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Recruitment and Differentiation of Conventional Dendritic Cell Precursors in Tumors

Jun Diao,* Jun Zhao,* Erin Winter,* and Mark S. Cattral*†

The origin of dendritic cells (DCs) in tumors remains obscure. Recent studies indicate that conventional DCs (cDCs) in lymphoid tissues arise from a distinct population of committed cDC precursors (pre-cDCs) that originate in bone marrow and migrate via blood. In this study, we show that pre-cDCs are precursors for cDCs in tumors. Pre-cDCs from tumors, bone marrow, and spleen exhibit similar morphologic, immunophenotypic, and functional properties. Adoptive transfer studies show that bone marrow pre-cDCs migrate from blood into the tumor where they generate cDCs. The chemokine CCL3, which is markedly upregulated in tumors, promotes pre-cDC recruitment. Both pre-cDCs and their cDC progeny actively proliferate within the tumor. cDCs that arise from pre-cDCs in tumors express lower levels of CD11c and MHC class II as compared with those in spleen; however, there was no difference in their abilities to respond to maturation stimuli or activate Ag-specific lymphocytes in vitro. Our study provides the first evidence supporting a role for pre-cDCs in DC development in tumors and suggests a potential target for cancer immunotherapy. The Journal of Immunology, 2010, 184: 1261–1267.

Cancer cells in humans and experimental murine tumor models create a unique inflammatory microenvironment consisting of various stromal elements and migrating hematopoietic cells, including dendritic cells (DCs) (1, 2). Many studies report that DCs in tumors are functionally defective and contribute to poor antitumor immune responses (3–5). Indeed, evidence suggests that tumor DCs induce tumor Ag-specific T cell anergy, a major obstacle to the success of tumor immunotherapy (6–8). Tumors produce a variety of cytokines and growth factors, including IL-10, IL-6, vascular endothelial growth factor, GM-CSF, and M-CSF (3). The effects of these factors on intratumor DC development remain unclear, however, because the immediate precursor(s) for tumor DCs has yet to be defined.

DCs comprise two main subpopulations: conventional DCs (cDCs) and interferon-producing plasmacytoid DCs (9). Recent studies have provided new insight into cDC ontogeny in lymphoid tissues. Under steady-state conditions, spleen cDCs arise from a distinct population of Lin−CD11c+ MHC class II−immature cDC precursors (pre-cDCs) (10–12). Pre-cDCs originate from bone marrow Lin−CD117int Flt3+CD115+cDC progenitors (13, 14) and transit rapidly in blood to spleen where they generate cDCs that can divide for several generations (11, 15, 16). A clonogenic macrophage-Dc precursor in bone marrow also has been shown to be the source of spleen cDCs (17, 18); however, recent evidence suggests that macrophage-Dc progenitors are precursors for common Dc progenitors and monocytes (16, 18).

Monocytes serve mainly as precursors for TNF-α and inducible NO synthase-producing DCs, which appear in spleen during some inflammatory conditions (19, 20).

In previous studies, we detected adoptively transferred pre-cDCs in nonlymphoid tissues (11), which raised the possibility that pre-cDCs might be a source of tumor cDCs. In this study, we report that tumors recruit replication-competent pre-cDCs from the circulation through a CCL3-dependent mechanism. Tumor pre-cDCs share the same morphologic, phenotypic, and functional properties as those isolated from spleen and bone marrow. Adoptively transferred bone marrow pre-cDCs generate proliferating cDCs in tumors. Tumor cDCs arising from pre-cDCs express lower levels of MHC class II than those in spleen; however, they respond appropriately to maturation stimuli and can present Ag to lymphocytes effectively. Collectively, our study establishes pre-cDCs as precursors for tumor cDCs.

Materials and Methods

Mice

Male C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). C57BL/6.SIL congenic mice and OT-II transgenic mice were purchased originally from Taconic Farms (Germantown, NY) or The Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility. Mice were maintained in pathogen-free conditions in accordance with institutional guidelines and used at 6–10 wks of age. The Animal Research Committee of University Health Network reviewed and approved the studies.

Tumor models

B16-F10 melanoma (B16), Lewis lung carcinoma (LLC), and CT26 colon carcinoma were purchased from American Type Culture Collection (Manassas, VA) and cultured in the recommended medium. To establish tumors in mice, 0.5–1 × 106 tumor cells in 50 μl of PBS were injected s.c. into the flank. The diameters of the s.c. tumors studied were ≤0.5 cm.

Abs and cytokines

Anti-CD11c (clone HL-3), I-A^d (KH74, 25-9-17), I-A^d (AMS-32.1), CD3 (17A2), CD19 (1D3), CD49b (pan-NK, DX5), Gr-1 (RB6-8C5), CD11b (M1/70), B220 (RA3-6B2), CD31 (MEC13.3), CD45 (30-F11), CD45.2 (104), CD45.1 (A20), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), Lysc (AL 21), CD25 (PC61), CD69 (H1.2F3), CD16/32 (2.4G2), Mac3 (553323), CD172a (p84), and CD135 (A2F10) were purchased from BD Pharmingen (San Diego, CA). Anti-F4/80 (A3-1) was purchased from Serotec (Oxford, U.K.). These Abs were unlabeled or conjugated to FITC.
PE, aliphophycocyanin, phycoerithrin-cyanine dye 7, or biotin as indicated. Biotinylated Abs were revealed with FITC, PE, aliphophycocyanin, Texas Red, cyanine dye 5, or cyanine dye 7. GM-CSF and Flt3 ligand (Flt3L) were purchased from BD Pharmingen.

Flow cytometry

Flow cytometry was performed with a Cytomation cytometer using Cytometrics software (Beckman Coulter, Fullerton, CA), as described previously (10). Briefly, cell suspensions were preincubated with anti-CD16/32 to block Fc receptors, then washed and incubated with the indicated mAb conjugates for 30 min at 4°C in a final volume of 100 μl of PBS containing 0.5% BSA and 2 mM EDTA. In all of the experiments, appropriate control isotype-matched mAbs were included to determine the level of background staining.

Cell isolation

Tumors and organs were minced, digested with collagenase and DNAse I for 0.5 h at 37°C, and incubated in PBS containing 2 mM EDTA and 5% FCS or 0.5% BSA for 10 min at room temperature. Mononuclear cells were isolated by Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) density gradient centrifugation and further enriched for CD11c+ cells by positive selection using MACS (Miltenyi Biotec, Auburn, CA) and CD11c+ immunomagnetic beads. Cells were stained with anti-I-Ab (FITC or PE), anti–the cell populations used was routinely

Flow cytometry and analysis software (DakoCytomation; Fort Collins, CO). The purity of the cell populations used was routinely ≥99% based on reanalyzed samples.

Cell culture

Sorted cells were cultured for 16–24 h in high-well U-bottom culture plates in 200 μl RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, 1 mM sodium pyruvate, 10 mM nonessential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin (complete medium) in the presence of GM-CSF (1000 U/ml) and Flt3L (100 ng/ml) in triplicate. The cells were recovered by gentle pipetting and incubated with 2 mM EDTA in PBS.

In stroma coculture experiments, sorted pre-cDCs (1 × 10^6) were labeled with CFSE and incubated on a confluent monolayer of irradiated (25 Gy) S17 tumor cells. Dead cells were excluded by propidium iodide staining. Flow cytometry was performed with a Cytomics cytometer using Cytomics Flow cytometry software (DakoCytomation; Fort Collins, CO). The purity of the cell populations used was routinely ≥99% based on reanalyzed samples.

Adoptive transfer studies of pre-cDCs

CFSE-labeled pre-cDCs (5–10 × 10^6) were injected i.v. into congeneric tumor-bearing recipients as described (11) or injected directly into the tumor or spleen. The spleen and tumor were removed from recipients 2–3 d later; mononuclear cells were isolated by Lympholyte-M density gradient centrifugation, stained with fluorochrome-conjugated Abs, and analyzed by flow cytometry. Dead cells were excluded by propidium iodide staining. Analysis of cell division (CFSE fluorescence) was limited to CFSE+ cells.

Chemokine and cytokine analysis

Proteome Profiler Antibody Arrays were performed using the Mouse Cytokine Array Panel A Array Kit (R&D Systems, Minneapolis, MN). Normal lung tissues, lungs with LLC tumor nodules (30–50 per lung), and s.c. LLC tumors (with or without draining lymph nodes) were collected. The tissues were homogenized in PBS with protease inhibitor and 1% Triton X-100. Tissue lysates containing equal amounts of protein (500 μg) were used. The pixel density data were collected by GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA) and analyzed by Quantity One 4.6.1 software (Bio-Rad Laboratories).

Generation of CCL3-producing B16 and LLC tumors

B16 and LLC tumor cells were transduced with a retrovirus encoding murine CCL3 (pmlcCCL3as) or a blank vector control (ploxn) and selected with G418 (1 mg/ml) for 2 wk (21). CCL3 expression in culture supernatants was confirmed by Western blot analyses. Stably transduced tumor cells were maintained in G418 (0.5 mg/ml) until used in vivo.

Blockade of pre-cDC migration with anti-CCL3 mAb

Mice bearing B16 tumors (0.1–0.2 cm^2) were injected with goat anti-mouse CCL3 Ab (100 μg i.p.; R&D Systems) or normal goat IgG daily for 3 d before recovery of tumors and spleen for analysis. The frequency of pre-cDCs in tumors and spleen were normalized to the total number of CD45^+ cells.

BrdU staining

BrdU (1 mg; Sigma-Aldrich, St. Louis, MO) was injected i.p. 3.5 h prior to tumor recovery. Surface staining for cDCs was performed with Abs against lineage markers (CD3, CD19, B220, and CD49b), CD11c+ and MHC class II. For pre-cDCs, the cells were stained with Abs against lineage markers (CD3, CD19, B220, and CD49b), CD11c+, MHC class II, and CD172a. The cells were resuspended in cold 0.15 M NaCl and fixed by dropwise addition of cold 95% ethanol. The samples were stored overnight at 4°C, stained with anti-BrdU Ab (BD Biosciences, San Jose, CA) as described by Kamath et al. (22), and analyzed by flow cytometry. Cells isolated from mice that were not injected with BrdU served as a control for background staining.

Allogeneic MLRs

Graded numbers of fresh-sorted or cultured (16–24 h in the presence of GM-CSF and Flt3L) pre-cDCs were seeded in triplicate in 96-well U-bottom culture plates (BD Biosciences, San Jose, CA). Responder spleen cells (1 × 10^6 cells per well) from BALB/c mice were added to the wells in a total volume of 200 μl RPMI 1640 complete medium and cultured for 3 d in a humidified atmosphere of 5% CO2 in air at 37°C. The culture was pulsed with 1 μCi [3H]thymidine (Amersham Biosciences, Arlington Heights, IL) 16 h before harvest and collected onto glass filter-filters (Millipore, Bedford, MA); [3H]thymidine incorporation was quantified using a scintillation counter (Beckman Coulter). Background controls with spleen cells or stimulator cells alone were included in all of the experiments. Results are expressed as the mean cpm of triplicate cultures.

Ag-specific T cell responses

CD4 T cells from spleen and lymph nodes of OT-II transgenic mice were enriched by depletion of CD19+, CD8+, B220+, CD11b+ CD11c+, TER119+, and MHC class II^+ cells. CFSE-labeled OT-II T cells (5 × 10^6 cells per well) were mixed with sorted tumor and spleen cDCs, which had been pulsed with OVA-MHC class II peptide (aa 323–339, 5 μM, 1 h at 37°C) or control peptide, and cultured for 3 d. OT-II T cell proliferation was assessed by CFSE dilution.

Statistical analysis

Continuous variables are expressed as mean ± SD and analyzed by two-tail Student t test. A p value <0.05 is considered statistically significant.

Results

Identification of pre-cDCs in tumors

We first analyzed tumor-infiltrating cells in s.c. B16 tumors and bone marrow and spleen cells from the same tumor-bearing mice by flow cytometry. Consistent with previous reports, plasmacytoid DCs (CD13−CD19−CD49b−B220+CD11c−MHC class II−) were rare in B16 tumors (data not shown). Lineage (CD3, CD19, B220, and Gr-1)−CD11c+ cells accounted for 7–15% of the total population of CD45+ tumor-infiltrating cells. Line−CD11c+ could be divided further into MHC class II^+ and MHC class II^− fractions (Fig. 1A). The MHC class II^+ fraction accounted for 15–20% of the Line−CD11c+ cells in tumor as compared with 60% in bone marrow and 15–20% in spleen. Our previous studies showed that Line−CD11c+ MHC class II^+ cells in bone marrow and secondary lymphoid tissues were pre-cDCs; however, this phenotype may be insufficient to distinguish pre-cDCs from monocytes or macrophages in nonlymphoid tissues (23, 24). Recent studies have further defined pre-cDCs in bone marrow and spleen as Line−CD11c^+ MHC class II^+ cells that are CD172a^−/lo (Sirpa−) and Flt3^+ (16). We therefore examined the expression level of CD172a and Flt3 in Line−CD11c^+ MHC class II^+ cells. We found that almost all of the Line−CD11c^+ MHC class II^+ cells in bone marrow were CD172a^−/lo, whereas the corresponding population in spleen contained a small proportion of CD172a^+ cells. CD172a^−/lo cells expressed higher levels of Flt3 than CD172a^+ cells in both spleen and bone marrow. In B16 tumors, Line−CD11c^+ MHC class II^+ cells comprised two distinct populations in spleen contained a small proportion of CD172a^−/lo cells expressed higher levels of Flt3 than CD172a^+ cells in both spleen and bone marrow. In B16 tumors, Line−CD11c^+ MHC class II^+ cells comprised two distinct
Characterization of tumor pre-cDCs

Characterization of tumor pre-cDCs

We next sorted tumor and bone marrow pre-cDCs for further analysis. Because almost all of the Lin\(^{-}\) CD11c\(^{+}\) MHC class II\(^{-}\) CD172\({\alpha}^{-}\) cells were Flt3\(^{+}\) (Fig. 1), Lin\(^{-}\) CD11c\(^{+}\) MHC class II\(^{-}\) CD172\({\alpha}^{-}\) cells were sorted as our working population. Sorted tumor pre-cDCs displayed a round shape with a large nucleus, similar to that of bone marrow pre-cDCs (Fig. 2A). After overnight culture in the presence of GM-CSF and Flt3L, pre-cDCs from tumors and bone marrow showed a veiled edge with a few long dendrites, expressed higher levels of MHC class II and CD86 (Fig. 2B and Ref. 11), and became potent stimulators of allogeneic lymphocytes (Fig. 2C).

Another defining feature of bone marrow and spleen pre-cDCs is their capacity to generate CD11c\(^{+}\) MHC class II\(^{+}\) DCs that continue to replicate in vivo and in vitro (11). To compare the proliferation capacities of tumor and bone marrow pre-cDCs, they were sorted, stained with the cytosolic dye CFSE, and cocultured on S17 monolayers in the presence of GM-CSF. At 3 d, the majority of the recovered cells derived from tumor and bone marrow pre-cDCs expressed CD11c and MHC class II and had completed several division cycles (Fig. 2D). Thus, on the basis of the current criteria used to define bone marrow and spleen pre-cDCs, we conclude that tumor Lin\(^{-}\) CD11c\(^{+}\)MHC class II\(^{-}\) CD172\({\alpha}^{-}/lo\) Flt3\(^{+}\) cells are pre-cDCs.

Migration of pre-cDCs to tumors

Previous studies have shown that bone marrow pre-cDCs migrate via blood to lymphoid tissues. We confirmed that pre-cDCs were present in the blood of tumor-bearing mice (Fig. 3A). Blood pre-cDCs express higher levels of CD62L than those in tumor or spleen, supporting the idea that pre-cDCs migrate via blood to the tumor.

Chemokines have been shown to regulate trafficking of tumor-infiltrating cells (25). To gain insight into the chemokine expression profile of tumor tissue, we assessed the levels of various...
CCL3 promotes migration of blood pre-cDCs to tumors. A, Identification of pre-cDCs in blood of tumor-bearing mice. Pre-cDCs were identified using the gating strategy shown in Fig. 1 and analyzed for CD62L expression. B, Cell lysates from lung tissue containing LLC and s.c. LLC tumors were prepared and analyzed for chemokine expression by chemokine arrays. Chemokine levels were normalized to levels detected in normal lung tissue. Results are representative of two independent experiments with three mice per group. C, Fold increase in the number of pre-cDCs and cDCs in B16 tumors transduced with retrovirus encoding murine CCL3 as compared with B16 tumors transduced with blank vector control. Results are representative of three independent experiments. D, Frequency of pre-cDCs in tumors and spleen in B16 tumor-bearing mice treated with anti-mouse CCL3 Ab or isotype control Ab for 3 d. Results are representative of four independent experiments. LLC, Lewis lung carcinoma.

Pre-cDCs differentiate into proliferating cDCs in tumors

Parabiotic and adoptive cell transfer studies have shown that pre-cDCs differentiate into MHC class II+ cDCs in lymphoid tissues. To determine the fate of pre-cDCs in tumors, we i.v. injected 1 × 106 FACS-purified bone marrow pre-cDCs from CD45.1 mice into congenic CD45.2 mice bearing LLC tumors and recovered the progeny of these cells from spleen and tumors 3 d later for analysis. For these experiments, we used LLC transduced with a retrovirus encoding CCL3 to increase the number of transferred cells in the tumor. As expected, pre-cDCs differentiated into CD11c+ MHC class II+ cDCs in spleen (Fig. 4A). Most cells arising from pre-cDCs in LLC tumors were CD11c+ and MHC class II+, although the expression levels of these markers were slightly lower than those in spleen.

We next examined whether differentiation of pre-cDCs into cDCs was accompanied by cell division. Sorted pre-cDCs were labeled with CFSE and directly injected into the tumor to increase the frequency of transferred cells for analysis. We injected CFSE-labeled pre-cDCs and B cells into the same tumor-bearing mice through blood or directly into spleen as controls. The route of cell delivery (i.e., direct injection into tissue or via blood) did not affect differentiation of pre-cDCs into cDCs in tumor or spleen (data not shown). At 3 d, ∼60% of the CD11c+ MHC class II+ progeny had undergone cell division in the tumor and spleen (Fig. 4B). As expected, transferred B cells did not divide in spleen or the tumor (Fig. 4C).

To evaluate proliferation of endogenous tumor DCs, we injected tumor-bearing mice with BrdU and recovered tumors 3.5 h later to analyze BrdU expression in tumor pre-cDCs and cDCs (Fig. 5). We found that 5.6 ± 1.3% of pre-cDCs and 2.5 ± 0.9% of cDCs were BrdU+ (n = 4). Cell cycle analysis confirmed that 4–6% of pre-cDCs and cDCs were in the S/G2/M phases of the cell cycle (data not shown).
CSFE+ CD11c+ MHC class II+ tumor and spleen cDCs that were
not shown). Thus, in situ proliferation of tumor pre-cDCs and
cDCs contributes to the generation of tumor cDCs.

Pre-cDCs generate competent cDCs in tumors

It remains controversial whether tumor cDCs have intrinsic defects
in Ag presentation (4, 5). To assess whether cDCs generated from
pre-cDCs in tumor are functional APCs, we sorted CD45.1+ (or
CSFE+) CD11c+ MHC class II+ tumor and spleen cDCs that were
derived from transferred pre-cDCs in tumor-bearing CD45.2+ mice and tested their capacities to stimulate Ag-specific T cell
proliferation. We also sorted endogenous CD11c+ MHC class II+ cDCs from the same tumor and spleen of each mouse. The
sorted cells were incubated with OVA peptide and mixed with purified CSFE-labeled transgenic CD4+ cells to
measure BrdU injection and analyzed by flow cytometry for BrdU and MHC class II staining. The dot plot quadrants were determined by isotype-matched controls. Data from control mice that did not receive BrdU treatment are shown in the left dot plots. The bar graph shows mean ± SD of three
determinations with three mice per group.

Discussion

Recent studies have established the importance of pre-cDCs in the
maintenance of splenic cDC homeostasis under steady-state condi-
tions (11, 15, 16, 29). Our study now extends their role to cDC development in s.c. tumors. Using criteria developed for the
identification and isolation of bone marrow and spleen pre-cDCs, we
show that pre-cDCs exist in a variety of experimental tumor models. We found that bone marrow pre-cDCs can migrate from
blood into the tumor, a process driven by aberrant chemokine
expression in tumor tissue. Within the tumor, pre-cDCs differen-
tiate into proliferating cDCs that have the ability to mature and
stimulate Ag-specific lymphocytes.

Our laboratory originally identified and isolated pre-cDCs from
bone marrow and subsequently in secondary lymphoid tissues based
on their Lin- CD11c+ MHC class II+ phenotype (10, 11). These
cells are distinct from monocytes and generate exclusively cDCs
in vitro and in vivo. Recent studies have further defined pre-cDCs as
Lin- CD11c+ MHC class II+ Flt3+ CD172α+ cells (12, 16). Our
previous studies demonstrated that pre-cDCs express functional
Flt3 based on their proliferative response to Flt3L, although the
expression level of Flt3 on sorted cells is low as determined by flow
cytometry (11). CD172α is a cell surface type 1 glycoprotein ex-
pressed on a small proportion of Lin- CD11c+ MHC class II+ cells in tumor, spleen, and blood.

It is notable that the low level of T cell proliferation in these experi-
ments was due to the low number of stimulators because the
limited number. We found that tumor and spleen cDCs derived from
transferred pre-cDCs induced similar levels of T cell proliferation;
there was also no difference between these cells and equivalent
numbers of endogenous tumor and spleen cDCs (Fig. 6A). It is
notable that the low number of T cell proliferation in these experi-
ments due to the low number of stimulators because the
proliferative response could be augmented by increasing the
number of endogenous cDCs.

The small number of pre-cDC-derived cDCs isolated from tumors
prevented us from fully examining their response to maturation
stimuli. However, we found that sorted endogenous tumor cDCs
upregulated surface expression of MHC class II and cos-
timulatory (CD40 and CD86) molecules after culture to levels
comparable to those expressed by splenic cDCs (Fig. 6B), in-
dicating that tumor cDCs were capable of maturation. Tumor and
spleen cDCs also stimulated comparable levels of lymphocyte
proliferation in an allogeneic MLR (data not shown). Collectively,
these findings indicate that pre-cDCs can generate competent
cDCs in tumors with functional properties similar to those of their
counterparts in spleen.

FIGURE 5. In situ proliferation of endogenous tumor pre-cDCs and
cDCs. Pre-cDCs and cDCs from B16 tumors were isolated 3.5 h after
BrdU injection and analyzed by flow cytometry for BrdU and MHC class II staining. The dot plot quadrants were determined by isotype-matched controls. Data from control mice that did not receive BrdU treatment are shown in the left dot plots. The bar graph shows mean ± SD of three
determinations with three mice per group.

FIGURE 6. Tumor cDCs arising from pre-cDCs are effective APCs. A, cDCs arising from transferred pre-cDCs and corresponding endogenous
cDCs were sorted from tumor and spleen, pulsed with OVA peptide, and
mixed with 5 × 10^5 naive CFSE-labeled OT-II T cells for 3 d. Histograms
display division of OT-II T cells stimulated by 2,500 or 10,000 cDCs. Numbers indicate the percentage of divided OT-II T cells. B, Flow cyto-
metric analysis of expression of indicated markers by endogenous tumor or
spleen cDCs before (gray histograms) and 24 h after culture in medium
containing GM-CSF and Flt3 (open histograms). Results are representative of three independent experiments.
We identified pre-cDCs in tumors derived from B16, LLC, and CT26 colon carcinoma. We also have detected pre-cDCs in tumors derived from A20 B cell lymphoma, C1498 myeloid tumor, and EMT6 breast carcinoma, suggesting that the ability to recruit pre-cDCs from circulation is a common property of transplantable tumors. The mechanisms that regulate pre-cDC release into blood and entry into spleen and lymphoid tissues under steady-state conditions have not been defined. Our finding that tumor tissues have increased expression of chemokines and proinflammatory cytokines (data not shown) suggests that tumors actively recruit circulating pre-cDCs. Bone marrow pre-cDCs express CCR1 and CCR5 and show positive chemotaxis to CCL3 in a dose-dependent manner (11). Several findings support the importance of CCL3 in pre-cDC migration to tumors: 1) CCL3 expression was highly upregulated in tumor tissue; 2) CCL3-transduced tumors contained more pre-cDCs and cDCs; and 3) blocking CCL3 with a neutralizing Ab decreased the frequency of tumor pre-cDCs. Notably, the tumor cells (B16 and LLC) used in our study do not produce CCL3 constitutively: tumor-infiltrating stromal and immune cells are the likely source. This finding suggests that the migration of pre-cDCs to tumors may represent a normal response to inflammation. Further studies are needed to delineate the role of pre-cDCs in other inflammatory processes and to compare them with monocytes, which are currently considered the chief source of inflammatory DCs in peripheral tissues (20, 30–33).

Our study shows that bone marrow pre-cDCs generate dividing MHC class II+ cDCs in tumors as in lymphoid tissues (11, 16). As compared with those in spleen, dividing tumor cDCs express lower levels of CD11c and MHC class II; whether this represents a specific effect of the tumor milieu or a normal characteristic of cDC development in nonlymphoid tissue is unclear. Despite this difference, cDCs arising from pre-cDCs in tumor and spleen stimulated Ag-specific T cells equally well; the stimulatory capacity of sorted endogenous tumor and spleen cDCs was also similar. Our results conflict with a previous study showing that tumor DCs are paralyzable and resistant to maturation (5). The criteria used to define and isolate tumor cDCs and the experimental conditions employed may account, at least partly, for the differences.

In various inflammation models, tissue-derived DCs have been shown to migrate from the inflammatory site via lymphatics to secondary lymphoid organs where they interact with lymphocytes (34). To what extent pre-cDC-derived tumor cDCs migrate to regional lymph nodes is unknown. Conceivably, they might remain in the tumor and eventually die or, alternatively, differentiate into other cell types (35, 36). We attempted to track pre-cDC-derived tumor cDCs at later time points (i.e., beyond 3–5 d), but this proved impossible because continuous rapid growth of the tumor diluted their frequency below a detectable level. Thus, although our study has revealed the recruitment of pre-cDCs to tumors and their differentiation into cDCs in situ, further studies are required to complete the life cycle of tumor cDCs and to define their role in the presentation of tumor-derived Ags in vivo.

There is a prevalent view that tumors inhibit DC differentiation from primitive hematopoietic progenitors in bone marrow, contributing to the generation of immunosuppressive myeloid populations (3). As compared with normal mice, however, we found no difference in the proportion or number of pre-cDCs and cDCs in bone marrow or blood of tumor-bearing mice. cDC homeostasis in spleen, which requires continuous influx of new pre-cDCs from the circulation (37), also appeared to be intact in tumor-bearing mice. We suspect that most tumors in our study were too small (∼0.5 cm in diameter) to influence the early stages of DC development in bone marrow. Interestingly, splenomegaly accompanied by increased numbers of splenic pre-cDCs and cDCs occurred in a few mice, presumably in response to tumor-associated necrosis and inflammation.

There is much interest in developing strategies to improve tumor immunotherapy (38): pre-cDCs offer a promising target. We have shown that augmenting intratumor CCL3 expression increases the number of tumor pre-cDCs significantly. Altering other aspects of the tumor milieu may enhance their capacity to stimulate antitumor immunity.

Disclosures
The authors have no financial conflicts of interest.

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