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*J Immunol* 2006; 177:6081-6090; doi: 10.4049/jimmunol.177.9.6081

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Incomplete Differentiation of Antigen-Specific CD8 T Cells in Tumor-Draining Lymph Nodes


CD8 T cells lacking effector activity have been recovered from lymphoid organs of mice and patients with progressing tumors. We explored the basis for lack of effector activity in tumor-bearing mice by evaluating Ag presentation and CD8 T cell function in lymphoid organs over the course of tumor outgrowth. Early after tumor injection, cross-presentation by bone marrow-derived APC was necessary for T cell activation, inducing proliferation and differentiation into IFN-γ-producing, cytolytic effectors. At later stages of outgrowth, tumor metastasized to draining lymph nodes. Both cross- and direct presentation occurred, but T cell differentiation induced by either modality was incomplete (proliferation without cytokine production). T cells within tumor-infiltrated nodes differentiated appropriately if Ag was presented by activated, exogenous dendritic cells. Thus, activated T cells lacking effector function develop through incomplete differentiation in the lymph nodes of late-stage tumor-bearing mice, rather than through suppression of previously differentiated cells. *The Journal of Immunology, 2006, 177: 6081–6090.

It is well established that CD8 T cells specific for tumor Ag can be recovered from both cancer patients and tumor-bearing mice. Despite the presence of these T cells, tumors frequently grow out and kill their host. Several mechanisms have been proposed to explain how tumors evade CD8 T cells. At one extreme, it has been reported that some tumors wall themselves off from the immune system and are ignored by CD8 T cells because tumor Ag presentation to naive cells in lymphoid organs never occurs (1–3). In cases in which tumor-specific CD8 T cells are activated, many tumors develop more active mechanisms of immune escape. Some tumors have been shown to down-regulate Ag or MHC class I molecule expression (4, 5) and can escape recognition by activated CD8 T cells. Other tumors express surface molecules such as Fas ligand (6, 7), which induces T cell apoptosis, or B7-H1 (8, 9), an inhibitory ligand for PD-1 that induces peripheral CD8 T cell tolerance (10).

In contrast to these mechanisms of tumor immune escape from functional CD8 T cells, a number of studies have demonstrated that tumor-specific CD8 T cells lack effector activity. In some cases, such dysfunctional CD8 T cells have been isolated from tumors (11–13). If such T cells are rested in vitro, they recover effector function (11, 12), suggesting that factors in the tumor microenvironment suppress the effector activity of otherwise fully differentiated T cells. Several tumors or cells infiltrating tumor masses secrete immunosuppressive cytokines such as TGF-β and IL-10 (14, 15), and these factors may induce local suppression of CD8 T cells. This idea of local immunosuppression within a tumor is supported by studies demonstrating that functional tumor-specific CD8 T cells can be recovered from PBL or secondary lymphoid compartments of mice with progressing tumors (13, 16). Other studies have demonstrated that tumor-specific CD8 T cells residing in secondary lymphoid compartments infiltrated by tumor also lack effector activity (17, 18). However, it is unclear whether these T cells acquired effector function before becoming suppressed or, alternatively, were never induced to become effectors at all.

Tumors could induce dysfunction of lymph node (LN)γ-resident CD8 T cells in a number of ways. First, the aforementioned local suppression associated with tumors may also influence already differentiated T cells residing in tumor-infiltrated LN. Alternatively, the differentiation of naive CD8 T cells in LN could be compromised by the nature of the APC or the microenvironment in which it occurs. Although one study showed that CD8 T cell activation required direct presentation by tumor (2), it has been argued that direct presentation is inherently defective because most tumors do not express costimulatory molecules necessary for T cell activation (19). LN-resident CD8 T cells lacking effector activity may therefore arise from nonprofessional direct presentation of Ag by tumor cells. Tumor cells may also alter the quality of cross-presenting host APC. Although cross-presentation of tumor Ag is required to induce CD8 T cell activation in some model systems (20, 21), many tumors have been shown to induce APC that are either poor stimulators of T cells in vitro or inducers of T cell anergy upon transfer into normal mice (22–27). Importantly, the in vivo relationship between cross-presentation, direct presentation, and LN-resident CD8 T cells that lack effector activity in tumor-bearing mice is largely unexplored. It remains unknown whether the quality of antitumor CD8 T cell responses depends upon the quality or nature of the APC and/or the stage of outgrowth at which Ag presentation occurs. Furthermore, it is unknown whether the basis for dysfunctional antitumor T cells in LN stems from defective

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3 Abbreviations used in this paper: LN, lymph node; β2m, β2-microglobulin; BM, bone marrow; DC, dendritic cell; BMDC, BM-derived DC; OVA, cytoplasmic chicken OVA; PT, paratracheal; Tyr369, amino acids 359–377 of tyrosinase.
activation of naïve T cells or local suppression of already differentiated effectors.

We have now studied the mechanism of tumor Ag presentation and the quality of the ensuing CD8 T cell response to two Ag expressed in B16 murine melanoma at both early and late stages of tumor outgrowth. There are conflicting data regarding immunity to this tumor. It has been reported that B16 is immunologically ignored by Ag-specific CD8 T cells due to barrier formation (2). In contrast, others have isolated activated Ag-specific CD8 T cells from B16 tumors (28, 29). Similarly, we have shown previously that B16 is partially controlled by tyrosinase-specific CD8 T cells in mice lacking tyrosinase and in tyrosinase-expressing mice immunized against this Ag (30). Importantly, however, these robust responses were not sufficient to eliminate the tumor, and it eventually killed the animal. Together, these data demonstrate that B16-associated Ag presentation can induce a tumor-specific CD8 T cell response, but that the tumor ultimately escapes it. Based on these observations, we investigated B16-associated Ag presentation in secondary lymphoid organs to understand whether the mechanism of tumor Ag presentation changes over the course of outgrowth and whether it influences the quality of the ensuing CD8 T cell response.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories. C57BL/6 Thy-1.1 congenic mice and bm1 mice were purchased from The Jackson Laboratory. OT-I RAG1−/− mice and β2-microglobulin (β2m)−/− mice were purchased from Taconic Farms. Tyrosinase (albino) mice on the C57BL/6 background expressing a chimeric MHC class I molecule composed of the α1 and α2 domains of HLA-A*0201 and the α3 domain of H2-Dβ (AAD) have been described previously (30). FH TCR transgenic mice on a C57BL/6 background express a chimeric MHC class I molecule composed of the α1 and α2 domains of HLA-A*0201 and the α3 domain of H2-Dβ (AAD) have been described previously (30). FH TCR transgenic mice on a C57BL/6 background express a T cell clone generated in an AAD

Peptides and MHC tetramers

Peptides comprising residues 257–264 of chicken OVA and 369–377 of murine tyrosinase (with Asp substituted for Asn at position 373) were synthesized at the University of Virginia Biomolecular Core Facility and purified to >98% purity by reverse-phase HPLC. Tetramers consisting of the Tyr369 epitope bound to HLA-A*0201 or the OVA257 epitope bound to H2-Kk were produced, as previously described (32).

Ex vivo analysis of Ag-specific T cell function

Single-cell suspensions were obtained from the peritoneal cavity (PT) LN by homogenization, and cells were cultured for 5 h at 37°C in the presence of IL-2 (20 U/ml) and monensin (10 μM) with appropriate stimulator cells. T2-Kk cells pulsed with 10 μM OVA257 were used for stimulation of OT-I cells, and the C1R human lymphoblastoid cell line transfected to express AAD (C1R-AAD) was pulsed with 10 μM Tyr369 peptide for stimulation of FH cells. Cells were then surface stained with PE-conjugated anti-Thy-1.2 (eBioscience) and PerCP-conjugated anti-CD8 (BD Pharmingen) Abs. All flow cytometric analyses were performed on a BD Biosciences FACScan using CellQuest software.

In vivo cytotoxicity assay

Splenocytes from C57BL/6 mice were harvested and divided into two populations. One population was pulsed with 1 μM OVA257 peptide for 90 min at 37°C, washed, and labeled with a high concentration of CFSE (5 μM). The other population was left unpulsed and labeled with a low concentration of CFSE (0.5 μM). A 50:50 mixture of 5 × 105 cells from each population was prepared and injected i.v. Lymphoid organs were harvested 12 h later, and percentage of targets killed was calculated by comparing the decrease in OVA257-labeled targets between naive vs tumor-bearing mice.

Results

Two different Ag associated with B16 melanoma stimulate specific CD8 T cells in vivo

To study the presentation of B16 melanoma-derived Ag to CD8 T cells in vivo, we took advantage of two distinct models: B16-cOVA, in which B16-F1 melanoma was transfected with the cytoplasmic version of chicken OVA, allowing the OVA257 epitope to serve as a model tumor Ag for recognition by OT-I TCR transgenic cells; and B16-AAD, in which the endogenous Tyr369 epitope from the melanocyte differentiation protein tyrosinase is presented on B16-F1 melanoma cells transfected with the rAAD class I MHC molecule (30). In the latter case, we have generated transgenic mice (designated as FH mice) using a TCR specific for this epitope isolated from AAD−/− albino mice. In these FH mice, ~88% of the CD8 T cells possess TCRs composed of Vα8 and Vβ11 sequences and bind tetramer composed of Tyr369 + HLA-A*0201 (L. Nichols, Y. Chen, T. Colella, C. Bennett, B. Clausen, and V. Engelhard, submitted for publication). In vitro, these OT-I and FH T cells each specifically recognize the relevant endogenously processed epitopes presented on B16-cOVA and B16-AAD, respectively (data not shown).

To evaluate whether presentation of Tyr369 and OVA257 occurred in mice bearing B16 tumors, we transferred CD8-enriched and CFSE-labeled TCR transgenic T cells from FH or OT-I mice
into AAD− tyrosinase− (albino) or C57BL/6 recipients, respectively. Two days later, these mice received $4 \times 10^3$ tumor cells, and 3 days subsequently CFSE dilution in TCR transgenic cells reisolated from spleen was assessed. CFSE dilution was evident in FH cells from mice bearing B16-AAD, but not from mice bearing AAD-transfected MC38, a colon carcinoma that does not express tyrosinase (Fig. 1). In keeping with this CFSE result, there was a ~5-fold increase in the percentage of CD8 T cells specific for Tyr369 in mice bearing B16-AAD. Similarly, significant CFSE dilution was observed in OT-I cells from mice bearing B16-cOVA, but not B16-F1 (Fig. 1). The percentage of CD8 T cells specific for OVA257 also increased ~27-fold in mice bearing B16-cOVA. These results establish that two different nonself Ag associated with B16 melanoma stimulate Ag-specific splenic CD8 T cells in vivo and that this tumor is not immunologically ignored.

**Presentation of Ag derived from i.v.-injected B16 is localized to spleen and PT LN**

Although the results above demonstrated that CD8 T cells in the spleen had encountered tumor Ag, they did not establish whether this was due to Ag presentation within the spleen or to recirculation of T cells activated elsewhere. To distinguish these possibilities, we assessed CD69 up-regulation on adoptively transferred OT-I cells only 24 h after tumor injection. CD69 was up-regulated on >50% of OT-I cells in the spleen and >35% of such cells in the PT LN (Fig. 2). However, CD69 was up-regulated on <1% of these cells in other LN, and no up-regulation was observed in either spleen or LN of mice injected with B16-F1. These results establish that presentation of B16-derived Ag within the first 24 h following i.v. tumor injection is localized to spleen and PT LN.

**Ag-specific CD8 T cell activation within 3 days of B16 injection is entirely due to cross-presentation by BM-derived APC**

We next evaluated whether the Tyr369 and OVA257 epitopes were directly presented to specific CD8 T cells by the tumor itself, or cross-presented by professional APC. We transferred CD8-enriched CFSE-labeled FH cells into AAD− albino mice and 2 days later injected these recipients i.v. with either B16-AAD or B16-F1. The latter cell does not express AAD and so cannot present Tyr369 directly. Three days later, CFSE dilution in splenic FH cells of B16-F1-injected mice was comparable to that of B16-AAD-injected mice, indicating that cross-presentation of Tyr369 to specific CD8 T cells occurs in the spleen of tumor-bearing mice. To assess whether Tyr369 could also be directly presented to FH cells, we generated chimeras in which AAD− albino mice were reconstituted with BM from AAD− albino donors, thereby eliminating AAD− APC that could cross-present Tyr369. These chimeras were injected with CFSE-labeled FH cells and subsequently injected i.v. with B16-AAD. There was no CFSE dilution in the labeled FH cells recovered from spleen (Fig. 3A) or PT LN (data not shown) of these mice, indicating that direct presentation was not occurring. Taken together, these results demonstrate that the only mechanism by which Tyr369 is presented to specific CD8 T cells within 3 days of i.v. B16 injection is cross-presentation by BM-derived APC.

Additionally, we immunized mice expressing a tumor-specific TCR (AAD−) with B16-F1 or B16-cOVA and determined whether OT-1 T cells could be activated in vivo in response to these tumors. No significant OT-1 T cell proliferation was observed. These results indicate that OT-1 T cells are not capable of responding to B16-F1 or B16-cOVA tumors in vivo.

**FIGURE 2.** Presentation of B16-derived Ag following i.v. injection is restricted to spleen and PT LN. OT-I cells were transferred into C57BL/6 mice. Two days later, mice were injected with tumor cells. Lymphoid organs were harvested 24 h later, and cells were stained for flow cytometry. Plots are gated on CD8+ lymphocytes. Numbers indicate percentage of tetramer+ cells that were also CD69+. LN evaluated were axillary (Ax), inguinal (Ing), brachial (Br), mesenteric (Mes), paraaortic (PA), and cervical (Cerv). Data are representative of three independent experiments.

**FIGURE 1.** Tyr369+ and OVA257+ specific CD8 T cells are activated in vivo following i.v. injection of cognate Ag-expressing B16 melanoma. FH or OT-I cells were transferred into AAD− albino or C57BL/6 mice, respectively. Two days later, mice were injected with tumor cells. Three days following tumor injection, splenocytes were isolated and analyzed by flow cytometry. Plots are gated on CD8+ tetramer+ lymphocytes. Data are representative of three independent experiments.
dependent experiments.

Based on the number of T cells activated, presentation of OVA257 epitope is required for the acti-

vation of specific CD8 T cells in spleen and PT LN shortly after i.v. injection of B16 tumors.

**CD8 T cells activated in the PT LN of 14-day-old tumor-bearing mice lack effecter function**

We next examined whether CD8 T cell activation at different stages of tumor outgrowth was associated with qualitatively dif-

ferent responses. When OT-I cells were labeled with CFSE and transferred into mice with either 1- or 14-day-old tumors, they underwent multiple rounds of division (Fig. 5A) and up-regulated the maturation marker CD44 (Fig. 5B) over 5 days in the PT LN of both types of mice. We next assessed the ability of these acti-

vated cells to produce IFN-γ as a measure of effecter activity. When Thy-1-mismatched OT-I cells were transferred into 1-day tumor-bearing mice and reisolated from PT LN 5 days later, a signiﬁcant percentage produced IFN-γ upon ex vivo stimulation with OVA257-pulsed T2-Kb cells. However, the percentage of IFN-γ-producing OT-I cells was dramatically decreased when these cells were reisolated from mice bearing 14-day-old tumors (Fig. 5C). OT-I cells from these late-stage tumor-bearing mice also did not produce either IL-4 or IL-10 after ex vivo stimulation (Fig. 5C), hallmark cytokines for Tc2-like and CD8 regulatory T cell differentiation programs, respectively (34, 35). Finally, in an in vivo cytotoxicity assay, the cytotoxic activity of OT-I cells against OVA257-pulsed splenocytes was signiﬁcantly decreased in mice with 14-day-old tumors compared with those with only 1-day-old tumors, despite an equal or greater number in the former of OT-I cells in the PT LN (Fig. 5D). Collectively, these results demon-

strate that CD8 T cells activated in the LN draining a well-established 14-day-old tumor do not completely differentiate into effectors.
CD8 T cell effector activity in late-stage tumor-bearing mice. We observed pigment in PT LN of a number of mice bearing tumor for 14–20 days (data not shown), suggesting that B16 cells had metastasized to this LN. In support of this, we found that OVA3257 was directly presented to OT-I cells in the PT LN of bm1→C57BL/6 chimeric mice that had been injected with B16-cOVA 14 days earlier (Fig. 6A, left). Over 50% of OT-I cells in the PT LN up-regulated CD69 24 h after transfer, a percentage equivalent to that seen in normal C57BL/6 mice bearing tumor for the same amount of time. Similar results were obtained in β2m−/− mice that lack stable MHC class I expression and thus cannot cross-present OVA3257 to OT-I cells (Fig. 6A, right). The absence of activation of OT-I cells transferred into either bm1→C57BL/6 chimeras or β2m−/− mice with 1-day-old tumors confirmed that T cell activation at this stage of outgrowth was entirely due to cross-presentation. These results demonstrate that a significant difference between the LN-draining 1- and 14-day-old tumors is the presence of tumor cells in the latter that present Ag directly to T cells.

To determine whether incomplete T cell differentiation in PT LN results when only direct presentation can occur, we assessed OT-I effector activity after transfer into 14-day tumor-bearing bm1→C57BL/6 chimeras (Fig. 6B, left) or β2m−/− mice (Fig. 6B, right). In both types of mice, OT-I cells underwent multiple rounds of division, but produced negligible IFN-γ by comparison with those activated in 1-day tumor-bearing C57BL/6 mice, in which OVA3257 must be cross-presented. These results demonstrate that incomplete CD8 T cell differentiation is associated with direct presentation within PT LN.

Cross-presentation of Ag derived from late-stage B16 tumors is also associated with incomplete CD8 T cell differentiation

Although the previous data demonstrated that CD8 T cells activated by direct presentation lack effector activity, the status of cross-presentation at a late stage of outgrowth remained to be established. Therefore, we evaluated cross-presentation of Tyr369 after injection of B16-F1, which expresses tyrosinase, but cannot directly present the epitope to FH cells. FH cells up-regulated CD69 24 h following transfer into either 1- or 14-day B16-F1 tumor-bearing AAD albino mice (Fig. 7A), demonstrating that cross-presentation of Ag still occurs in 14-day tumor-bearing mice. Interestingly, the percentage of proliferating FH cells that secreted IFN-γ 5 days after transfer into mice bearing 14-day-old B16-F1 tumors was significantly reduced in comparison with that of FH cells isolated from mice bearing B16-AAD for only 1 day (Fig. 7B). Similar results were obtained in the B16-cOVA model system. Direct presentation of OVA3257 by B16-cOVA could not readily be eliminated while still allowing the possibility of cross-presentation. Therefore, we transferred OT-I cells into mice that had been injected with B16-cOVA 1 day earlier as well as B16-F1 14 days earlier. In this design, B16-F1 establishes a 14-day-old tumor-draining LN microenvironment, whereas OVA3257 from the 1-day-old B16-cOVA can only be cross-presented. IFN-γ production by OT-I cells activated in these mice was significantly less than that observed in mice bearing only B16-cOVA for 1 day and similar to that of cells activated in 14-day B16-cOVA tumor-bearing mice (Fig. 7C). Together, these results demonstrate that cross-presentation of Ag at a late stage of tumor outgrowth is also associated with incomplete CD8 T cell differentiation.

Incomplete CD8 T cell differentiation is localized to tumor-draining LN

To determine whether incomplete CD8 T cell differentiation was restricted to tumor-draining LN, we injected soluble OVA protein 1 day following OT-I transfer into mice bearing B16-F1 for either

The LN-draining 14-day-old tumors contain B16 cells that directly present Ag to T cells

We next wished to understand what differences between PT LN draining 1- and 14-day-old tumors could account for the lack of...
In this paradigm, the tumor grew progressively and infiltrated PT LN, but did not serve as a local source of OVA257 epitope. In tumor-free mice, soluble OVA injection resulted in the systemic presentation of OVA257 and activation of a large fraction of OT-I cells to both proliferate and secrete IFN-γ (Fig. 8). Similar results were seen in both tumor-draining (PT) and nontumor-draining (inguinal) LN of mice injected with B16-cOVA tumors. Five days following transfer, OT-I cell proliferation and IFN-γ production were evaluated. Numbers indicate percentage of CFSE+ cells that were also IFN-γ+. Plots are gated on CD8+ CFSE+ lymphocytes. Data are representative of two independent experiments.

Optimally activated exogenous APC induce complete CD8 T cell differentiation in tumor-draining LN

Incomplete CD8 T cell differentiation in late-stage tumor-draining LN might be due to tumor or tumor-derived factors acting directly on CD8 T cells to block their differentiation. Alternatively, the tumor might affect the T cell differentiation indirectly via an influence on cross-presenting APC. To distinguish these possibilities, we injected mice i.v. with B16-F1, which does not express OVA, and transferred OT-I cells either 1 or 14 days later. One day following OT-I transfer, we immunized these mice with OVA257-pulsed CD40L-activated BMDC. Using this maneuver, Ag was presented by an immunogenic APC, but not by either the tumor or endogenous APC. However, B16 still infiltrated the LN of 14-day-old tumor-bearing mice (data not shown). Regardless of the residence time of the B16-F1 tumor, there was no defect in IFN-γ production by OT-I cells in the PT LN 5 days following DC immunization (Fig. 9). Because i.v. DC immunization primes T cells in both PT LN and spleen, it was possible that the IFN-γ-producing OT-I cells in the PT LN had either differentiated within that compartment or, alternatively, had differentiated in the spleen and then redistributed to the PT LN. To distinguish these possibilities, DC-immunized, tumor-bearing mice were treated with FTY720, a pharmacological analog of sphingosine 1-phosphate that inhibits T cell egress from lymphoid compartments by binding to sphingosine 1-phosphate receptors (36). We have used this compound to limit the redistribution of activated T cells among LN (S. Sheasley-O’Neill, C. Brinkman, and V. Engelhard, submitted for publication). However, the fraction of activated OT-I cells producing IFN-γ in the PT LN of 14-day tumor-bearing mice was not diminished by FTY720 treatment (Fig. 9). This indicates that OT-I cells are capable of differentiating within late-stage tumor-draining PT LN when stimulated by well-activated DC, and suggests that incomplete CD8 T cell differentiation results from an effect of the tumor on the cross-presenting APC, rather than on the differentiating T cell.
Discussion
In this study, we evaluated the mechanism of B16 melanoma-specific Ag presentation to CD8 T cells and the quality of the ensuing T cell response at early and late stages of tumor outgrowth. Shortly after i.v. injection of B16 cells, presentation of Tyr369 and OVA257 was confined to spleen and lung-draining PT LN. T cell activation at this stage was entirely due to cross-presentation of Ag by host BM-derived APC and involved both proliferation and differentiation into IFN-γ-secreting, cytolytic effectors. At later stages, tumor metastasis to PT LN resulted in direct presentation of Ag, but did not abrogate cross-presentation. CD8 T cells activated at this point proliferated normally, but failed to acquire effector function. However, if Ag was presented by activated, exogenous DC, T cells within late-stage tumor-draining LN did acquire effector activity. Therefore, CD8 T cells lacking effector activity within these LN do not result from suppression of previously differentiated cells, but instead reflect incompletely differentiated cells that arise following Ag presentation by either tumor cells themselves or APC that acquired Ag from late-stage tumors.

Although it is well established that induction of CD8 T cell immunity against pathogens depends upon cross-presentation of Ag (37), the mechanism by which tumor Ag presentation occurs has been controversial. Several studies demonstrated that cross-presentation of tumor Ag was required to activate naive CD8 T cells (20, 21, 38, 39). Others have reported that cross-presentation does not occur, resulting in either a failure of T cells to become activated in lymphoid organs (1, 2, 40), or alternatively, a requirement for tumor cells to infiltrate lymphoid organs and present Ag.
FIGURE 9. Induction of complete CD8 T cell differentiation in tumor-draining LN by optimally activated exogenous DC. OT-I cells were transferred into C57BL/6 Thy-1.1 congenic mice bearing 1- or 14-day-old B16-F1 tumors, and mice were immunized i.v. 1 day later with OVA257-pulsed CD40L-activated BMDC. Five days later, OT-I cell IFN-γ production was evaluated. FTY720 treatment was added to the experimental design as described in Materials and Methods. Numbers indicate percentage of Thy-1.2+ cells that were also IFN-γ+. Plots are gated on CD8+ lymphocytes.

directly (2). It has been unclear whether these apparent disparities are due to differences in tumor barrier formation (2, 41), metastasis to LN (17), interference with APC function (22), or level of Ag expression (39). In addition, these studies evaluated presentation at a single time point, and it has not been established previously whether direct and cross-presentation depend on the stage of tumor outgrowth.

We have now demonstrated that two distinct B16 melanoma-derived Ag are presented to naive CD8 T cells in secondary lymphoid organs by both direct and cross-presentation pathways. Importantly, one of these Ag is derived from the highly glycosylated melanocyte differentiation protein tyrosinase and is highly homologous to a well-characterized human melanoma Ag. Cross-presentation of tumor-derived Ag has been demonstrated by the use of transgenes, for which a normal expression level cannot be determined. Although we are using a transgenic restriction element for cross-presentation of Tyr369 (AAD), its expression is comparable to that of endogenous murine class I MHC molecules (42). Therefore, because cross-presentation depends on the level of Ag expression (39), our results with tyrosinase confirm that this is a relevant presentation mechanism for endogenous Ag as well. Our results also establish that cross-presentation is robust regardless of the stage of tumor outgrowth. It has been argued elsewhere that barrier formation, which is typical of late-stage tumors, precludes Ag presentation (2, 41). An interesting possibility is that barrier formation eliminates primary tumor-infiltrating APC as a source of cross-presented Ag, and that cross-presentation at a late stage of outgrowth is based on uptake of Ag from LN-infiltrating tumor cells by resident APC. Although this possibility remains to be evaluated, our characterization of the induction of CD8 T cell immunity at different stages of tumor outgrowth provides a more complete understanding of tumor Ag presentation.

Tumor-specific CD8 T cells lacking effector activity have been isolated from progressing tumors (11–13), as well as tumor-infiltrated LN of melanoma patients (17, 18). In most of these reports, the basis for dysfunction was not established, although the ability to detect these cells in significant numbers suggests that they were able to proliferate in response to Ag at some point. In two studies, it was found that CD8 T cells proliferated and were cytotoxic in tumor-draining LN (13, 16). In one of these, it was demonstrated that the cells lost effector activity upon infiltration of the tumor (13). This suggests either that they were locally suppressed or underwent clonal exhaustion. In contrast, we have demonstrated that T cells activated at an early stage of outgrowth in a tumor-free LN by cross-presenting APC acquire effector activity, whereas a large percentage of those activated at a late stage in a tumor-infiltrated LN does not. The differences between our results and those of previous studies may reflect the specific tumor evaluated, the failure of the tumor to metastasize to LN (16), or the measurement of effector function only after in vitro restimulation (13). The incomplete CD8 T cell differentiation that we observe is localized, not systemic, consistent with either a direct influence of the tumor in the LN, or an influence of tumor-derived factors or tumor-altered APC that drain to that LN from the lungs. Importantly, our observation of diminished effector activity did not require that the tumor itself express Ag, suggesting that it did not reflect clonal exhaustion. Thus, our results establish that T cell proliferation and acquisition of effector activity can be altered independently in the environment of a late-stage tumor.

Incomplete differentiation could result from tumor-derived or tumor-induced immunoregulatory factors acting directly on CD8 T cells. B16 does not produce IL-10, but does secrete TGF-β (43). However, although blockade of TGF-β signaling in T cells in vivo augments tumor immunity, this was associated with enhanced CD8 T cell proliferation (44). The robust proliferation of Ag-specific T cells we observe is inconsistent with a strong effect of TGF-β on CD8 T cells. Similarly, tumor-associated immature myeloid cells and plasmacytoid DC with immunosuppressive activity have also been described. However, these cells also block T cell proliferation (15, 27, 45, 46). Although we cannot exclude the possibility that another tumor-derived factor is directly suppressing CD8 T cell differentiation, immunization with exogenous, activated DC-presenting Ag induced complete CD8 T cell differentiation in LN-draining late-stage tumor. This suggests that the tumor affects cross-presenting APC, which in turn are responsible for incomplete T cell differentiation. Several groups have isolated immature APC from tumor-bearing animals or patients (23–26). Tumors have also been shown to inhibit the maturation of APC in vitro (24–26, 47), or induce an immunosuppressive phenotype (23, 48). Importantly, however, these APC fail to induce T cell proliferation. It is therefore very likely that the tumor-associated immature/immunosuppressive APC described in other systems are not responsible for the incomplete T cell differentiation that we have observed.

An important observation in the present work is that incomplete CD8 T cell differentiation at a late stage of tumor outgrowth occurs following either direct or cross-presentation of Ag, suggesting a commonality between professional APC and nonprofessional tumor cells presenting Ag. B16 expresses the inhibitory molecule B7-H1 (9) in our unpublished data), the ligand for PD-1 on T cells, and increasing expression of this molecule on B16 inhibited cytokine production by preactivated CD8 T cells in vitro (49). Immature DC also express B7-H1 (9) and could potentially have similar effects on T cells, although a recent study showed in vivo that immature DC induce CD8 T cell anergy through a PD-1-dependent mechanism (10). Alternatively, tumor cells and cross-presenting APC may fail to deliver signal 3 necessary to induce complete CD8 T cell differentiation. The phenotype of CD8 T cell proliferation, but diminished or absent effector function, has been observed in some studies of Ag presentation leading to tolerance,
but the cells apoptose and/or fail to accumulate (50, 51). Proliferation leading to accumulation in the absence of effector function has been described for CD8 T cells activated by artificial APC (52). This can be reversed by addition of IL-12 or type I IFN that has been described for CD8 T cells activated by artificial APC and leading to accumulation in the absence of effector function but the cells apoptose and/or fail to accumulate (50, 51). Proliferation leading to accumulation in the absence of effector function has been described for CD8 T cells activated by artificial APC and leading to accumulation in the absence of effector function but the cells apoptose and/or fail to accumulate (50, 51).

Acknowledgments

We thank Sarah Lewis and Janet Gorman for technical assistance and the rest of the Engerhard laboratory for insightful discussions and advice.

Disclosures

The authors have no financial conflict of interest.

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