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Differential Development of Murine Dendritic Cells by GM-CSF versus Flt3 Ligand Has Implications for Inflammation and Trafficking[1]

Yuekang Xu,[2*§] Yifan Zhan,[†] Andrew M. Lew,[†] Shalin H. Naik,[†‡] and Michael H. Kershaw[2*§]

To gain ample numbers of dendritic cells (DCs) for investigation, or for immunotherapy, the culture of DC precursors from bone marrow in either GM-CSF and IL-4 (GM/IL4-DCs) or Flt3L (FL-DCs) has often been used. Despite their common use, the relationship of these culture-derived DCs to those in vivo, and their relative potential for use in immunotherapy, needs further elucidation. In this study we found that in contrast to FL-DCs, highly purified GM/IL4-DCs were larger and more granular, surface Mac-3$^+$, and were comprised of two populations (CD24^{low}CD11b^{high} and CD24^{high}CD11b^{low}). Functionally, although comparable in T cell activation, GM/IL4-DCs produced more inflammatory mediators including TNF-α, IL-10, CCL-2, and NO than FL-DCs upon TLR ligation. However, FL-DCs migrated more efficiently to draining lymph nodes after s.c. injection and produced a different profile of cytokines to GM/IL4-DCs. Developmentally, unlike GM/IL4-DCs, FL-DCs cannot be differentiated from CD11b^{high}Ly6C^{high}Ly6G$^-$ monocytes. Collectively, these data suggest that the GM/IL4-DCs are the equivalents of the TNF-α and inducible NO synthase producing DCs in vivo that emerge after inflammation whereas FL-DCs better represent the steady-state resident DCs. The differences between GM/IL4-DCs and FL-DCs have serious implications for DC-based immunotherapeutic strategies. *The Journal of Immunology,* 2007, 179: 7577–7584.

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Many studies have demonstrated the importance of dendritic cells (DCs)[3] in an immune response, which is ascribed largely to their unique ability to affect T cell fate. In the activated state, DCs present Ag to cognate naive or memory T cells and induce their proliferation and effector function. In the nonactivated state, DCs can induce T cell anergy or deletion to prevent autoimmunity. These aspects of DC biology have been embraced in a series of applications using DCs for active immunotherapy against cancer, infection, and autoimmune disease, with mixed success (1).

A variety of DC subsets exist in vivo as distinguished by cell surface markers. In the mouse, these subtypes can be broadly categorized into conventional DC (cDC) that already have dendritic form and exhibit DC functions in the steady-state, and the immediate precursors of DC (pre-DC) that can have their own intrinsic immune functions but require further development to become DC (2). Steady-state cDCs include the migratory DC (Langerhans cells and dermal DCs) and lymphoid tissue-resident DC (such as CD8$^+$ cDC and CD8$^-$ cDC). Types of pre-DC include plasmacytoid pre-DC (pDC) that, upon activation, secrete large amounts of type I IFN and convert into a DC form (3–5), as well as monocytes, which generate DCs in vivo in the presence of inflammation (6–8). In some cases, such monocyte-derived "inflammatory" DCs are thought to correspond to the TNF-α and inducible NO synthase-producing DCs (Tip-DCs) that emerge in certain inflammatory states after psoriasis (9) or infection with certain intracellular bacteria, such as *Listeria monocytogenes* (10).

The relative number of DCs in vivo is low compared with most other lineages, and their isolation in sufficient numbers for the clinic or for comprehensive studies can be logistically burdensome. Therefore, the majority of applications currently rely on the in vitro generation of DCs from blood monocytes, CD34$^+$ progenitors or bone marrow (BM) cells with the appropriate hemopoietic growth factors, usually GM-CSF plus IL-4 (11–16) or *fms*-like tyrosine kinase 3 ligand (Flt3L) (2, 17, 18). However, there are many distinct DC subtypes in the mouse, each specialized for a particular set of functions (2). It is therefore important to determine how various cytokine-cultured DCs reflect individual DC subsets in vivo. Recently, the culture of BM with Flt3L was reported to generate three distinct DC subtypes that were functionally and phenotypically equivalents to steady-state DCs in vivo, namely pDC, CD8$^+$ cDC and CD8$^-$ cDC (18). However, the in vivo counterpart of GM-CSF/IL-4-derived-DC (GM/IL4-DCs), and how they compare with Flt3L-derived DCs (FL-DCs), is still not clear. In addition, culture in alternative cytokines can produce DCs with distinct characteristics that may differ in their utility for therapeutic purposes. Which of these two most popular in vitro culture systems gives rise to a more suitable DC form for use in particular therapeutic applications also needs clarification.

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[3] Abbreviations used in this paper: DC, dendritic cell; cDC, conventional DC; pre-DC, precursors of DC; pDC, plasmacytoid DC; Tip-DC, TNF-α and inducible NO synthase-producing DCs; BM, bone marrow; Flt3L, *fms*-like tyrosine kinase 3 ligand; GM/IL4-DC, DC derived from GM-CSF/IL-4 culture; FL-DC, DC derived from Flt3L culture.

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In this study, we performed a side-by-side comparison between GM/IL4-DCs and FL-DCs and found several striking differences. These included differences in cell morphology; the profiles of cytokine, chemokine, and inflammatory mediator secretion; ability to migrate to lymphoid organs; and developmental origin. We also found that GM/IL4-DCs display features akin to Tip-DCs. Thus, we propose in vitro GM/IL4-DCs represent inflammatory DCs normally found in vivo. Both GM/IL4-DCs and FL-DCs have their unique characteristics and therapeutic potential.

Materials and Methods

Mice

C57BL/6 and BALB/c female mice, aged 7–11 wk were obtained from The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, and housed in a specific pathogen-free environment in the Peter MacCallum Cancer Centre (PMCC) animal facilities. All procedures conducted on mice were in accordance with the conditions specified by the PMCC Animal Experimentation Ethics Committee.

Cell preparation and cultures

BM cells from C57BL/6 mice were isolated by flushing femurs with 5 ml PBS supplemented with 2% heat-inactivated FBS (ThermoTrace). The BM cells were centrifuged once and then resuspended in tris-ammonium chloride at 37°C for 3 min to lyse RBC. The cells were centrifuged again and then strained through a 70-μm filter before being resuspended in RPMI 1640 supplemented with 2.5 mM HEPES, 5.5×10^{-5} M 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM glutamine, and 10% FBS. For GM/IL4-DC culture, BM cells were resuspended at 1×10^6/ml containing 10 ng/ml GM-CSF (supernatant derived from a murine erythroleukemia cell line stably transfected to express mouse GM-CSF, a gift from Dr. Helene Martin (Walter and Eliza Hall Institute for Medical Research, Australia)) and 0.3 ng/ml IL-4 (supernatant from X63/0 hybridoma cells stably transfected with an IL-4 expression vector, a gift from Dr. Andreas Strasser (Walter and Eliza Hall Institute for Medical Research, Australia)) and seeded at 5 ml/well in 6-well tissue culture plates. After 3 days, the cells were passaged 1/2 in fresh medium with cytokines added and replated. Loosely adherent cells were then collected on day 6 and replated in fresh medium before being harvested on day 7. In some experiments, as indicated, DCs were cultured as above but in medium containing GM-CSF but lacking IL-4 to produce GM-DCs. For FL-DC culture, BM cells were resuspended at 2×10^6/ml in medium containing 200 ng/ml human recombinant Flt3L (Amgen), plated at 5 ml/well in 6 well plates and cultured for 9 days without disturbing. Maturation of the DCs was induced by adding LPS (Sigma-Aldrich) at 1 μg/ml during last 20 h of culture. All cells were incubated at 37°C with 10% CO_2.

Flow cytometry

In vitro generated DCs were incubated with the rat anti-mouse FcγRII/FcγRIII mAb (2.4G2) for 15 min at 4°C, to block nonspecific binding of Abs, before staining with varying combinations of mAbs to CD11c (N418-PE-Cy5.5), CD11b (M1/70-FITC), B220 (RA3–6B2-APC), CD24 (M 1/69-PE), Ly6G (1A8-PE), Mac-3 (M3/84-PE), I-A/I-E (2G9-FITC), Ly6C (AL-21-biotin), CD86 (GL1-biotin), CD40 (3/23-biotin), and with second-stage staining with streptavidin-FITC (BD Pharmingen). Cell analysis was performed on an LSR flow cytometer (BD Biosciences).

Morphological analysis

Cells from GM-CSF/IL4 or Flt3L cultures were positively selected by magnetic separation using CD11c MACS beads (Miltenyi Biotec) according to the manufacturer's instructions. Purified GM/IL4-DCs and FL-DCs were centrifuged at room temperature onto slides at 100,000 per slide. Slides were air-dried and stained with May Grünwald Giemsa for morphological analysis. Observations of cells were made by means of an inverted microscope (Zeiss, 73447 Oberkochen, Germany) at 400× magnification.

In vitro stimulation and quantitation of cytokine production

MACS-purified GM/IL4-DCs, FL-DCs, or sorted splenic DCs were resuspended at 0.5×10^6/ml in fresh medium in the presence or absence of a single TLR agonist. The following panel of TLR agonists was used: Pam3Cys (1 μg/ml) (Invivogen), ODN 1826 (2 μg/ml) (Coley Pharmaceutical), Poly I:C (50 μg/ml), and LPS (1 μg/ml) (Sigma-Aldrich). DCs were plated at 1 ml/well in 48-well plates and cultured for 20 h before supernatants were collected and analyzed for TNF-α, IL-12p70, IL-10, RANTES/CCL5, MCP-1/CCL-2 using ELISA according to the manufacturer's instructions (BD Biosciences).

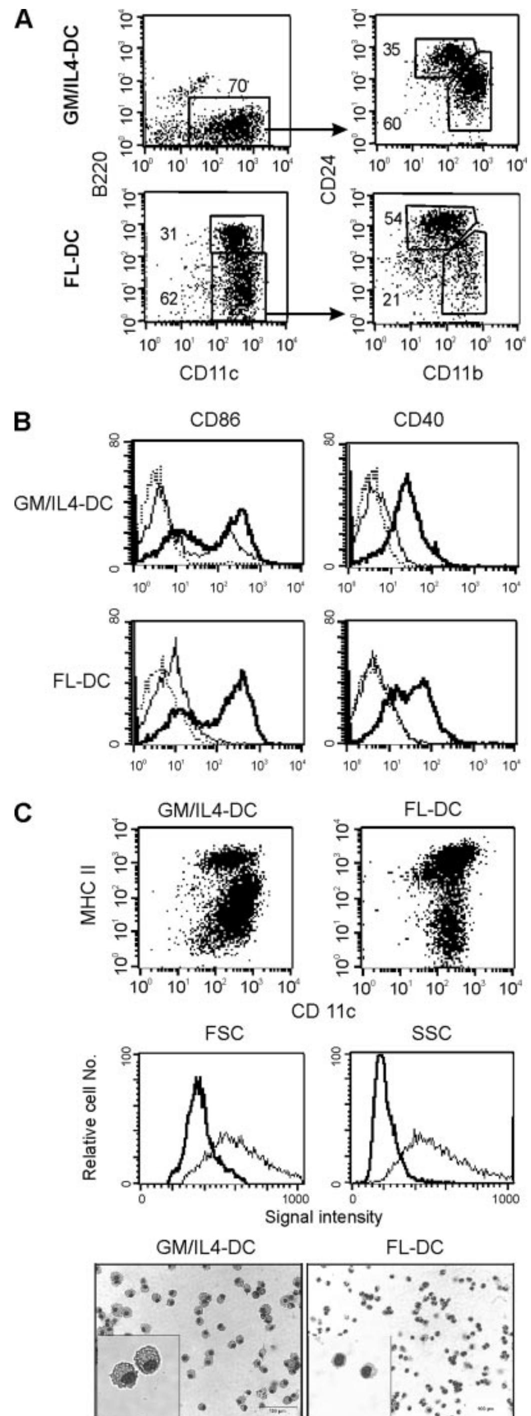

FIGURE 1. Phenotypes of DCs generated from the two different culture systems. *A*, BM-derived DCs cultured in presence of GM-CSF/IL4 (GM/IL4-DC) or Flt-3 ligand (FL-DC), as described in *Materials and Methods*, were harvested and stained with mAbs specific for the indicated cell surface markers. Numbers depict the percentage of total cells within the respective regions. *B*, Cells were also gated on the $CD11c^+$ population and their expression of T cell costimulatory molecules determined in the presence (thick line) or absence (thin line) of LPS using flow cytometry. The dotted line represents the background staining using an isotype control Ab. *C*, The MHCII vs CD11c expression, size, and granularity (thin line for GM/IL4-DCs and thick line for FL-DCs) of purified DCs were examined by FACS after dead cell exclusion or cytospins prepared and examined by light microcopy (3× magnified portions were embedded in the pictures, and Bar = 100 μm). All experiments were performed at least four times with similar results.

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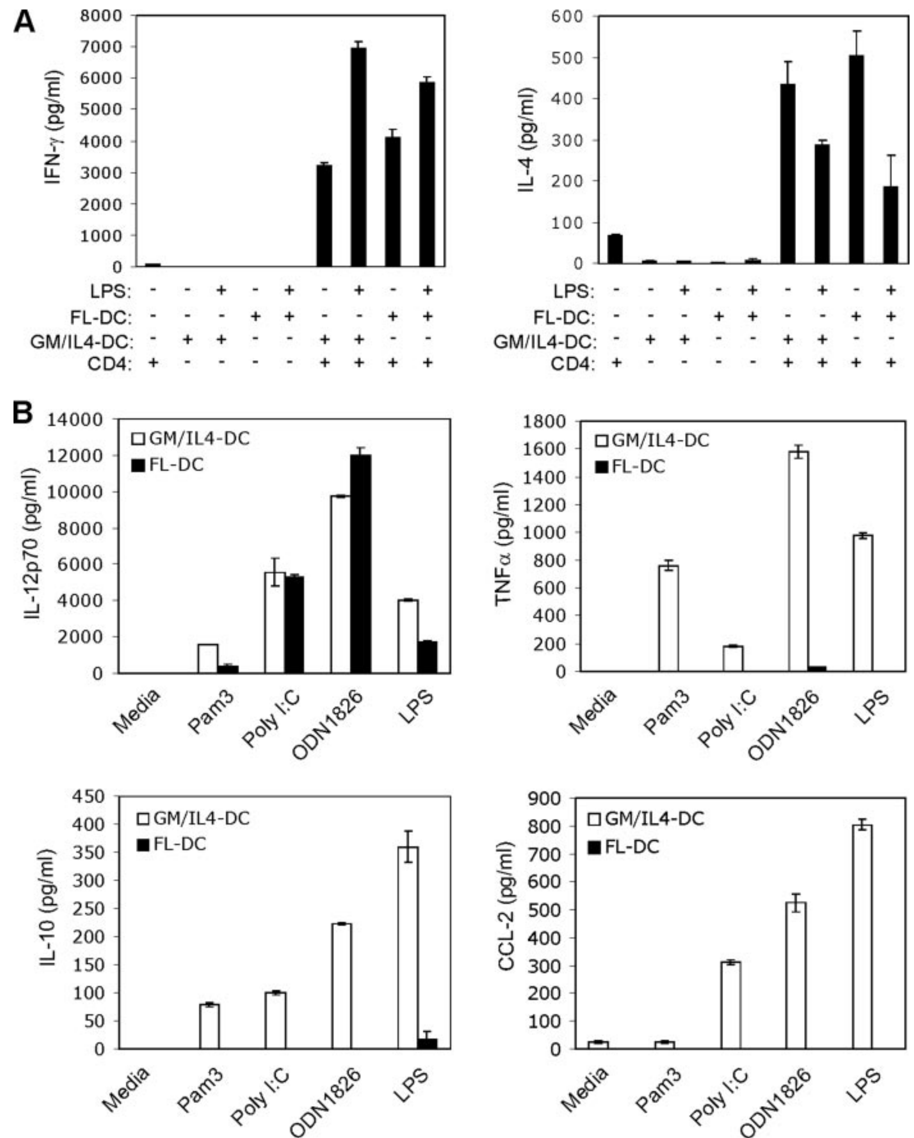

FIGURE 2. T cell cytokine profiles of GM/IL4-DCs and FL-DCs. *A*, 1×10^5 purified $CD4^+$ T cells from BALB/C mice were mixed with 1×10^4 purified DCs from C57BL/6 mice and cultured for 72 h. The level of IFN-γ and IL-4 in the supernatants was assayed using ELISA. Values are the average ± SD of duplicate wells, and are representative of four independent experiments performed. *B*, Highly purified DCs were incubated with or without the indicated TLR agonists for 20 h. Supernatants were analyzed for the indicated cytokines or chemokines using ELISA. Values are the average ± SD of duplicate wells. The results are representative of five independent experiments.

For T cell cytokine quantitation, purified $CD4^+$ T cells from BALB/c mice were resuspended in supplemented RPMI at 1×10^6 and added to round-bottom 96-well plate at 100 μl/well in the presence of 1×10^4 in vitro cultured DCs from C57BL/6 mice. The mixture of cells was cultured for 3 days before supernatants were collected for IL-4 and IFN-γ analysis by ELISA (BD Biosciences).

NO measurement

Highly purified GM/IL4-DCs or FL-DCs were added into 96-well round-bottom plate (2×10^5/well) in a titration of LPS, and incubated for 48 h at 37°C. NO production was assayed by measurement of the nitrite concentration in the cell culture using the Griess assay (19). In brief, supernatants (100 μl) were added to 100 μl of a 1/1 mixture of 1% sulfanilamide dihydrochloride and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4. Plates were incubated at room temperature for 30 min and the absorbance at 550 nm was measured with a microplate reader. Nitrite concentrations were calculated using a sodium nitrite standard curve as reference.

Listeria monocytogenes *infection*

Mice were injected i.v. with 1×10^4 *Listeria monocytogenes* for 24–48 h. Spleens from infected and uninfected mice were digested for 20 min at room temperature with collagenase-DNase and then treated for 5 min with EDTA to disrupt T cell-DC complexes. Light density cells (<1.077 g/cm^3 at mouse osmolarity) were selected by centrifugation in a Nycodenz (Nycomed Pharma AS) medium (20). Selected cells were stained for CD11c and CD11b, washed, and subjected for analysis and sorting on FACSAria (BD Biosciences).

In vitro differentiation of inflammatory monocytes

Freshly isolated BM cells from C57/BL6 mice were depleted of B cells and DCs by CD19/CD11c MACS Beads (Miltenyi Biotech). Inflammatory monocytes were sorted by gating on $Ly6G^-$ $CD11b^{high}$ $Ly6C^{high}$ population. Highly purified inflammatory monocytes were then cultured at 1×10^6 cell/ml in 96-well plates in the absence or presence of GM-CSF/IL-4 or Flt3L at the same concentration as mentioned above for GM/IL4-DCs or FL-DCs cultures. Cells were harvested at indicated time and the kinetics of CD11c vs MHCII expression were examined by FACS.

DC trafficking

To analyze the migration of in vitro cultured DCs in vivo, 1×10^6 cultured DCs were labeled with CFSE at 2.5 μM at 37°C for 8 min and then washed three times with PBS before being injected into the footpad of syngeneic C57BL/6 mice. From 4 h onwards after DC injection, mice were culled and skin in the injection areas, and the draining popliteal lymph node were extracted, minced into small fragments, and treated with collagenase-IV and DNase at 37°C for 30 min. Cells were passed through a 70-μm mesh, washed in PBS, and stained with propidium iodide to distinguish viable cells before flow cytometric analysis.

Statistics

The nonparametric Mann-Whitney *U* test was used to determine the significance of observed differences using StatsDirect software. A *p* value <0.05 was considered significantly different.

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Results

Different types of DCs are generated from GM-CSF/IL-4 and Flt3 ligand cultures

DCs derived from GM-CSF/IL-4 or Flt3L cultures were first characterized for phenotypes. Both cultures were assessed for CD11c, B220, CD24, and CD11b expression, markers known to distinguish the DC subtypes in culture. FL-DC cultures contained three distinct subtypes; B220$^+$ pDC, CD24^{high}CD11b^{low} DCs (CD8$^+$ cDC equivalents), and CD24^{low}CD11b^{high} DCs (CD8$^-$ cDC equivalents) (Fig. 1*A*), as previously described (17, 18, 21). Within GM/IL4-DC cultures, most DC were B220$^-$, so did not contain any pDC, and the majority were CD24^{low}CD11b^{high} DC. GM/IL4-DCs are often referred to as "myeloid" DC because of this CD11b^{high} phenotype (Fig. 1*A*). We did note a population of CD24^{high}CD11b^{low} GM/IL4-DCs in smaller percentage, although this population was not further dissected in this study.

T cell costimulatory molecules such as CD86 and CD40 are important activation markers for DC, and their up-regulation in response to bacteria product LPS stimulation defines different functional properties of the APCs. Fresh culture-derived DCs, and those stimulated 1 day prior with LPS were harvested and stained with Abs specific for the above-mentioned molecules for FACS analysis. After gating on the CD11c$^+$ population, GM/IL4-DCs have slightly more mature population than FL-DCs in terms of CD86 expression although both types of DCs have negligible expression of CD40. Following LPS activation, however, enhanced expression of these T cell costimulation markers was readily observed in both BM-derived DCs to a comparable level (Fig. 1*B*).

Because cells of lineages other than DCs, such as macrophages and monocytes, can also survive in these cytokine-supplemented cultures, we enriched for CD11c$^+$ cells by MACS to restrict our investigation to DCs for all subsequent experiments. The purity of CD11c$^+$ DCs after enrichment was >98% (data not shown). Enriched DCs contained both immature (MHCII^{low}) and mature (MHCII^{high}) DCs (Fig. 1*C*). Interestingly, GM/IL4-DCs were notably larger and more granular than FL-DCs as determined by flow cytometry (Fig. 1*C*). By light microscopy, the larger size appeared to be due more abundant cytoplasm (Fig. 1*C*). These results highlighted that GM/IL4-DCs and FL-DCs were different not only by surface phenotype, but also by morphology.

Different cytokine profiles of GM/IL4-DCs and FL-DCs following TLR ligation

An important characteristic of DCs is their ability to elicit cytokine secretion from T cells, which can vary according to the DC subset and their activation status. Because cytokine secretion by CD4$^+$ T cells is a consequence of DC stimulation, we determined the levels of both Th1 (IFN-γ) and Th2 cytokines (IL-4) in a MLR as a readout of T cell-stimulating ability of the two types of DCs. T cells and DCs by themselves did not secret IFN-γ, and only a limited amount of IL-4 was detected in the T cell culture alone. After coculture with DCs, however, both GM/IL4-DCs and FL-DCs could induce CD4$^+$ T cells to secrete both the Th1 cytokine IFN-γ and the Th2 cytokine IL-4. Activation of the DCs by LPS significantly increased their ability to influence T cell function, as determined by the increase of IFN-γ ($p = 0.043$ for GM/IL4-DCs; $p = 0.029$ for FL-DCs) and reduction of IL-4 ($p = 0.021$ for GM/IL4-DCs; $p = 0.038$ for FL-DCs) secretion from CD4$^+$ T cell. However, there was no significant difference between GM/IL4-DCs and FL-DCs in eliciting IFN-γ and IL-4 production from CD4$^+$ T cells either before or after LPS activation. (Fig. 2*A*).

Cytokine and chemokine production in response to TLR ligation is another important function of DCs that can differ between different DC types. We therefore determined the relative ability of GM/IL4-DCs and FL-DCs to secrete a variety of cytokines and chemokines in response to a range of TLR agonists. We found that both GM/IL4-DCs and FL-DCs secreted heterodimeric IL-12 p70 in response to TLR agonists (Fig. 2*B*), but the amount was not significantly different between GM/IL4-DCs and FL-DCs ($p = 0.315$). However, in the same culture supernatants after TLR stimulation, significant differences were found between GM/IL4-DCs and FL-DCs in their production of TNF-α ($p < 0.021$) and IL-10 ($p < 0.02$) for all four TLR agonists. GM/IL4-DCs secrete both the Th1 cytokine TNF-α and the Th2 cytokine IL-10 with all four TLR agonists, while FL-DCs did not secrete any substantial levels of TNF-α or IL-10. For chemokine secretion, both GM/IL4-DCs and FL-DCs were able to secrete RANTES in response to stimulation through TLRs (data not shown), but only GM/IL4-DCs produced CCL-2, the ligand for CCR-2, in comparison to FL-DCs ($p < 0.002$) (Fig. 2*B*). Thus, in response to TLR stimulation, GM/IL4-DCs secreted a distinct set of mediators, TNF-α, IL-10, and CCL-2, which were not produced by FL-DCs.

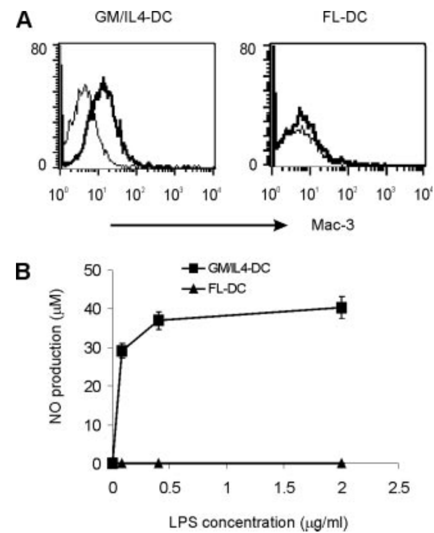

FIGURE 3. Tip-like DC features on GM-CSF/IL-4 cultured DCs. *A*, DCs cultured in either GM-CSF/IL4 or Flt3L were enriched by MACS beads for the CD11c$^+$ population and stained for Mac-3 expression (thick line). The thin line represents background staining of an isotype control Ab. The experiments were repeated three times with similar results. *B*, NO production by the purified DCs was also determined following incubation with the indicated concentration of LPS for 24 h. Shown here is the result ± SD of duplicate wells in one experiment, which is representative of four independent experiments.

Because culture of DC precursors in GM-CSF and IL-4 can generate a mixed population of immature and relatively mature DCs, we were also interested to determine how DCs generated in GM-CSF alone (GM-DCs) (a less mature population) compared functionally with FL-DCs. Following stimulation with a panel of TLR agonists, GM-DCs were also found to secrete greater amounts of TNF-α, IL-10, and CCL-2 than FL-DCs (data not shown).

GM/IL4-DCs share characteristics of inflammatory Tip-DCs

The higher level of TNF-α production by GM/IL4-DCs indicated that they might represent the Tip-DCs, a type of inflammatory DCs that is induced after infection with *Listeria monocytogenes* (10). Such DCs and similar DC types generated from monocytes in inflammatory conditions (6) have an unique CD11c^{int}CD11b^{high}Mac-3$^+$ phenotype. When examined for surface expression, GM/IL4-DCs were

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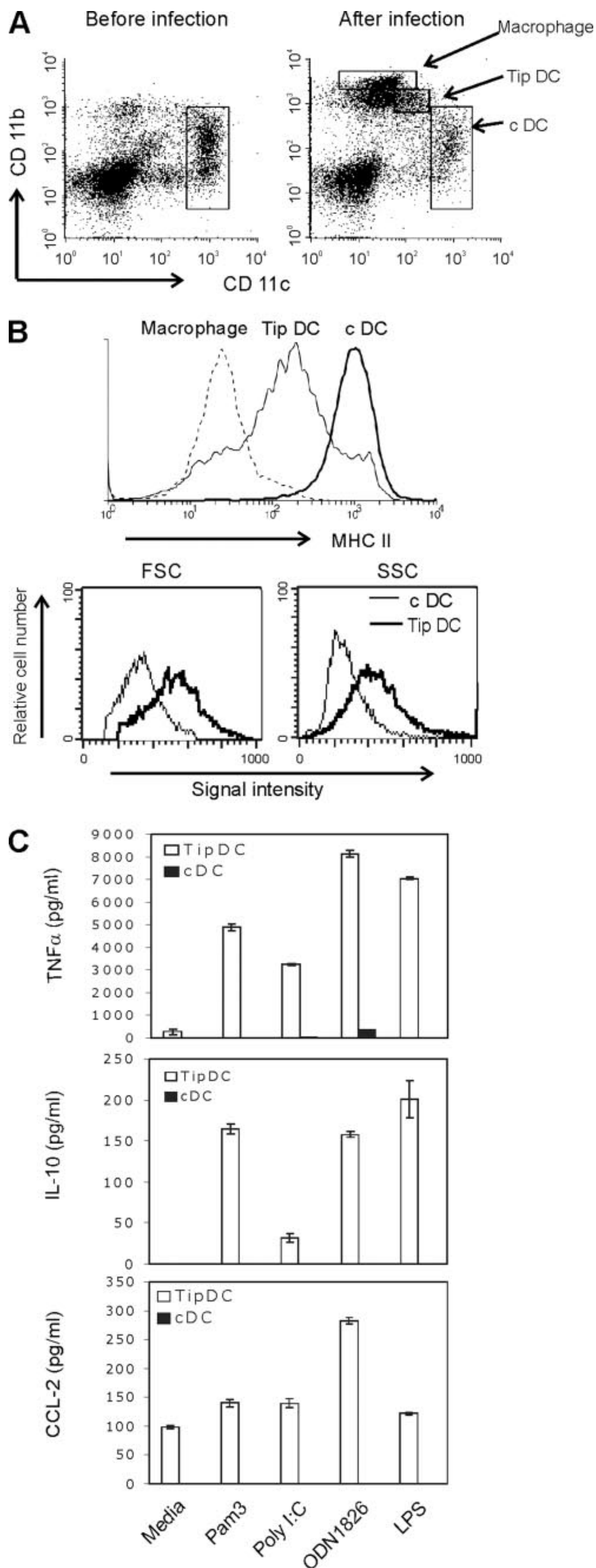

FIGURE 4. Isolation and cytokine profiles of splenic Tip-DC and cDC. Mice were infected with 10000 *L. monocytogenes*, and spleens were harvested at 24–48 h postinjection and collagenase digested. *A*, CD11b and CD11c expression on live splenocytes before and after *L. monocytogenes* infection. Regions were depicted to denote the gates used for sorting of Tip-DC and cDC respectively. *B*, Sorted Tip-DC, cDC, and macrophage were examined for MHCII expression. The size and granularity of Tip-DC (thick line) and cDC (thin line) following incubation in culture medium without cytokine overnight were determined by FACS. *C*, Cytokine secretion of sorted Tip-DC and cDC was detected by ELISA after stimulation with the indicated TLR agonists for 16–20 h. All results are representative of three independent experiments, and values shown are mean ± SD of duplicate wells.

found to express surface Mac-3. FL-DCs, in contrast, were mostly Mac-3^- (Fig. 3*A*).

The induction of NOS expression by LPS on the two types of DCs was also examined by measuring the generation of its product, NO. Highly purified DCs were stimulated with different concentrations of LPS for 48 h, and then NO production was measured in culture supernatants by using Griess reagent. Neither GM/IL4-DCs nor FL-DCs produced NO above background in the absence of exogenous stimuli. However, upon stimulation by LPS, nitrite was produced by GM/IL4-DCs in a dose-dependent manner (Fig. 3*B*). By contrast, FL-DCs did not produce any NO at any concentration of LPS tested ($p = 0.0002$). Collectively, these data suggest that GM/IL4-DCs demonstrate features characteristic of Tip-DCs.

Tip-DCs secrete similar cytokines to GM/IL4-DCs upon TLR stimulation

To directly test our hypothesis that, in contrast to FL-DCs (the representative of steady state DCs in vivo), GM/IL4-DCs are more like Tip-DC in vivo that are induced under inflammatory conditions, mice were injected with *Listeria monocytogenes* for 24–48 h to generate Tip-DCs as described (10). Twenty-four hours after infection of *Listeria monocytogenes*, a much increased population of $CD11c^{int}$ $CD11b^{high}$ $MHCII^+$ was confirmed in the spleen (Fig. 4, *A* and *B*, *top panel*). Further analysis demonstrated that this population was Mac-3^+ and secreted NO as expected, while cDC was not (data not shown), supporting its Tip-DC identity. Like cDC, the sorted Tip-DC also displayed dendrite-like shape under the microscope. However, Tip-DCs were larger and more granular than cDCs by FACS (Fig. 4*B*, *bottom panel*), as was observed with GM/IL4-DCs compared with FL-DCs (Fig. 1*C*, *middle panel*).

Finally, the cytokine profiles that were unique to GM/IL4-DCs in comparison to FL-DCs (TNF-α/IL10/CCL-2) were compared between Tip-DCs and splenic cDCs following TLR agonist stimulation. Besides producing TNF-α as previously reported, Tip-DCs were also found to produce significantly greater amounts of IL-10 and CCL-2 than splenic cDCs ($p < 0.02$) (Fig. 4*C*). Thus, the novel finding of similar inflammatory mediator profile between Tip-DC and GM/IL4-DC further suggested that they are equivalent cell types.

GM/IL4-DC, but not FL-DC, developed from $CD11b^{high}Ly6C^{high}Ly6G^-$ monocytes

The striking difference between the DCs derived from these two cytokine-supplemented culture systems prompted us to examine the potential for different developmental origins. Because $CD11b^{high}Ly6C^{high}$ monocytes in the BM are reported to be precursor cells for the inflammatory Tip-DCs (22), we reasoned that GM/IL4-DC could also come from the same parental cells that generated Tip-DCs. To isolate such monocytes, BM cells were depleted of cells expressing CD19 (B cells) and CD11c (DCs), gated on SSC^{low} and $Ly6G^-$ cells, and then sorted for $CD11b^{high}Ly6C^{high}$ monocytes to 99% purity (Fig. 5). In the presence of GM-CSF/IL4, $CD11b^{high}Ly6C^{high}$ monocytes developed into $CD11c^+MHCII^+$ DCs over time. In contrast, the cells died by

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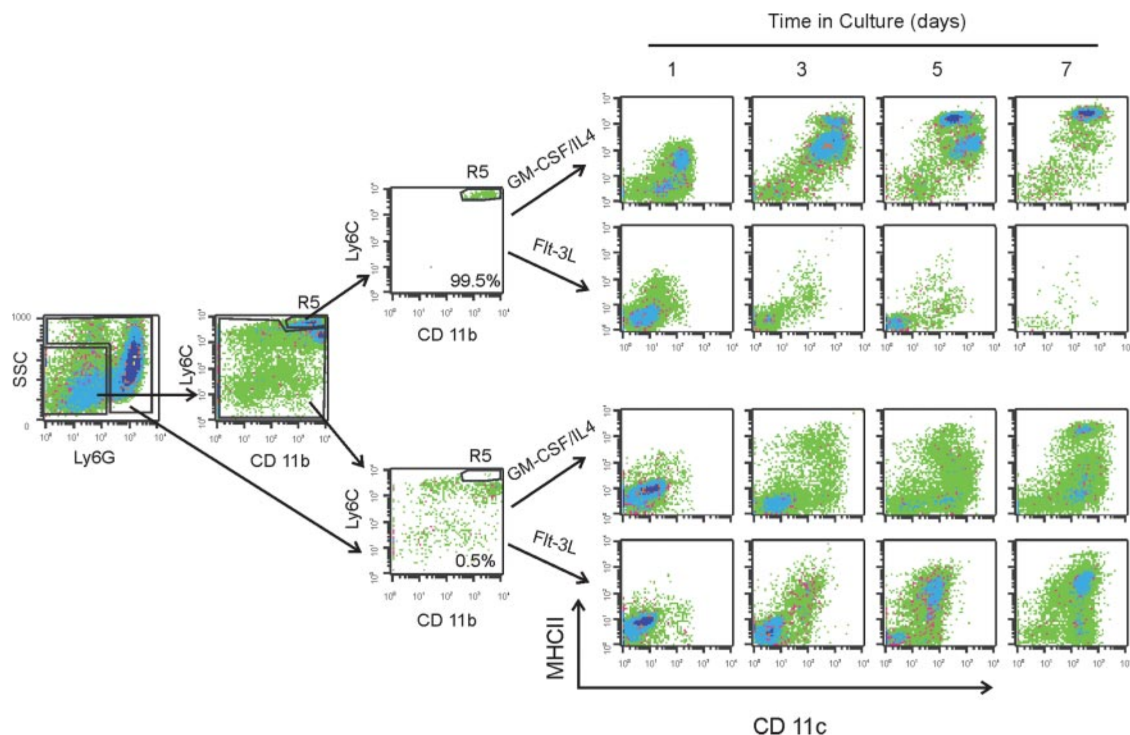

FIGURE 5. GM/IL4-DCs, but not FL-DCs, are developed from inflammatory monocytes in BM. Inflammatory monocytes were sorted by gating on Ly6C^{high} CD11b^{high} Ly6G^{-} population from freshly isolated BM cells after DC and B cell depletion. Both sorted and remaining cells in the BM were then cultured in presence of either GM-CSF/IL4 or Flt3L. Kinetics of CD11c vs MHCII expression on the cultured cells was examined by FACS at indicated time points. The percentage in the figure represented the percentage of Ly6C^{high}CD11b^{high} population (R5). Two independent experiments were performed with similar results.

days 4–7 under Flt3L, presumably from "neglect", as this reduced viability was similar to that observed in medium alone (data not shown). By day 7 of GM-CSF/IL4 culture, a much purer population of CD11c^{+}MHCII^{+} DCs was formed. Phenotypic examination showed that they were B220^{-} Mac-3^{+} and NO-secreting in response to LPS stimulation (data not shown); this was thus similar to GM/IL4-DC developed from whole BM cells (Fig. 5, *upper panel*). Interestingly, the culture of cells other than CD11b^{high}Ly6C^{high} with GM-CSF/IL4 led to the generation of a similar population of CD11c^{+}MHCII^{+} DCs, but with slower kinetics compared with the culture of CD11b^{high}Ly6C^{high} cells. This was presumably from yet earlier nonmonocyte progenitors. In Flt3L culture, however, the development patterns of CD11b^{high}Ly6C^{high}-depleted BM cells are strikingly different from that of Flt3L-cultured CD11b^{high}Ly6C^{high} monocytes in that they are differentiated normally into CD11c^{+}MHCII^{+} DCs from day 7 (Fig. 5, *bottom panel*). These results suggested that GM/IL4-DCs and FL-DCs are of different precursor origins. Therefore, not only have we demonstrated the functional similarity between GM/IL4-DC and Tip-DC, but also we unveiled a developmental linkage between GM/IL4-DC and Tip-DC. Moreover, the different lineage between GM/IL4-DC and FL-DC might explain their distinct functions.

FL-DCs migrate more effectively to draining lymph nodes whereas GM/IL4-DCs remain at the site following s.c. injection

An important question with regard to the therapeutic use of DCs (as tolerance or immune induction presumably occurs in the lymphoid organs) is how well they migrate following injection. We therefore compared the trafficking of GM/IL4-DCs and FL-DCs after s.c. injection. DCs were labeled with the fluorescent dye CFSE and injected into the footpads of syngeneic mice. When samples of the injection site were analyzed following tissue dis-

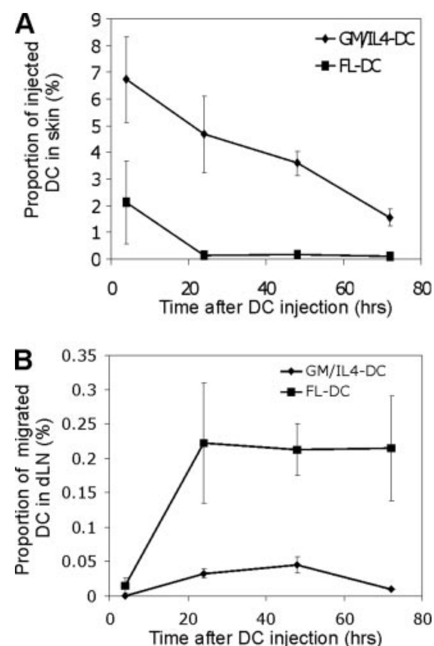

FIGURE 6. DC trafficking after s.c. injection. *A*, Skin retention. One million DCs cultured in presence of either GM-CSF/IL-4 or Flt3L were labeled with CFSE and injected into footpads of syngeneic mice. At the indicated time points after DC injection, the skin at the injection area was taken and digested with collagenase and DNase for 2 h. The resulting cell suspension was stained with propidium iodide to determine viable cells before FACS analysis. The percentage of live cells that was CFSE^{+} over total skin cell population is shown. *B*, Migration to draining lymph node. DCs were labeled and injected as above, and the percentage of CFSE^{+} cells over total cells in draining popliteal lymph node determined at different times after DC injection. Values shown are the mean ± SEM of four experiments.

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sociation, it was clear that GM/IL4-DCs were preferentially retained at the injection site when compared with FL-DCs (Fig. 6*A*) beyond 24 h after injection ($p = 0.03$). In contrast, FL-DCs quickly moved to the draining lymph nodes by 24 h and remained in that tissue at least for 72 h (Fig. 6*B*), whereas GM/IL4-DCs had a much lower efficacy in this migration ($p = 0.028$).

Discussion

In this study, we provided evidence that DCs derived in vitro with GM-CSF/IL-4 are the equivalents of the induced "inflammatory" or Tip-DCs in vivo, rather than those of the steady-state DCs. We demonstrated this by their equivalent features in phenotype, morphology, function, and developmental origin. DCs generated with Flt3L, in contrast, are distinct from GM/IL4-DCs by these measures and better represent the resident DC subtypes, as previously shown (18). These points are important to note case-by-case, when considering the source of DCs for investigation. Additionally, while both of these in vitro-derived DCs can stimulate T cells to a comparable level, several features including cytokine secretion and migratory ability differ significantly between GM/IL4-DCs and FL-DCs. This may be important when making the choice of DCs in the context of human immunotherapy.

Several lines of evidence, both published and now described in this study, support the relationship between GM/IL4-DCs and inflammatory/Tip-DCs. First, the "inflammatory" DCs that emerge after GM-CSF-dependent inflammation (6) or infection with *Listeria monocytogenes* (Tip-DCs) (10) can be distinguished readily by their $CD11c^{+}CD11b^{high}Mac\text{-}3^{+}$ phenotype (10). Our observations that GM/IL4-DCs are also $CD11c^{+}CD11b^{high}Mac\text{-}3^{+}$, and secrete TNF-$\alpha$ and NO, suggest they are likely to be the in vitro equivalents of such inflammatory Tip-DC, rather than the equivalents of any resident DC subtype. Second, serum GM-CSF levels are distinctively absent in the steady-state (23–25), but rapidly increase after infection with *Listeria* at a time when Tip-DCs emerge (23). Thus, the inflammatory nature of this cytokine during *Listeria* infection fits with its ability to drive GM/IL4-DC development in vitro. Third, we show in this study that GM/IL4-DCs, but not FL-DCs, can be developed from highly purified $CD11b^{high}Ly6C^{high}$ $Ly6G^{-}$ monocytes, the same precursors for Tip-DCs (10, 22) and inflammatory DCs in vivo (6, 26), demonstrating not only a developmental linkage between Tip-DC and GM/IL4-DCs, but also separated developmental pathways for GM/IL4-DCs and FL-DCs. This is consistent with the published observation that $Ly6C^{high}$ monocytes are the final precursor stage en route to generation of GM-CSF-cultured DCs from total BM (27, 28), but not for resident cDCs in vivo (6, 29, 30). Our finding that GM/IL4-DCs could also be generated from culture of cells other than $CD11b^{high}Ly6C^{high}$ cells but with slower kinetics, suggests that these cells, in response to GM-CSF/IL4, gave rise to $CD11b^{high}Ly6C^{high}$ cells, which in turn developed into DCs, which is consistent with a previous study (27). Fourth, GM/IL4-DCs and Tip-DCs have the same signature cytokine profiles, an indispensable function for the inflammation-induced DCs. Finally, our hypothesis for GM/IL4-DCs as an in vitro counterpart of Tip-DCs fits well with mouse in vivo data. GM-CSF and GM-CSF receptor null mice have normal steady-state resident DC numbers (31) and normal FL-DC generation (32), which suggests GM-CSF is not an obligatory cytokine for steady-state DC. By contrast, defects in the Flt3L pathway lead to reduced steady-state DC numbers (33, 34), but have normal monocyte numbers (35) and in vitro GM/IL4-DC generation (34). It will be interesting to explore the role of GM-CSF in inflammatory DC generation. Preliminary evidence suggests GM-CSF receptor$^{-/-}$ monocytes effectively migrate to the spleen but have defects in MHC II up-regulation and T cell activation during inflammation (Naik, S.H., unpublished observation).

That GM/IL4-DCs and Tip-DCs may represent a different branch from the steady-state DC subtypes raises another issue: What is the relationship between lymphoid-tissue resident $CD8^{-}$ cDCs and in vitro-derived GM/IL4-DCs? These DC subtypes are often considered as equal and referred to as "myeloid" DC due to their $CD11b^{high}$ phenotype, although little direct evidence links these two cell types. We observed that all FL-DC subtypes (of which the $CD8^{-}$ cDC equivalents are one type), were much smaller in size and had a reduced granularity compared with GM/IL4-DCs. Moreover, GM/IL4-DCs (present study) and Tip-DCs (10) can produce TNF and NO in response to LPS, whereas $CD8^{-}$ cDC (36, 37), and their FL-DC equivalents (present study) cannot. Finally, and as already mentioned, the precursors differ between the DC types: Tip-DC/GM/IL4-DCs derive from $CD11b^{high}$ $Ly6C^{high}$ monocytes, whereas $CD8^{-}$ cDCs derive from a non-monocyte pre-cDC in vivo (6, 38), and a similar precursor for their equivalents in Flt3L cultures (Fig. 5, *bottom panel*). Further work will be required to dissect whether $CD8^{-}$ cDCs and GM/IL4-DCs/Tip-DCs represent the same, related, or unrelated subtypes.

Interestingly, both GM/IL4-DCs and Tip-DCs secrete CCL-2 following stimulation via TLRs, whereas FL-DCs and cDCs did not. CCR-2, the receptor for CCL-2, is expressed on $CD11b^{high}Ly6C^{high}$ monocytes, and important for their recruitment for Tip-DC generation (10, 22). Local secretion of CCL-2 by the Tip-DCs could provide an important self-amplification loop in recruitment of more Tip-DCs and/or their precursor cells to the site of infection when the stimulation is present. In addition, CCL-2 is also known to negatively impact T cell differentiation and function (39, 40). As for the IL-10 production, it might be linked with the shut-down mechanisms of cytokine storm following pathogen clearance. It implies that the same proinflammatory cells can dampen inflammation via IL-10 production. Clearly, the influence of Tip-DC cytokine secretion in an immune response will be important to further dissect.

The different capacities in inflammatory cytokine secretion by these two cultured DCs might give clues regarding the optimal type of DC for use in the clinical setting. Currently, there is much interest in the use of mature DCs in vaccine design to stimulate T cells, leaving their innate properties, such as inflammatory cytokine secretion largely ignored. One of the main hurdles in using immunotherapy against cancer is that tumor cells arise from self-tissue, which, in most circumstances, is not immunogenic. Local introduction of inflammation by DCs that can be retained in peripheral tissue and secrete inflammatory cytokines and chemokines might be important to initiate a host immune response against tumors. In our study, we found that after s.c. injection, naive GM/IL4-DCs retained in the skin better than FL-DCs. The poor migration of GM/IL4-DCs to the draining lymph node may be due to this retention, or to death in situ, or migration to other tissues. Our use of naive immature GM/IL4-DCs may account for this phenomenon, as LPS activated GM/IL4-DCs were found to be able to traffic efficiently to the draining nodes after s.c. injection (41). The different trafficking ability of the BM-derived DCs could have important implications for the priming of T cells in the context of therapy.

In conclusion, we directly compared the BM-derived DCs in the two most frequently used culture systems and found that the products generated are inherently different. GM/IL4-DCs have Tip-DC-like properties whereas FL-DCs possess lymphoid organ resident DC characteristics. Ongoing clinical trials and basic research often use GM/IL4-DCs derived from monocytes or $CD34^{+}$ progenitors. Therefore, it will be important to establish whether GM/

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IL4-DCs or FL-DCs are the ideal in vitro-derived DC subtype for the individual context of immunotherapy. The differences described herein provide important guidance for the therapeutic application, as well as evidence of the in vivo equivalents of these cells in these culture systems.

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Disclosures

The authors have no financial conflict of interest.

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