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Characterization of an Immediate Splenic Precursor of CD8⁺ Dendritic Cells Capable of Inducing Antiviral T Cell Responses¹

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Mouse spleens contain three major dendritic cell (DC) populations: plasmacytoid DC, conventional CD8⁺CD24⁺ DC (CD8⁺ DC), and conventional CD8[−]CD24[−] DC (CD8[−] DC). We have previously shown that CD8⁺ DC are the major cross-presenting subtype *in vivo* and are the main inducers of antiviral cytotoxic T lymphocyte responses. Here we show that after depletion of CD8⁺ DC, the only DC capable of viral Ag presentation was a small subset that expresses CD24 but not CD8. This CD8[−]CD24⁺ DC population is greatly expanded in mice treated with the DC growth factor FMS-like tyrosine kinase 3 ligand. The CD8[−]CD24⁺ DC represent an immediate precursor of CD8⁺ DC, as demonstrated by their expression pattern of characteristic markers of CD8⁺ DC, their capacity to cross-present *in vitro*, and their conversion into CD8⁺ DC upon adoptive transfer into recipient mice. Accordingly, the lifespan of transferred CD8[−]CD24⁺ DC *in vivo* was greatly enhanced as compared with terminally differentiated CD8⁺ DC. Moreover, in a vaccination protocol, CD8[−]CD24⁺ DC induced stronger T cell responses and accelerated viral clearance of HSV-1 compared with CD8⁺ DC. Our results demonstrate that the ability to cross-present first appears in an immediate precursor population of CD8⁺ DC that does not yet express CD8. The enhanced capacity of CD8[−]CD24⁺ DC to induce immune responses upon adoptive transfer makes them an attractive novel tool for DC-based immunotherapies. *The Journal of Immunology*, 2009, 182: 4200–4207.

Dendritic cells (DC)⁴ are heterogeneous. Several subtypes can be distinguished by their differential expression of surface molecules. In mouse spleens, two major subtypes can be defined under steady-state conditions. Plasmacytoid DC (pDC) express CD45RA and intermediate levels of CD11c, while conventional DC (cDC) are characterized by higher levels of CD11c and the absence of CD45RA. cDC can be further separated into CD8⁺CD24⁺ DC (CD8⁺ DC hereafter) and CD8[−]CD24[−] DC (CD8[−] DC). Numerous studies have revealed important differences in origin and function among these DC subsets (1–4). Functional features that distinguish the DC subtypes include their

migratory properties (5), their capacity to respond to different pathogen products (6–8), and their ability to secrete immunostimulatory cytokines such as type I IFN or IL-12 (9–11). One function that has received particular attention in recent years is the ability to cross-present Ags (i.e., to present exogenous Ags on MHC class I molecules). Using diverse forms of model Ags such as cell-associated, soluble, bacterial, DC-specific Ab-coupled, or particle-bound, the CD8⁺ DC have been shown to be the main, often sole, DC subtype capable of cross-presentation (12–16). These observations correlate with the superior ability of CD8⁺ DC to prime CD8⁺ T cells against viral infections, a process thought to be largely mediated by cross-presentation (17–19). Moreover, mice deficient in *Batf3*, a transcription factor required for CD8⁺ DC development, are defective in cross-presentation and induce poor antiviral immune responses (20).

In this study, we wanted to address whether the dominant role of the CD8⁺ DC in presentation of viral Ags (17, 21, 22) masked the contribution of other APCs. To achieve this, we measured antiviral responses in mice after *in vivo* Ab depletion of CD8⁺ DC. Ag-specific T cell proliferation was not abrogated in the absence of CD8⁺ DC, which led us to identify an immediate precursor of this population that is expanded upon depletion of CD8⁺ DC. This precursor is able to contribute to viral Ag presentation and has the capacity to cross-present Ags. Here, we describe its phenotype and highlight the advantages it provides for vaccination strategies.

Materials and Methods

Mice and virus

C57BL/6 (B6), B6.CH-2^{bm-1} (bm1), MHC II-deficient (23), OT-I (24), OT-II (25), and 2.1 gBT-I (26) mice were bred and maintained under specific pathogen-free conditions at The Walter and Eliza Hall Institute animal breeding facility according to institutional guidelines. B6 mice were left untreated or injected s.c. with 5×10^6 B16 melanoma cells secreting murine FMS-like tyrosine kinase 3 ligand (Flt3-L) (27) and killed after 9–10

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⁴ Abbreviations used in this paper: DC, dendritic cell; pDC, plasmacytoid DC; cDC, conventional DC; Flt3-L, FMS-like tyrosine kinase 3 ligand; B6, C57BL/6.

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days. HSV-1 KOS (HSV-1) strain (28) was propagated and titrated using Vero cells (CSL) grown in MEM10 (MEM containing 10% FCS, 23.83 g/L HEPES, 4 ml L-glutamine, 50 μ M 2-ME, and antibiotics). Mice were infected with 1×10^6 PFU of HSV-1 by i.v. or cutaneous infection as previously described (28).

Cell isolation

OT-I, OT-II, and gBT-I T cells were isolated from the lymph nodes of OT-I, OT-II, or gBT-I transgenic mice, respectively. Cells were purified after a depletion step using Abs against CD11b (M1/70), F4/80, Ter-119, Gr-1 (RB6), MHC class II (M5/114), and CD4 (GK 1.5) or CD8 (YTS 169.4), followed by incubation with anti-rat IgG-coupled magnetic beads (DynaL Biotech) following the manufacturer's protocols. T cell preparations were 90–95% pure as determined by flow cytometry. For adoptive transfer or in vitro experiments, T cells were labeled with 2.5 μ M CFSE (Sigma-Aldrich). DC were isolated from mouse spleens as described (29, 30). For in vitro Ag-presentation assays, cells were further purified by cell sorting with a FACSAria (BD Biosciences) or Mo-Flo (Dako) instrument. For in vivo experiments, CD8⁺ DC were isolated from the spleens of Flt3-L tumor-bearing mice by positive selection using immunomagnetic beads (MACS; Miltenyi Biotec) after staining with anti-CD8 (YTS 169.4) Ab, and CD8[−]CD24⁺ cells were isolated by negative selection using MACS beads after staining with anti-CD8 (YTS 169.4) and anti-CD11b (M1/70) Abs. Cells were 95–98% pure as assessed by flow cytometry.

In vivo CD8⁺ DC depletion

CD8⁺ DC, expressing the CD8.2 allele, were depleted in vivo by i.p. injections of an anti-CD8.2 Ab (mAb 2.43) (31). For in vivo Ag presentation assays, the Ab was injected three times over 7 days (first dose: 1 mg at day 0, second and third dose: 0.5 mg at day 2 and day 7) and for ex vivo Ag presentation assays, the Ab was injected twice (first dose, 1 mg at day 0; second dose, 0.5 mg at day 2). The efficiency of depletion of CD8⁺ cells in the spleen was assessed by flow cytometry.

In vivo Ag presentation assay

Untreated B6 mice or treated B6 mice were injected with 1×10^6 CFSE-labeled CD8.1 gBT-I T cells at day 4 after the first injection of mAb 2.43. The following day, mice were either infected with 1×10^6 PFU of HSV-1 by i.v. or cutaneous infection or left uninfected. Proliferation of gBT-I cells was measured in the spleen 5 days after their transfer.

Ex vivo Ag presentation assay

B6 mice were infected with HSV-1 at day 4 after the first injection of mAb 2.43. The following day, spleens were removed, cut into small fragments, and digested in collagenase II (1 mg/ml; Worthington Biochemical) and DNase I (0.1%; Boehringer Mannheim). Other non-DC splenic populations were depleted using Abs and magnetic beads as described (22). DC were further purified by cell sorting with a FACSAria. Serial dilutions of the sorted DC subtypes were coincubated with 5×10^4 CFSE-labeled gBT-I and proliferation was assessed 60 h later by flow cytometry.

Flow cytometry analysis

Cells were labeled with propidium iodide to assess viability and Abs anti-CD11c (N418), CD8 (YTS 169.4), CD24 (M1/69), CD172a (p84), CD11b (M1/70), CD205 (NLDC-145), CD36 (63), CD54 (YN1/1.7.4), CD45.2 (S450 15.2), V α 2 (B20.1), or H-2K^b gB_{497–505} tetramer and analyzed on a FACSCalibur or LSR instrument (BD Biosciences).

Western blot analysis

Total cell lysates were run on a 4–12% SDS-PAGE gel (Invitrogen) and transferred onto nitrocellulose membrane. Rabbit antisera anti-actin (Sigma-Aldrich) and anti-cystatin C (Dako) and HRP-conjugated anti-rabbit IgG Ab (Sigma-Aldrich) were used for protein detection.

In vitro Ag presentation assay

MHC II-deficient or bm1 spleen cells were gamma-irradiated (1500 rad), washed, incubated with 10 mg/ml OVA (Sigma-Aldrich) in RPMI 1640 medium for 10 min at 37°C, and washed three times with RPMI 1640 medium supplemented with 3% FCS. The amount of OVA associated with the cells was previously quantitated to be 8 ng of OVA per 10^6 cells (32). DC populations were added to 96-well Costar (Corning) plates at 2.5×10^4 cells per well with different numbers of OVA-coated splenocytes at 37°C in RPMI 1640 medium supplemented with 10% FCS, 50 μ M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 200 U/ml GM-CSF (PeproTech). CFSE-labeled OT-I or OT-II cells (5×10^4) were

added in each well. Proliferation was analyzed by flow cytometry after 60–65 h of culture as described (29). Each determination was performed in duplicate.

In vivo DC transfer assay

CFSE-labeled CD45.2⁺ DC (5×10^6) were injected i.v. into CD45.1⁺ B6 mice. At different time points after adoptive transfer, mice were killed and their spleens collected. Splenic cells were digested with DNase and collagenase, enriched for light-density cells by centrifugation in 1.077 g/cm³ Nycodenz as described (30), and stained for analysis by flow cytometry.

Vaccination assay

Purified CD8[−]CD24⁺ DC and CD8⁺ DC from Flt3-L tumor-bearing mice were incubated with 1 μ g/ml gB_{497–505} peptide (SSIEFARL) (Auspep) for 45 min at 37°C and washed twice. DC (1×10^6) were injected i.v. into recipient B6 mice. 14 days later, mice were either 1) sacrificed and the expansion of endogenous gB-specific T cells was measured in the spleens by tetramer staining, 2) injected i.v. with 2×10^5 PFU HSV-1, or 3) infected on the flank with 1×10^6 PFU HSV-1. Seven days after infection, the expansion of endogenous gB-specific T cells was measured in the spleens by tetramer staining. Alternatively, 7 days after flank infection, viral replication in the skin was determined using standard PFU assays on confluent Vero cell monolayers as previously described (28). Ten-fold serial dilutions were tested for plaque formation to determine viral titers in the original tissue sample. As controls, naive B6 mice were infected i.v. with 2×10^5 PFU HSV-1 either on day 0 or 14, or they were infected on the flank with 1×10^6 PFU HSV-1 on day 14.

Results

In vivo depletion of CD8⁺ DC reveals a minor DC subset that contributes to MHC I presentation of viral Ag

To analyze the effect of CD8⁺ DC elimination on MHC I-restricted presentation of viral Ags, we depleted CD8⁺ DC in B6 mice by successive injections of the 2.43 Ab (mAb 2.43), which is selective for the allelic variant of CD8 termed CD8.2 (31). CD8⁺ DC were efficiently depleted over a period of 10 days (Fig. 1A). Depleted and control mice were adoptively transferred with 1×10^6 CFSE-labeled transgenic gBT-I T cells (gBT-I), which express a TCR specific for an H-2K^b-restricted immunodominant epitope derived from HSV glycoprotein B (gB epitope). The gBT-I T cells were purified from transgenic mice that express the CD8.1 allele (26), which is not recognized by mAb 2.43, so these cells were not affected by the presence of the depleting Ab in the recipient animals. The mice were infected i.v. with HSV-1 (28) on the following day, and the proliferation of gBT-I cells in the spleen was measured by flow cytometry 5 days later. Depletion of CD8⁺ DC reduced but did not abrogate the proliferation of gBT-I cells in response to HSV-1 infection (Fig. 1B). This suggested that gBT-I cells proliferated in the depleted, HSV-1-infected mice in response to cross-presentation by a non-CD8⁺ DC. Homeostatic factors might also have contributed to gBT-I expansion, but if this were the case, such factors only appeared to play a role in HSV-1-infected mice because transferred gBT-I cells did not proliferate in uninfected mice, regardless of whether the mice had been depleted of CD8⁺ cells (data not shown).

To identify which splenic DC population was responsible for the presentation of the gB epitope in control (undepleted) and CD8⁺ DC-depleted mice, we purified splenic CD8⁺ and CD8[−]CD24[−] DC from control mice and CD8[−]CD24[−] DC from mAb 2.43-treated mice and cultured them in vitro with CFSE-labeled gBT-I cells. After 60 h, we measured the proliferation of gBT-I cells as a read-out of viral Ag presentation. The CD8⁺ DC presented the viral Ag as expected, but the CD8[−]CD24[−] DC from either the control or the CD8⁺ DC-depleted mice did not (Fig. 1C), indicating they were not responsible for the induction of gBT-I proliferation in mice that were devoid of CD8⁺ DC. Analysis of pDC purified from the spleens of control or CD8⁺ DC-depleted mice

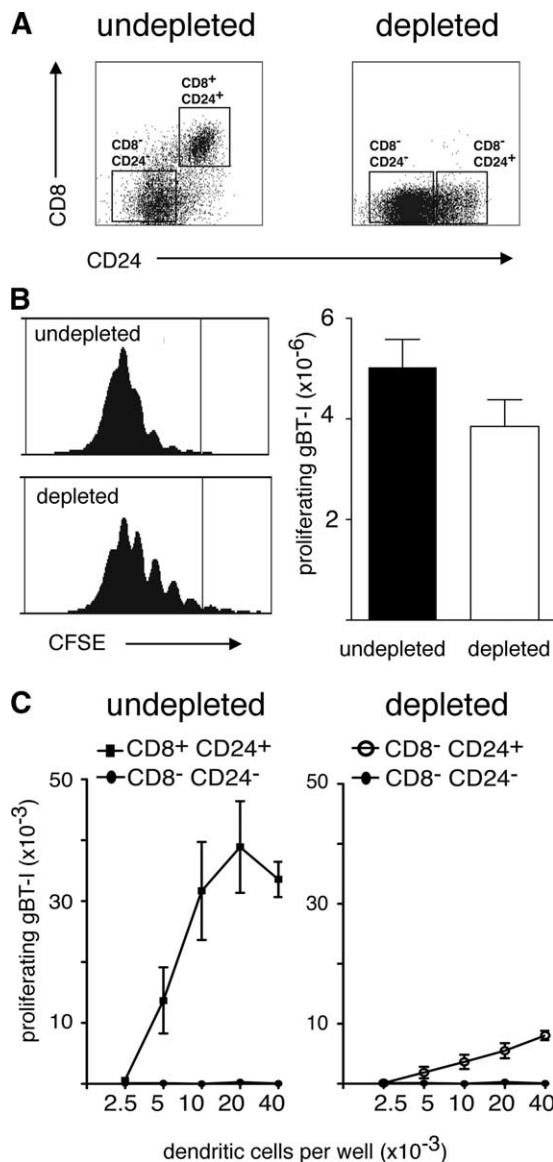


FIGURE 1. In vivo depletion of CD8⁺ DC fails to affect priming of virus-specific CD8 T cells and reveals a new population of splenic DC. **A**, B6 mice were depleted of CD8⁺ DC by successive i.p. injections of anti-CD8.2 Ab (at days 0, 2, and 7). To assess depletion efficiency, splenic DC from depleted and undepleted mice were purified and stained for CD11c, CD8, and CD24 at day 10. Representative dot plots (gated on CD11c⁺ cells) are shown. **B**, Control mice (undepleted) or treated mice (depleted) were adoptively transferred with 1×10^6 CFSE-labeled CD8.1 gBT-I at day 4 after the first injection of Ab, and infected i.v. with 2×10^5 PFU HSV-1 at day 5. The proliferation of gBT-I T cells was measured in the spleen at day 10 by flow cytometry. A representative histogram of CFSE dilution is shown. The number of proliferating gBT-I cells in the spleen is shown (mean \pm SEM, $n = 9$ in three independent experiments). **C**, CD8⁺CD24⁺ and CD8⁻CD24⁺ from undepleted HSV-1 infected mice and CD8⁻CD24⁺ and CD8⁻CD24⁻ from depleted HSV-1 infected mice were purified and their ability to induce proliferation of CFSE-labeled gBT-I ex vivo was measured after 60 h of coculture by flow cytometry. Number of gBT-I T cells that have undergone at least one division as assessed by CFSE dilution is shown (mean \pm SEM in three independent experiments).

likewise demonstrated that this DC population was not responsible for presentation of HSV Ags (data not shown).

We then turned our attention to a population of CD11c^{high} CD8⁻CD24⁺ cells that could be clearly discerned in CD8⁺ DC-

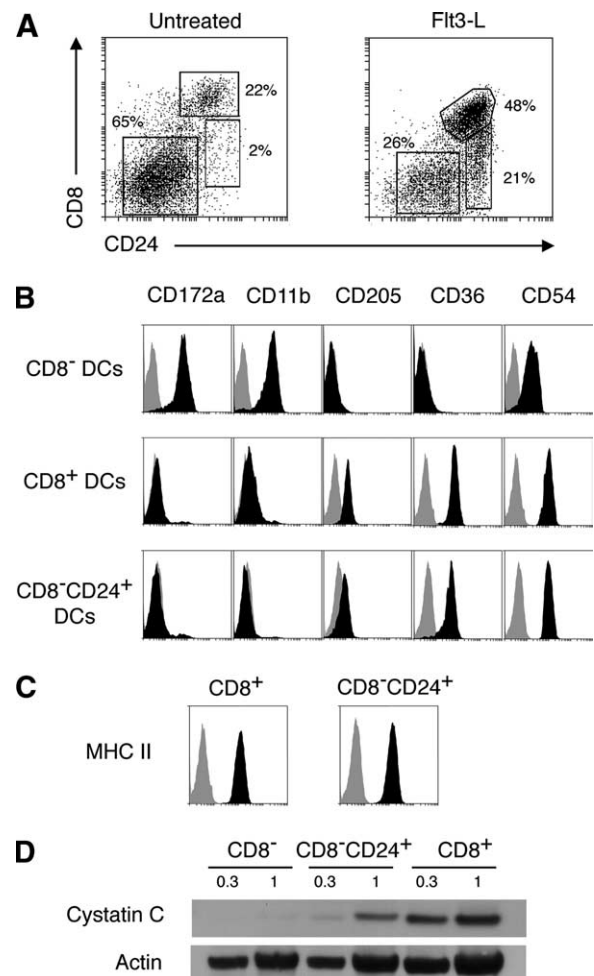


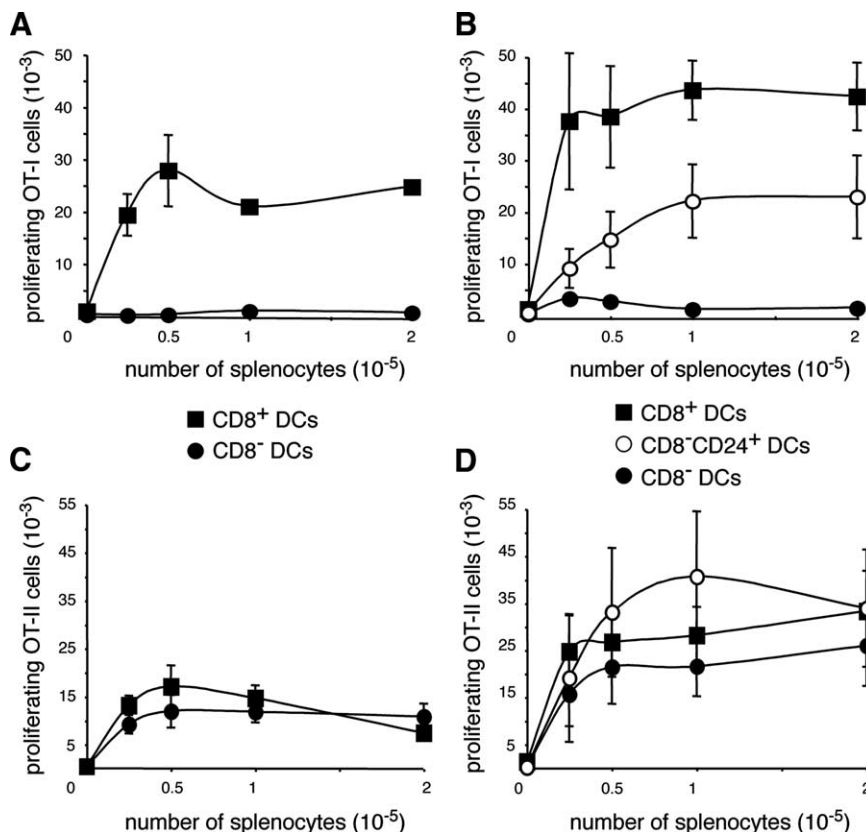
FIGURE 2. CD8⁻CD24⁺ DC display a similar phenotype to CD8⁺ DC. **A**, DC were isolated from the spleens of untreated mice or mice injected with Flt3-L-secreting B16 melanoma cells and analyzed by flow cytometry for their expression of CD8 and CD24. Percentage of each population is shown. **B**, DC subsets from Flt3-L-treated mice, as defined on panel A, were analyzed by flow cytometry for their expression of CD172a, CD11b, CD205, CD36, and CD54. Gray histograms represent isotype control staining. **C**, CD8⁺ and CD8⁻CD24⁺ DC from Flt3-L-treated mice were analyzed by flow cytometry for their expression of MHC II molecules. Gray histograms represent isotype control staining. **D**, Total cell lysate from 0.3 or 1×10^6 DC was submitted to electrophoresis. The expression of actin and cystatin C was assessed by Western blot analysis. Results from one representative experiment out of multiple independent experiments are shown.

depleted mice (Fig. 1A). High levels of CD24 expression is a feature of the CD8 DC lineage (33), so it was possible that this population was responsible for cross-priming of HSV-specific T cells in these mice. Indeed, the CD8⁻CD24⁺ DC purified from HSV-infected mice induced gBT-I proliferation, although they were less efficient than were the CD8⁺ DC from control mice (Fig. 1C). We hypothesized that the CD8⁻CD24⁺ DC might correspond to intrasplenic precursors of CD8⁺ DC, which had already acquired the capacity to cross-present viral Ags. To test this, we conducted a more extensive phenotypic and functional analysis of this population.

CD8⁻CD24⁺ DC have a similar phenotype to CD8⁺ DC

Analysis of CD8⁻CD24⁺ DC was hampered by their rarity (Fig. 2A). However, we reasoned that if these cells were indeed CD8⁺

FIGURE 3. $CD8^-CD24^+$ DC can cross-present cell-associated Ag. Purified DC from untreated mice (A and C) or mice injected with Flt3-L-secreting B16 melanoma cells (B and D) were incubated in vitro with different numbers of OVA-coated splenocytes from bm1 (A and B) or MHC II-deficient (C and D) mice and CFSE-labeled OT-I (A and B) or OT-II (C and D) T cells. T cell proliferation was analyzed by flow cytometry after 60–65 h of culture. Number of T cells that have undergone at least one division as assessed by CFSE dilution is shown (mean \pm SEM in two independent experiments).



DC precursors, their number should increase in mice exposed to the DC growth factor Flt3-L, which drives DC differentiation, and especially the production of $CD8^+$ DC (34). Therefore, we injected mice with a B16 melanoma cell line that secretes Flt3-L (27). Splenic DC were isolated from Flt3-L-secreting tumor-bearing mice, and expression of CD8 and CD24 was analyzed by flow cytometry (Fig. 2A). The absolute number of DC in the spleens of these mice increased >15-fold (data not shown), of which nearly one-half were $CD8^+$ DC (Fig. 2A). Strikingly, the proportion of $CD8^-CD24^+$ DC increased by >10-fold in Flt3-L-treated mice compared with control mice, supporting the hypothesis that they correspond to $CD8^+$ DC precursors.

Next we analyzed the expression of CD172a, CD11b, CD205, CD36, and CD54 in $CD8^-CD24^+$ DC (Fig. 2B). These markers are differentially expressed between $CD8^-$ and $CD8^+$ DC (30, 35–37). The pattern of expression of these surface molecules on $CD8^-CD24^+$ DC was identical to that on $CD8^+$ DC in Flt3-L-treated mice (Fig. 2B) and untreated mice (data not shown). Moreover, $CD8^-CD24^+$ DC and $CD8^+$ DC expressed similar levels of MHC II (Fig. 2C). Another characteristic difference between $CD8^-$ and $CD8^+$ DC is that the cysteine protease inhibitor cystatin C is only expressed in the latter (7, 38). Western blot analysis of total cell lysates revealed that $CD8^-CD24^+$ DC express cystatin C, although at lower levels than do $CD8^+$ DC (Fig. 2D). These results show that, for all markers tested, $CD8^-CD24^+$ DC display a phenotype closely resembling that of $CD8^+$ DC, except for the lack of CD8 expression.

$CD8^-CD24^+$ DC can cross-present cell-associated Ag

$CD8^+$ DC have a much higher capacity to cross-present various forms of Ag than do $CD8^-$ DC (12, 14–16), probably explaining their dominant role in MHC I presentation of viral Ags (18). Since the $CD8^-CD24^+$ DC could contribute to viral Ag presentation

(Fig. 1), we also tested their capacity to cross-present cell-associated OVA. We purified $CD8^-$, $CD8^+$, or $CD8^-CD24^+$ splenic DC from Flt3-L-treated mice, and $CD8^-$ and $CD8^+$ DC from control mice (untreated), and incubated them with OVA-coated bm1 splenocytes, as a source of Ag, and H-2K^b-restricted OVA-specific OT-I T cells, labeled with CFSE, as a responding T cell population. Bm1 splenocytes express H-2^{bm1} molecules unable to present OVA (39), so in this assay OT-I cells can only respond if the DC cross-present the Ag. After 60–65 h of culture, T cell proliferation was assessed by flow cytometry. $CD8^+$ DC from control or Flt3-L-treated mice cross-presented the Ag efficiently as measured by T cell proliferation, whereas $CD8^-$ DC did not (Fig. 3, A and B). $CD8^-CD24^+$ DC were able to induce OT-I T cell proliferation, although less efficiently than $CD8^+$ DC (Fig. 3B). All DC populations efficiently presented MHC II-restricted OVA peptides when incubated with OVA-coated splenocytes from MHC II-deficient mice (Fig. 3, C and D). These results indicate that $CD8^-CD24^+$ DC are capable of cross-presenting cell-associated Ag, albeit not as efficiently as $CD8^+$ DC, a result that correlates with the relative ability of these two subsets to present viral Ags encountered in vivo (Fig. 1).

$CD8^-CD24^+$ DC differentiate in vivo into $CD8^+$ DC

To definitively address whether $CD8^-CD24^+$ DC were direct precursors of $CD8^+$ DC, we first adoptively transferred CFSE-labeled splenic $CD8^-CD24^+$ DC from Flt3-L-treated CD45.1 mice into CD45.2 recipient mice and analyzed their CD8 expression at different time points after transfer (Fig. 4A). Transferred CD45.1 $CD8^-CD24^+$ DC began up-regulating CD8 expression 2 days after injection, and by day 4 >80% of the transferred DC expressed surface CD8 (Fig. 4B). $CD8^-CD24^+$ DC did not proliferate after transfer, as shown by the absence of CFSE dilution (Fig. 4C). We then compared the survival of transferred $CD8^-CD24^+$ DC with

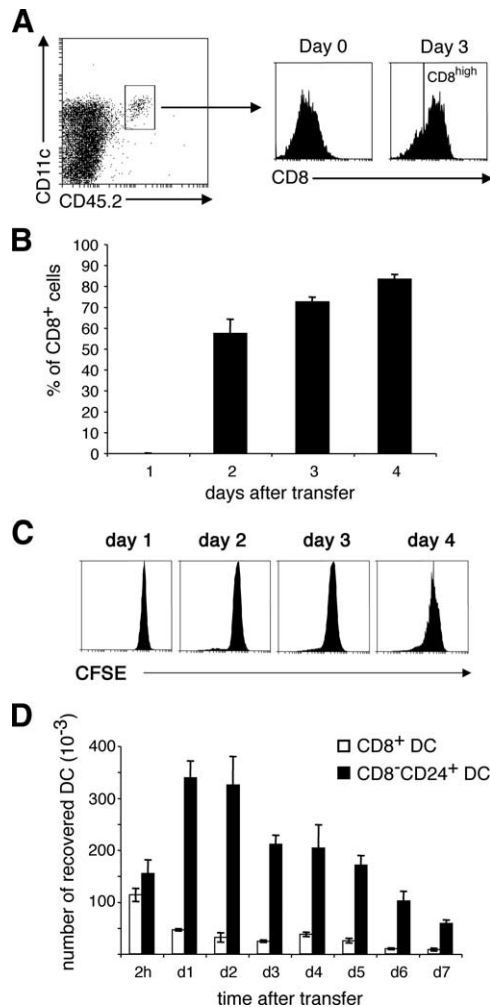


FIGURE 4. CD8⁻CD24⁺ DC are a precursor form of CD8⁺ DC. CFSE-labeled CD45.2⁺ DC (5×10^6) were isolated from the spleens of Flt3-L-treated mice and injected i.v. into CD45.1 recipient mice. At different time points, spleens were collected and donor DC were analyzed by flow cytometry. Dead cells were excluded from the analysis by propidium iodide staining. *A*, Transferred DC were identified by their expression of CD45.2. Up-regulation of CD8 expression was assessed for transferred CD8⁻CD24⁺ DC. Staining of injected cells is also shown (day 0). *B*, Percentage of CD8 expressing cells (as defined in *A*) among transferred CD8⁻CD24⁺ DC is shown (mean \pm SD, $n = 9$ in three independent experiments). *C*, CFSE profile of transferred DC at different times after injection. Data from one representative mouse are shown ($n = 9$ in three independent experiments). *D*, Recovery of transferred cells in the spleen is shown for CD8⁺ DC and CD8⁻CD24⁺ DC at different times after injection: 2 h (2h) or 1–7 days (d1–d7) (mean \pm SD; $n = 4$ –6 in two to three independent experiments).

that of terminally differentiated CD8⁺ DC; if the CD8⁻CD24⁺ cells were precursors of CD8⁺ DC, the former would be expected to survive longer after adoptive transfer than the latter. We injected equal numbers of CD8⁺ DC or CD8⁻CD24⁺ DC from Flt3-L-treated CD45.1 mice into CD45.2 recipients and determined the number of CD45.1 DC contained in the spleens of the recipient mice at different times postinjection. Two hours after adoptive transfer, the number of donor-derived DC was equivalent in both groups of mice, indicating that the two populations had a similar ability to home into the spleen (Fig. 4*D*). However, whereas the number of DC derived from the initial CD8⁺ DC inoculum dropped rapidly, the number of DC derived from CD8⁻CD24⁺ DC increased further 1 day after injection, and only started to

decrease from day 3 onward. Because transferred cells did not proliferate, the increase in the number of DC derived from CD8⁻CD24⁺ DC precursors observed between 2 h and 1 day in the spleens of the recipient mice was probably due to increased accumulation rather than to expansion. These results show that CD8⁻CD24⁺ DC represent a long-lived, nondividing precursor of CD8⁺ DC that has not yet up-regulated CD8 expression and exhibits an intermediate capacity to cross-present Ags.

CD8⁻CD24⁺ DC induce stronger T cell recall responses than do CD8⁺ DC

DC loaded ex vivo with Ags can be used as vaccination vectors, as they are capable of eliciting immune responses upon inoculation in vivo. The efficiency of this strategy depends on multiple factors, with two factors being the ability of the DC to home to lymphoid organs and the duration of Ag presentation by the DC vaccine (40). We reasoned that since the CD8⁻CD24⁺ DC precursors survived in lymphoid organs much longer than did fully developed DC, they might induce better immune responses. To address this question, we first measured the ability of CD8⁺ and CD8⁻CD24⁺ DC purified from Flt3-L-treated mice to induce a primary endogenous gB-specific response upon injection into naive mice. To ensure equal presentation of the gB epitope by both DC groups, the cells were incubated with a synthetic peptide corresponding to the gB epitope recognized by gBT-I cells (residues 497–505). Fourteen days after injection of the DC, we used tetramer staining to measure the magnitude of gB-specific T cell response following DC vaccination. These responses were compared with those induced by i.v. HSV-1 infection. Peptide-pulsed CD8⁺ and CD8⁻CD24⁺ DC induced similar primary T cell responses, although less efficiently than viral infection (Fig. 5*A*). DC did not induce gB-specific T cell proliferation unless loaded with peptide (data not shown).

We then assessed the induction of secondary CD8 T cell responses against i.v. HSV-1 infection in mice that had been vaccinated 14 days earlier with peptide-pulsed CD8⁺ or CD8⁻CD24⁺ DC. A separate group of naive mice was also infected at the same time to compare these secondary responses to the primary response. Both groups that had been vaccinated with DC before the viral challenge developed a stronger secondary response compared with the primary response (Fig. 5, *B* and *C*). Furthermore, vaccination with CD8⁻CD24⁺ DC induced greater expansion of gB-specific memory T cells than that induced by CD8⁺ DC. We then examined whether the increase in magnitude of gB-specific CD8 T cells induced by DC vaccination correlated with enhanced capacity to clear viral infection. HSV-1 only replicates in epithelial cells, so to carry out this test we employed our well-characterized flank skin infection model (28). We vaccinated mice with peptide-pulsed CD8⁺ or CD8⁻CD24⁺ DC as above, and after 14 days infected them in the skin. Seven days later we measured the viral titers in the infection site. The results showed that the viral load was significantly reduced in mice vaccinated with CD8⁻CD24⁺ DC as compared with unvaccinated mice (Fig. 5*D*, note that the results are plotted using a logarithmic scale). The viral load was also reduced in mice vaccinated with CD8⁺ DC, but this reduction was not statistically significant (Fig. 5*D*). The phenotype of responding gB-specific T cells was similar in mice injected with either CD8⁺ or CD8⁻CD24⁺ DC in terms of surface markers (CD62L, CD127, CD25, CD27), expression of granzyme B, and cytokine secretion (IL-2, TNF- α , IFN- γ) (data not shown), indicating quantitative effects of our DC vaccination protocol rather than qualitative differences. The CD8⁻CD24⁺ DC thus proved superior to the CD8⁺ DC at inducing a protective recall T cell response in a vaccination

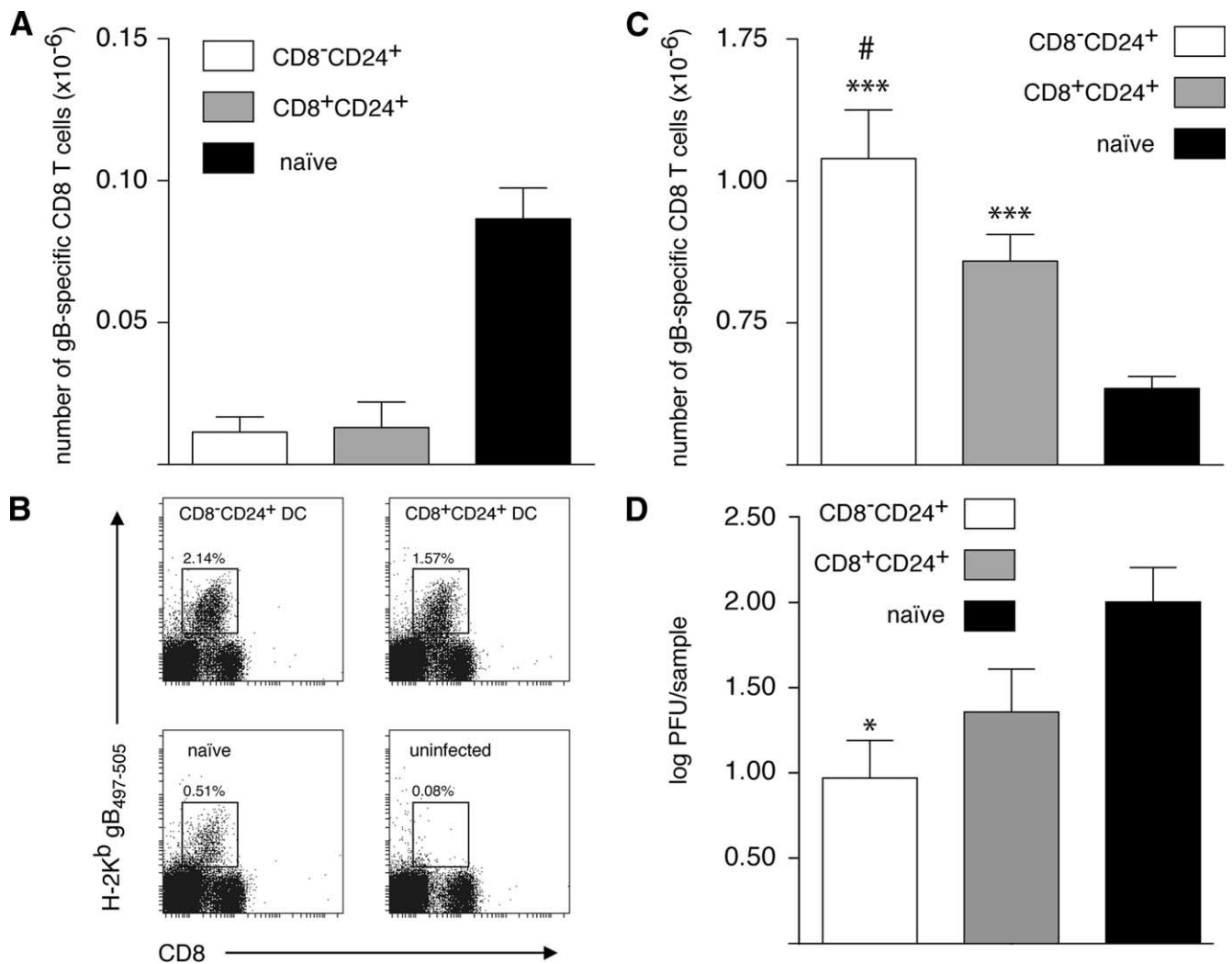


FIGURE 5. CD8⁻CD24⁺ DC induce potent immune responses in a vaccination setting. **A**, Purified gB-pulsed CD8⁻CD24⁺ and CD8⁺CD24⁺ DC from Flt3-L-treated mice were i.v. injected into naive B6 mice and 14 days later expansion of endogenous gB-specific CD8 T cells was determined by H-2K^b gB_{498–505} tetramer staining and compared with expansion of gB-specific CD8 T cells in mice infected i.v. 14 days earlier with 2×10^5 PFU HSV-1. Number of gB-specific T cells in the spleen is shown (mean \pm SEM; $n = 9$ in three independent experiments); the background (number of gB-specific T cells in the spleen of naive mice) has been subtracted from all measurements. **B** and **C**, Mice transferred 14 days earlier with gB-pulsed CD8⁻CD24⁺ and CD8⁺CD24⁺ DC from Flt3-L-treated mice were challenged i.v. with 2×10^5 PFU HSV-1 and 7 days later expansion of gB-specific CD8 T cells was determined by tetramer staining. Naive mice were infected at the same time and served as controls for the magnitude of the primary response. Uninfected mice were included as a control for background tetramer staining. Dot plots showing the percentage of tetramer-positive cells among total splenic CD8⁺ cells for one representative mouse in each group (**B**) and number of gB-specific T cells in the spleen (**C**) are shown (mean \pm SEM; $n = 9$ in three independent experiments, background subtracted). ***, $p < 0.0001$ between experimental group (DC vaccination) and control (HSV-1 infection). #, $p < 0.05$ between CD8⁻CD24⁺ DC and CD8⁺CD24⁺ DC. **D**, Mice vaccinated 14 days earlier with gB-pulsed CD8⁻CD24⁺ and CD8⁺CD24⁺ DC were infected in the skin with 1×10^6 PFU HSV-1, and 7 days later samples of the skin from the infection sites were excised for viral titer determination. Naive mice were infected at the same time as a control. Viral titer expressed as PFU per sample is shown (mean \pm SEM; $n = 15$ – 19 in four independent experiments). *, $p < 0.05$.

protocol, most likely due to their superior ability to induce accumulation of protective antiviral T cells.

Discussion

In this work, we have characterized an immediate precursor of the splenic CD8⁺ DC population. CD8⁻CD24⁺ DC express the same surface markers as CD8⁺ DC, except for CD8 (30, 35–37), and they also express the cysteine protease inhibitor cystatin C, which is selectively expressed by CD8⁺ DC (38). CD8⁻CD24⁺ DC convert in vivo into CD8⁺ DC without dividing, and they have an enhanced lifespan compared with fully differentiated CD8⁺ DC.

Previous studies have described an intrasplenic DC precursor (termed pre-cDC) able to differentiate into all conventional splenic

DC types (33, 41, 42). Pre-cDC are heterogeneous for CD24 expression, and those expressing the highest level of this marker are restricted to CD8⁺ DC (33). The precursor we describe herein appears to be downstream of the pre-cDC along the CD8⁺ DC differentiation pathway. This conclusion is based on the following differences between pre-cDC and CD8⁻CD24⁺ DC: 1) pre-cDC proliferate upon transfer in vivo but the CD8⁻CD24⁺ DC did not; 2) pre-cDC do not express MHC II and only express low levels of CD205, whereas the CD8⁻CD24⁺ DC expressed both of these molecules at levels similar to those found on CD8⁺ DC; 3) pre-cDC can generate both CD8⁻ and CD8⁺ DC, but CD8⁻CD24⁺ DC only generated the latter. We conclude that CD8⁻CD24⁺ DC represent a developmental intermediate in the CD8⁺ DC lineage,

located downstream of pre-cDC and already committed to conversion into CD8⁺ DC. A previous report described a small population (<5% of total DC) of splenic DC that do not express CD8 but express high levels of CD24 and produce IFN- γ when stimulated (43). It seems likely that these cells correspond to the CD8⁻CD24⁺ population that we have characterized herein.

CD8⁻CD24⁺ DC are greatly amplified by exposure to Flt3-L, and our results thus draw caution to using CD8 as the only marker to isolate CD8⁺ and CD8⁻ DC from Flt3-L-treated mice. In such mice, nearly half of the CD8⁻ DC are CD8⁺ DC precursors (Fig. 2A), so studies of CD8⁻ DC purified from Flt3-L-treated mice might wrongly attribute to this population phenotypic and functional properties that are in fact only present in the CD8⁺ DC lineage.

CD8⁻CD24⁺ DC efficiently cross-present Ags both in vivo and in vitro, a functional ability that has been shown to be largely restricted to CD8⁺ DC (12–16, 20). Our results show that the cross-presentation pathway is functional in cells of the CD8 DC lineage before they are terminally differentiated. Our results also demonstrate that pDC do not participate in priming of CD8⁺ T cell responses against HSV-1 infection, even when the major contributors to this presentation, namely CD8⁺ DC (20), are absent. This result is consistent with previous reports that failed to demonstrate a contribution by pDC to CD8⁺ T cell priming against a variety of viral infections, including HSV, influenza virus, and vaccinia virus (21). These responses are thought to be mediated by cross-presentation, so the lack of participation of pDC to this function correlates with the previously reported poor cross-presenting capacity of mouse pDC (44). This contrasts with studies of human DC, which have concluded that pDC can be as efficient at cross-presentation as conventional DC (45). It is possible that the pDC of the two species are differentially equipped with cross-presentation machinery (3). Alternatively, it is possible that neither the pDC nor the human cDC types analyzed so far are as efficient at cross-presentation as is the putative human equivalent of murine CD8⁺ DC, which has as of yet not been identified (1, 2). This is a question that remains for future investigation.

When used in a vaccination protocol, CD8⁻CD24⁺ DC induced stronger priming of virus-specific CD8 T cells than do CD8⁺ DC. Notably, the increased magnitude of virus-specific CD8 T cells also corresponded to increased viral clearance. The higher potency of CD8⁻CD24⁺ DC is likely a consequence of their longer half-life upon injection in the vaccinated animals, because expression of the surrogate phenotypic markers that we used to assess the quality of the T cell response was similar in both groups of vaccinated mice. Large-scale production of CD8⁺ DC precursors may thus provide a better vector for vaccination purposes than fully developed CD8⁺ DC, especially when efficient cross-priming is the desired outcome. Our results also suggest that targeting Ags to DC using Abs specific for surface receptors may be more effective if the targeted molecule is already expressed at the DC precursor stage, which would allow targeting of both precursors and fully differentiated DC. In conclusion, characterization of the developmental pathways of DC may reveal unanticipated new targets for vaccine development that may result in improved immunotherapies.

Disclosures

The authors have no financial conflicts of interest.

References

- Shortman, K., and S. H. Naik. 2007. Steady-state and inflammatory dendritic-cell development. *Nat. Rev. Immunol.* 7: 19–30.
- Villadangos, J. A., and P. Schnorrer. 2007. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat. Rev. Immunol.* 7: 543–555.
- Villadangos, J. A., and L. Young. 2008. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* 29: 352–361.
- Young, L. J., N. S. Wilson, P. Schnorrer, A. Proietto, T. ten Broeke, Y. Matsuki, A. M. Mount, G. T. Belz, M. O'Keeffe, M. Ohmura-Hoshino, et al. 2008. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. *Nat. Immunol.* 9: 1244–1252.
- Villadangos, J. A., and W. R. Heath. 2005. Life cycle, migration and antigen presenting functions of spleen and lymph node dendritic cells: limitations of the Langerhans cells paradigm. *Semin. Immunol.* 17: 262–272.
- Edwards, A. D., S. S. Diebold, E. M. Slack, H. Tomizawa, H. Hemmi, T. Kaisho, S. Akira, and C. Reis e Sousa. 2003. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 α^+ DC correlates with unresponsiveness to imidazoquinolines. *Eur. J. Immunol.* 33: 827–833.
- Naik, S. H., A. I. Proietto, N. S. Wilson, A. Dakic, P. Schnorrer, M. Fuchsberger, M. H. Lahoud, M. O'Keeffe, Q. X. Shao, W. F. Chen, et al. 2005. Cutting edge: generation of splenic CD8⁺ and CD8⁻ dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J. Immunol.* 174: 6592–6597.
- Schulz, O., S. S. Diebold, M. Chen, T. I. Naslund, M. A. Nolte, L. Alexopoulou, Y. T. Azuma, R. A. Flavell, P. Liljestrom, and C. Reis e Sousa. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433: 887–892.
- Maldonado-Lopez, R., T. De Smedt, P. Michel, J. Godfroid, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain, and M. Moser. 1999. CD8 α^+ and CD8 α^- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J. Exp. Med.* 189: 587–592.
- Hochrein, H., K. Shortman, D. Vremec, B. Scott, P. Hertzog, and M. O'Keeffe. 2001. Differential production of IL-12, IFN- α , and IFN- γ by mouse dendritic cell subsets. *J. Immunol.* 166: 5448–5455.
- Colonna, M., G. Trinchieri, and Y. J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat. Immunol.* 5: 1219–1226.
- Schulz, O., and C. Reis e Sousa. 2002. Cross-presentation of cell-associated antigens by CD8 α^+ dendritic cells is attributable to their ability to internalize dead cells. *Immunology* 107: 183–189.
- Dudzinski, D., A. O. Kamphorst, G. F. Heidkamp, V. R. Buchholz, C. Trumpf, S. Yamazaki, C. Cheong, K. Liu, H. W. Lee, C. G. Park, et al. 2007. Differential antigen processing by dendritic cell subsets in vivo. *Science* 315: 107–111.
- Pooley, J. L., W. R. Heath, and K. Shortman. 2001. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8⁻ dendritic cells, but cross-presented to CD8 T cells by CD8⁺ dendritic cells. *J. Immunol.* 166: 5327–5330.
- den Haan, J. M., S. M. Lehar, and M. J. Bevan. 2000. CD8⁺ but not CD8⁻ dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* 192: 1685–1696.
- Schnorrer, P., G. M. Behrens, N. S. Wilson, J. L. Pooley, C. M. Smith, D. El-Sukkari, G. Davey, F. Kupresanin, M. Li, E. Maraskovsky, et al. 2006. The dominant role of CD8⁺ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc. Natl. Acad. Sci. USA* 103: 10729–10734.
- Belz, G. T., K. Shortman, M. J. Bevan, and W. R. Heath. 2005. CD8 α^+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J. Immunol.* 175: 196–200.
- Heath, W. R., G. T. Belz, G. M. Behrens, C. M. Smith, S. P. Forehan, I. A. Parish, G. M. Davey, N. S. Wilson, F. R. Carbone, and J. A. Villadangos. 2004. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol. Rev.* 199: 9–26.
- Sigal, L. J., S. Crotty, R. Andino, and K. L. Rock. 1999. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* 398: 77–80.
- Hildner, K., B. T. Edelson, W. E. Purtha, M. M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B. U. Schraml, E. R. Unanue, M. S. Diamond, et al. 2008. *Batf3* deficiency reveals a critical role for CD8 α^+ dendritic cells in cytotoxic T cell immunity. *Science* 322: 1097–1100.
- Belz, G. T., C. M. Smith, D. Eichner, K. Shortman, G. Karupiah, F. R. Carbone, and W. R. Heath. 2004. Cutting edge: conventional CD8 α^+ dendritic cells are generally involved in priming CTL immunity to viruses. *J. Immunol.* 172: 1996–2000.
- Allan, R. S., C. M. Smith, G. T. Belz, A. L. van Lint, L. M. Wakim, W. R. Heath, and F. R. Carbone. 2003. Epidermal viral immunity induced by CD8 α^+ dendritic cells but not by Langerhans cells. *Science* 301: 1925–1928.
- Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis. 1991. Mice lacking MHC class II molecules. *Cell* 66: 1051–1066.
- Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76: 17–27.
- Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based α - and β -chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34–40.
- Mueller, S. N., W. Heath, J. D. McLain, F. R. Carbone, and C. M. Jones. 2002. Characterization of two TCR transgenic mouse lines specific for herpes simplex virus. *Immunol. Cell Biol.* 80: 156–163.
- Mach, N., S. Gillissen, S. B. Wilson, C. Sheehan, M. Mihm, and G. Dranoff. 2000. Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res.* 60: 3239–3246.

28. van Lint, A., M. Ayers, A. G. Brooks, R. M. Coles, W. R. Heath, and F. R. Carbone. 2004. Herpes simplex virus-specific CD8⁺ T cells can clear established lytic infections from skin and nerves and can partially limit the early spread of virus after cutaneous inoculation. *J. Immunol.* 172: 392–397.
29. Wilson, N. S., D. El-Sukkari, G. T. Belz, C. M. Smith, R. J. Steptoe, W. R. Heath, K. Shortman, and J. A. Villadangos. 2003. Most lymphoid organ dendritic cell types are phenotypically and functionally immature. *Blood* 102: 2187–2194.
30. Vremec, D., J. Pooley, H. Hochrein, L. Wu, and K. Shortman. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J. Immunol.* 164: 2978–2986.
31. Sarmiento, M., A. L. Glasebrook, and F. W. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytotoxicity in the absence of complement. *J. Immunol.* 125: 2665–2672.
32. Li, M., G. M. Davey, R. M. Sutherland, C. Kurts, A. M. Lew, C. Hirst, F. R. Carbone, and W. R. Heath. 2001. Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin in vivo. *J. Immunol.* 166: 6099–6103.
33. Naik, S. H., D. Metcalf, A. van Nieuwenhuijze, I. Wicks, L. Wu, M. O’Keeffe, and K. Shortman. 2006. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat. Immunol.* 7: 663–671.
34. Maraskovsky, E., K. Brasel, M. Teepe, E. R. Roux, S. D. Lyman, K. Shortman, and H. J. McKenna. 1996. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J. Exp. Med.* 184: 1953–1962.
35. Anjuere, F., P. Martin, I. Ferrero, M. L. Fraga, G. M. del Hoyo, N. Wright, and C. Ardavin. 1999. Definition of dendritic cell subpopulations present in the spleen, Peyer’s patches, lymph nodes, and skin of the mouse. *Blood* 93: 590–598.
36. Belz, G. T., D. Vremec, M. Febbraio, L. Corcoran, K. Shortman, F. R. Carbone, and W. R. Heath. 2002. CD36 is differentially expressed by CD8⁺ splenic dendritic cells but is not required for cross-presentation in vivo. *J. Immunol.* 168: 6066–6070.
37. Lahoud, M. H., A. I. Proietto, K. H. Gartlan, S. Kitsoulis, J. Curtis, J. Wettenhall, M. Sofi, C. Daunt, M. O’Keeffe, I. Caminschi, et al. 2006. Signal regulatory protein molecules are differentially expressed by CD8⁺ dendritic cells. *J. Immunol.* 177: 372–382.
38. El-Sukkari, D., N. S. Wilson, K. Hakansson, R. J. Steptoe, A. Grubb, K. Shortman, and J. A. Villadangos. 2003. The protease inhibitor cystatin C is differentially expressed among dendritic cell populations, but does not control antigen presentation. *J. Immunol.* 171: 5003–5011.
39. Nikolic-Zugic, J., and M. J. Bevan. 1990. Role of self-peptides in positively selecting the T-cell repertoire. *Nature* 344: 65–67.
40. Melief, C. J., and S. H. van der Burg. 2008. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. *Nat. Rev. Cancer* 8: 351–360.
41. Liu, K., C. Waskow, X. Liu, K. Yao, J. Hoh, and M. Nussenzweig. 2007. Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat. Immunol.* 8: 578–583.
42. Diao, J., E. Winter, C. Cantin, W. Chen, L. Xu, D. Kelvin, J. Phillips, and M. S. Cattral. 2006. In situ replication of immature dendritic cell (DC) precursors contributes to conventional DC homeostasis in lymphoid tissue. *J. Immunol.* 176: 7196–7206.
43. Vremec, D., M. O’Keeffe, H. Hochrein, M. Fuchsberger, I. Caminschi, M. Lahoud, and K. Shortman. 2007. Production of interferons by dendritic cells, plasmacytoid cells, natural killer cells, and interferon-producing killer dendritic cells. *Blood* 109: 1165–1173.
44. Sapozhnikov, A., J. A. Fischer, T. Zaft, R. Krauthgamer, A. Dzionek, and S. Jung. 2007. Organ-dependent in vivo priming of naive CD4⁺, but not CD8⁺, T cells by plasmacytoid dendritic cells. *J. Exp. Med.* 204: 1923–1933.
45. Hoeffel, G., A. C. Ripoche, D. Matheoud, M. Nascimbeni, N. Escriou, P. Lebon, F. Heshmati, J. G. Guillet, M. Gannage, S. Caillat-Zucman, et al. 2007. Antigen crosspresentation by human plasmacytoid dendritic cells. *Immunity* 27: 481–492.