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Tumors Suppress In Situ Proliferation of Cytotoxic T Cells by Promoting Differentiation of Gr-1+ Conventional Dendritic Cells through IL-6

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

Cancers are often accompanied by inflammation, which can promote tumor growth, invasion, and metastases. We show that the tumor microenvironment induces the development of a Gr-1+ conventional dendritic cell (cDC) subpopulation that is functionally defective. Gr-1+cDCs differentiated from recruited immediate precursors of cDCs, a process supported by the inflammatory cytokine milieu in tumors. Inhibition of Gr-1+cDC differentiation enhanced intratumor expansion of cytotoxic CD8+ T cells (CTLs), resulting in suppression of tumor growth. Diphtheria toxin treatment of CD11c–diphtheria toxin receptor chimeras revealed the importance of intratumor cDCs in stimulating CTL proliferation in situ. Our study demonstrates a key role of intratumor cDCs in determining antitumor CTL responses and suggests that they may be an appropriate target for tumor immunotherapy. 

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Inflammation is a well-recognized component of various types of cancer in patients and in animal tumor models (1). Altered expression of chemokines, cytokines, and growth factors in tumors contributes to an influx of infiltrating cells and generation of various stromal elements; in these respects, tumors resemble a chronic “smoldering” wound (2). Cellular stress, aberrant growth, and death of cancer cells generates many tumor-associated factors that can enhance inflammatory responses including high mobility group box 1, matrix proteins (fibronectin, heparan sulfate, versican), heat shock proteins, ATP, and uric acid (3–5). Tumor-associated inflammation promotes tumorigenesis, angiogenesis, local invasion, metastases, and evasion of immune surveillance (4, 6).

Dendritic cells (DCs) provide a critical link between innate and adaptive immune responses (7). DCs capture and process Ags in peripheral tissues and migrate to lymphoid tissues where they stimulate Ag-specific lymphocytes. In addition, recent evidence suggests that tissue DCs stimulate effector and memory T cells in situ, enhancing T cell expansion and differentiation (8–10). In cancer-bearing patients, defective DC function is considered a key cause of impaired immunity to Ags expressed by tumors and an obstacle to immunotherapy (11). Published reports document reduced numbers and immaturity of DCs in peripheral blood and tumors, which correlates with prognosis for some cancers (12, 13). Whether these factors alone account for DC dysfunction has been challenged, however (14, 15). Much remains unclear about the nature of DCs in tumors, the mechanisms by which tumors inhibit their function, and how they affect antitumor T cell responses in vivo.

In this study, we show that the intratumor inflammatory milieu skews differentiation of conventional DC (cDC) toward a Gr-1+ subpopulation. This transformation altered the composition and immunogenicity of tumor cDCs and was tumor dependent. These changes influenced the proliferation and expansion of Ag-specific CTLs in tumors. Further, inhibition of Gr-1+ cDC differentiation increased intratumor CTL expansion and the success of adoptive T cell immunotherapy.

Materials and Methods

Mice

Male C57BL/6 and BALB/c mice were purchased from Charles River (Wilmington, MA). C57BL/6.SJL congenic mice, OT-I, OT-II, and CD11c–diphtheria toxin receptor (CD11c-DTR) transgenic mice, and Il-6−/− mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (Germantown, NY) and bred in our animal facility. Mice were maintained in pathogen-free conditions in accordance with institutional guidelines and used at 2–3 mo 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. For some studies, we used B16 tumors that express the model tumor Ag OVA ([B16-OVA; kindly provided by R.W. Dutton, Trudeau Institute (16)]. To establish tumors in mice, 0.5 × 106 to 106 tumor cells in 50 μl PBS were injected s.c. into the flank or i.v. The diameter of s.c. tumors was measured by caliper, and tumor size was calculated by multiplying 1/2 length and width.

Abs and cytokines

Anti-CD11c (clone HL3), I-Ab (M5/C11.1), I-Ad (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 (1043D6), CD45.1 (16-2.1), and invariant NK T cell marker (clone 6H9) (Biolegend) were used for flow cytometry analysis. The intratumor cytokine milieu was characterized by ELISA and Western blotting. The online version of this article contains supplemental material.

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The online version of this article contains supplemental material.

Abbreviations used in this article: B16, B16-F10 melanoma; cDC, conventional dendritic cell; DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; iNOS, inducible NO synthase; LLC, Lewis lung carcinoma; LLC-CM, Lewis lung carcinoma conditioned medium; MDCSC, myeloid derived suppressor cell; MHC II, MHC class II; pre-cDC, immediate precursor of cDC.

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Supplemented with 10% FBS, 50 with or without LPS (1 Biotec) and CD11c+-immunomagnetic beads. Cells were stained with anti–

Burlington, ON, Canada) density gradient centrifugation, stained with fluo-

cirtschaft as described (18). The spleen and tumor were removed from recipients 3–

Flow cytometry

Flow cytometry was performed with a Cytomics cytometer using Cyto-

mics software (Beckman Coulter, Miami, FL), as described previously (17). 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 PBS containing 0.5% BSA and 2 mM EDTA. In all experiments, appropriate control isotype-matched mAbs were included to determine the level of background staining. For intracellular cytokine detection, surface Ab-labeled cells were fixed, permeabilized, and stained with anti-cytokine Abs according to the instruction manual from the BD Cytofix/Cytoperm kit.

Tumor DC isolation

Tissues 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 for 10 min at room temperature. Mononuclear cells were isolated by Lympholyte-M or Nycodenz density gradient centrifugation and further enriched for CD11c+ cells by positive selection using MACS (Miltenyi Biotec) and CD11c+–immunomagnetic beads. Cells were stained with anti–I-A^K (FITC or PE), anti-CD11c (FITC, PE, or allophycocyanin), anti–Gr-1 (PE, allophycocyanin, or PC7), and biotin-labeled anti-lineage markers (anti–CD3-, anti–CD19-, anti–B220, and anti–CD49b-biotin-streptavidin-allo-

peocyanin/PC5 or PC7). Desired populations were sorted on a MoFlo High-speed Cell Sorter using Summit acquisition and analysis software (DakoCytomation, Fort Collins, CO). The purity of the cell populations used was routinely ≥99% based on reanalyzed samples.

Sorted DCs were cultured in 96-well plates in 200 µl RPMI 1640 supplemented with 10% FBS, 50 µM 2-mercaptoethanol, 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 Flt3 ligand (100 ng/ml).

Adoptive transfer of immediate precursors of cDCs

CFSE-labeled CD45.2 immediate precursors of cDCs (pre-cDCs) (5 × 10^5 to 10^6) were injected i.v. into congenic CD45.1 tumor-bearing recipients, as described (18). The spleen and tumor were removed from recipients 3–4 d later; mononuclear cells were isolated by Lympholyte-M (Cedarlane, Burlington, ON, Canada) density gradient centrifugation, stained with fluo-

rochrome-conjugated Abs, and analyzed by flow cytometry.

Cytokine assays

Proteome Profiler Ab Arrays was performed on normal lung tissues, lungs with LLC tumor nodules (30–50 per lung), and s.c. LLC tumors (weight, 0.5–1 g) with the Mouse Cytokine Array Panel A Array kit (R&D Sys-

tems). Pixel density data were collected by GS-800 Calibrated Densito-

meter and analyzed by Quantity One 4.6.1 software.

Gr-1+ and Gr-1 cDCs were sorted from LLC tumors and incubated with or without LPS (1 µg/ml) for 24 h. Culture supernatants were assayed for IL-10, IL-6, TGF-β, IL-4, TNF-α, and IL-12p70 by ELISA (R&D Systems).

Tumor conditioned media

Supernatant collected from LLC tumor cells cultured in DMEM for 24 h was filtered through a 0.22-µm filter, stored at −80˚C, and thawed im-

ediately before use. Pre-cDCs were cultured in 25% Lewis lung carci-

noma conditioned medium (LLC-CM) for 3 d; in some experiments, cy-

tokines (10 ng/ml) or neutralizing anti-cytokine Abs (10 µg/ml) were added to the culture medium.

Virus vectors

Adenoviral vectors encoding OVA or GFP were prepared, purified, and titrated as described previously (19).

Allogeneic mixed leukaemic reactions

Graded numbers of fresh-sorted or 24-h cultured Gr-1+ and Gr-1– cDCs were seeded in triplicate in U-bottom 96-well culture plates (Becton Dickinson, Franklin Lakes, NJ). Responder spleen cells (1 × 10^6/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 at-

mosphere of 5% CO2 in air at 37˚C. The culture was pulsed with 1 µCi [H]thymidine (Amersham, Arlington Heights, IL) 16 h before harvest and collected onto glass fiber filters (Millipore, Etobicoke, ON, Canada); [H] thymidine incorporation was quantified using a Beckman scintillation counter. Background controls with spleen cells or stimulator cells alone were included in all experiments.

Ag-specific T cell responses

OT-II T cells (5 × 10^5/well) were incubated in triplicate for 3–5 d with sorted, OVA-pulsed Gr-1+ cDCs or Gr-1– cDCs (OVA, 500 µg/ml with LPS 1 µg/ml for 2 h at 37˚C). T cell proliferation was assessed by tritiated thymidine incorporation and cell counting. Anti–IL-10R mAb (10 µg/ml) or an isotype control Ab was included in the culture medium in some ex-

periments.

To test T cell responses in vivo, sorted Gr-1+ cDCs and Gr-1– cDCs were pulsed with OVA protein (500 µg/ml, 2 h, 37˚C) for OT-II T cells and OVA MHC class I peptide (amino acids 257–264; 10 µg/ml; 30 min at 37˚C) for OT-I T cells in the presence of GM-CSF (1000 U/ml) and LPS (1 µg/ml). Equal numbers of Ag-loaded cDCs (1 × 10^5 to 1 × 10^6) were injected s.c. into mice that had received 1 × 10^6 OT-I or OT-II cells 24 h earlier. Three days later, cells were recovered from lymph nodes, and T cell pro-

liferation was determined by CFSE dilution. To assess intracellular cyto-

kine production, OT-II T cells were restimulated with OVA (250 µg/ml) for 12 h, and OT-I T cells were stimulated with peptide (1 µg/ml) for 6 h in the presence of GolgiStop (BD). In some experiments, divided (peak 2–6) and nondivided T cells from lymph nodes were pooled from three to four mice, sorted, and restimulated with Ag as described above.

Generation of effector OT-I T cells

OT-I transgenic mice were injected i.p. with 1 × 10^7 viral particles of the adenovirus vector encoding OVA. Effector CD8+ T cells were isolated 3 d later by negative selection using magnetic beads.

Antitumor CTL responses in vivo

Effector OT-I cells (1 × 10^6 to 5 × 10^6) were labeled with CFSE and injected i.v. into IL-6−/− and wild-type mice inoculated with 5 × 10^4 B16-

OVA or B16 tumor cells 7 d earlier. Polyclonal CTLs from wild-type B6 mice immunized with an adenovirus encoding GFP were used as control. Mice were killed at various time points to assess the frequency and pro-

liferation in tumors and lymphoid tissues by flow cytometry. Intracellular IFN-γ production in OT-I CTLs was assessed after restimulation with OVA peptides in vitro. For detection of OT-I-1 CTL apoptosis in vivo, lymphocyte- 

M centriﬁguration of tumor cell suspensions was eliminated to avoid re-

moval of apoptotic cells. For tumor survival studies, 1 × 10^6 OT-I-1 CTLs or control CTLs were injected into mice 5 d after tumor inoculation.

DC depletion in vivo

We generated CD11c-DTR and control B6 chimeras as described (20). Briefly, 2 × 10^6 bone marrow cells from CD45.2 CD11c-DTR transgenic or wild-type B6 mice were injected into gamma-irradiated SJL (CD45.1) mice (550 cGy twice with a 2-h interval between irradiations); flow cy-

tometric analysis of blood at 2 mo conﬁrmed a >95% reconstitution rate. B16-OVA tumor cells were injected into CD11c-DTR and B6 chimeras 10 d before starting diphtheria toxin (DT) treatment. DT was injected i.p. (100 ng/g body weight) 1 d before and 1 d after i.v. injection of 1 × 10^8 CFSE-labeled OT-I-1 CTLs. Cell division of OT-I-1 CTLs in tumors and lymphoid tissues was evaluated 3 d after CTL injection.

Statistical analysis

Statistical signiﬁcance (p < 0.05) of data was determined by the Student t test. Kaplan–Meier survival curves were compared by the log-rank test. Data were analyzed with GraphPad Prism software.

Results

Composition of cDCs in tumors

We analyzed the composition of cDCs in s.c. tumors derived from LLC and B16 in B6 mice and CT26 colon carcinoma in BALB/c
mice by flow cytometry. CD11c-enriched cells dissociated from tumors were stained with Abs to lineage markers (CD3, CD49b, B220, and CD19), CD11c, and MHC class II (MHC II). Consistent with previous reports (14), these tumors contained few B220+ plasmacytoid DCs. We defined cDC in the Lin⁻ population based on the expression of CD11c and MHC II. We found that tumor cDCs consisted of two subsets that could be distinguished by cell surface expression of Gr-1 (Fig. 1A), which is an epitope of Ly6C and Ly6G (21). Further phenotypic analysis revealed that neither subset expressed CD115, Ly6G, Mac-3, CD31, or CD45RB; some cells from both subsets expressed Ly6c; and most cells from both subsets expressed CD11b and F4/80 (Fig. 1B). This analysis indicated that Gr-1⁺ cDCs were distinct from Gr-1⁺ myeloid-derived suppressor cells (MDSCs), which do not express CD11c and MHC II (22, 23).

Gr-1⁺ cDCs accounted for 23.5 ± 8.4% of intratumor cDCs in B16 (n = 8), 34.8 ± 13.9% in LLC (n = 8), and 16.1 ± 2% in CT26 (n = 4) (Fig. 1C). In normal mice, Gr-1⁺ cDCs were undetectable in lung, liver, spleen, thymus, bone marrow, s.c. and mesenteric lymph nodes, and Peyer’s patches. However, we found that Gr-1⁺ cDCs appeared in draining lymph nodes and spleen of tumor-bearing mice; the frequency of Gr-1⁺ cDCs in lymphoid tissues correlated directly with tumor size (Fig. 1D) and increased markedly when they contained tumor metastases (Fig. 1E). We also found Gr-1⁺ cDCs in lungs with B16 or LLC tumor metastases (data not shown). These data suggested that Gr-1⁺ cDCs represent a novel population of cDC that is abundant in transplantable tumors irrespective of tumor type and location and mouse strain.

**Characterization of Gr-1⁺ cDCs and Gr-1⁻ cDCs**

To understand the nature of Gr-1⁺ cDCs, we compared tumor Gr-1⁺ cDCs with Gr-1⁻ cDCs sorted from the same tumors. Although similar in shape and size, Gr-1⁺ cDCs exhibited fewer and shorter dendrites than Gr-1⁻ cDCs (Fig. 2A). After overnight culture, about half of the Gr-1⁻ cDCs had adhered to the plastic culture well with an irregular cell shape, whereas most Gr-1⁻ cDCs floated freely with typical DC morphology (data not shown). Cell survival at the end of culture was ~60% for both populations.

To compare the phagocytic capacity of Gr-1⁺ cDCs and Gr-1⁻ cDCs, we incubated them with OVA conjugated to allophycocyanin and measured uptake at 2 h by flow cytometry. Gr-1⁺ cDCs and Gr-1⁻ cDCs acquired similar amounts of OVA (Fig. 2B). This result agreed with our finding that both populations isolated from B16 tumors contained a similar number of melanin-derived granules in their cytoplasm (data not shown).

We compared the stimulatory capacity of Gr-1⁺ cDCs and Gr-1⁻ cDCs in mixed allogeneic lymphocyte reactions. Freshly isolated Gr-1⁻ cDCs, but not Gr-1⁺ cDCs, stimulated BALB/c lymphocyte proliferation. Overnight culture increased the stimulatory capacity of Gr-1⁻ cDCs significantly but had little effect on the potency of Gr-1⁺ cDCs (Fig. 2C). We examined the expression of costimulatory molecules by flow cytometry (Fig. 2D). CD80 expression was higher in Gr-1⁺ cDCs (61 versus 29%), whereas both populations expressed similar levels of CD86 and CD40. In addition, both expressed similar levels of the coinhibitor molecule PD-L1. Both populations upregulated cell surface expression of MHC II and CD86 after overnight culture, but this change was much less in Gr-1⁺ cDCs (Fig. 2E). We obtained similar results by culturing the cells with LPS and TNF-α (data not shown).

Freshly isolated Gr-1⁺ cDCs expressed more IL-10 constitutively than Gr-1⁻ cDCs (22 ± 9.5% versus 2 ± 0.6%; n = 6, p < 0.001) (Fig. 2F). IL-12p40 expression was low but marginally higher in Gr-1⁺ cDCs (8.6 ± 0.5% versus 1 ± 0.8%). Neither population expressed IFN-γ, IL-6, or TNF-α, and ~25% of both populations expressed iNOS (data not shown). We also assessed cytokine levels in supernatants after 18-h culture and found that Gr-1⁺ cDCs spontaneously produced higher amounts of IL-10 than Gr-1⁻ cDCs; this difference persisted even after treatment with LPS (Fig. 2G). IL-6 production by Gr-1⁺ cDCs and Gr-1⁻ cDCs was undetectable in basal conditions but increased to similar levels after LPS stimulation (Fig. 2H). We did not detect TNF-α, TGF-β, IL-12p70, or IFN-γ in culture supernatants from either population with or without LPS stimulation (data not shown). These findings indicated that tumor Gr-1⁺ cDCs were distinct from inflammatory Gr-1⁺ “tip” DCs, which typically produce large amounts of TNF-α and iNOS (24).

To determine whether preferential production of IL-10 by Gr-1⁺ cDCs contributes to T cell hyporesponsiveness, we stimu-

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**FIGURE 1.** Identification of Gr-1⁺ cDC. **A.** CD11c⁺ cells from B16 tumors were enriched by anti-CD11c magnetic beads and analyzed by multicolor flow cytometry. The number adjacent to the outlined areas in the far right dot-plot indicates the percentage of Gr-1⁺ cells in the Lin⁻CD11c⁺MHC II⁺ population. **B.** Flow cytometry of freshly isolated Lin⁻CD11c⁺MHC II⁺ cells for the expression of the indicated markers (open) or isotype control (shaded). **C-E.** Percentage (mean ± SD) of Gr-1⁺ cDCs among total cDCs in the indicated s.c. tumors (C), in lymph nodes and spleen of normal mice and mice bearing B16 tumors <0.5 cm or >1 cm in diameter (D), and in normal spleen and spleen containing visible B16 tumor metastases (E). Results are representative of more than four independent experiments.
Tumors induce pre-cDCs to differentiate into Gr-1+ cDCs

The distribution pattern of Gr-1+ cDCs suggested that the tumor microenvironment was critical for their development. Recent studies indicate that circulating pre-cDCs (Lin− CD11c+MHC II+ Gr-1+Flt3+Sirp-α−) are an important source of tumor cDCs (14). To test whether tumors could induce recruited pre-cDCs to differentiate into Gr-1+ cDCs, we adoptively transferred CFSE-labeled bone marrow CD45.1 pre-cDCs into tumor-bearing CD45.2 congenic mice and analyzed their progeny 3 d later. Consistent with previous reports (14), ~60% of the cDCs generated from pre-cDCs had divided, as determined by CFSE dilution. We detected de novo Gr-1 expression in 16.3 ± 8% (n = 6) of the cDCs arising from pre-cDCs in LLC (Fig. 3A); similar results were obtained in mice bearing B16 tumors. In normal mice and in mice bearing small s.c. tumors, pre-cDCs generated exclusively Gr-1+ cDCs in spleen and lung (Fig. 3B), whereas Gr-1+ cDCs could be detected in lungs with B16 or LLC tumor metastases (Fig. 3C). The frequency of Gr-1+ cDCs in lungs correlated directly with tumor burden; Gr-1+ cDCs constituted 4% of the total number of cDCs in lungs with 100–150 macroscopic (1- to 3-mm diameter) B16 tumors. These findings indicated that tumors can induce pre-cDCs and/or their cDC progeny to differentiate into Gr-1+ cDCs.

Tumor-associated cytokines regulate Gr-1+ cDC differentiation

To understand how tumors drive differentiation of pre-cDCs into Gr-1+ cDCs, we performed a cytokine and growth factor screen of normal lungs, lungs containing LLC tumor metastases, and LLC tumors. Compared with normal lung tissue, we identified increased expression of various cytokines and growth factors in lungs with tumors and in LLC tumors (Supplemental Fig. 1). The expression levels of IL-6, IL-1β, IL-3, IL-10, G-CSF, GM-CSF, and M-CSF showed the greatest increase. We next cultured pre-cDCs for 3 d with LLC tumor supernatants in vitro in the presence of the cytokines and growth factors that showed the greatest change in vivo and with cytokines known to affect DC development (e.g., TGF-β and IL-4) (Fig. 4A). About 20–25% of the cells expressed Gr-1 de novo with tumor supernatant alone; this percentage increased with the addition of IL-6, IL-1β, IL-10, and G-CSF and decreased with the addition of GM-CSF, IL-3, IL-4, TGF-β, and TNF-α. We found that adding a neutralizing anti–IL-6 Ab to culture medium containing tumor supernatant alone decreased the frequency of Gr-1+ cDCs by 50% (Fig. 4B) and improved their capacity to stimulate allogeneic lymphocyte pro-
liferation (data not shown); by contrast, blocking Abs to G-CSF, IL-1β, and IL-10 had little or no effect. We therefore focused our attention on assessing the relevance of IL-6 in Gr-1+ cDC development in vivo.

We found a 3-fold reduction in the frequency of tumor Gr-1+ cDCs in IL-6−/− mice, which was associated with an increase in the MHC II expression level by the total tumor cDC population (Fig. 4C, 4D). This response appeared to be confined to the tumor because the frequency and phenotype of cDCs in secondary lymphoid tissue were similar in IL-6−/− mice and wild-type mice, as reported previously (25) (Supplemental Fig. 2). The absence of IL-6 did not affect the intratumor frequency of CD4+ T cells, CD8+ T cells, and NK cells (Fig. 4E). We also found no difference in the frequency of MDSCs or Foxp3+ T cells in tumors (data not shown). Collectively, these data indicated that the absence of IL-6 inhibits Gr-1+ cDC differentiation in tumors.

Gr-1+ cDCs induce defective T cell responses in vivo

Because Gr-1+ cDCs produce IL-10 and were poor stimulators of lymphocytes in vitro, we investigated the effect of tumor Gr-1+ cDCs on T cell responses in vivo. OVA-pulsed tumor Gr-1+ and Gr-1− cDCs were injected s.c. into mice that had received CFSE-labeled OT-II CD4+ or OT-I CD8+ T cells 24 h earlier. We recovered lymph nodes at 3 d to analyze OT-I and OT-II T cell proliferation by CFSE dilution and cytokine production after in vitro restimulation. OT-II cells from Gr-1+ cDC-injected mice proliferated less than those from Gr-1− cDC-injected mice (Fig. 5A). Moreover, primed (CFSE-diluted) OT-II T cells from Gr-1+ cDC-injected mice produced 3-fold less IL-2 and IFN-γ (Fig. 5B). The proliferative response of OT-I T cells mirrored that of OT-II T cells, but the differences in IFN-γ production were more striking (Fig. 5C, 5D). These data indicate that T cells primed by Gr-1+ cDCs in vivo are functionally impaired.

Tumor cDCs promote intratumor CTL proliferation

Recent studies indicate that cognate interactions between Ag-experienced T cells and tissue DCs can enhance T cell proliferation and function (8, 9). To investigate whether the frequency of tumor Gr-1+ cDCs influences CTL activity, we transferred OT-I CTLs into wild-type and IL-6−/− mice with B16 or B16-OVA tumors and recovered tumors, lymph nodes, and spleen at various time points for analysis. We found preferential accumulation of OT-I CTLs and a corresponding increase in IFN-γ+ CD8+ cells in B16-OVA tumors but not in control tumors; this difference increased further by 3- to 4-fold in IL-6−/− mice compared with that in wild-type mice (Fig. 6A–D). Spleen and lymph nodes in

FIGURE 3. Pre-cDCs generate Gr-1+ cDCs. A, Flow cytometry of the progeny of pre-cDCs in s.c. LLC-CCL3 tumors 3 d after i.v. transfer of 1 × 10^6 CFSE-stained bone marrow pre-cDCs; cells were analyzed for the expression of CFSE and the indicated cell surface markers. B, Expression of Gr-1 and MHC II by cells derived from transferred pre-cDCs in spleen and lung of mice bearing s.c. LLC tumors. The results from A and B are representative of six independent experiments. C, Flow cytometry of lung DCs arising from bone marrow pre-cDCs 3 d after i.v. transfer in mice with B16 lung metastases. Results are representative of three independent experiments. Numbers in dot-plots indicate percentage Gr-1+ cDCs.

FIGURE 4. Tumor-associated cytokines regulate Gr-1+ cDC development. A, Effect of adding various cytokines to LLC-CM on the percentage of Gr-1+ cDCs generated from pre-cDCs after 3 d of culture. Data are representative of more than three independent experiments. B, Flow cytometry of Gr-1 and MHC II expression of pre-cDCs cultured in LLC-CM for 3 d in the presence of isotype control or anti–IL-6 Abs. C–E, The frequency of Gr-1+ cDCs (C), the expression of MHC II by cDCs (D), and the frequency of CD8, CD4, and NK cells (E) in s.c. B16 tumors growing in wild-type or IL-6−/− mice.
both IL-6−/− and wild-type mice contained a low but similar frequency of OT-I CTLs regardless of tumor OVA expression, which suggested that the absence of IL-6 was not directly responsible for the increased frequency of tumor OT-I CTLs (Supplemental Fig. 3A). On a per cell basis, OT-I CTLs in B16-OVA tumors and lymphoid tissues expressed comparable levels of IFN-γ, suggesting that intratumor OT-I CTLs were functionally competent (Supplemental Fig. 3B).

In a tumor survival study, the increased intratumor frequency of IFN-γ+ OT-I CTLs detected in IL-6−/− mice correlated with significantly lower B16-OVA tumor growth rates and longer tumor-free survival (Fig. 6E, 6F). Without CTL therapy, B16-OVA tumors grew similarly in IL-6−/− and wild-type mice, indicating that IL-6 does not directly affect B16-OVA tumor growth in vivo.

We next explored potential mechanisms for the increased accumulation of intratumor CTLs in IL-6−/− mice. The similar frequency of OT-I CTLs in the lymphoid tissues of IL-6−/− and wild-type mice argued against a larger reservoir of transferred cells in IL-6−/− mice. Moreover, we detected no difference in the frequency of OT-I CTLs in their blood (Fig. 7A). The rate of OT-I CTL apoptosis in tumors (<1%) and lymph nodes and spleen (5–10%) in IL-6−/− and wild-type mice was also similar (Fig. 7B). Consistent with the importance of OT-I CTL proliferation, we found that intratumor OT-I CTLs had completed multiple rounds of cell division at 7 d as determined by CFSE dilution, which was more extensive in IL-6−/− mice (Fig. 7C). Ag-specific OT-I CTL proliferation in lymph nodes and spleen was limited in both IL-6−/− and wild-type mice, suggesting that proliferation occurred predominately in the tumor. Using a 1-h BrdU pulse assay, we confirmed that a high proportion of CTLs in tumors were actively dividing at 7 d (Fig. 7D).

To investigate whether tumor cDCs contribute to CTL proliferation, we generated CD11c-DTR bone marrow chimeras, in which CD11c+ cells can be ablated by treatment with DT. Preliminary experiments revealed that a higher dosage of DT was required to deplete cDCs in tumors compared with that in lymphoid tissues (Supplemental Fig. 4 and Ref. 20). We assessed the rate of OT-I CTL proliferation 3 d after transfer in tumor-bearing CD11c-DTR and B6 chimeras that were treated with DT 1 d before and 1 d after OT-I CTL injection. DT treatment caused a 50–60% reduction in the frequency of tumor cDCs in CD11c-DTR chimeras (Fig. 8A). Despite this incomplete depletion, the level of OT-I CTL proliferation decreased significantly in B16-OVA tumors from CD11c-DTR chimeras compared with that in those from control chimeras (Fig. 8B). A low level of OT-I CTL proliferation persisted in the lymph nodes and spleen of both chimeras, indicating that OT-I CTL proliferation in these tissues is mostly autonomous and independent of the presence of DCs. Notably, activated CD8+ T cells express CD11c (26); however, the adoptively transferred OT-I CTLs are unaffected by DT because they lack DTR.

To establish further the involvement of tumor cDCs in CTL proliferation, we selectively sorted DC-depleted CD45+ infiltrating cells from B16-OVA tumors, stained them with CFSE, and cultured them together with or without sorted cDCs from the same tumor. We found that OT-I CTLs proliferated only in the presence of tumor cDCs (Fig. 8C). We considered the possibility that tumor cDCs were providing non-Ag-specific support for T cell proliferation; however, normal spleen cDCs failed to stimulate CTL proliferation unless they were pulsed with OVA peptide (data not shown). Tumor cDCs isolated from B16-OVA tumors in IL-6−/− mice stimulated higher rates of proliferation, consistent with the lower frequency of Gr-1+ cDCs in these tumors. It was possible that the higher proliferative response to tumor cDCs from IL-6−/− mice might be related to differences in the suppressive activity of tumor myeloid cells or lymphoid cells contained in the DC-depleted population (27). However, we obtained the same results when DC-depleted tumor-infiltrating cells from IL-6−/− mice and wild-type mice were combined together and stimulated with sorted tumor cDCs from wild-type or IL-6−/− mice.

**Discussion**

Our study has revealed that cancer-associated inflammation changes the composition and immunogenicity of tumor cDCs by inducing the differentiation of Gr-1+ cDCs. We show that the development of this subpopulation is associated with defects in antitumor T cell immune responses and uncover the unanticipated importance of tumor cDCs in the stimulation of CTL proliferation, a key determinant of intratumor CTL frequency and efficacy.

Many embrace the view that all cDCs in tumors are functionally defective. Our study shows that the impairment resides mainly in the Gr-1+ cDC population. Gr-1− cDCs are indistinguishable from cDCs in normal lymphoid tissues with respect to their ability to capture Ag, mature, and stimulate T cell responses in vitro and in vivo (14). Gr-1+ cDCs first appear in tumors at an early stage of tumor development and subsequently increase in frequency in draining lymph nodes and spleen with tumor growth. Gr-1+ cDCs in lymphoid tissues are similar to those in tumors and likely arise...
through migration from the tumor, the local effects of tumor metastases, and the systemic effects of soluble molecules released from tumors. Several characteristics of Gr-1+ cDCs help explain their suppressive activity: low expression levels of MHC II and costimulatory molecules, resistance to maturation stimuli, and constitutive production of IL-10. Gr-1+ cDCs differ from Gr-1+ MDSCs, a heterogeneous population of neutrophils, macrophages, and primitive myeloid cells that accumulates in tumors, bone marrow, and lymphoid tissues of tumor-bearing mice. MDSCs lack expression of CD11c and MHC II and directly suppress MLRs through the release of NO, arginase, and other metabolites (28–30). By contrast, neither Gr-1+ cDCs nor Gr-1+ cDCs can inhibit the stimulatory activity of third-party APCs in vitro (data not shown). Gr-1+ cDCs are also distinct from “tip” DCs, a specialized population of inflammatory Gr-1+ DCs that arise from monocytes during some inflammatory processes (24).

FIGURE 6. Increased frequency of tumor Ag-specific CTLs in tumors from IL-6−/− mice decreases tumor growth and prolongs survival. A and B, Frequency of OT-I CTLs in B16 and B16-OVA tumors 7 d after adoptive transfer of CD45.1 OT-I CTL into wild-type and IL-6−/− CD45.2 mice. Numbers in dot-plots indicate percentage OT-I CTL in gate. Error bars are mean ± SD. Data are representative of >6 experiments. C and D, Frequency of IFN-γ+ CD8+ T cells in B16 and B16-OVA tumors 7 d after adoptive transfer of CD45.1 OT-I CTLs into wild-type and IL-6−/− CD45.2 mice. E, B16-OVA tumor size in wild-type and IL-6−/− mice that received OT-I CTL or control T cells 5 d after tumor inoculation. Data are representative of three independent experiments (n = 5 mice per group). *p < 0.01 (paired Student t test). F, Tumor-free survival of mice from E. p = 0.012 (log-rank test).

FIGURE 7. Increased proliferation of tumor Ag-specific CTLs in tumors from IL-6−/− mice. CFSE-labeled CD45.1/CD45.2 OT-I CTLs were transfected into CD45.2 wild-type or IL-6−/− mice bearing no tumor or a tumor derived from B16 or B16-OVA. Seven-day tumors, blood, and tissues were collected for analysis. A, Frequency of OT-I CTLs in blood (mean ± SD, p = NS). B, OT-I CTL apoptosis as determined by annexin V staining. C, OT-I CTL proliferation by analysis of CFSE dilution. Data are representative of three independent experiments with four mice per group. D, BrdU uptake by transferred OT-I CTLs in spleen, lymph nodes, and tumors in wild-type mice bearing B16-OVA tumors. Data are representative of two independent experiments with three mice per experiment.
Pre-cDCs are considered the main immediate precursor for cDCs in lymphoid tissues under steady-state conditions, and recently their role in DC development has been extended to various organs and tumors (14, 17, 18, 31–34). By monitoring the fate of pre-cDCs in tumors and in tumor-conditioned medium, we confirmed that pre-cDCs generate Gr-1+ cDCs, which were similar in all respects to endogenous Gr-1+ cDCs. Pre-cDCs upregulate MHC II expression rapidly when cultured in vitro, and at this stage these replication-competent cDCs are Gr-1
18
(18). Because Gr-1+ cDCs were undetectable in normal mice, we believe they signify a pathologic response of pre-cDCs and their immediate Gr-1
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 cDC progeny to the inflammatory milieu in tumors. Thus, Gr-1+ cDC transformation represents a tumor-controlled process that decreases cDC immunogenicity. Whether pre-cDCs are the only source for Gr-1+ cDCs is unclear. We found that monocytes, the most likely alternative precursor, fail to generate cDCs in tumors after adoptive transfer, suggesting that the tumor microenvironment either inhibits, or lacks key supportive signals for, DC differentiation.

Although pre-cDCs and cDCs integrate numerous signals in the tumor microenvironment, our study suggests that altering the expression of a single cytokine—IL-6—upsets the balance and hinders differentiation of Gr-1+ cDCs. Other studies have shown that IL-6 regulates MHC II assembly in DCs by activating the transcription factor STAT3 (35, 36), and inhibits monocyte differentiation into DCs (37). IL-6 has been documented to be a factor in tumorigenesis and tumor progression in many animal tumor models (4, 38). High serum concentrations of IL-6 occur in patients with various epithelial and lymphoid cancers and correlate with poor outcome and abnormal immune responses (39, 40). In patients with hepatocellular carcinoma, low expression of microRNA-26a in the tumor is associated with high IL-6 expression and poor survival (41). Many tumor-associated molecules and metabolites act as endogenous “danger signals,” which can initiate and enhance expression of inflammatory cytokines and chemokines in tumor cells and host-derived infiltrating cells (42). Versican, an extracellular matrix protein that is upregulated in LLC tumors and some human cancers, stimulates macrophages to produce IL-6 by signaling through TLR2 and its coreceptors TLR6 and CD14 (4). Whether this mechanism participates in Gr-1+ cDC differentiation remains to be defined.

The success of CTL therapy for cancer relies on achieving sufficient numbers of functional CTLs in the tumor (43, 44). Our study indicates that transferred CTLs undergo multiple rounds of cell division in tumors, which was tumor Ag dependent. A small proportion of CTLs divided in lymphoid tissues; however, this also occurred in the absence of cognate Ag, consistent with autonomous proliferation. CTLs in tumors and lymphoid tissues expressed similar levels of IFN-γ, suggesting that CTLs in tumors maintained functional competence. The antitumor efficacy of CTL treatment in IL-6
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 mice was better than that in wild-type mice; this response correlated directly with a 4-fold higher frequency of CTLs in their tumors. Greater intratumor CTL proliferation appeared to be the main cause for the difference. Tumor Ags can be presented to intratumor T cells by tumor cells directly or by APCs via cross-presentation pathways (45). The DC depletion experiments in CD11c-DTR chimeras and the in vitro Ag presentation studies with tumor cDCs demonstrated a link between tumor cDC and CTL proliferation. Further, the in vitro studies indicated that tumor cDCs could stimulate CTL proliferation even in the presence of other myeloid and lymphoid cells contained in...
the tumor-infiltrating population. The absence of IL-6 had no effect on OVA expression in B16 tumors, nor did it alter the frequency of MDSCs, B cells, NK cells, or Foxp3^+ regulatory T cells in tumors or lymphoid tissues. Although we do not exclude the possibility that subtle changes of other immune cells in IL-6^-/- mice might increase intratumor CTL proliferation, our data strongly suggest that the reduced frequency of Gr-1^- DCs in tumors was an important factor.

Reports of recent studies in viral infection models indicate that Ag-experienced T cells require cognate interactions with tissue DCs for local expansion and acquisition of effector functions (8, 9, 46). The concept that tumor stromal cells participate in cross-presentation of tumor-derived Ags and serve as targets for immune destruction by CTLs is well supported by previous studies (45, 47, 48); however, the identity of the APCs in these studies was not defined. To our knowledge, our study establishes for the first time the critical role of intratumor DCs in regulating CTL frequency by stimulating CTL proliferation. Further, we highlight the significance of defective DC differentiation as a tumor-specific mechanism for impairing CTL efficacy. These findings have implications for adoptive T cell therapy: In settings where tumor-derived Ags are “weak” or have limited presentation in lymphoid tissues, intratumor DCs offer a promising target to improve antitumor CTL responses.

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Disclosures

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References


