Mature Human Langerhans Cells Derived from CD34+ Hematopoietic Progenitors Stimulate Greater Cytolytic T Lymphocyte Activity in the Absence of Bioactive IL-12p70, by Either Single Peptide Presentation or Cross-Primming, Than Do Dermal-Interstitial or Monocyte-Derived Dendritic Cells

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Gudrun Ratzinger, 2,3*, Jan Baggers, 2*† Maria A. de Cos, 4*, Jianda Yuan, *, Tao Dao, *, John L. Reagan, *, Christian Münz, 3 Glenn Heller, 5§ and James W. Young 6, *†‡¶

The emerging heterogeneity of dendritic cells (DCs) mirrors their increasingly recognized division of labor at myriad control points in innate and acquired cellular immunity. We separately generated blood monocyte-derived DCs (moDCs), as well as Langerhans cells (LCs) and dermal-interstitial DCs (DDC-IDCs) from CD34⁺ hematopoietic progenitor cells. Differential expression of CD11b, CD52, CD91, and the CD1 isoforms proved useful in distinguishing these three DC types. All mature DCs uniformly expressed comparable levels of HLA-DR, CD83, CD80, and CD86, and were potent stimulators of allogeneic T cells after exposure either to recombinant human CD40L trimmer or a combination of inflammatory cytokines with PGE₂. moDCs, however, required 0.5–1 log greater numbers than LCs or DDC-IDCs to stimulate comparable T cell proliferation. Only moDCs secreted the bioactive heterodimer IL-12p70, and moDCs phagocytosed significantly more dying tumor cells than did either LCs or DDC-IDCs. LCs nevertheless proved superior to moDCs and DDC-IDCs in stimulating CTL against a recall viral Ag by presenting passively loaded peptide or against tumor Ag by cross-priming autologous CD8⁺ T cells. LCs also secreted significantly more IL-15 than did either moDCs or DDC-IDCs, which is especially important to the generation of CTL. These findings merit further comparisons in clinical trials designed to determine the physiologic relevance of these distinctions in activity between LCs and other DCs. The Journal of Immunology, 2004, 173: 2780–2791.
functional distinctions between these three conventional or myeloid DC types. This area merits investigation because of the distinct cytokine profiles, chemokine responsiveness, and functional segregation of at least LC and DDC-IDC emigrés to discrete T cell or B cell predominant areas of secondary lymphoid organs (21–23). A bias has also emerged in some vaccine trials that inclusion of CD34+ HPC-derived DCs is beneficial, presumably because these include LCs (24). The basis for such benefit, however, has not been established; and in practice, the blood monocyte precursors of moDCs are more readily available and more widely used than are CD34+ HPCs.

Taking advantage of established approaches for generating these three types of DCs in vitro with defined cytokines in the absence of FCS, we undertook a detailed characterization of phenotype and function in standard assays of T cell proliferation and CTL development. We further explored the cytokine secretory profile of these DCs with respect to supporting Ag-specific CTL stimulation. Approach the use of DCs for cross-presentation, we also quantified the phagocytic uptake of dying tumor cells by these three different DC types. More importantly, we compared the capacity of LCs with moDCs to prime Ag-specific T cell responses against cross-presented Ag from dying tumor cells, as an approximation of MHC-restricted Ag presentation relative to phagocytic uptake.

Materials and Methods

Media, sera/plasma, and noncytokine supplements

Complete RPMI 1640 and complete IMDM were supplemented with 10 mM HEPES, 1% penicillin/streptomycin (Media Laboratory, Memorial Sloan-Kettering Cancer Center (MSKCC), New York, NY), 50 μM 2-ME (Invitrogen Life Technologies, Carlsbad, CA), 1% L-glutamine (Invitrogen), 1% t-glutamine (Invitrogen Life Technologies), and heat-inactivated, autologous, or single donor human plasma or serum (1, 10, or 20% v/v as specified for a particular experiment). X-VIVO 15 (BioWhittaker, Walkersville, Maryland) was used as manufactured without additives. All media and reagents were endotoxin-free.

Cytokines

Sterile recombinant, endotoxin-, pyrogen-, and mycoplasma-free human cytokines were used to support generation of moDCs, LCs, and DDC-IDCs in vitro, with exact doses specified within those procedures below. These included GM-CSF (sargramostim, leukine; Immunex, Seattle, WA; now Berlex, Wayne/Montville, NJ); FLT-3 ligand (FL), IL-4, TNF-α, TGF-β, GM-CSF (1000 IU/ml), TNF-α (5 ng/ml), c-kit ligand (20 ng/ml), and FLT-3 ligand (50 μg/ml). Cultures were reconstituted with cytokines and media on day 3. On days 5–6, the developing progeny were thoroughly washed and then recultured at 2 × 10^7/3 ml/well in the same cytokines, but without c-kit ligand and FLT-3 ligand from days 5–6 onward. The DDC-IDC cultures were also transferred to X-VIVO 15 at this time. Thereafter, cytokines and medium were replenished every other day for the duration of the culture.

The following additions were made for the respective generation of LCs vs DDC-IDCs (6–10, 25). To support LC development, TGFβ1 (10 ng/ml) was added to the common cytokines throughout the entire culture period (9, 19, 25). For the specific generation of DDC-IDCs, IL-4 (500 IU/ml) was added to suppress macrophage differentiation (25, 26) when the cells were recultured on days 5–6 in X-VIVO 15 with GM-CSF and TNF-α, but without c-kit ligand and FLT-3 ligand. The DDC-IDC cultures were never exposed to TGFβ1.

Maturation and activation of DCs (Fig. 1)

Immature DCs (days 5–6 moDCs; days 11–12 LCs or DDC-IDCs) were used for assessment of dying tumor cell uptake and as flu-infected target cells for CTL assays where indicated. Otherwise, terminal maturation was essential to ensure optimal activation of DC progeny (1, 13, 27). This was accomplished from days 6–8 for moDCs and from days 12 to 14 for LCs and DDC-IDCs by exposure either to CD40L (28, 29) (soluble rhuCD40-L trimer, 0.5 μg/ml; Immunex) or to a combination of inflammatory cytokines (IL-1β (2 ng/ml), IL-6 (1000 IU/ml), TNF-α (10 ng/ml) and PGE2 (5 mM/ml) (27). Either method resulted in mature, large forward scatter (FSC), CD14^neg, HLA-DR^+^HLA-DP^+^HLA-DQ^+^HLA-ND^+^CD80^+^CD86^+^DCs of all three types, which did not revert to less mature forms or alternatively differentiate into adherent macrophages upon removal of cytokines.

Optional DC enrichment

Certain experiments required high purity achieved by one or both of two methods to ensure equivalent numbers of different DC types in functional assays. Developing DC clusters were sedimented by gravity over HSA columns (10), but at the expense of overall cell yield. Cluster formation was most apparent in the LC cultures (10, 16), but to the extent that macrophage differentiation was sufficiently inhibited by IL-4, developing DDC-IDC and moDC clusters were also separable over HSA columns. The bulk cultures or the recultured cluster populations could also be sorted by flow cytometry for large FSC, HLA-DR^high, CD83^+, CD80^+, CD86^+ DCs of all three types, which did not revert to less mature forms or alternatively differentiate into adherent macrophages upon removal of cytokines.

T lymphocytes

T cells were obtained from tissue culture plastic nonadherent PBMCs, then further purified by nonadherence and elution from nylon wool columns.
Cytokine assays

Direct hemacytometer counts and flow cytometry quantification of HLA-DR<sup>expression</sup>, CD14<sup>+</sup>, CD83<sup>+</sup>, CD86<sup>+</sup> were performed on day 7. Similarly, the percentage and absolute number of mature DCs were determined by flow cytometry. The expression of maturation markers was analyzed after 5 days of culture by gating on live events. For analysis of specific cytokines, human recombinant IL-12 and IL-15 were measured using commercial ELISA according to the manufacturer’s protocol (IL-12p70, Human IL-12 US Immunose assay kit, BioSource International; IL-12p70, Human IL-12p70 Immunop assay kit, BioSource International; IL-15, Quantikine; R&D Systems, Minneapolis, MN). Measurement of secreted, rather than membrane-bound IL-15 first required 10-fold concentration by ultrafiltration with Vivaspin concentrators (10 kDa exclusion size; Vivascience, Goettingen, Germany). A number of other cytokines were evaluated by a flow cytometry-based assay (no. 551811, Human Inflammation Cytokine Bead Array; BD Biosciences, San Diego, CA).

Mixed leukocyte reactions (MLRs)

DCs were cocultured with 10<sup>5</sup> purified allogeneic T cells (allo-MLRs) in triplicate round-bottom microwells (Costar 3799; Corning) at variable ratios from 30:1 to 3000:1 (T-DC), in complete RPMI 1640 supplemented with 10% autologous or single donor serum or plasma. DCs were extensively washed to remove cytokines and irradiated 1500 c/13<sup>3</sup> before addition to T cells. The resulting proliferation of responder T cells was based on the incorporation of methyl-[<sup>3</sup>H]thymidine ([<sup>3</sup>H]Tdr, 1 uCi/well; New England Nuclear, Division of PerkinElmer Life Sciences, Boston, MA) during the last 8–12 h of a 4–5 day culture, as measured in a beta scintillation counter (Betaplate, Wallac, Division of PerkinElmer Life Sciences, Wellesley, MA).

Induction and measurement of influenza virus-specific CTL

Mature DCs from HLA-A*0201 donors were washed and resuspended in cytokine and serum-free RPMI 1640, then either loaded directly with influenza matrix peptide (fluMP) or infected with live influenza virus. For peptide pulsing, mature DCs were separated cultured with 10 μM fluMP (HLA-A2.1 restricted 9-mer fluMP, GILGFVFTL; ResGen, Division of Inivtrogen, Carlsbad, CA) for 1 h at room temperature. For direct infection, mature DCs were exposed to influenza virus strain PR8/34 (SFAPAS, Preston, CT) at a dose of 1000 hemagglutinin U/ml per 5–10×10<sup>5</sup> DCs/ml serum-free RPMI 1640 for 60 min at 37°C (31). DCs were washed three times after either peptide pulsing or infection. IfuMP-pulsed or influenza-infected mature DCs of each type were cocultured with 10<sup>5</sup> purified allogeneic T cells in triplicate round-bottom microwells (Costar no. 3799 96-well plates; Corning) at variable ratios from 30:1 to 1000:1 (T-DC) in complete RPMI 1640–10% autologous or single donor serum or plasma. After 6–7 days, T cells were assayed for the amount of cytolytic activity generated per primary culture by direct addition of 51Cr-labeled (75 uCi of Na<sup>2</sup>CrO<sub>4</sub>/2×10<sup>5</sup> targets at 37°C for 1 h followed by four washes; New England Nuclear) target cells, which were infected with virus (as described above) or infected, untreated, immature and matured moDCs. Some experiments also used T2 cells or A*0201-transduced K562 cells (a kind gift of T. Wolfel, University of Mainz, Mainz, Germany) as targets for peptide pulsing, although the T2 cells tended to release variable amounts of cytolytic activity.

Uptake of dying cells by DCs

A melanoma cell line (SKMel29; SKMel) that expressed HLA-A*0201 was proven mycoplasma-free by PCR (mAb Core Facility, SKMel). Viable, growing tumor cells were adherent to tissue culture plastic but were released by gentle rinsing after a 3–5 min incubation at 37°C in prewarmed trypsin (trypsin 0.25%; ICN Biomedical, Aurora, OH) diluted 1/1 with prewarmed RPMI 1640. After two washes, the cells were recultured in RPMI 1640–10% FCS (FCS; Gemini BioProducts, Woodland, CA) at 3×10<sup>6</sup> cells/3 ml/35-mm tissue culture well of a six-well plate and induced to

HUMAN LCs ARE SUPERIOR STIMULATORS OF CTL

(paraformaldehyde, Sigma-Aldrich) and permeabilized in 50% v/v PBS (MSKCC Media Laboratory) + 50% v/v 0.2% Triton X-100 (Sigma-Aldrich). Cells were stained first by anti-Ki-67 (no. IM 0066; Immunotech, Marseille, France, and the DNA was subsequently stained with 7-aminoactinomycin D (no. 9400, Sigma-Aldrich). Cells were analyzed by flow cytometry for cell cycle entry into S/G2/M phase as a measure of proliferation (30).

Phenotypic analyses by confocal microscopy

Cells for confocal microscopy were centrifuged onto glass slides (15,000 cells/slide) at 900 rpm × 5 min (Cytospin 3; Shandon, Pittsburgh, PA), air-dried overnight, placed in slide boxes with desiccant (Drierite, anhydrous calcium sulfate; W. A. Hammond Drierite, Xenia, OH), sealed with Parafilm (American National Can, Neenah, WI) to prevent condensation, and stored at ~70°C until use. Just before staining, slides were brought to room temperature while still sealed in the slide box with desiccant. The cytocentrifuged cells were fixed and permeabilized by exposure to cold 90°C acetone for 10 min. Slides were air-dried again and then stained at room temperature using unconjugated primary mAbs against CD207 (Langerin; kindly provided by Schering-Plough, Dudilly, France) or CD208 (DC-LAMP; Schering-Plough) for 30 min, followed by an additional 30–min incubation with Texas Red-conjugated goat-anti-mouse Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) without exposure to ambient light. For two-color staining, the cells were then quenched with 10% normal mouse serum in PBS, followed by the addition of anti-HLA-DR FITC (clone L243; BD Pharmingen), CD1a/b/c/d PE (Immunotech, Beckman Coulter, Marseille, France) and anti-CD83-PE (Serotec, Raleigh, NC). Unconjugated mAbs included anti-e-cadherin (clone HECD-1; R&D Systems); and anti-CD1a/b/c/d (generous gifts of Dr. S. A. Porcelli, Albert Einstein College of Medicine, Bronx, NY). Isotype controls included the appropriate fluorochrome conjugated or unconjugated mouse IgG1 or IgG2a (DAKO, Carpinteria, CA) or rat IgG2b-FITC (Serotec). Unconjugated primary IgGs were secondarily stained with biotinylated goat anti-mouse Ig followed by streptavidin-FITC or -PE (BioSource International, Camarillo, CA). Flow cytometry studies used a FACScan (BD Immunocytometry Systems, San Jose, CA), gating for collection and analysis of live events. For analysis of specific epitope expression by DCs, candidate cells were gated for viable, large FSC, HLA-DR bright cells, and 10,000 events were collected.

with PGE<sub>2</sub> matured the DCs. Supernatants were collected after 18–20 h of maturation, immediately frozen, and thawed once for assay. The correct maturation phenotype (HLA-DR<sup>expression</sup>, CD14<sup>+</sup>, CD83<sup>+</sup>, CD86<sup>+</sup>) was confirmed at the conclusion of each culture.

IL-12 and IL-15 were measured using commercial ELISA according to the manufacturer’s protocol (IL-12p40, Human IL-12 US Immunoss ay kit, BioSource International; IL-12p70, Human IL-12p70 Immunassay kit, BioSource International; IL-15, Quantikine; R&D Systems, Minneapolis, MN). Measurement of secreted, rather than membrane-bound IL-15 first required 10-fold concentration by ultrafiltration with Vivaspin concentrators (10-kDa exclusion size; Vivascience, Goettingen, Germany). A number of other cytokines were evaluated by a flow cytometry-based assay (no. 551811, Human Inflammation Cytokine Bead Array; BD Biosciences, San Diego, CA).

DC precursors, DC progeny, and T cells could be cryopreserved at 5–10×10<sup>6</sup> cells/ml in autologous serum (90% final v/v) or HSA (12.5% final v/v in RPMI 1640; Swiss Red Cross, distributed by Alpine Biologies) plus DMSO (10% final v/v; Sigma-Aldrich, St. Louis, MO), by controlled rate freezing at ~1 to ~3°C/minute (Model 9000; Gordinier Electronic, Ros-
Induction and measurement of tumor-specific CTL by ELISPOT after cross-priming

moDCs, which are more actively phagocytic than either LCs or DDC-ITCs, were compared with LCs as the more immunogenic of the two DC types generated from CD34+ HPCs. These DCs were matured by the inflammatory cytokine mixture with PGE$_2$ for ~48 h, beginning 24 h after the start of unlabeled apoptotic tumor cell uptake as above. The cell suspension was then collected and replated with autologous T cells at a DC:T ratio of 1:30 in RPMI 1640–10% autologous or single donor serum or plasma. After 7 days, the T cells were harvested, washed, counted, and restimulated for 5 days with fresh autologous, mature DCs that had also phagocytosed SKMel29 cells. No exogenous cytokines, e.g., IL-2, were ever added to these controls. Control wells for the afferent stimulation included autologous T cells stimulated by mock-loaded DCs handled exactly as above.

After these two rounds of 7 and 5 days of stimulation, CD8$^+$ T cells were positively selected using anti-CD8 microbeads (Miltenyi Biotec) and analyzed for IFN-$\gamma$ production by an enzyme-linked immunosorbsent spot assay (ELISPOT) for human IFN-$\gamma$, Mabtech, DiaPharma Group, West Chester, OH. A total of 10$^5$ purified CD8$^+$ T cells followed by 10$^4$ target cells were added to each well in triplicate. Target cells comprised either HLA-A*0201 SKMel29 tumor cells if the donor also expressed HLA-A*0201, or immature autologous DCs loaded with dying SKMel29 tumor cells if the donor did not express HLA-A*0201. Nonmelanoma tumor cells (prostate cancer cell line; HLA-A*0201-positive LeCIm) or immature DCs that had not taken up dying tumor cells were added as control targets to separate wells. Wells were read by an automated ELISPOT reader (Automated ELISPOT Reader System, Carl Zeiss Vision) to give the number of spot-forming cells (SFC) per 10$^5$ input cells. Mean values were calculated from triplicate wells. Adjusted numbers of SFCs were calculated by subtracting the IFN-$\gamma$ release from both of the two types of control wells (T cells stimulated by mock-loaded DCs and tested against tumor Ag-expressing targets; T cells stimulated by loaded DCs and tested against empty DC or LeCIm targets), from that of the experimental wells.

Statistics

A permutation test was used to compare differences in DC stimulatory capacity for allogeneic T cell proliferation or influenza-specific, autologous CTL generation. The null hypothesis for this test was that any two types of DCs being compared exerted no differential stimulation of responder T cells. The statistic used to test this hypothesis was the sum of the squared differences between mean cpm (T cell proliferation in the allo-MLRs) or mean percent-specific lysis values (CTL generation in autologous, flu-specific MLRs), summed over all dose levels. This generated a p value corresponding to the proportion of all test statistics from the permutation distribution that were equal to or more extreme than the test statistic observed. Pairwise comparisons of multiple single readouts from independent experiments used either the Student t test or the nonparametric Wilcoxon rank sum test as indicated.

Results

moDCs and CD34$^+$ HPC-derived LCs and DDC-ITCs are phenotypically distinct

All three DCs achieved comparable expression of HLA-DR, CD86, CD45RO (not shown), CD80 (not shown), and CD83 after maturation (Fig. 2A). The combination of IL-1-β, IL-6, TNF-$\alpha$, and PGE$_2$ (27) achieved phenotypic maturation of all DC types comparable to that effected by rhuCD40L-trimer (Fig. 2B). Maturation of both DDC-ITCs and LCs increased DC-LAMP/CD208 (32), although some was already detectable at day 12 because of physical manipulation and activation of the cells. The distribution of CD208 merges with that of HLA-DR in mature DDC-ITCs but not LCs, the basis and implications of which remain unknown.

Several epitopes also distinguished moDCs and DDC-ITCs from each other, as well as from LCs (Fig. 2, A and D). Only moDCs expressed significant amounts of CD52 (20), which is targeted by the humanized mAb alemtuzumab. CD91, which is the highly conserved α-2-macroglobulin receptor for heat shock protein gp96 (33) implicated in the uptake of dying cells by DCs, was also restricted to moDCs. All of these human DCs expressed the myeloid marker CD11c (not shown), which helps to distinguish them from CD11c$^{\text{neg}}$ plasmacytoid DCs (34); whereas only moDCs and DDC-ITCs expressed CD11b. LCs were consistently negative for CD11b, whether examined when immature or after maturation. e-cadherin expression was limited to LCs and was readily detected before exposure to terminal maturation stimuli (Fig. 2A). CD207/Langerin was also only expressed by LCs as previously reported (17, 18), but predominantly by less mature forms and with apparent asynchrony during differentiation in culture (Fig. 2D). Neither DDC-ITCs (see inset, Fig. 2D) nor moDCs (not shown) ever expressed CD207.

Contrary to initial results, there were no consistent distinctions in expression of CD137L (35) or CD137 (36) by any one type of DC or maturation state, using improved mAbs (see Materials and Methods) that became available during the course of the study.

Finally, we examined expression of the CD1 isoforms (Fig. 2A). CD1a was originally considered an LC-specific epitope, but it has long been known that GM-CSF exposure will induce CD1a/b/c on monocytes (37). All three DC types actually expressed CD1a, and LCs down-regulated this epitope with maturation more than did the other two. CD34$^+$ HPC-derived LCs expressed little to none of the other three isoforms, whereas CD1b, CD1c, and notably CD1d expression by moDCs paralleled that of DDC-ITCs (38).

moDCs, having arisen from a more uniform starting population of committed precursors, generally achieved a final purity of ~80–90%, the principal contaminants being B cells from the initial plastic adherence of PBMCs. Without intermediate enrichment steps, LCs and DDC-ITCs derived from pluripotent CD34$^+$ HPCs achieved a final purity of ~65–70%. The remaining cells comprised immature granulocytic cells, especially immature eosinophils due to the high concentrations of GM-CSF.

In summary (Table I), we find that among the large FSC, HLA-DR$^{\text{bright}}$, CD14$^{\text{neg}}$, CD83$^+$ population, the presence of both CD11b and CD1d distinguishes CD34$^+$ HPC-derived DDC-ITCs from LCs. The further presence of both CD52 and CD91 distinguishes moDCs from the CD34$^+$ HPC-derived DDC-ITCs. Furthermore, either CD40L or the combination of inflammatory cytokines were equally effective in inducing the maturation or activation epitopes shared by the three DC types under study, e.g., class II MHC$^{\text{bright}}$, CD83$^+$, CD86$^+$, CD80$^{+/-+/-}$, CD45RO$^+$, CD208$^{+/-+/-}$. The single exception was that mature LCs expressed CD25 only after exposure to the inflammatory cytokines but not after exposure to CD40L, whereas mature moDCs and DDC-ITCs expressed this epitope after either maturation stimulus (data not shown). The function of the CD25 epitope on mature, activated DCs remains unknown.
LCs and DDC-IDCs achieve comparable cell cycle entry during expansion from proliferating CD34⁺ HPCs

moDC precursors, like all postmyelocyte cells, no longer divide or proliferate, but only differentiate. In contrast, serial cell cycle analyses documented similar entry into S/G2/M phase for CD34⁺ HPCs developing into either LCs or DDC-IDCs (Fig. 3) in the absence of FCS (30). The first 5–6 days supported the greatest expansion of both populations, as previously reported for FCS-supplemented cultures (6). The absence of FCS compromised overall cell yields by ~5-fold, but this was offset by increased purity of the DC progeny and reduced endogenous cytokine exposure in the presence of serum-containing media, especially those

**FIGURE 2.** Distinct epitope expression differentiates moDCs and CD34⁺ HPC-derived LCs and DDC-IDCs. A, Blood monocytes and CD34⁺ HPCs were isolated and cultured with cytokines as described. Terminal maturation and activation was accomplished by exposure to inflammatory cytokines, and the resulting progeny were stained with mAbs for the indicated epitopes on days 7–8 for moDCs and days 13–14 for LCs and DDC-IDCs. The exception was e-cadherin (see *e-cad*), which is lost with maturation, and was therefore stained on days 11–12 before final exposure to the inflammatory cytokine mixture. Fluorescent intensity of single epitopes (thick line histograms) compared with an isotype control (thin line histograms) is indicated along the x-axes (log10), and cell frequency is depicted against the y-axes. B, Maturation by either rhuCD40L or an inflammatory cytokine mixture including IL-1β, TGF-α, IL-6, and PGE₂, achieved comparable phenotypic up-regulation of maturation/activation markers CD83 and class II MHC. Furthermore, the expression of CD83 and HLA-DR (as well as CD86 and CD40, not shown) was similar between LCs, DDC-IDCs, and moDCs. Correlation coefficients were >0.9 for each single type of DC (LCs, DDC-IDCs, moDCs), when comparing that single DC type matured with either rhuCD40L or the inflammatory cytokines in the same assay of immunostimulatory function (allo-MLR or induction of fluMP-specific CTL; not shown). C, Cytocentrifuged LCs and DDC-IDCs were stained for CD208 (DC-LAMP; Texas Red) and HLA-DR (FITC) on days 12 and 14 of culture. DC-LAMP expression increased with maturation of both populations and merged with the HLA-DR in the DDC-IDCs. D, Cytocentrifuged specimens were stained for CD207 (Langerin; Texas Red) and HLA-DR (FITC) at serial time points during culture and assessed by confocal microscopy. CD207 expression decreased with maturation of LCs. DDC-IDCs (see inset on day 12) never expressed this LC molecule, which supports the formation of Birbeck granules. The overlapping and distinct epitopes that aid phenotypic distinction of these three DC types are summarized in Table I.
with FCS. LCs also had a distinct requirement for provision of exogenous TGFβ1 in the absence of FCS (9, 19, 25, 39, 40), which further compromised final LC yields by about half when compared with DDC-IDCs.

All three DC types exert potent immunostimulatory activity; but CD34+ HPC-derived LCs and DDC-IDCs are more active than moDCs

We compared moDCs with the CD34+ HPC-derived LCs and DDC-IDCs for their ability to stimulate resting allogeneic T cells in the MLR (allo-MLR), as this is a standard assay for DC function. DCs were added in serial 3-fold dilutions to a constant number of $10^5$ purified T cells (Fig. 4, x-axis). The proliferative response of the allogeneic T cells indicated that all three DC types were potent stimulators based on their mature, activated phenotypes (Fig. 4, note y-axis log2 scale depicting T cell division and proliferation).

That said, moDCs proved to be somewhat less active overall on a cell-for-cell basis insofar as moDCs required about a half-log greater number than either LCs or DDC-IDCs to stimulate the same level of T cell proliferation (LCs or DDC-IDCs vs moDCs, $p < 0.01$ for both; LCs vs DDC-IDCs, $p = 0.23$, NS). DCs of each type had been added in the same numbers based on screening flow cytometry for large FSC, HLA-DRbright, CD83+ cells and confirmation by direct hemacytometer counts of cells with typical morphology by inverse phase microscopy. Because the moDCs are derived from a more committed and partially differentiated starting population than are the LCs and DDC-IDCs, their purity is always greater. Hence any errors in equating the DC counts between the three types under study should have favored the moDCs.

LCs are more potent than moDCs in stimulating CTL against a viral recall Ag after presentation of passively loaded peptide, but similar after direct influenza infection

We explored the capacity of moDCs, LCs, and DDC-IDCs to stimulate flu-specific CTLs after a single round of stimulation without addition of exogenous cytokines. fluMP-pulsed LCs were more potent on a cell-for-cell basis than either fluMP-pulsed DDC-IDCs (Fig. 5, top, $p < 0.01$, $n = 16$ independent experiments) or moDCs

FIGURE 3. CD34+ HPC-derived LCs and DDC-IDCs achieve comparable cell cycle entry for expansion in the absence of FCS-supplemented media. CD34+ cells were cultured as described under Materials and Methods using conditions that supported the distinct generation of LCs or DDC-IDCs, but notably in the absence of FCS. Cells were stained for Ki-67 and 7-aminoactinomycin D at the indicated time points along the x-axis. The double-positive cells by cytofluorography represented the percent of the total population in S/G2/M phase, plotted along the y-axis. Cell cycle entry was similar for the two types of DCs, even though overall expansion was compromised compared with FCS-supplemented media and similar cytokines (3- to 10-fold vs 40- to 100-fold). Representative of three experiments assessed in this manner.

FIGURE 4. LCs, DDC-IDCs, and moDCs are all potent stimulators of resting allogeneic T cells, with LCs exerting somewhat greater activity despite comparable phenotypic maturation and activation among all three DC types. Mature moDCs, LCs, and DDC-IDCs were added in equivalent graded doses, based on mature phenotype (large FSC, HLA-DRbright, CD14neg, CD11c+ and typical morphology (FACS corroborated by typical DC morphology on direct hemacytometer counts), to separate triplicate cultures of $10^5$ allogeneic T cells per round-bottom microwell in a 96-well plate. DC doses ranged from 3000 to 100 cells per well, yielding starting DC:T ratios of 1:30 to 1:100. [3H]Tdr uptake by proliferating allogeneic T cells over the last 12 h of a 4–5 day culture was measured as an index of DC immunogenicity. The averaged triplicate means ± SEM for [3H]Tdr Incorporation by T cells stimulated in independent allo-MLRs by 16 pairs of LCs and DDC-IDCs from the same donor, 10 of which also included moDCs from the same donor, are depicted logarithmically (log2) against the y-axis. By the permutation test, $p = 0.23$ (NS) for LCs vs DDC-IDCs, but $p < 0.01$ for moDCs vs either LCs or DDC-IDCs.

### Table I. Epitopes that can distinguish different DC types

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<td>Immature pos (asynchronous), decreases with maturation</td>
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*All conventional myeloid, or nonplasmacytoid, human DCs are class II MHC brightly, CD80+CD86+, CD14neg, and CD11c+. Immature DCs express class II MHC, CD80, and CD86, and these increase with maturation. CD14 is lost with differentiation of moDCs from monocyte precursors and of DDC-IDCs from CD34+ HPCs via a CD14+ intermediate.*
LCs are more potent on a cell-for-cell basis than DDC-IDCs or moDCs in stimulating CTL against an HLA-A*0201-restricted viral peptide (fluMP), a disparity that is abrogated by direct flu infection. LCs, DDC-IDCs, and moDCs were separately generated from the same HLA-A*0201 donor. CD34+ HPCs served as the starting population for LCs and DDC-IDCs, and adherent blood monocytes were used to generate moDCs, all with defined cytokines and media as described in Materials and Methods. The resulting HLA-A*0201 DCs were sorted cytofluorographically to select equivalent numbers of mature CD83 MHC IIbright progeny of each DC type. Separate cytofluorographic analyses confirmed the distinct phenotype of each DC type as shown in Fig. 2. Each population was pulsed separately with fluMP (top panel) or separately infected with influenza virus (bottom panel), then added in serial doses to triplicate microwells containing 1 × 10^5 autologous T cells. On days 6–7, the amount of influenza-specific cytolytic activity per primary culture was determined by adding 5 × 10^5 fluMP-pulsed, 51Cr-labeled target cells, and measuring 51Cr release 4–6 h later. The y-axes depict the adjusted mean percent-specific 51Cr release (after subtracting the lysis of control targets) ± SEM from 16 independent experiments using fluMP-pulsed LCs vs DDC-IDCs (p < 0.01), 4 of which also included moDCs (LCs vs moDCs, p = 0.40; DDC-IDCs vs moDCs, p = 0.40, NS). Ten independent experiments also compared flu-infected LCs vs DDC-IDCs (p = 0.54, NS), 5 of which also included moDCs (LCs vs moDCs, p = 0.49, NS; DDC-IDCs vs moDCs, p = 0.52, NS). The primary autologous stimulatory T-DC ratios (not E:T ratios) are depicted along the x-axes. 51Cr-labeled target cells were flu-infected vs uninfected autologous immature moDCs, or flu-MP vs gp100-pulsed K562-HLA*A201 transfectants or T2 cells.

FIGURE 5. LCs are more potent on a cell-for-cell basis than DDC-IDCs or moDCs in stimulating CTL against an HLA-A*0201-restricted viral peptide (fluMP), a disparity that is abrogated by direct flu infection. LCs, DDC-IDCs, and moDCs were separately generated from the same HLA-A*0201 donor. CD34+ HPCs served as the starting population for LCs and DDC-IDCs, and adherent blood monocytes were used to generate moDCs, all with defined cytokines and media as described in Materials and Methods. The resulting HLA-A*0201 DCs were sorted cytofluorographically to select equivalent numbers of mature CD83 MHC IIbright progeny of each DC type. Separate cytofluorographic analyses confirmed the distinct phenotype of each DC type as shown in Fig. 2. Each population was pulsed separately with fluMP (top panel) or separately infected with influenza virus (bottom panel), then added in serial doses to triplicate microwells containing 1 × 10^5 autologous T cells. On days 6–7, the amount of influenza-specific cytolytic activity per primary culture was determined by adding 5 × 10^5 fluMP-pulsed, 51Cr-labeled target cells, and measuring 51Cr release 4–6 h later. The y-axes depict the adjusted mean percent-specific 51Cr release (after subtracting the lysis of control targets) ± SEM from 16 independent experiments using fluMP-pulsed LCs vs DDC-IDCs (p < 0.01), 4 of which also included moDCs (LCs vs moDCs, p = 0.49; DDC-IDCs vs moDCs, p = 0.40, NS). Ten independent experiments also compared flu-infected LCs vs DDC-IDCs (p = 0.54, NS), 5 of which also included moDCs (LCs vs moDCs, p = 0.49, NS; DDC-IDCs vs moDCs, p = 0.52, NS). The primary autologous stimulatory T-DC ratios (not E:T ratios) are depicted along the x-axes. 51Cr-labeled target cells were flu-infected vs uninfected autologous immature moDCs, or flu-MP vs gp100-pulsed K562-HLA*A201 transfectants or T2 cells.

FIGURE 6. LCs, DDC-IDCs, and moDCs exhibit distinct cytokine secretion profiles during maturation, especially with respect to IL-12p70 and IL-15. Immature LCs, DDC-IDCs, and moDCs were separately generated from their respective precursors using defined cytokines and media as described in Materials and Methods, then densely recultured for maturation using soluble rhuCD40L trimer. Supernatants were concentrated 10-fold before assaying. The mature, activated phenotype was confirmed for each DC type as in Fig. 2 at the end of a 48-h maturation. Cytokine levels (picograms per milliliter) are plotted against the y-axis. The Student t test was used to compare differences between the groups, and the p values are shown for each paired combination.

Immature moDCs, LCs, and DDC-IDCs were densely replated at 10^6 cells/ml for maturation using soluble rhuCD40L trimer. Supernatants were collected at serial time points during the 2 days of maturation, with peak cytokine detection at ~18–20 h in pilot assays. We found a wide disparity between the secretion of IL-12p40 and the bioactive heterodimer IL-12p70 (Fig. 6). Although all three DCs
moDCs are more actively phagocytic of dying tumor cells than are either LCs or DDC-IDCs

Cross-presentation is an important mechanism by which DCs can obtain Ag from a third party cell and present that Ag in a self MHC-restricted fashion to autologous Ag-specific T cells (45–54). An important first step is uptake of an Ag source, and one means is by phagocytosis.

We used a flow cytometry based method to compare the phagocytic capacity of immature day 5–6 moDCs, compared with immature day 11–12 LCs and DDC-IDCs, to take up apoptotic tumor cells. Each immature DC type was confirmed to lack the maturation phenotype, including absence of surface CD83. Tumor cells from the SKMel29 cell line labeled with a green fluorescent vital dye (PKH67) were rendered apoptotic by mitomycin C, and cocultured for 24 h at 37°C with each immature DC type by direct addition to the DC-containing wells. It was important not to physically manipulate the DCs excessively, especially the LCs, as this induced some maturation. After harvesting the cocultured cells and staining with anti-HLA-DR-PE, we gated for HLA-DR+ cells and determined the fluorescence in the FITC channel to quantify the amount of PKH67-labeled, apoptotic tumor cells that each DC type had phagocytosed (Fig. 7A). Phagocytosis was also confirmed by direct inspection of the cells under a fluorescence microscope to rule out simple adhesion. Uptake also did not occur at 4°C as confirmed by flow cytometry (not shown).

moDCs were the most actively phagocytic by almost 2 logs greater MFI for PKH67, compared with LCs and DDC-IDCs, which had comparable activity in this regard. By cytofluorographic profiles in Fig. 7A, one could detect a minor subpopulation of both LCs and DDC-IDCs that was highly phagocytic like the moDCs. The average MFI from multiple independent experiments like the one shown in Fig. 7A are shown in Fig. 7B (n = 10 for moDCs, and n = 9 for LCs and DDC-IDCs). Longer incubation time between DCs and apoptotic tumor, or the addition of apoptotic tumor cells in excess of 1:1 tumor cells-DCs, did not result in greater uptake.

Phagocytic uptake of Ag does not translate directly into Ag presentation and stimulation of T cells

Although Ag uptake is a critical first step to Ag processing and presentation, especially for cross-priming or cross-presentation on class I MHC molecules, the alternative fate of phagocytosed Ag is sequestration and degradation. Hence, the two processes are not necessarily directly correlated. We therefore compared the highly phagocytic moDCs with the more potent LCs of the two DC types generated from CD34+ HPCs, with respect to induction of CTLs after cross-presentation of tumor and tumor-associated Ags. Immature moDCs, LCs, or DDC-IDCs, were cocultured 1:1 with SKMel29 tumor cells that had been membrane-labeled with PKH67 and rendered apoptotic (~80% at onset of coculture) by mitomycin C exposure. At the end of a 24-h incubation at 37°C, cells were harvested, counterstained with anti-HLA-DR-PE and then gated for large FSC, HLA-DR-positive cells on a flow cytometer. The uptake by DCs in this gate of PKH67+ tumor cells, detected in the FITC channel, is shown along the x-axis (log10 fluorescence) for one representative experiment in A. Replicate MFIs for tumor cell uptake ± SD are shown in B (n = 10 experiments for moDCs, and n = 9 experiments each for LCs and DDC-IDCs), with p values for pairwise comparisons by the Wilcoxon rank sum statistic indicated on the figure.

FIGURE 7. moDCs are the more actively phagocytic by at least one log than are either LCs or DDC-IDCs. Immature moDCs, LCs, or DDC-IDCs, were cocultured 1:1 with SKMel29 tumor cells that had been membrane-labeled with PKH67 and rendered apoptotic (~80% at onset of coculture) by mitomycin C exposure. At the end of a 24-h incubation at 37°C, cells were harvested, counterstained with anti-HLA-DR-PE and then gated for large FSC, HLA-DR-positive cells on a flow cytometer. The uptake by DCs in this gate of PKH67+ tumor cells, detected in the FITC channel, is shown along the x-axis (log10 fluorescence) for one representative experiment in A. Replicate MFIs for tumor cell uptake ± SD are shown in B (n = 10 experiments for moDCs, and n = 9 experiments each for LCs and DDC-IDCs), with p values for pairwise comparisons by the Wilcoxon rank sum statistic indicated on the figure.
never been loaded with apoptotic tumor cells. Target controls for tumor-loaded DCs were mock-loaded DCs, and controls for tumor cell targets were an HLA-A*0201-positive cell line bearing irrelevant tumor Ag (prostate cancer cell line, LnCAP). The SFCs induced by each of these control cultures were both subtracted from the SFCs generated by tumor-loaded DCs for plotting final results (Fig. 8).

In three independent experiments, both moDCs and LCs primed IFN-γ-secreting CD8+ T cells that were specific for the inciting tumor and tumor-associated Ags. Fig. 8 shows the averaged means of triplicate wells ± SEM from three separate experiments, after subtracting the SFCs of both sets of control replicates from the SFCs of experimental wells. The replicates from which the means were calculated for a single DC type were quite close within a single experiment. The difference between moDCs vs LCs was also clear within a single experiment, although LCs proved more variable relative to each other between different experiments. Despite the lower phagocytic activity of LCs compared with moDCs, the mean CD8+ T cell response primed by LCs cross-presenting tumor and tumor-associated Ag was ~4-fold greater than that primed by moDCs (p = 0.01 by Wilcoxon rank sum).

**FIGURE 8.** LCs more effectively cross-prime autologous T cells to secrete IFN-γ in response to melanoma Ags than do moDCs. Immature moDC and LCs generated from healthy donors were cocultured as above with dying SKMel29 tumor cells for 24 h, followed by maturation with the inflammatory cytokine mixture. These mature, tumor-loaded DCs were then cocultured with autologous T cells at a DC-T ratio of 1:100 for 7 days. Thereafter, the cells were harvested, washed, and restimulated for 5 additional days with fresh tumor-loaded DCs of the same type as used for the first stimulation at a DC-T ratio of 1:30. Exogenous cytokines were never added to these cultures. At the end of 12 days total, CD8+ T cells underwent positive immunomagnetic selection and were tested for IFN-γ secretion by rechallenge in ELISPOT assays with SKMel29 cells (n = 1 for the single donor who was HLA-A*0201-positive, and n = 2 for DCs of the same type used for the two rounds of stimulation and loaded with apoptotic SKMel29). The number of SFCs per 10^5 input CD8+ T cells was determined in triplicate and averaged for each experiment by an automated, standardized ELISPOT plate reader. Nonspecific IFN-γ release from controls for both the afferent stimulation and efferent target recognition (see text) were subtracted from the experimental values to give an adjusted number of SFCs/10^3 input CD8+ T cells, and these values are plotted as averaged triplicate means ± SEM from three independent experiments (p > 0.01 by Wilcoxon rank sum statistic). Although the LCs exhibited a greater variability than the moDCs, the middle value was from rechallenge of the HLA-A*0201-positive donor by the HLA-A*0201-positive SKMel29 cells, whereas the flanking values were from rechallenge by autologous LCs that had taken up dying SKMel29 cells.

**Discussion**

We have generated phenotypically and functionally distinct populations of LCs, DDC-IDCs, and moDCs in vitro using defined precursors and cytokines in the absence of FCS. LCs stimulate the greatest CTL activity, even without the bioactive p70 heterodimeric form of IL-12, whether passively presenting a viral recall antigenic peptide or cross-presenting tumor and tumor-associated Ag to autologous CD8+ T cells. Among a variety of parameters investigated to account for the superior activity of mature LCs in vitro, secreted IL-15 protein is the greatest and most significantly detected difference between LCs and the other two DC types under study. Our phenotypic and functional data also demonstrate that DDC-IDCs and moDCs can no longer be considered interchangeable, even though both develop from a CD14+ intermediate or precursor.

All three DC types express comparable maturation phenotypes, while other epitopes distinguish one from the other (summarized in Table I). All three types of DCs are also potent stimulators in allogeneic MLRs, even though moDCs require half log higher numbers than either LCs or DDC-IDCs to stimulate the same degree of T cell proliferation. This is despite somewhat greater purity of moDCs than LCs or DDC-IDCs, which should have favored the activity of moDCs in case of any counting errors when the same numbers of CD83+, HLA-DRbright DCs were added to allogeneic T cells.

The superior activity of LCs in stimulating autologous CD8+ CTL against a recall viral Ag was not the anticipated result. Direct influenza infection of the mature DCs, which is not as cytopathic as it is for immature DCs, enables all three to generate robust and equivalent CTL. This mitigates the differences observed when presenting a class I MHC-restricted peptide alone to a responder population containing both CD8+ and CD4+ T cells, perhaps by providing helper epitopes or by releasing type I IFNs in a paracrine fashion that could enhance activation of other DCs and equalize their activities (55). Although the starting population of T cells was ~90–95% CD3+, NK cells were not specifically depleted at the outset. Given the emerging role of DCs in stimulating NK cells (41, 56), perhaps virally infected DCs could stimulate NK cells to secrete additional cytokines that would enhance their own immunogenicity and help polarize toward type I immune responses (57, 58).

As noted above, LCs secrete significantly more IL-15 than either DDC-IDCs or moDCs, as well as somewhat more IL-1β, both of which are pertinent to the initiation of primary immune responses and the generation of primary and memory CD8+ CTL (59–62). Prior studies have shown that both LCs and DDC-IDCs express IL-15 mRNA (22), however, and a blocking mAb against IL-15 can inhibit some functions of moDCs (63). We therefore consider soluble IL-15 to be a surrogate measurement of bioactive IL-15, which functions at the membrane in picomolar concentrations in both cis and trans toward responder T cells (44), as a 10-fold concentration of the supernatants was required to detect IL-15 in our experiments (64). Our efforts using blocking mAbs to determine more precisely the contribution or hierarchy of individual cytokines did not yield clear results, perhaps reflecting the redundancy often found for factors supporting the immunogenicity of DCs. We also did not have convincing positive controls to confirm inhibition by the anti-cytokine mAbs. Similarly, attempts to ascribe normalization of virally infected LC and DDC-IDC activity to normalization of cytokine profiles did not yield a clear pattern, suggesting that other factors are operative.

Our data indicate that there is no absolute requirement for IL-12p70 in the generation of class I MHC-restricted CTL activity or
IFN-γ secretion by CD8+ T cells. Only moDCs secrete measurable IL-12p70, while LCs and DDC-IDCs secrete little to none at any time point during maturation, even after an optimal stimulus delivered by rhuCD40L, trimer (29, 42). We have corroborated similar patterns of IL-12p70 secretion by moDCs, but not LCs or DDC-IDCs, after maturation by inflammatory cytokines with PGE2 in the absence of FCS, although the amounts are about a log lower than after maturation by rhuCD40L (41). Mature LC and DDC émigrés from human skin also do not secrete IL-12p70 (42). LCs are nevertheless more potent in eliciting CTL after presenta- tion of a passively loaded peptide or after cross-presentation of Ag(s) taken up from dying tumor cells. These data lend further credence to recent commentary that the requirement for IL-12, while optimal for Th1 and CTL responses, may have been overemphasized by inadvertent detection of IL-12p40 rather than p70 (65).

The mechanics of these types of experiments unfortunately cannot account for cross-priming and -presentation of Ags restricted to class I MHC, as well as helper epitopes from autologous Ags (54) expressed by dying cells. LCs, even around type I adaptive immune responses (57, 66). Theoretically, this could also apply to early activation of NKT cells that would produce IFN-γ in response to glycolipid Ags presented by CD1d, which was solely expressed by moDCs and DDC-IDCs, but never by LCs (38).

Investigators are increasingly interested in cross-priming and cross-presentation by DCs for both immunity and tolerance (48–52, 67–69). This provides an important means for DCs to process and present exogenous Ags from the microenvironment, in addition to classic processing and presentation of endogenous Ags on class I MHC. Cross-priming and -presentation of Ag from a third party cell can also expand potential Ag sources, obviate the need to use defined peptides with known HLA-restrictions, and simultaneously provide epitopes for class I and II MHC presentation.

By quantifying the uptake of apoptotic tumor cells, we have shown that moDCs are efficiently phagocytic as expected, while LCs and DDC-IDCs are more indolent in this respect. LCs are on average 4-fold more potent than moDCs, however, in cross-priming autologous T cells to melanoma and associated Ags derived from apoptotic tumor cells without requiring exogenous cytokines. The mechanics of these types of experiments unfortunately cannot exclude the introduction of additional (e.g., allogeneic or even xenogeneic (cell lines cultured in the presence of FCS)) Ags from tumor cell lines. We assume these provided additional immunogenic Ags restricted to class I MHC, as well as helper epitopes presented on class II MHC to CD4+ T cells in the responder popu- lation. These experimental issues applied evenly to both moDCs and LCs, yet the LCs remained superior even after subtraction of controls for both the afferent stimulation and effector recognition.

Several points are therefore worth noting. Obviously, the balance between processing and presentation vs sequestration and degradation of endocytosed Ag is not entirely concordant, at least for moDCs (54). This may be one rationale underlying the benefit of targeting Ags to specific receptors or intracellular organelles for enhanced cross-presentation by DCs, especially human moDCs (51–54, 67, 68). LCs, which are less phagocytic than moDCs, may nevertheless acquire sufficient Ag for cross-presentation by this means, or by another process altogether that LCs may or may not share with other conventional DCs. The outcome of phagocytosis for cross-presentation may also differ from that of pinocytosis of soluble, fluid phase Ags (54).

Although our finding that LCs (and DDC-IDCs) do not express CD91 is far from a comprehensive assessment of heat shock protein receptors, Ag uptake by LCs at least does not occur by ac- quisition of gp96 (33) expressed by dying cells. LCs, even around day 11 before maturation, also express very little if any CD36 (not shown), which is the thrombospondin receptor expressed by immature moDCs that is involved in their uptake of dying cells (49). Hence, LCs deserve specific further study of the cell biology of Ag processing that occurs between Ag uptake and presentation, as has been focused on mouse DCs and human moDCs (67, 70–72).

Our findings that LCs have a superior capacity to stimulate CTL against a single peptide or cross-presented tumor and tumor-assoc- iated Ags in vitro help explain a published report that CD34+- derived DCs are superior to moDCs in stimulating an Ag-specific CD8+ T cell line (73). Such CD34+ HPC-derived DCs would have included LCs (6–8), and our results indicate that the stimulatory properties specifically exerted by LCs are equally true for circulating T lymphocyte responders as for T cell lines. Lastly, our results also support the findings of some investigators (24) asserting advantages to the administration of DC vaccines that include LCs, as opposed to those that use only moDCs.

Published and ongoing clinical trials using peptide-pulsed moDCs, which are more readily obtained than CD34+ HPC-de- rived DC populations have proven the efficacy of moDCs in stimulat- ing Ag-specific T cell responses in vivo (74, 75). The results reported here neither negate nor discount those important findings. Our results do, however, beg the question as to whether the differ- ences observed in vitro between mature LCs and either DDC- IDCs or moDCs remain valid in vivo. Functional differences iden- tified in this study in vitro could simply reflect a requirement for numerically more DDC-IDCs or moDCs than LCs to exert similar CTL stimulatory activity. Our data also suggest that methods of Ag loading, including those that provide helper epitopes and/or simulta- neously stimulate NK cells, merit further attention. On balance, conventional, myeloid-type DCs have not only shared but also distinct phenotypes and functions, which may prove physiologi- cally important to a multipronged activation of both innate and adaptive cellular immunity.

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CORRECTIONS


In Materials and Methods, in the third sentence of the third paragraph under the heading Cell purification and generation of DCs (Fig. 1), an error was made in the cytokine dosage. The dosage for FLT-3 ligand is nanograms per milliliters instead of micrograms per milliliters. The correct sentence is shown below.

Specific cytokine supplements used in common for generating both LCs and DDC-IDCs included GM-CSF (1000 IU/ml), TNF-α (5 ng/ml), c-kit ligand (20 ng/ml), and FLT-3 ligand (50 nanograms/ml).


In Materials and Methods, in the second sentence of the third paragraph under the heading Endothelial cell culture and transient transfection, the nucleotide sequence of ID3 shRNA is incorrect. The correct sequence is shown below.

TCGGATCCAACACTGCTACTCCCGCCTGTTCAAGAGACAGGCGGGAGTAGCAGTGGTTTTTTGGAAAA

GCTTGG.


In Results, the F4/80 B6 panel of Fig. 5A was inadvertently duplicated in the F4/80 lpr/lpr panel. The figure legend is correct as originally published and the data and conclusions in the manuscript are not affected. The correct figure is shown below.


The second author’s last name is misspelled. The correct name is Karen McConachie.