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Tumor-Infiltrating Dendritic Cells Are Potent Antigen-Presenting Cells Able to Activate T Cells and Mediate Tumor Rejection

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Dendritic cells (DC) play a major role in orchestrating immune responses. They are characterized by their ability to capture and present Ags to naïve T cells in a MHC class I- or class II-restricted fashion, thus initiating Ag-specific immune responses (1). The presence of cells with a dendritic morphology and/or expression of DC markers has been reported in numerous tumors (reviewed in Ref. 2).

Despite the presence of such tumor-infiltrating DC (TIDC), tumor cell outgrowth often occurs, indicating that immunity against tumor cells is either improperly induced or bypassed by the tumor. This raises questions regarding the status of TIDC. Studies have shown that tumor cells produce molecules that inhibit DC maturation such as IL-10 (3), vascular endothelial growth factor (4), PGE₂ (5), and TGF-β (6). More recently, purified TIDC were shown to be refractory to ex vivo maturation stimuli because of autocrine production of IL-10 (7). This observation is consistent with their inability to induce appropriate allogeneic T cell activation (8). Taken together, the above reports suggest that the tumor milieu is immunosuppressive for DC and has the ability to selectively modulate or recruit TIDC to produce IL-10 (7, 9). Despite the reported immunosuppressive properties of the tumor milieu, evidence in favor of the presence of functional APC in tumor-bearing hosts also exists. Indeed, host APC have been shown to potently cross-prime T cells specific for tumor Ags in several murine tumor models (10, 11). Furthermore, Spiotto et al. (12) have demonstrated that tumor-infiltrating cells derived from the bone marrow could induce such T cell cross-priming, suggesting that TIDC are capable of effective Ag presentation.

Given the conflicting reports in the literature, we performed a detailed study of the phenotype and functional status of DC infiltrating several types of mouse melanoma, with an effort to minimize the ex vivo procedures preceding cell analysis.

Materials and Methods

Mice

Six to 8-wk C57BL/6 and C3H × C57BL/6 F₁ (B6C3F₁) mice were bred in-house. The Tyr-ras transgenic mouse has been described elsewhere (13). In this mouse, topical application of 50 µg of DMBA in 100 µl of acetone (1 application/wk to the dorsal skin) for 5 wk induced palpable cutaneous melanoma within 4–5 mo. K14-ΔNΔC β-catenin transgenic mice (provided by Dr. H. Fuchs, Rockefeller University New York, NY) spontaneously developing epithelialomas have already been described (14). Procedures involving animals and their care were conducted in conformity with the Swiss Veterinary Office.

Tumors

The B16F10 melanoma cell line (H-2b) was provided by L. Fidler (University of Texas, Houston, TX). The K17-35 melanoma cell line (H-2k) was provided by D. B. Werhle-Haller (Geneva University, Geneva, Switzerland). MC38 and CMT93 colon carcinoma cell lines were purchased from American Type Culture Collection. To obtain OVA-expressing K17-35 melanoma, full-length OVA, provided by Dr. M. Bevan (University of Washington, Seattle, WA), was subcloned into pCDNA3.1(+). A cytoplasmic form of OVA was obtained by PCR-created deletion of the first 45 aa required for OVA secretion. The primers used were 5'-CCGC TCGAGTTCACCATGGGTGCAAAAGACAGCAC-3' (forward primer) and 5'-CGGGTGACTCTAAAGGGGAACACATCTG-3' (reverse primer).

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4 Abbreviations used in this paper: DC, dendritic cell; TIDC, tumor-infiltrating DC; FSC-H, forward light scatter-high; SSC-H, side scatter-high; MHC II, MHC class II.
K17-35 cells were transfected by electroporation with 10 μg of plasmid DNA and selected with 0.2 mg/ml G418 (Invitrogen Life Technologies). Tumor cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS. Cells were detached from culture dishes in PBS with 2 mM EDTA, and 2 × 10^6 tumor cells were injected s.c. in 50 μl of HBSS (Invitrogen Life Technologies). Tumor growth was monitored daily using a caliper. TIDC were analyzed when tumor diameter ranged between 0.5 and 1 cm. After resection, tumors were diced in Ca^2+- or Mg^2+-free HBSS and incubated with 1 mg/ml type IV collagenase (Sigma-Aldrich) for 90 min at room temperature and under constant stirring. EDTA (2 mM) was added to the mixture for 30 additional min before filtration of the cell suspension on 70-μm cell strainers (BD Biosciences). The cell suspension was finally washed twice in HBSS before analysis.

**Normal skin dissociation**

Whole skin was incubated overnight at 4°C in medium with trypsin 0.25% (Invitrogen Life Technologies). The epidermis was then separated from the dermis and dissociated mechanically before incubation with 10 mM EDTA for 30 min at 37°C. Dermis was dissociated with type IV collagenase + EDTA as described above. Single-cell suspensions from dermis and epidermis were mixed before analysis.

**Flow cytometry**

The fluorochrome-conjugated Abs used were against CD45, I-A, CD11c, CD8α, B220 (CD45R), CD40, CD80, CD86, CD11b (all from BD Pharmingen), IL-12p70, IL-12p40 (eBioscience), and DEC205 (Cedarlane Laboratories). K^b^-SIINFEKL tetramer (a gift from Dr. A. Donda, Swiss Institute of Biochemistry, Epalinges, Switzerland) was used to detect CD8^+ T cells specific for the immunodominant epitope of OVA (OVA257–264). For surface staining and separation by flow cytometry with forward light scatter-high (SSC-H) parameters analyzed on a logarithmic scale. Cells positive for microbeads were then assessed for CD11c and (FSC-H)/side scatter-high (SSC-H) parameters analyzed on a logarithmic scale. Cells positive for microbeads and AutoMACS (Miltenyi Biotec; procedure recommended by the manufacturer). A total of 1 × 10^6 OVA-pulsed TIDC in 50 μl of HBSS was injected in the dermis of syngeneic mice. The same day, mice received 2 × 10^6 CFSE-labeled splenocytes (1.25 μM; Sigma-Aldrich) i.v. from transgenic OT-I and/or OT-II mice (provided by Dr. W. Heath (Eliza Hall Institute of Biochemistry, Victoria, Australia)). In some control animals, B6C3F1 mice were immunized with 80 μg of OVA emulsified in CFA (Sigma-Aldrich). Three days later, lymph nodes were collected, mechanically dissociated, stained with appropriate Abs, and CFSE dilution was analyzed by flow cytometry in gated cells. For tumor protection experiments, OVA-pulsed TIDC were injected twice, 1 wk apart, in naive B6C3F1 mice. Two weeks after the last injection, mice were challenged with K17-35-OVA melanoma.

**Results**

Mouse melanomas are infiltrated by DC

The presence of cells with a DC phenotype was investigated in two transplantable (B16F10 and K17-35) and one spontaneous mouse melanoma model. Upon tumor dissociation, leukocytic cells (CD45^+ ) could be distinguished by FACS analysis from malignant cells by their size (FSC-H) and morphology (SSC-H) (Fig. 1A). In the leukocytic cell population, CD11c^-/MHC II^- cells were visualized, indicating the presence of cells with a DC phenotype (Fig. 1B). In this experiment, PBS perfusion of mice before tumor removal and dissociation did not decrease the recovery of CD11c^- cells (data not shown), indicating that these were not circulating DC present in the tumor vasculature but TIDC. CD11c immunostaining of tumor sections confirmed the presence of CD11c^- cells within the tumor bed (Fig. 1C). The recovery of TIDC was not restricted to melanomas because we could also recover such cells from tumors of different origin, including colon carcinomas (MC38 and CMT93) and a spontaneous epithelioma (Fig. 1D). Despite such ubiquitous presence of TIDC in tumors, the level of infiltration was variable and dependent on the tumor type. TIDC represented 4.9 ± 0.22% of the total cell suspension from the K17-35 melanoma, whereas they represented 0.02 ± 0.004 and 0.13 ± 0.07%, respectively, in spontaneous and B16F10 melanomas (Fig. 1D). In some tumors, the percentage of TIDC was even higher than that observed in normal skin (Fig. 1D).

**Different DC subsets infiltrate mouse melanomas**

The phenotypic characteristics of TIDC were investigated using the markers CD11c, CD8α, and B220 as described by Allan et al. (15). Exclusion of CD3^- cells to eliminate DCTL doublets (observed in preliminary experiments) and elimination autofluorescent cells by flow cytometry were applied (16). With these settings, two major DC populations, namely B220^-/CD8α^- and B220^+ , were constantly found (Fig. 2A). The B220^-/CD8α^- cells corresponded to myeloid DC, and the B220^+ cells corresponded to plasmacytoid DC (15). Among the latter, some expressed CD8α in accordance with that reported by others (15). Finally, in the B16F10 melanoma, a third minor CD8α^-/B220^- population was also found. To confirm these observations, an additional staining with DEC205 was performed. A majority of the TIDC were CD8α^- with or without expression of DEC205, confirming the predominance of the myeloid subset (Fig. 2B). Small amounts of DEC205^-/CD8α^- cells were observed in the B16F10 melanoma, confirming the presence of lymphoid DC. The CD8α^-/DEC205^- population likely represented the plasmacytoid subset described above. In these experiments, we did not observe cells expressing high levels of DEC205, as observed in skin preparations (data not shown), thus excluding the presence in these melanomas of cells with a phenotype of Langerhans cells. In conclusion, the myeloid subset constituted >80% of the total CD11c^- cells in the two transplantable melanomas, whereas they were less predominant in the spontaneous melanoma (39%). Immunostaining of other tumor types showed that the myeloid subset was also predominant in MC38 and CMT93 colon carcinomas (data not shown), thus confirming that the transplantable tumors analyzed herein contain TIDC that mostly have the phenotypic characteristics of myeloid DC.
**TIDC have endocytic properties**

To assess the functional status of TIDC, we performed an in vivo particle uptake assay by injecting nondiffusible fluorescent 1-µm microbeads. In this experimental setting, the presence of fluorescent events in the cellular gate is indicative of particle uptake (17–19) (Fig. 3A). Similarly to these previously reported in vivo phagocytic assays, fluorescent microbeads were associated with cells specifically stained with the CD11c Ab in both B16F10 and K17-35 melanomas (Fig. 3B). CD11c+ cells (27.5 ± 19.5 and 12 ± 4.2%) from the B16F10 and K17-35, respectively, had taken up one or more microbeads. Some CD11c− cells that had taken up microbeads expressed CD11b, indicating that infiltrating macrophages also internalized beads (Fig. 3B). Taken together, these observations indicate that a sizeable fraction of TIDC are able to internalize particles in vivo, thus demonstrating their endocytic capacity and indicating an immature state.

**TIDC rapidly acquire a fully mature phenotype after tumor dissociation**

To assess the functional status of TIDC, we analyzed their surface expression of the activation markers MHC class II (MHC II), CD40, CD80, and CD86. To minimize signaling events that could occur during the purification procedure, TIDC were immunostained immediately after tumor dissociation. In these conditions, MHC II, CD40, CD80, and CD86 were found to be constitutively expressed on the majority of TIDC, indicating a certain state of activation. After overnight culture of the tumor cell suspension at 37°C, further up-regulation of costimulatory molecules on TIDC from B16F10 melanomas (Fig. 4A, left panel), K17-35 melanomas (Fig. 4A, middle panel), and spontaneous melanomas (Fig. 4A, right panel) was observed when compared with cells stained immediately after tumor dissociation. This up-regulation was observed for all the costimulatory molecules analyzed (MHC II, CD40, CD80, and CD86) on TIDC. In the K17-35 melanoma, only CD40 and CD86 were up-regulated on TIDC, whereas MHC II and CD80 expression did not increase significantly. This is consistent with an intermediate activation phenotype of TIDC, with some markers being already expressed at a level observed on mature cells and indicates that TIDC were able to mature upon ex vivo culture in the dissociated tumor milieu. It is noteworthy that addition of LPS + anti-CD40 Ab in the medium during overnight culture did not further up-regulate the expression of costimulatory...
molecules when compared with that observed after overnight culture alone (data not shown). The maturation status of the different TIDC subsets was also analyzed in this experiment, with CD40 used as activation marker. Myeloid TIDC (B220<sup>-</sup>/H11002) and plasmacytoid TIDC (B220<sup>+</sup>/H11001) from B16F10 melanoma up-regulated CD40 after overnight culture at 37°C (Fig. 4B), showing that both TIDC subsets acquired a phenotype of mature cells. In addition, intracellular IL-12 accumulation could be detected in the majority of CD11c<sup>-</sup> cells from B16F10 and K17-35 melanomas after overnight culture (Fig. 4C). Taken together, these results indicate that TIDC have the capacity to spontaneously mature upon tumor dissociation and ex vivo culture. Furthermore, under these conditions, DC activation was potent enough to drive IL-12 production.

**FIGURE 3.** TIDC internalize particles in vivo. A. Free microbeads and cells were separable by flow cytometry analysis according their size (FSC-H) and morphology (SSC-H) using a logarithmic scale. B. Analysis of the phagocytosis of fluorescent microbeads injected in B16F10 or K17-35 melanoma. Microbead<sup>-</sup> cells in B16F10 melanoma were gated and analyzed for CD11b/CD11c expression.

We next investigated the capacity of TIDC to process a soluble Ag and to activate naive T cells in vivo. In this experiment, K17-35 melanoma was used to recover a sufficient number of TIDC for vaccination purposes. Tumor cell suspensions were pulsed with OVA for 4 h, and purified CD11c<sup>-</sup>/H11001 cells were subsequently injected into the mouse dermis. This resulted in specific proliferation of transferred OT-I and OT-II cells as observed in draining lymph node 3 days after injection (Fig. 5A). This experiment confirms the endocytic property of TIDC previously observed and further demonstrates their Ag processing and presentation capacity. To demonstrate that TIDC effectively migrated from the injection site to the draining lymph node, OVA-pulsed CD11c<sup>-</sup> cells were exposed to Bordetella Pertussis toxin before injection into the dermis. This toxin has been shown to inhibit DC migration via irreversible blockade of the α subunit of G proteins (20, 21). In this
setting, OT-1 cell proliferation was fully abrogated (Fig. 5B). Furthermore, intradermal injection of CFSE-labeled TIDC resulted 3 days later in the presence of CFSE+/H11001 fluorescent events in draining lymph nodes (Fig. 5C). Before injection into the dermis, TIDC were labeled with CFSE. Three days later, the presence of CFSE+/CD11c+ cells was investigated by flow cytometry in draining lymph nodes.

FIGURE 5. TIDC process soluble OVA, migrate to lymph nodes, and activate naive T cells. A, TIDC were recovered from K17-35. Mice were challenged with OVA-pulsed purified TIDC, and the proliferation of transferred CFSE-labeled OT-I and OT-II was analyzed 3 days later in lymph nodes. CFSE dilution was analyzed by flow cytometry in CD8+/CD4− for OT-I and CD8+/CD4+ cells for OT-II. B, The same experiment as in A using OT-I cells. Pertussis toxin (200 ng/ml) was added to the cell suspension during the pulse with OVA. C, Before injection into the dermis, TIDC were labeled with CFSE. Three days later, the presence of CFSE+/CD11c+ cells was investigated by flow cytometry in draining lymph nodes.

FIGURE 6. OVA-loaded TIDC induce in vivo T cells expansion. Mice were vaccinated with OVA-pulsed TIDC from K17-35 or PBS. Two weeks later, the percent of tetramer+ cells in CD8+ T cells was investigated. *, A significant statistical difference according to the Student t test (p < 0.01).

TABLE 1. Immunodominant OVA epitope.

Discussion
Herein, we have analyzed the number, phenotype, and functional characteristics of DC present in murine tumors. The presence of significant numbers of TIDC was observed in several tumors. In some tumors, the percentage of TIDC was higher than in healthy skin, which is indicative of an active mobilization of these cells to tumors. Langerhans cells (DEC205high) were not found, and most TIDC did not harbor a phenotype of dermic DC (DEC205−), suggesting that melanomas do not specifically recruit local DC present in the skin. The level and quality of TIDC infiltration was reproducible for individual tumors of same type between experiments but was dependent on the tumor type. The biggest difference was that observed between transplantable and spontaneous melanomas. Such a difference may reflect modifications induced at the site of implantation by the needle trauma in the case of transplantable tumors. The difference in levels of TIDC infiltration between different types of murine tumors is consistent with the variations described in cancer patients (2, 22, 23), suggesting that the tumor milieu varies in its capacity to mobilize DC (24).

FIGURE 7. OVA-pulsed TIDC protect against a tumor challenge. OVA-pulsed TIDC were isolated from the K17-35 melanoma and injected twice (one injection per week) into the skin of groups of tumor-free B6C3F1 mice. Two weeks later, mice vaccinated with OVA-pulsed TIDC from K17-35 were challenged with K17-35-OVA. Tumor growth was monitored each day. Their size was calculated by multiplying the length (centimeters) × the width (centimeters). n indicates the number of mice for each group.
TIDC were mostly constituted by myeloid and to a lesser extent plasmacytoid DC. In accordance with this observation, both myeloid and plasmacytoid DC have been shown to infiltrate human tumors (25–27). In our experimental setting, we observed that TIDC have characteristics of immature DC in vivo and retain the capacity to mature into fully competent APC ex vivo. This was simply achieved in the tumor milieu upon dissociation and without the need for addition of cytokines or bacterial products. In this respect, our results contrast with the previously reported resistance of TIDC to ex vivo maturation signals in the absence of anti-IL-10R treatment (7, 28). Experimental differences between the two studies may be the cause of this discrepancy. First, we did not purify TIDC before assessing ex vivo maturation, whereas others immunomagnetically purified TIDC with an anti-CD11c Ab. Second, TIDC were left in contact with dissociated tumor cells in our protocol. The tumor cells may provide an important maturation signal in culture, especially if a certain level of tumor cell death is induced during the dissociation procedure (29, 30). This is in accordance with the fact that TIDC maturation could not be enhanced by LPS and anti-CD40 treatment.

Our experiments constitute the first demonstration that DC naturally infiltrating tumors have the capacity to process a soluble exogenous Ag ex vivo and subsequently migrate to lymphoid organs after s.c. injection to activate both naïve CD4+ and CD8+ T lymphocytes. Hence, one could ask why efficient antitumor T cell priming is not more often observed spontaneously in tumor-bearing subjects. Three important features relative to the tumor environment could explain the absence of spontaneous T cell immunity despite the presence of TIDC. First, immunosuppressive factors such as IL-10, TGF-β, vascular endothelial growth factor, or PGE2 acting on TIDC and T cells may represent a significant barrier in vivo (31). Second, in vitro-generated DC injected into a s.c. transplanted tumor have been shown not to migrate into lymphoid organs (32), indicating that tumors secrete factors that sequester (33) or even retain DC, possibly via intercellular interactions (34). Third, the loading of TIDC with tumor Ags may be insufficient to induce T cell priming. Mobilized TIDC following tumor transduction with GM-CSF and CD40L have been shown to take up tumor-associated Ags and efficiently present them to T cells (35), but this has never been achieved with DC infiltrating untransduced tumors. In addition to the limited amount of tumor Ag available, TIDC may be inefficient at taking up cell-associated tumor Ags. In this context, it is interesting to note that lymphoid DC are the subset that are known to be the most competent in cross-presentation of cell-associated Ags to T cells (36, 37), whereas myeloid DC are more competent in cross-presentation of soluble Ag to T cells (38). Since it is widely accepted that for extralymphatic tumors, T cell priming occurs via cross-presentation (our unpublished observations) and in accordance with the subset distribution observed in this study, the low relative frequency of lymphoid cells infiltrating the tumor may explain in part the absence of efficient T cell cross-priming. Finally, Van Mierlo et al. (39) have recently shown that lymph node CD11c+ cells are sufficiently loaded with tumor Ags to activate T cells. However, this study did not determine whether TIDC were loaded locally with Ags within the tumor lesion or whether cell-free tumor Ags diffused to the lymph node to load resident DC.

Herein, we have shown that naturally occurring TIDC from melanoma are functionally competent to process Ag and prime T cells when an exogenous Ag is given to them ex vivo. This confirms that tumors do not irreversibly impair the ability of infiltrating DC to generate specific immune responses as previously reported (7) and opens perspectives for in vivo therapeutically oriented manipulations targeting TIDC. Indeed, intratumoral injection of DC-activating agents has been shown to induce antitumor T cell immunity (7, 28). However, based on the present study, in vivo manipulations aimed at increasing the loading of TIDC, such as induction of tumor cell death, could theoretically replace the ex vivo pulse we have performed here and thereby induce efficient T cell priming. Along this line, it is interesting to note that systemic administration of a chemotherapeutic drug has been shown to increase tumor Ag presentation in draining lymph nodes, and this leads to significant antitumor immunity (40). In addition, local tumor irradiation has also been shown to induce potent antitumor CD4+ and CD8+ T cell immunity in the B16F10 model (41). Taken together, these results reinforce the idea that suitable treatment modalities aimed at inducing tumor cell death and, as a consequence, maturation of TIDC, while preserving immune cell functions, may provide an improvement to current immune-based cancer treatments.

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Disclosures

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