yet, we were not able to detect any effect of anti-mouse DCAL2 when used alone or with TLR ligands on cytokine or receptor expression by mouse splenic DCs (e.g., Supplemental Figs. 1 and 2). This difference between mouse and human studies [27, 33] may be a species difference or reflect differences in the mAb used. However, like human DCAL2, mouse DCAL2 is internalized rapidly upon mAb binding (Supplemental Fig. 3) [27].

CD8α⁻ DCs can be subdivided further based on expression of DCAL2 and DCIR2

As our anti-DCAL2 mAb had no detectable effect on DCs, we tested whether it might be useful for identifying and isolating cells expressing different levels of DCAL2. Besides a CD8α⁺DCAL2⁺ population, we found two distinct populations within the CD8α⁻ DC subset: CD8α⁻DCAL2⁺ and CD8α⁻DCAL2⁻. CD8α⁻DCAL2⁻ DCs correspond to the CD8α⁻DCIR2⁺ population found in spleen and mesenteric LNs but not in inguinal LNs (**Fig. 2A**). In this study, we focused on splenic DC subsets.

The CD8α⁻ DC subsets defined, based on the relative expression of DCAL2 and DCIR2, differ from the CD8α⁻ DC subsets defined based on CD4 expression. CD8α⁻CD4⁺ DCs expressed little or no DCAL2, whereas CD8α⁻CD4⁻ DN DCs contain DCAL2⁺ and DCAL2⁻ populations (Fig. 2B). Thus, although the CD8α⁻DCAL2⁺ subset falls mainly within the CD8α⁻CD4⁻ subset, the CD8α⁻CD4⁻ and the CD8α⁻CD4⁺

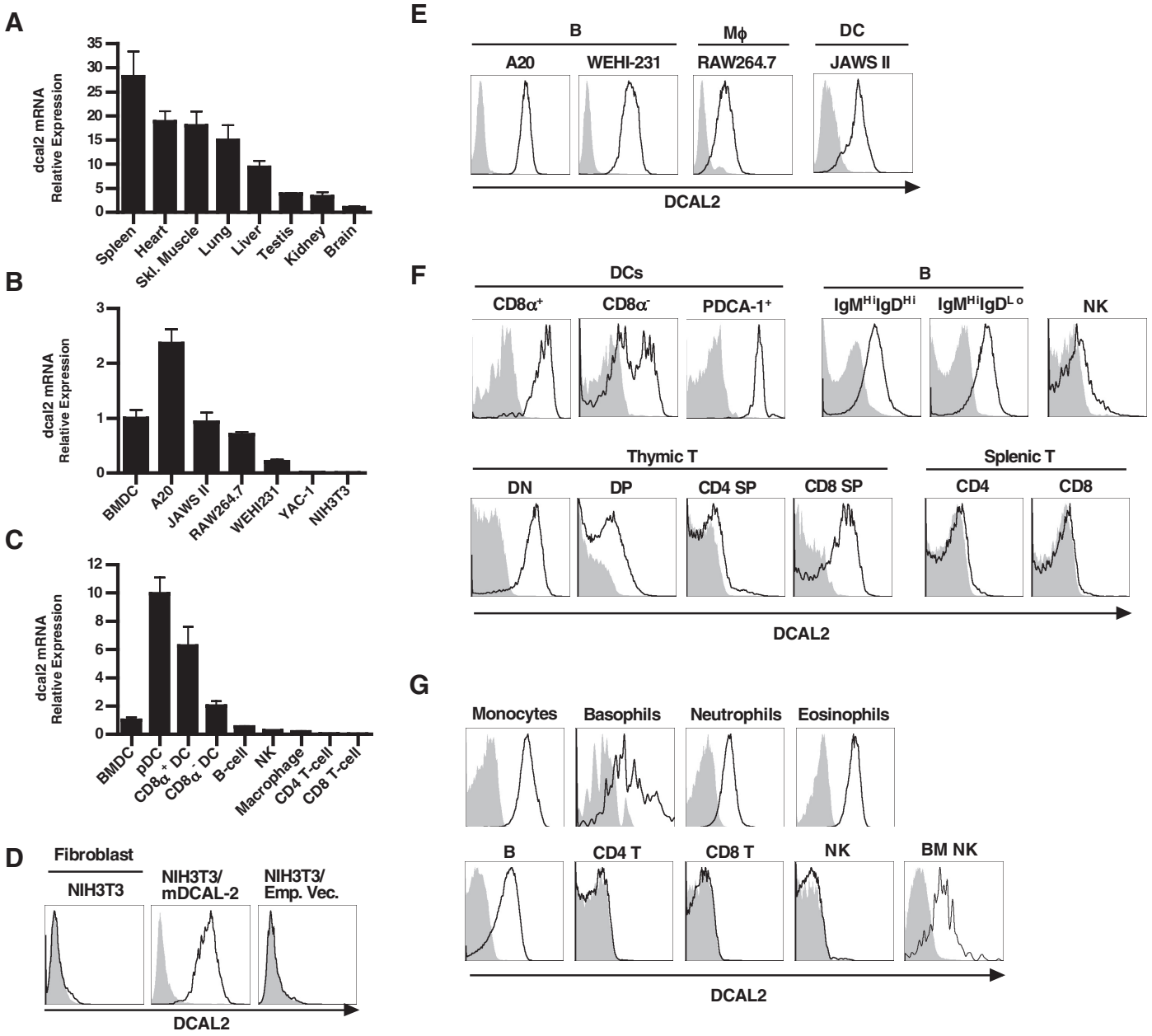

Figure 1. Distribution of DCAL2 in tissues and cells. (A) Relative expression levels of DCAL2 mRNA in various mouse tissues were examined by real-time PCR. The expression level of brain was defined as 1.0. (B) As in A, DCAL2 mRNA expression in cell lines was examined. The expression level of BMDC was adjusted to 1.0. From left, A20 (mature B cell line), JAWS II (DC line), RAW264.7 (macrophage line), WEHI231 (immature B cell line), YAC-1 (T cell line), and NIH3T3 (fibroblast). (C) As in B, DCAL2 mRNA expression in sorted primary immune cells from C57BL/6J mice was measured. The expression level of BMDC was adjusted to 1.0. Representative data from three independent experiments with similar results (A–C). (D–G) Distribution of DCAL2 protein on immune cells. DCAL2 mAb were developed (Materials and Methods), and expression of DCAL2 on immune cells from C57BL/6J mice was analyzed by flow cytometry. As in B, various cell lines were also examined for DCAL2 protein expression. (D) NIH3T3 cells were transfected with a *Dcal2* carrying plasmid and selected for high expression of the DCAL2 protein. Splenocytes were costained for Dectin-1 and DCAL2 to confirm that DCAL2 mAb did not cross-react to Dectin-1. mDCAL-2, Mouse DCAL2. (E) DCAL2 expression on cell lines detected by flow cytometry. Mϕ, Macrophage. (F) DCAL2 expression on splenic cell populations. DCs were subdivided into $CD8\alpha^+$, $CD8\alpha^-$, and $PDCA\text{-}1^+$ (pDC) populations. B cell populations were subdivided into a $IgM^{High}IgD^{High}$ population, which includes T2 B cells, or a $IgM^{High}IgD^{Low}$ population, which contains T1 and MZ B cells. $NK1.1^+$ cells were identified as NK cells. Thymic T cells were subdivided into CD4 and CD8 DN, double-positive (DP), and SP. $CD3^+$ splenic CD4 and CD8 T cells were also analyzed. (G) DCAL2 expression on blood leukocytes and BM NK cells. Monocytes ($CD11b^+F480^+$), neutrophils ($7/4^{high}Gr\text{-}1^{high}$), basophils ($SSC^{high}IgE^+$), and eosinophils ($7/4^{low}F4/80^+SSC^{high}$) were examined. As in F, blood T ($CD3^+NK1.1^-$), B ($CD19^+MHC\text{-}II^+$), and NK cells ($DX5^+NK1.1^+$) and BM NK cells ($DX5^+NK1.1^+$) were stained for DCAL2. Representative data of more than five independent experiments with similar results.

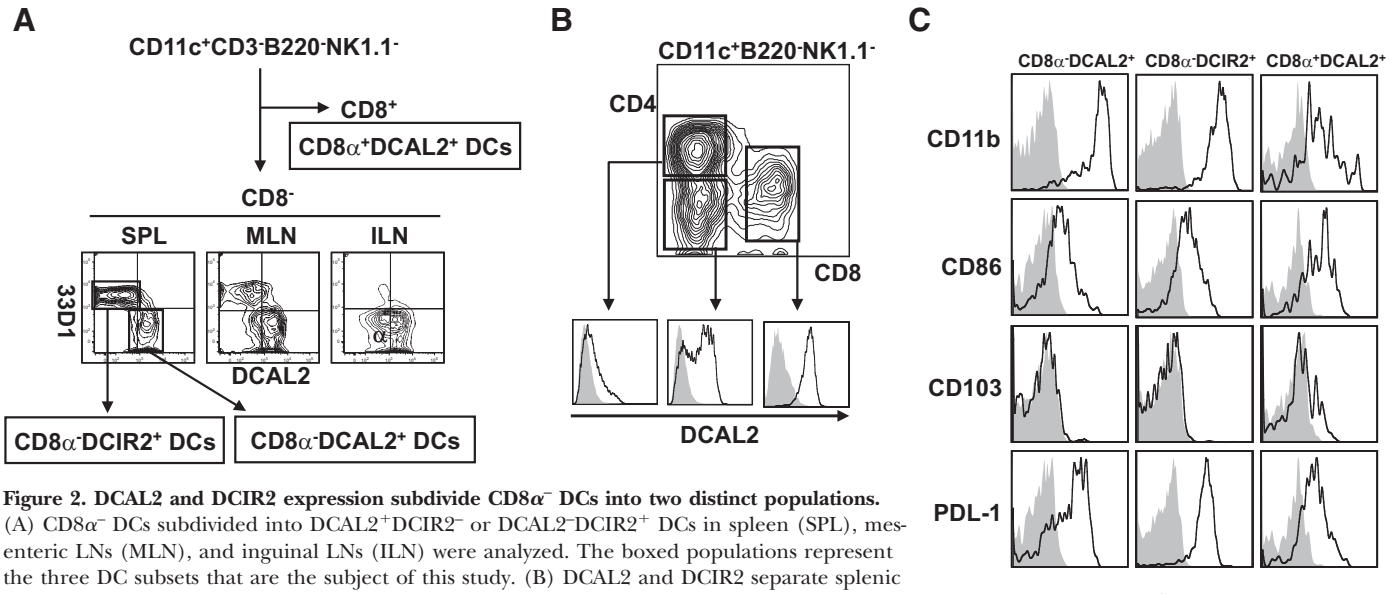

Figure 2. DCAL2 and DCIR2 expression subdivide CD8α⁻ DCs into two distinct populations. (A) CD8α⁻ DCs subdivided into DCAL2⁺DCIR2⁻ or DCAL2⁻DCIR2⁺ DCs in spleen (SPL), mesenteric LNs (MLN), and inguinal LNs (ILN) were analyzed. The boxed populations represent the three DC subsets that are the subject of this study. (B) DCAL2 and DCIR2 separate splenic DCs differently from the conventional way using CD4 and CD8α expression. Splenic DCs were identified by gating CD11c⁺CD3⁻B220⁻NK1.1⁻ cells and examined for the expression of DCAL2 on the CD8α⁺, CD4⁺, or DN population. (C) Cell-surface phenotype of unstimulated DC subsets examined ex vivo. Expression of adhesion molecules and costimulatory molecules was examined on each DC subset by flow cytometry. Representative data of three experiments with similar results (A–C).

subsets do not correspond to the CD8α⁻DCAL2⁺ and CD8α⁻DCIR2⁺ DC subsets. To further characterize the CD8α⁻ DC subsets, we analyzed their expression of adhesion and costimulatory molecules (Fig. 2C). CD8α⁻DCAL2⁺ DCs expressed slightly higher levels of CD11b than CD8α⁻DCIR2⁺ DCs (Fig. 2C). CD103 was weakly expressed on CD8α⁺DCAL2⁺ DCs but not on CD8α⁻DCAL2⁺ or CD8α⁻DCIR2⁺ DCs. CD86 levels were higher on CD8α⁺DCAL2⁺ DCs than on the other subsets, but the results were opposite in the case of PDL-1 expression, which was higher on CD8α⁻DCAL2⁺ and CD8α⁻DCIR2⁺ DCs but lower on CD8α⁺DCAL2⁺ DCs (Fig. 2C). Levels of CD62L, CD40, PDL-2, and CCR7 expression were not different among these three subsets (data not shown).

Expression of PRRs is different among CD8α⁺DCAL2⁺, CD8α⁻DCAL2⁺, and CD8α⁻DCIR2⁺ DC subsets

The selective differences in expression of adhesion and costimulatory molecules suggested that the CD8α⁻ DC subsets may have distinct functions, such as different responses to pathogenic stimuli. To investigate this possibility, we first measured the expression in the DC subsets of several PRRs, including TLRs and some intracellular receptors that recognize RNA (RIG-I) or activate inflammasomes (Nod1 and Ipaf/NLRC4). Splenic DCs were sorted as shown in **Fig. 3A**, and the purity of each subset was confirmed to be >98%. The ratio of subset within the DC gate (CD11c⁺B220⁻NK1.1⁻) was ~20% for CD8α⁻DCAL2⁺ DCs, 50% for CD8α⁻DCIR2⁺ DCs, and 18% for CD8α⁺DCAL2⁺ DCs (Fig. 3A), whereas the ratio of pDCs within the CD11c⁺ population was 15–20% (data not shown). In other words, the number of splenic CD8α⁻DCAL2⁺ DCs was similar to the number of the well-characterized CD8α⁺DCAL2⁺ DC subset.

We measured the levels of PPRs expressed in DC subsets by RT-PCR (Fig. 3B); CD8α⁻DCAL2⁺ DCs expressed higher levels of TLR2 and TLR4 than the other subsets, whereas CD8α⁻DCIR2⁺ DCs showed higher expression of TLR5 than the other subsets. TLR3 expression was considerably higher in CD8α⁺DCAL2⁺ DCs, consistent with earlier studies [38]. Nod1, Nod-like receptor family caspase-activating recruitment domain-containing protein 4 (Ipaf), and RIG-I levels were highest in CD8α⁻DCIR2⁺ DCs and lowest in CD8α⁺DCAL2⁺ DCs.

CD8α⁻DCAL2⁺ DCs produce cytokines, including IL-12, in response to TLR stimuli

The differences in PRR expression suggested that each DC subset may respond differently to certain PAMPs. To test this, we isolated CD8α⁺DCAL2⁺, CD8α⁻DCAL2⁺, and CD8α⁻DCIR2⁺ DCs, stimulated them with TLR ligands in vitro, and measured cytokine production. As has been reported [9], CD8α⁺DCAL2⁺ DCs produced significant amounts of IL-12; however, CD8α⁻DCAL2⁺ DCs also produced significant levels of IL-12p40 and IL-12p70 when stimulated with CpG (**Fig. 4A**). In addition, CD8α⁻DCAL2⁺ DCs secreted significantly higher levels of TNF-α, IL-6, and IL-10 than the other subsets in response to CpG or zymosan. Although flagellin increased OX40L expression (see below), it had no effect on cytokine expression by any of the DC subsets (data not shown). The difference among the DC subsets could not be attributed to binding of anti-DCAL2, as the anti-DCAL2 mAb used for sorting did not affect the levels of these cytokines compared with an isotype control antibody (Supplemental Fig. 2). The CD8α⁻DCIR2⁺ DCs, a large proportion of which express CD4 (Fig. 2B), produced minimal cytokines in response to CpG or zymo-

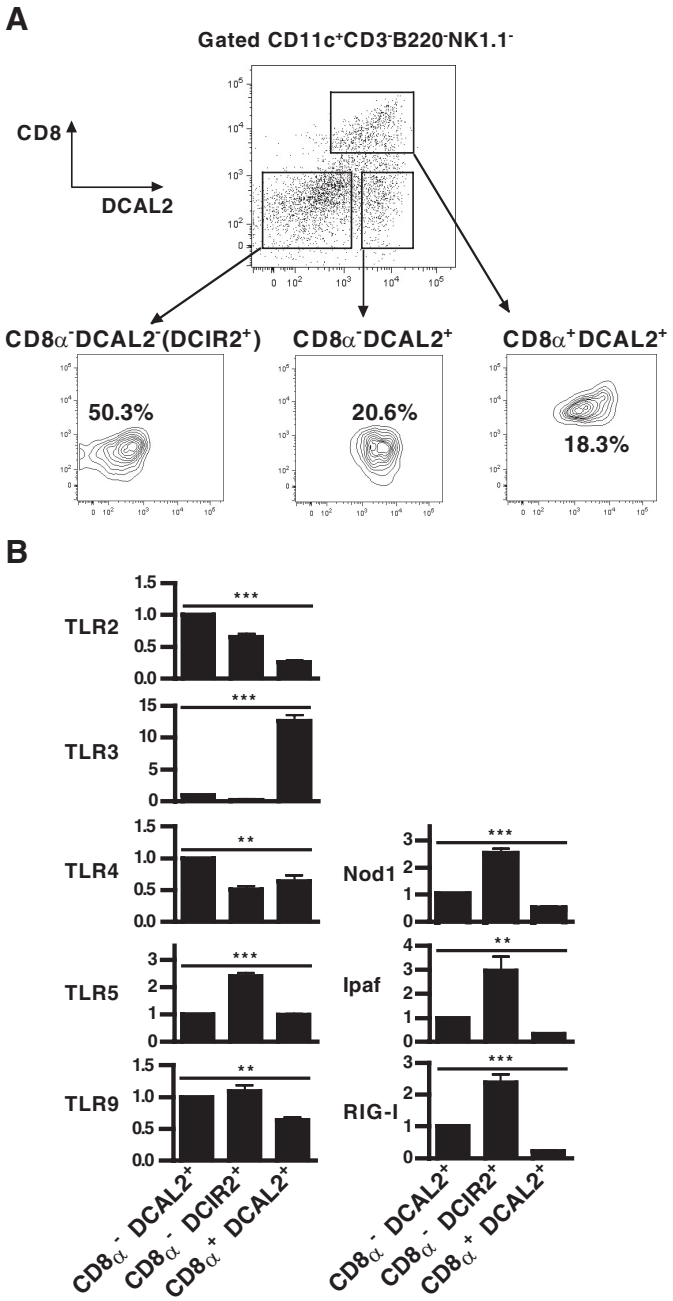

Figure 3. Differential expression of PRRs in DC subsets. (A) Sorting strategy used to separate CD8α⁻DCAL2⁺ DCs, CD8α⁻DCIR2⁺ DCs, and CD8α⁺DCAL2⁺ DCs. Splenic DC subsets were sorted as described in Materials and Methods. The boxed populations were sorted, and the purity of each population was >98%. (B) Relative expression of TLR and intracellular PRR mRNA in DC subsets. Total mRNA was extracted from sorted DC subsets and examined for the expression levels of mRNA for TLRs, Nod1, Ipaf, and RIG-I by real-time PCR. Relative expression levels were standardized to 18S ribosomal RNA, and expression levels in CD8α⁻DCAL2⁺ DCs were adjusted to 1.0. Data are mean ± SD of three independent experiments; One-way ANOVA with the Bonferroni's multiple comparison test; $^{**}P < 0.01$; $^{***}P < 0.001$.

san; this result is similar to an earlier study showing that CD8α⁻CD4⁺ DCs produce low amounts of cytokines upon stimulation [9]. Whereas CD8α⁺DCAL2⁺ DCs produced similar levels of IL-12p40 and lower levels of IL-12p70 compared with CD8α⁻DCAL2⁺ DCs in response to CpG, they produced larger amounts of IL-12p40 and -p70 in response to zymosan. The amount of IL-12p70 produced by DCs in our experiments was somewhat lower than that reported by other groups [9, 11], but this difference may be a result of the fact that in these earlier studies, IL-4- and/or CD40L-expressing cells were added to cultures with TLR agonists.

We next tested if CD8α⁻DCAL2⁺ DCs can be activated in vivo to produce IL-12. Splenic DC subsets from CpG-injected mice were sorted and cultured ex vivo without further stimulation. CD8α⁻DCAL2⁺ DCs produced higher levels of IL-12p40 than other DC subsets ex vivo (Fig. 4B). CD8α⁺DCAL2⁺ DCs also produced IL-12p40, but production levels were more than twice lower than that of CD8α⁻DCAL2⁺ DCs, which is consistent with our in vitro data (Fig. 4A). CD8α⁻DCIR2⁺ DCs did not produce IL-12p40 in response to CpG in vitro and produced little IL-12p40 after stimulation in vivo. These data show that CD8α⁻DCAL2⁺ DCs produce IL-12p40 in vivo and suggest that they may have a capacity to induce Th1 responses.

CD8α⁻DCIR2⁺ DCs are predisposed to inducing IL-4-producing T cells when *Leishmania major* homologue of receptors for activated C kinase antigen is targeted with anti-DCIR2 (33D1) to CD8α⁻DCIR2⁺ DCs [25]. Thus, we hypothesized that CD8α⁻DCIR2⁺ DCs, but not CD8α⁻DCAL2⁺ DCs, may preferentially promote Th2 responses. Consistent with this model, CD8α⁻DCIR2⁺ DCs up-regulated OX40L in response to TLR ligands, most strongly to flagellin, whereas CD8α⁻DCAL2⁺ DCs failed to express OX40L in response to any TLR ligands tested (Fig. 4C). The inability of CD8α⁻DCAL2⁺ DCs to up-regulate OX40L is not a result of impaired cell viability, as CD8α⁻DCAL2⁺ DCs were able to up-regulate CD80 upon stimulation (Fig. 4C). Thus, these three splenic DC subsets differentially respond to certain pathogenic stimuli, suggesting that the type of pathogenic stimuli and DC subset may determine the quality of CD4 T cell responses.

CD8α⁻DCAL2⁺ DCs preferentially induce Th1 cells, whereas CD8α⁻DCIR2⁺ DCs induce Th2 cells in vitro

One paradigm is that CD8α⁺ DCs, rather than CD8α⁻ DCs, direct the development of Th1 responses [14, 15]. However, as CD8α⁻DCAL2⁺ DCs are able to produce large amount of IL-12 in vitro and in vivo (Fig. 4A and B), we hypothesized that CD8α⁻DCAL2⁺ DCs were also capable of inducing Th1 responses.

To test if CD8α⁻DCAL2⁺ DCs could induce IFN-γ production by CD4 T cells, we cocultured splenic DC subsets with CD4 T cells in the presence of anti-CD3, along with CpG or flagellin (**Fig. 5A** and **B**). CD8α⁻DCAL2⁺ DCs and CD8α⁺DCAL2⁺ DCs induced IFN-γ in the presence of CpG but not flagellin; the CD8α⁻DCAL2⁺ DCs were consistently more effective than CD8α⁺DCAL2⁺ DCs, as measured by ELISA (Fig. 5A). In contrast, CD8α⁻DCIR2⁺ DCs did not

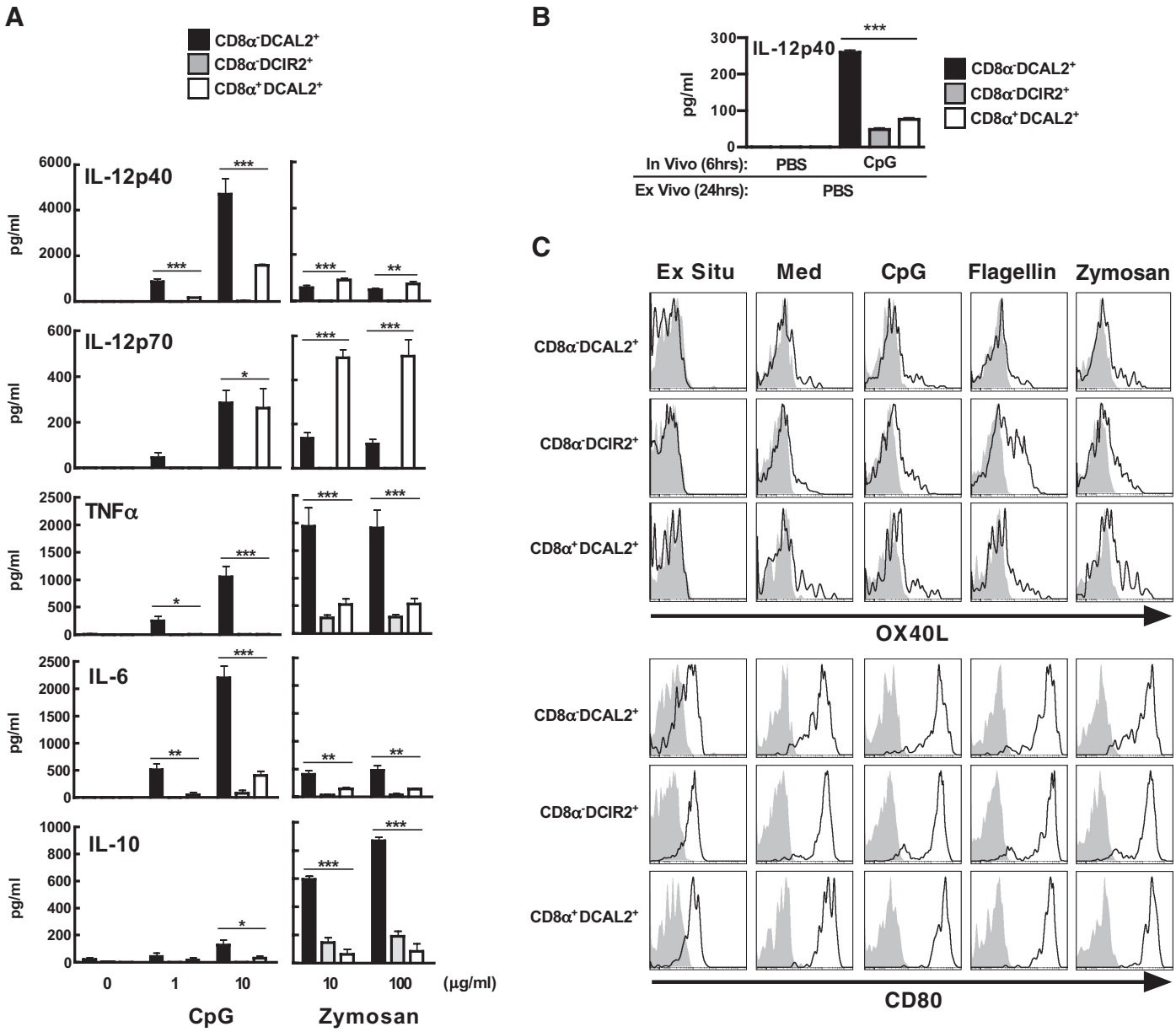

Figure 4. Functional difference among DC subsets in response to TLR stimuli. (A) Cytokine production by DC subsets. As in Fig. 3A, splenic DCs were enriched, sorted, and stimulated with CpG or zymosan for 24 h. Anti-DCAL2 mAb, which we used for sorting, did not affect the levels of cytokine that we measured (Supplemental Fig. 2). Supernatants were tested for the amount of TNF-α, IL-6, IL-10, IL-12p40, and IL-12p70 by ELISA. Data are the mean of three independent experiments; one-way ANOVA with the Bonferroni's multiple comparison test; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (B) In vivo priming of the DC subset for IL-12p40 production. Mice were injected twice with100 μg CpG at −6 h and −2 h, followed by spleen harvested at 0 h. DC subsets were sorted as in Materials and Methods and unstimulated or cultured with CpG for 24 h. Five mice were used for each injection, and splenocytes were pooled before sorting. Representative data of two experiments with similar results; one-way ANOVA with the Bonferroni's multiple comparison test; $***P < 0.001$. (C) Up-regulation of OX40L in response to CpG, flagellin, or zymosan. Splenic DCs were enriched with magnetic beads and stimulated with CpG (10 μg/ml), flagellin (100 ng/ml), or zymosan (100 μg/ml) for 24 h. Expression of OX40L was analyzed by flow cytometry. Although DCIR2 expression levels were slightly diminished after DC maturation, these DC subsets were stable enough to perform flow cytometry analysis (data not shown). Representative data of five experiments with similar results. Med, Media.

induce IFN-γ, regardless of the stimulus. CD8α⁻DCAL2⁺ DCs and CD8α⁻DCIR2⁺ DCs induced low levels of IL-4 in the coculture without TLR stimuli, but only CD8α⁻DCIR2⁺ DCs showed enhanced IL-4 induction in the presence of flagellin. CD4 T cells also expressed higher levels of GATA-3 after culturing with CD8α⁻DCIR2⁺ DCs compared with other DC subsets (Fig. 5C). We then compared the ability of OVA-pulsed DC subsets to induce IFN-γ in anti-

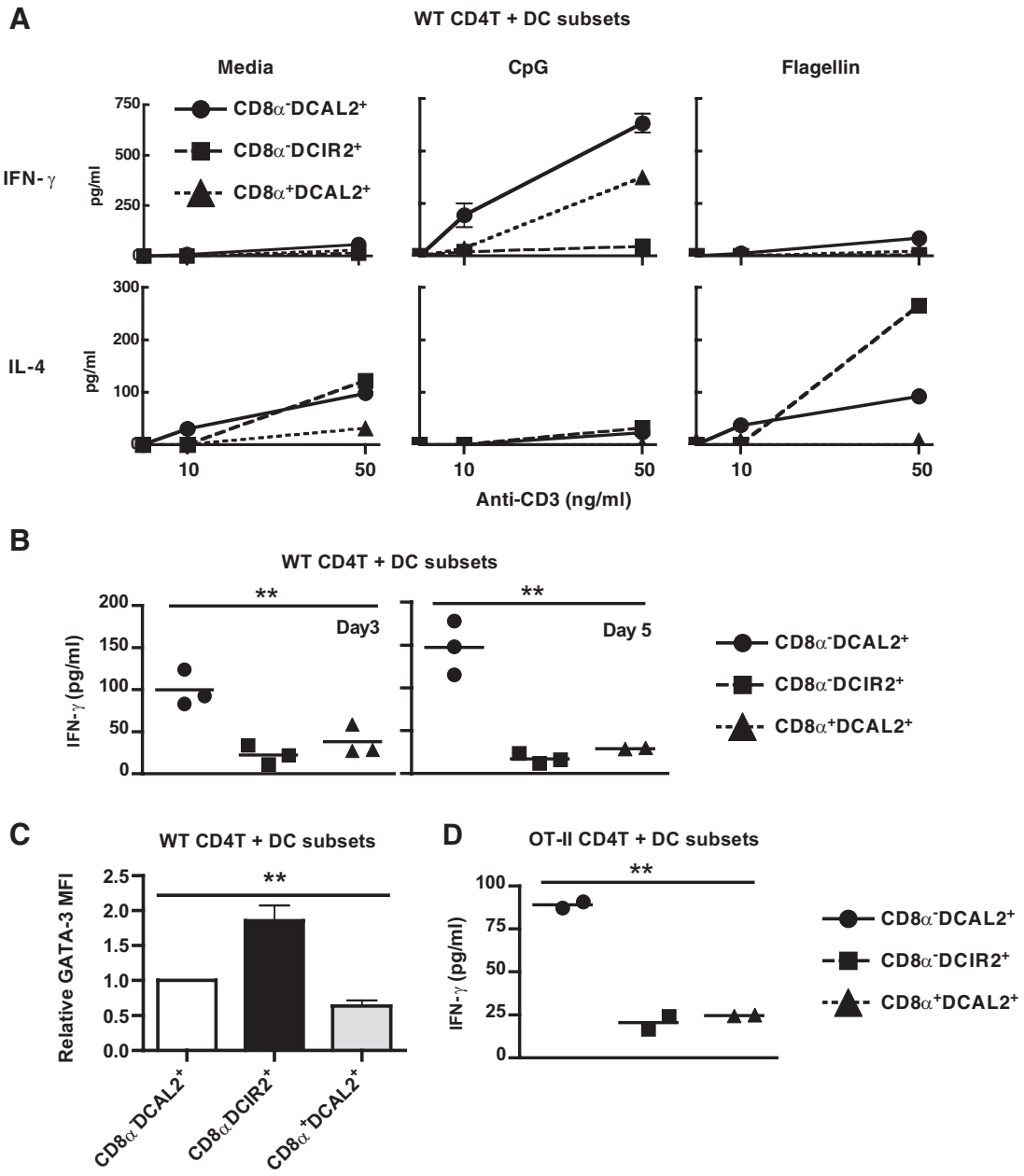

Figure 5. Distinct function of DC subsets to induce Th1 or Th2 in response to CpG or flagellin in vitro. As described in Materials and Methods, splenic DCs were enriched and sorted into three subsets. (A) CD4 T cells from C57BL/6J mice were purified using magnetic separation. DCs (5×10^4) and CD4 T cells (1×10^5) were cocultured in the presence of two different doses of anti-CD3 mAb (10 and 50 ng/ml), together with CpG (10 μg/ml) or flagellin (1 ng/ml). After 3 days, supernatants were harvested, and the amounts of IFN-γ and IL-4 were measured by ELISA. Representative data of five experiments. (B) As in A, CD4 T cells were cocultured with sorted DC subsets in the presence of anti-CD3 mAb (100 ng/ml) and CpG (10 μg/ml). After 3 and 5 days, ELISA was performed for IFN-γ and IL-4. Data are the mean of three independent experiments; one-way ANOVA with the Bonferroni's multiple comparison test; $^{**}P < 0.01$. (C) As in A, CD4 T cells were cocultured with sorted DC subsets in the presence of anti-CD3 mAb (100 ng/ml) and CpG (10 μg/ml) for 3 days. Cells were restimulated with ionomycin (1 μM) and PMA (50 ng) in the presence of GolgiStop for 4 h, and GATA-3 was stained for flow cytometric analysis. Mean fluorescence intensities (MFI) were standardized and presented as relative expressions. Data are the mean of three independent experiments; one-way ANOVA with the Bonferroni's multiple comparison test; $^{**}P < 0.01$. (D) OT-II CD4 T cells were purified and cocultured with sorted DC subsets in the presence of 2.5 μM OVA peptide (323–339) and CpG (10 μg/ml). After 4 days, supernatants were harvested, and the amounts of IFN-γ and IL-4 were measured by ELISA. Data are the mean of two independent experiments; one-way ANOVA with the Bonferroni's multiple comparison test; $^{**}P < 0.01$.

gen-specific OT-II CD4 T cells; once again, the CD8α^-DCIR2$^+$ DCs induced higher levels of IFN-γ compared with the other subsets (Fig. 5D). These data suggest that CD8α^-DCAL2$^+$ DCs can induce Th1 responses, whereas CD8α^-DCIR2$^+$ DCs are more prone to support Th2 responses by up-regulating GATA-3 in CD4 T cells.

We also examined whether Th17 responses were preferentially induced by different splenic DC subsets. Although there was a trend that CD8α^-DCAL2$^+$ DCs induced higher levels of IL-17 production from CD4 T cells in response to zymosan, the differences among each DC subset to induce Th17 cells were not as significant as those for the induction of Th1 and Th2 cells (data not shown).

Differential cytokine regulation by CD8α^- DC subsets in vivo

We next examined if CD8α^-DCAL2$^+$ DCs and CD8α^-DCIR2$^+$ DCs differ in their ability to promote Th1 and Th2 responses in vivo. We pulsed DC subsets with OVA in the presence of CpG or flagellin, washed the cells to remove the CpG or flagellin, and adoptively transferred them into naive mice. After 8–14 days, splenocytes were harvested and

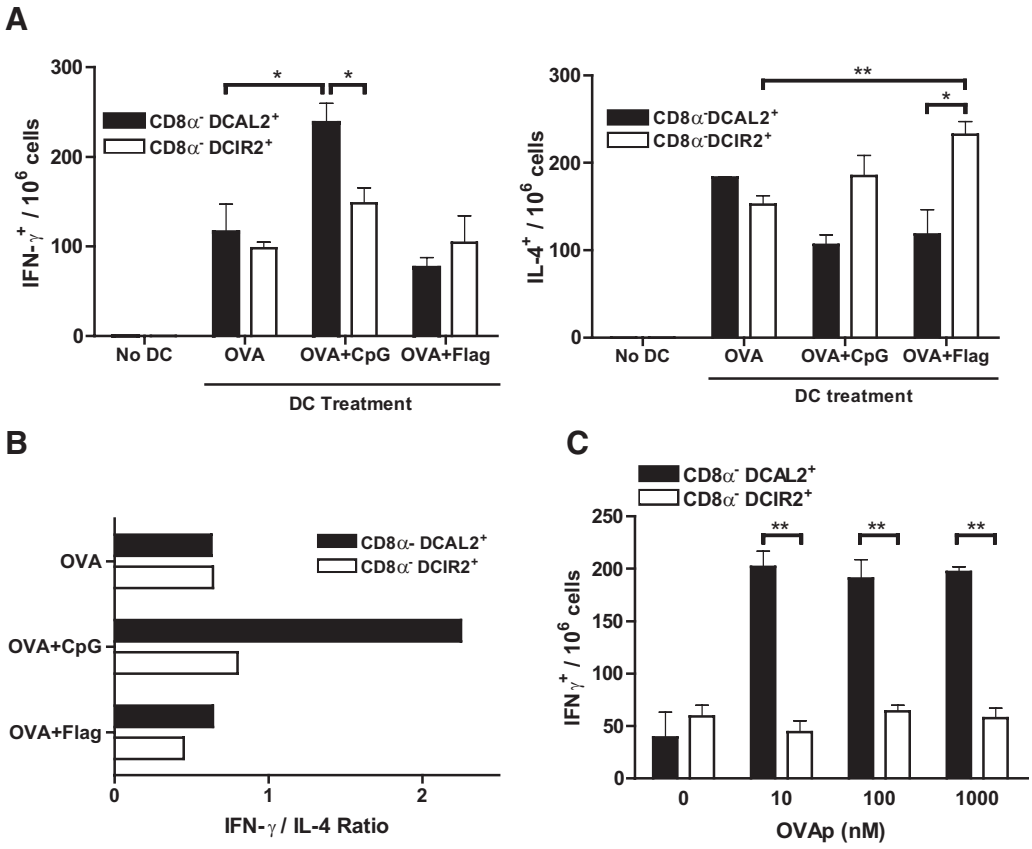

Figure 6. Regulation of IFN-γ and IL-4 response by DC subsets in vivo. Splenic DCs were enriched and sorted into CD8α⁻DCAL2⁺ and CD8α⁻DCIR2⁺ subsets. DCs were pulsed overnight with soluble OVA with CpG or flagellin (Flag), washed, and adoptively transferred into naive mice. (A) Splenocytes were harvested 8 days after DC transfer, and the frequencies of IFN-γ- and IL-4-producing cells were measured by ELISPOT after 24 h of restimulation with OVA peptide (323–339). The number of IFN-γ- and IL-4-positive cells/10^6 cells is presented. Data are mean ± SD of three independent mice/group; representative data of three experiments with similar results; Student's *t*-test; $*P < 0.05$; $**P < 0.01$. (B) IFN-γ to IL-4 ratio. Based on the spot number shown in A, an IFN-γ:IL-4 ratio was calculated. (C) Splenocytes were harvested from recipients 14 days after transfer of DCs pulsed with OVA and CpG, and the frequencies of IFN-γ-producing cells induced in the presence of graded doses of OVA peptide (323–339) were measured as in A. Data are mean ± SD of three independent mice/group; representative data of two experiments with similar results; Student's *t*-test; $**P < 0.01$.

restimulated with an OVA peptide that activates antigen-specific CD4–T cells, and the numbers of responding cells were measured by IFN-γ and IL-4 ELISPOT.

When CD8α⁻DCAL2⁺ DCs were pulsed with OVA, together with CpG, they induced a higher number of IFN-γ⁺ cells than when they were pulsed with OVA alone ($P<0.05$; **Fig. 6A**). However, CpG did not induce a significant increase of IFN-γ⁺ cells triggered by CD8α⁻DCIR2⁺ DCs (Fig. 6A). Consistent with the in vitro data, CD8α⁻DCAL2⁺ DCs induced more IFN-γ⁺ cells than CD8α⁻DCIR2⁺ DCs when stimulated with CpG ($P<0.05$; Fig. 6A). Flagellin did not affect CD8α⁻DCIR2⁺ DCs in their ability to induce IFN-γ⁺ cells, and if anything, flagellin tended to promote CD8α⁻DCAL2⁺ DCs to induce fewer IFN-γ⁺ cells (Fig. 6A).

CD8α⁻DCIR2⁺ DCs, when pulsed with OVA with flagellin, unlike pulsed CD8α⁻DCAL2⁺ DCs, induced more IL-4⁺ cells than when pulsed with OVA alone ($P<0.01$; Fig. 6A). In addition, similar to the in vitro data, CD8α⁻DCIR2⁺ DCs, when stimulated with flagellin, were superior to CD8α⁻DCAL2⁺ DCs in inducing IL-4⁺ cells ($P<0.05$; Fig. 6A). CpG did not have a statistically significant effect on either DC subset for the induction of IL-4⁺ cells (Fig. 6A).

Overall, CpG increased the ratio of IFN-γ to IL-4 spots from three- to fourfold for CD8α⁻DCAL2⁺ DC-injected mice but had little effect on CD8α⁻DCIR2⁺ DC-injected mice (Fig. 6B). Flagellin had no effect on the IFN-γ:IL-4 ratio for CD8α⁻DCAL2⁺ DC-injected mice (Fig. 6B). However, flagellin slightly decreased the IFN-γ:IL-4 ratio for CD8α⁻DCIR2⁺ DC-injected mice (Fig. 6B).

The strong effect of CpG on the proportion of IFN-γ- versus IL-4-producing cells induced by antigen-pulsed CD8α⁻DCAL2⁺ DCs was even more pronounced 14 days after DC injection (Fig. 6C). Spleen cells from mice injected with CD8α⁻DCAL2⁺ DCs, pulsed with OVA with CpG, unlike cells from mice inoculated with pulsed CD8α⁻DCIR2⁺ DCs, had significant increases in IFN-γ-producing cells at all doses of OVAp restimulation ($P<0.01$; Fig. 6C). Together with the in vitro data, these results suggest that CD8α⁻DCAL2⁺ DCs preferentially induce IFN-γ-producing cells in response to CpG, whereas CD8α⁻DCIR2⁺ DCs are better at supporting IL-4 responses.

DISCUSSION

CD8α⁻ DCs can be divided into functionally distinct subsets based on their level of expression of the CLRs, DCAL2, and DCIR2. Whereas CD8α⁻DCAL2⁺ DCs can produce IL-12 and support Th1 cells in response to CpG, CD8α⁻DCIR2⁺ DCs can up-regulate OX40L and promote Th2 cells in response to flagellin.

One way to classify mouse DC subsets is based on relative CD8α and CD4 expression; splenic DCs can be subdivided into CD8α⁺CD4⁻DEC205⁺CD11b^{lo} (also known as CD8α⁺

DCs), $CD8\alpha^-CD4^+DEC205^-CD11b^{hi}$ ($CD4^+$ DCs), and $CD8\alpha^-CD4^-DEC205^-CD11b^{hi}$ ($CD8\alpha$/CD4 DN DCs) [7, 39, 40]. These subsets are found in distinct anatomical locations; $CD8\alpha^+$ DCs reside in splenic T cell areas, whereas $CD4^+$ and DN DCs are found in MZs. Furthermore, these DC subsets appear to play different roles in inducing T cell responses [40]. $CD8\alpha^+$ DCs are a major producer of IL-12 [9, 14, 15] and thus, induce Th1 responses, whereas $CD4^+$ DCs are generally low cytokine producers [9, 11]. DN DCs may be tolerogenic and produce TGF-β and induce regulatory T cells [13] or immunogenic and induce Th1 and Th17 cells [41]. Some studies have suggested that $CD8\alpha^-$ DCs could also induce Th1 responses, but the precise mechanisms and cells within the $CD8\alpha^-$ population responsible were not made clear [13, 42]. For example, $CD8\alpha^-$ DCs, which induce Th1 responses, could not be identified based on relative CD4 expression [34]. However, DCAL2 is a marker that helps distinguish a $CD8\alpha^-$ DC subpopulation, which produces significant levels of IL-12 and induces Th1 responses. The $CD8\alpha$/CD4 DN DCs are heterogenous, as they contain DCAL2-high and -low/negative populations. DCAL2 expression is useful for classifying and isolating $CD8\alpha^-$ DC subsets, which differentially induce Th1 and Th2 responses. In addition, DCAL2 mAb are useful for isolating $CD8\alpha^-DCIR2^+$ DCs, which make up about one-half of all splenic DCs, without ligating DCIR2.

CLRs on DCs play important roles in immunity, such as pathogen-capturing, costimulation, adhesion, and signaling [17, 37, 43]. The function and ligand(s) of DCAL2 remain to be identified. Ligating human DCAL2 is expressed on DCs and can induce protein tyrosine phosphorylation, MAPK activation, and IL-6 and IL-10 production but not full DC maturation [27]. Antibody cross-linking itself did not induce human DC maturation but induced up-regulation of CCR7 expression [27]. The effect of anti-DCAL2 appeared to differ depending on whether DCs were receiving signals from TLR4 or CD40. The ITIM of DCAL2 can recruit protein tyrosine phosphatases SHP-1 and SHP-2 in cell lines expressing DCAL2/Dectin-1 chimeric receptors [29]. However, antibody cross-linking of mouse DCAL2 did not modulate DC maturation or cytokine production (Supplemental Figs. 1 and 2, and see ref. [30]).

Dudziak et al. [24] characterized intrinsic differences of two splenic DC subsets: $CD8\alpha^+DEC205^+$ DCs and $CD8\alpha^-DCIR2^+$ DCs, both of which are distinct from $CD8\alpha^-DCAL2^+$ DCs. $CD8\alpha^+DEC205^+$ DCs express MHC-I-associated genes and are efficient in presenting antigen to CD8 T cells, whereas $CD8\alpha^-DCIR2^+$ DCs up-regulate MHC-II-related genes and are specialized for activating CD4 T cells [24]. They concluded that these differences in antigen presentation were subset-intrinsic and not dependent on the receptor signaling [24]. The $CD8\alpha^-DCAL2^+$ DCs are $DEC205^-DCIR2^-$ and distinct from the two DC populations described by Dudziak et al. [24]. They express different amounts of PRR-high levels of TLR2, -4, and -9 and produce a different set and quantity of cytokines compared with the other subsets. $CD8\alpha^-DCIR2^+$ DCs express the highest levels of TLR5 and higher levels of intracellular PRRs, such as Nod1, Ipaf, and RIG-I (Fig. 3B). These differences in PRR expression levels within $CD8\alpha^-$ DCs are not evident when CD4 expression is used to subdivide $CD8\alpha^-$ DCs [38].

Our data reveal that $CD8\alpha^-DCAL2^+$ DCs are as capable of producing IL-12 as $CD8\alpha^+DCAL2^+$ DCs (Fig. 4) and can effectively support Th1 responses (Figs. 5 and 6). After treatment with CpG, the $CD8\alpha^-DCAL2^+$ DCs produced IL-12p40 in vitro, and the $CD8\alpha^-DCAL2^+$ DCs did not (Figs. 3 and 4A). The $CD8\alpha^-DCIR2^+$ DCs may require a second signal to induce IL-12p40, as they were able to produce low levels of IL-12p40 after stimulation with CpG in vivo (Fig. 4B). Although $CD8\alpha^+DCAL2^+$ DCs and $CD8\alpha^-DCAL2^+$ DCs produced detectable levels of IL-12p70 in vitro, we did not detect IL-12p70 production ex vivo under conditions where IL-12p40 was produced (Fig. 4B; data not shown). This may be because IL-12-p70 production requires exogenous cytokines [9]. $CD8\alpha^-DCAL2^+$ DCs produced lower amounts of IL-12p70 than $CD8\alpha^+DCAL2^+$ DCs in response to zymosan, perhaps as they produced IL-10 (Fig. 4B), which can suppress IL-12p70 production from DCs [44]. Interestingly, before this study, there has been little evidence suggesting that $CD8\alpha^-$ DCs produce sufficient IL-12 to support Th1 responses [42, 45, 46]. Skokos and Nussenzweig [42] reported a Delta-4-dependent, IL-12-independent, LPS-mediated Th1 induction by $CD8\alpha^-$ DCs, but Delta-4 accounted for only 10–15% of the total Th1 responses, as a result of the functional redundancy with IL-12. $CD8\alpha^-DCAL2^+$ DCs produced high levels of IL-12 in response to CpG, which is very likely to be contributing to Th1 induction. This discrepancy could be a result of the difference between TLR4 and TLR9 signaling in programming DCs to use Delta-4 for Th1 induction.

The question remains as to why $CD8\alpha^-DCAL2^+$ DCs are able to produce more IL-12 than the other DC subsets, although prior literature suggested $CD8\alpha^-$ DCs produced less IL-12 than $CD8\alpha^+$ DCs. One possibility is that $CD8\alpha^-DCIR2^+$ DCs regulate $CD8\alpha^-DCAL2^+$ DCs and prevent them from producing IL-12. However, addition of increasing numbers of $CD8\alpha^-DCIR2^+$ DCs to $CD8\alpha^-DCAL2^+$ DC cultures had no effect on IL-12 production (data not shown). A more likely possibility is that in previous $CD8\alpha^-$ DC studies, some cytokine responses by $CD8\alpha^-DCAL2^+$ DCs were not detected, as splenic $CD8\alpha^-DCIR2^+$ DCs are ~2.5-fold more frequent than $CD8\alpha^-DCAL2^+$ DCs (Fig. 3).

In response to flagellin, $CD8\alpha^-DCIR2^+$ DCs up-regulated IL-4-producing cells in vitro and in vivo (Figs. 5 and 6). They appear to be well-equipped for inducing IL-4-producing cells, as they express high levels of the flagellin sensors TLR5 and Ipaf and in response to flagellin, selectively up-regulate OX40L (Figs. 3 and 4), which plays a key role in stimulating primary and memory Th2 responses in vivo [47]. In addition, targeting of antigen to $CD8\alpha^-DCIR2^+$ DCs results in induction of Th2 responses in vivo [25]. Moreover, flagellin induces MyD88-dependent, DC-mediated Th2 in vivo by promoting the production of IL-4 and IL-13 from antigen-specific CD4 T cells, as well as IgG1 responses [48]. Thus $CD8\alpha^-DCIR2^+$ DCs appear to be designed for responding to pathogens that induce Th2 cells. Although

non-DCs, such as basophils, can produce IL-4 and also promote Th2 responses [49, 50], DCs are absolutely required to induce Th2 responses in certain contexts, such as infections with the parasitic helminth, *Schistosoma mansoni*, as depletion of DCs severely disrupts Th2 responses [51]. Further studies are needed to determine how the CD8α⁻DCIR2⁺ subset is programmed and in turn, regulates protective Th2 immunity.

AUTHORSHIP

S.K. performed experiments, analyzed data, and wrote this manuscript. E.A.C. is the principal investigator and provided intellectual and material/institutional resources and revised this manuscript.

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KEY WORDS:
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