Early Tumor-Infiltrating Dendritic Cells Change their Characteristics Drastically in Association with Murine Melanoma Progression

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Dendritic cells (DCs) have a critical effect on the outcome of adaptive immune responses against growing tumors. Tumor-infiltrating dendritic cells (TIDCs) play diverse roles in the regulation of tumor regression or growth, but the characteristics that distinguish those effects are obscure. In this study, we investigated the frequency, phenotype, and function of TIDCs over time from early stages of melanoma growth in mice. Flow cytometric analysis revealed that the tumors were infiltrated by a significant population of $CD11c^+$ major histocompatibility complex II^+ DCs, especially at an early stage of tumor growth. The allogeneic stimulatory capacity of TIDCs increased with tumor growth, whereas this capacity of DCs in lymph nodes decreased. TIDCs harvested at an early stage of melanoma (early TIDCs) accelerated tumor growth, but those harvested at a late stage (late TIDCs) delayed tumor progression when they were coinjected with melanoma cells. Furthermore, coinjection of early TIDCs failed to induce full immunocompetent maturation of $CD8^+$ T cells, with much lower expression of IFN-γ, granzyme B, and perforin within the tumor microenvironment. In conclusion, TIDCs change their characteristics from an immunoinhibitory to an immunostimulatory phenotype over time in association with tumor progression.

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INTRODUCTION

Dendritic cells (DCs) are potent antigen-presenting cells that play key roles in initiating and regulating adaptive immune responses against growing tumors (Liu and Nussenzweig, 2010; Steinman and Idoyaga, 2010). Although it is generally assumed that the presence of phenotypically mature DCs in a tumor should promote protective antitumor immunity, evidence to the contrary has been obtained (Jensen et al., 2012; Sandel et al., 2005). To escape from antitumor immune responses that DCs orchestrate, tumors have equipped various mechanisms to impair the immunostimulatory functions of DCs or even to manage them to promote tumor growth and progression (Engelhardt et al., 2012; Gottfried et al., 2006). The development of melanoma models in mice has greatly contributed to the molecular understanding of melanoma immunobiology, but the precise nature and molecular mechanisms of tumor-infiltrating DCs (TIDCs) have not been elucidated, and there is surprisingly little consensus in the field with respect to the frequency, composition, and function of TIDCs (Klarquist and Janssen, 2012). One of the reasons for this might be that few studies have aimed to elucidate the nature of TIDCs infiltrating early stages of melanoma growth and their functional changes over time. Thus, in this study, we performed quantitative, phenotypic, and functional analyses of TIDCs obtained from early to late stages of murine melanoma cells inoculated with Matrigel.

RESULTS

Murine melanomas are infiltrated by a significant population of DCs, especially at an early stage of tumor growth

To investigate the intratumor dynamics of DCs, we first examined the precise proportion and number of TIDCs during tumor growth after melanoma inoculation. We began our analysis 2 days after tumor inoculation, using tumors injected intradermally with Matrigel, to develop a more complete understanding of the characteristics of TIDCs from the early tumor microenvironment. Mice were assessed for the proportions and numbers of TIDCs over time after tumor injection (Figure 1a). Although tumors are first macroscopically visible at about days 10−14 in the usual model, we could see tumors and harvest them even at an early stage of tumor growth using this Matrigel method (Figure 1b, Supplementary Figure S1 online). In murine melanoma, TIDCs are identified by their high expression levels of CD11c and major histocompatibility complex (MHC) class II (Figure 1c). As shown in Figure 1d, flow cytometric analysis of tumor-infiltrating cells revealed that the tumors were infiltrated by a significant population of $CD11c^+$ MHC II^+ DCs, especially at early stages of tumor growth. TIDCs infiltration constituted

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Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; DCs, dendritic cells; LNs, lymph nodes; PD-L1, programmed death-ligand 1; rDCs, resident DCs; TIDCs, tumor-infiltrating dendritic cells; VEGFR-2, vascular endothelial growth factor receptor 2

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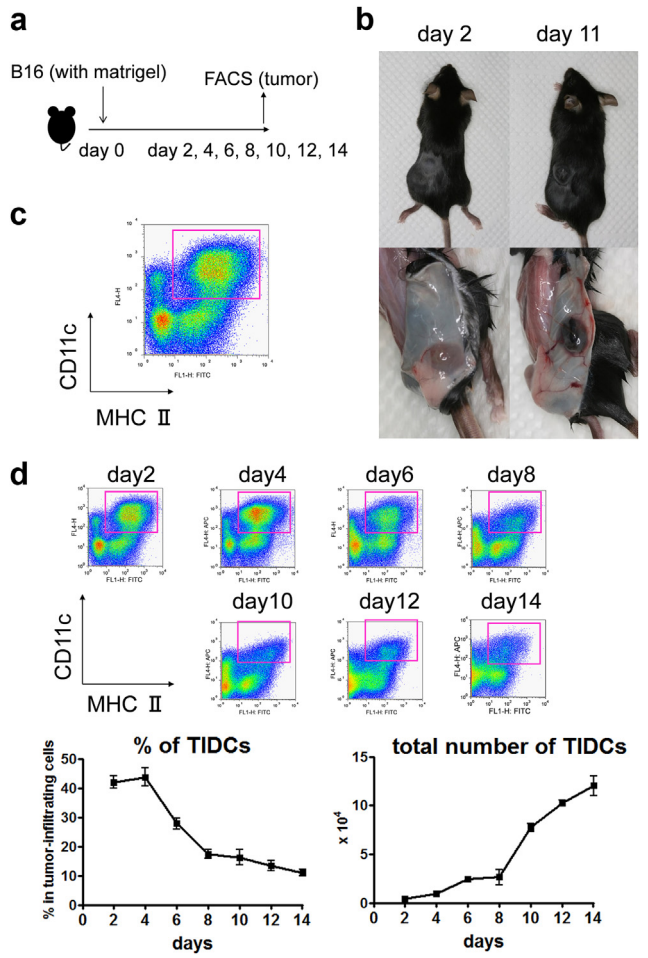

Figure 1. Murine melanomas are infiltrated by a significant population of TIDCs. (**a**) B16 cells were suspended in Matrigel and inoculated into the flanks of C57B/6 mice. After harvesting of tumors, tumor-infiltrating $CD45^+$ cells were isolated by density gradient centrifugation and were assessed for the proportion and number of TIDCs by FACS. (**b**) Representative images of melanoma are shown from mice 2 days or 11 days after tumor inoculation. (**c**) TIDCs in melanoma stained for CD11c and MHC class II. (**d**) Time course of the proportion and total number of TIDCs ($CD11c^+$/MHC class II^+) in tumor-infiltrating cells during melanoma growth. All values represent the mean ± SE of 3 to 12 mice. Representative data from three separate experiments are shown. MHC, major histocompatibility complex; TIDCs, tumor-infiltrating dendritic cells.

approximately 40% of all tumor-infiltrating $CD45^+$ cells harvested from the tumor at day 2. The proportion of TIDCs increased until day 4 and then gradually decreased until day 14. On the other hand, the absolute number of TIDCs increased over the course of tumor growth. In contrast with the proportion of TIDCs, that of $CD8^+$ T cells kept increasing during tumor growth, resulted in the increase of the ratio of $CD8^+$ T cells to TIDCs (Supplementary Figure S2 online).

TIDCs are phenotypically more mature than DCs in draining lymph nodes (LNs) during tumor growth

In subcutaneous LNs, migratory DCs are skin-derived DCs and have a $CD11c^{int}$ MHC class II^{high} phenotype, whereas resident DCs (rDCs) have a $CD11c^{high}$ MHC class II^{int} phenotype (Figure 2a) (Nakahara et al., 2010). Compared with the dynamic change in the proportion of TIDCs, the proportion of DCs in draining or nondraining LNs did not change significantly during tumor growth (Figure 2b). Therefore, the dynamic change of TIDCs was much more prominent than that of DCs in LNs. Next, we examined the phenotypic maturation status of rDCs in draining LNs and TIDCs. rDCs in draining LNs expressed somewhat higher levels of CD86 than rDCs of naive control mice from days 2 to 8 after tumor inoculation. After day 10, the expression of CD86 by rDCs in draining LNs decreased to the level of rDCs of naive control mice. Meanwhile, TIDCs expressed persistently higher levels of CD86 than rDCs of naive control mice during tumor growth (Figure 2c). Notably, TIDCs were phenotypically more mature than rDCs in draining LNs at each time point, especially at later time points of tumor growth (Figure 2d). These results reveal that TIDCs are likely to be more active participants in our melanoma model than rDCs in LNs.

TIDCs become more potent immunostimulatory DCs during tumor growth

We next examined the immunostimulatory function of TIDCs and DCs from draining LNs by measuring the proliferation of allogeneic T cells in mixed lymphocyte reaction at early (day 4) and late (day 11) time points of tumor growth. At day 4, there was little difference in allogeneic stimulatory capacity between TIDCs and DCs from draining LNs. On the other hand, TIDCs had a more potent allogeneic stimulatory capacity than DCs from draining LNs at day 11 (Figure 3a). These results indicated that TIDCs increased their allogeneic stimulatory capacity with time during melanoma growth, whereas that was not the case for DCs from draining LNs. In fact, late TIDCs had a more potent allogeneic stimulatory capacity than early DCs when they were compared directly (Figure 3b). In subsequent experiments, we used these early (day 4) and late (day 11) TIDCs.

Early TIDCs express abundant immunoinhibitory molecules

Although it was once thought that phenotypically mature DCs exclusively promote T-cell activation, this paradigm has been challenged by the discovery of immunoinhibitory molecules (Reis e Sousa, 2006). Although the CD86 expression levels of early and late TIDCs were similar, early TIDCs were less immunostimulatory than their later counterparts. Therefore, we next evaluated the expression of vascular endothelial growth factor receptor 2 (VEGFR-2) and programmed death-ligand 1 (PD-L1), both of which are representative immunoinhibitory molecules (Dikov et al., 2005; Krempski et al., 2011; Mimura et al., 2007). As shown in Figure 4a, early TIDCs expressed much higher levels of VEGFR-2 and PD-L1 than rDCs from draining LNs. Although late TIDCs also expressed higher levels of VEGFR-2 and PD-L1 than rDCs from draining LNs, their expression levels were significantly lower than those of early TIDCs. Actually, early TIDCs expressed higher levels of VEGFR-2 and PD-L1 than late TIDCs when they were compared directly (Figure 4b). We also examined the expression levels of some other immunoinhibitory genes such as indoleamine 2,3-dioxygenase 1, indoleamine 2,3-dioxygenase 2, forkhead box O3, signal transducer and activator of transcription 3, and heme oxygenase-1, which are known to be associated with immunosuppressive functions of DCs (Moreau et al., 2009; Tran Janco et al., 2015). Interestingly, early TIDCs expressed higher levels of such kinds of immunoinhibitory