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Defective Function of Langerhans Cells in Tumor-Bearing Animals Is the Result of Defective Maturation from Hemopoietic Progenitors

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Langerhans cells (LC), the APCs in the skin, serve as a model for investigation of dendritic cell (DC) function in tissues. DC play a crucial role in the generation of antitumor immune responses. In this study, we investigated the effect of the presence of tumor in vivo on the ability of LC to take up Ag, migrate to draining lymph nodes, and stimulate primary T cell responses. In two animal models, these functions were substantially inhibited. This effect was not restricted to LC located in the skin near a tumor but was also seen at sites distant from the tumor. The duration of tumor exposure, and not its ultimate size, were found to be important, suggesting that tumors could be inhibiting the maturation of LC rather than directly inhibiting their function. Model experiments with radiation chimeras supported this hypothesis. To investigate the potential role of vascular endothelial growth factor (VEGF) in these effects we used anti-VEGF-neutralizing Ab to treat animals bearing tumors. Treatment with the Ab at a dose of 10 μg i.p. per mouse, twice a week for 4 wk, significantly improved the number and function of LC as measured by their ability to migrate to lymph nodes and stimulate primary T cell responses, even at doses that do not affect the growth of these established poorly immunogenic tumors. Thus, inhibition of VEGF signaling may improve DC function in tumor-bearing hosts and possibly serve to improve the efficacy of cancer immunotherapy. The Journal of Immunology, 1998, 161: 4842–4851.

Antigen-presenting cells and particularly dendritic cells (DC) are important elements of antitumor immune responses. Defects in DC function could be responsible in part for the failure of the host to control tumor growth. Langerhans cells (LC) belong to the family of DC and are the major APCs in the skin. On stimulation with Ags, they take up and deliver Ags to sites of lymphocyte stimulation (lymph nodes) (1, 2). These cells thus may serve as a model for the in vivo study of DC function in tissues. The investigation of DC function in cancer is important not only for improved understanding of the mechanisms of tumor escape but also in connection with the growing interest in the immunotherapy of cancer using skin application of tumor-specific Ags. Recently, we and others reported impaired function of peripheral blood and tumor-associated DC, as well as DC in tumor-bearing mice (3–7) and that one of the responsible factors was tumor-derived vascular endothelial growth factor (VEGF). This defective function may be an important factor contributing to immune nonresponsiveness in cancer. However, previous studies did not evaluate the competency of DC in tumor-bearing hosts to take up Ag, transport it to draining lymph nodes, and present it to T cells in vivo, the natural chain of events in Ag presentation and immunity induction. These studies have only taken DC out of peripheral blood or tumor and assessed various functions in vitro. Specifically, it is not known whether there might be a defect in the ability of DC to transport Ag to nodes or whether this defect was restricted to the site of the tumor or generalized. The mechanism of these defects is also unclear. To answer these questions, we studied the function of LC using a model in which the skin is painted with contact sensitizers. This model depends on the ability of LC to take up cutaneous Ags, migrate from the skin to lymph nodes, and stimulate primary T cell responses. The use of FITC as an Ag gives an additional opportunity to directly detect by fluorescence those cells in lymph nodes that have taken up the Ag. Previous experiments demonstrated that LC are almost the only cells capable of carrying FITC to lymph nodes in this setting (8, 9).

Here, we demonstrate a systemic defect in these LC functions in tumor-bearing mice. We show that those defects are closely associated with length of time that the animal was exposed to the tumor and most likely due to the effect of tumor-derived factors on the process of LC maturation. We also demonstrate that these defects can be partially reversed by inhibiting one of these tumor-derived factors by the administration of anti-VEGF Ab.

Materials and Methods

Animals

Female BALB/c and A/J mice, 6 to 8 wk old, were purchased from Harlan (Indianapolis, IN) and were housed in specific pathogen-free units of the Division of Animal Care at Vanderbilt University Medical Center.

Cell lines and Abs

Two tumor cell lines have been used. D459 tumor cells were constructed by transfection of BALB/c 3T3 cells with EJ ras and mutant human p53 open reading frame. Details of this cell line were described elsewhere (10, 11). MethA sarcoma cells was obtained from Dr. L. J. Old. This is a transplantable 3-methylcholanthrene-induced sarcoma of BALB/c origin passaged as an ascitic tumor (12). The following Ab-producing hybridomas were obtained from the American Type Culture Collection (Manassas, VA) and used as culture supernatants: anti-CD4 (L3T4, TIB-207), anti-CD8

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3 Abbreviations used in this paper: DC, dendritic cells; LC, Langerhans cells; VEGF, vascular endothelial growth factor.
I-A<sup>+</sup> and I-A<sup>+</sup> Abs (PharMingen) were used in LC analysis. Neutralizing goat anti-mouse VEGF Ab was purchased from R&D Systems (Minneapolis, MN). Control goat Ig was obtained from Sigma (St. Louis, MO).

Cell preparation

Cells were prepared as described earlier (3). Briefly, a single-cell suspension was prepared from inguinal, axillary, and brachial lymph nodes by pressing the tissues through a wire mesh. Cells were then washed and layered onto a metrizamide gradient (Nygaard, Oslo, Norway) (gradient 14.5 g plus 100 ml of RPMI 1640 medium) and centrifuged for 30 min at 600 × g (metrizamide can be also obtained from Sigma). Cells at the interface were washed once and resuspended in complete culture medium (RPMI 1640 (Life Technologies, Gaithersburg, MD) with 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 1 × 10<sup>-8</sup> M 2-mercaptoethanol, and 10% FCS (HyClone, Logan, UT)). DC were identified by their distinctive morphology and by labeling with N418 Ab. In some experiments, FITC-positive cells and PE-conjugated anti-I-Ad<sup>+</sup> Abs were used in flow cytometry were purchased from Becton Dickinson, Mountain View, CA). Pelleted cells from the lymph node suspension were passed through nylon wool columns to obtain >90% pure T cells.

Tumor induction and assay of LC migration

Two hundred thousand D459 cells or 6 × 10<sup>3</sup> MethA sarcoma cells were injected s.c. into the shaved back of mice. LC migration was measured at different times after tumor inoculation as described earlier (13). Briefly, mice were painted on the shaved back or abdomen with 50 μl of 1% FITC (isomer 1; Sigma) dissolved in a 50:50 (v/v) acetone-dibutylphthalate mixture. Twenty-four hours after painting with FITC or vehicle, mice were killed and single-cell suspensions were prepared from inguinal, axillary, and brachial lymph nodes. The DC fraction was enriched by separation on a metrizamide gradient. Cells were labeled with PE-conjugated anti-I-A<sup>+</sup> or B7-2 Abs and analyzed with the gate set around the cluster of large cells using a FACStar flow cell sorter (Becton Dickinson, Mountain View, CA).

Epidermal sheet preparation and LC identification

Epidermal sheets were prepared from abdominal skin using the EDTA separation procedure described earlier (13–15). The ventral trunk skin was shaved and the mice killed by cervical dislocation. The remaining hair was removed by chemical depilation with a thioglycolate-based commercial depilatory cream. The keratin layer was then removed by two or three applications of cellophane tape, and the skin was surgically excised while firmly attached to fresh cellophane tape. The skin was incubated for 2.5 h at 37°C in PBS containing 20 mM EDTA (pH 7.3) and 0.001% trypsin. The cellophane tape, with the epidermis attached, was separated from the dermis, washed, and incubated overnight at 4°C with biotinylated anti-I-A<sup>+</sup> or anti-I-A<sup>4</sup> Abs. After that time, these sheets were washed and incubated with streptavidin-peroxidase (PharMingen) for 2 h at room temperature. I-A<sup>+</sup> cells were visualized in 0.7% mg/ml, 3,3'-diaminobenzidine containing 2 mg/ml H<sub>2</sub>O<sub>2</sub> (Sigma FAST DAB tablet set; Sigma) for 5 min at room temperature. The sheets were then washed, lightly dried, and mounted on slides. I-A<sup>+</sup> cells were counted in 1 mm<sup>2</sup> by counting 10 separate, randomly selected fields.

Assessment of LC development using radiation bone marrow chimeras

Control and tumor-bearing BALB/c mice (D<sup>+</sup>, K<sup>+</sup>, L<sup>+</sup>, I-A<sup>+</sup>, I-E<sup>+</sup>) were irradiated with two fractions of radiation to a total dose of 1080 cGy (two doses of 540 cGy with a 3-h interval). Mice were then immediately injected with 3 to 4 × 10<sup>6</sup> bone marrow cells obtained from AJ mice (D<sup>+</sup>, K<sup>+</sup>, L<sup>+</sup>, I-A<sup>+</sup>, I-E<sup>+</sup>) after it was depleted for T-, B-, and MHC class II-positive cells using mAbs and complement (Low-Tox-M Guinea Pig Complement; Cedarslane, Hornby, Ontario, Canada). From 3 to 4 wk later, LCs in skin were analyzed as described above using a biotinylated I-A<sup>+</sup> Ab. For control of experimental conditions bone marrow also was injected into nonirradiated BALB/c mice.

T cell proliferation assay

DC were obtained from lymph nodes of FITC-painted mice as described above. Cells were irradiated (2000 cGy) and added in triplicate to 5 × 10<sup>4</sup> T cells obtained from healthy BALB/c mice at different DC:T cell ratios.

After a 3-day incubation in 96-well U-bottom plates, the cultures were pulsed with 1 μCi of [<sup>3</sup>H]thymidine (Amersham, Arlington Heights, IL). [<sup>3</sup>H]Thymidine uptake was counted using a liquid scintillation counter.

Results

The presence of tumor substantially affects the ability of LC to migrate to draining lymph nodes

To study the effect of tumor on LC migration and function, BALB/c mice were inoculated with 2 × 10<sup>6</sup> D459 cells. Six weeks later, when tumors reached 15 to 17 mm in diameter (220–280 mm<sup>3</sup>), mice were painted with FITC. In an initial series of experiments, we compared the effect of painting the skin around the tumor in the dorsal trunk (“local site”) and painting the skin at a distant site (the ventral trunk). The number of DC in lymph nodes was counted using morphologic criteria and then confirmed by analysis of cell surface molecule expression. Since there is no single marker that can identify all lymph node DC, we used several different markers. The percentage of cells positive for each marker was counted. We found no statistically significant differences between the percentage of CD11c<sup>+</sup> and B7-2 MDS-2<sup>+</sup> cells in all samples. These two markers were used in most of the experiments. The changes in FITC-painted tumor-bearing mice were also confirmed by staining with MHC class II Abs. Figure 1A demonstrates the results of a typical experiment looking at the fraction of CD11c-positive cells in the lymph nodes of FITC-painted mice.

Twelve forty hours after skin painting with the contact sensitizer, a twofold increase in the total number of leukocytes in lymph nodes was observed in both control and tumor-bearing mice (Fig. 1B). The number of DC in lymph nodes obtained from control animals was also increased more than twofold. However, almost no increase was detected in tumor-bearing mice. No differences were found between mice painted with FITC near the tumor or at a distant site. The percentage of FITC-positive DC in lymph nodes was analyzed using flow cytometry. Lymph node DC were labeled with PE conjugated anti-B7-2 or anti-I-A<sup>+</sup>. Double-positive (FITC and PE) cells represent LC that had taken up FITC and migrated to the lymph nodes. Tumor-bearing mice had a 2.5-fold reduced percentage of FITC-positive DC compared with control animals (p < 0.01, Fig. 2). This was observed in mice painted both near the tumor and at a distant site (data not shown). We then asked whether the observed decrease in the number of FITC-positive cells was also associated with a decreased ability of DC to stimulate primary FITC-specific T cell responses. DC were isolated from the lymph nodes of painted tumor-bearing or control mice and irradiated, and their number was adjusted to equal levels using morphologic criteria confirmed by staining with anti-CD11c Ab. These DC were then incubated with T cells from control untreated BALB/c mice. DC from control FITC-painted mice stimulated significant T cell proliferation, whereas very low responses were detected when DC were isolated from tumor-bearing mice (Fig. 3). Thus, the presence of a tumor was associated with a defect in the ability of LC to migrate from the skin to draining lymph nodes in response to the topical application of an Ag. Additionally, LN DC from tumor-bearing animals were significantly less capable of stimulating primary Ag-specific T cell responses to the cutaneous Ag. These defects were observed even when the skin distant from tumor was painted. Since there were no differences between mice painted around tumor or in a distant site, mice were painted on the ventral trunk in all subsequent experiments.

Effect of tumor on function and number of LC in skin

Next, we attempted to identify the possible mechanism of the observed defective LC migration to lymph nodes. We have previously shown in an animal model that defective function of DC in
cancer was associated with a reduced expression of MHC class II and the costimulatory molecule B7 on the surface of DC (3,4). B7 expression is also low in tumor-associated DC in patients with cancer (7). Here, we investigated the expression of MHC class II and B7-2 molecules on DC from FITC-painted mice. Control and tumor-bearing mice were painted with FITC as described in Materials and Methods. After 24 h, single-cell suspensions from lymph nodes were prepared. The total number of leukocytes per lymph node was counted on a hemocytometer. DC were enriched on a metrizamide gradient, counted using morphologic criteria, and confirmed by staining with anti-B7-1, CD11c, and I-A<sup>d</sup> Abs. The numbers of cells per lymph node in five independently performed experiments is shown. *, Statistically significant differences (p < 0.05) from mice painted with vehicle alone. TB, tumor-bearing mice; FL1-H, FITC-positive cells; FL2-H, PE-positive cells.

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** The presence of tumor affects LC migration from skin to lymph nodes. A, Example of staining of DC in lymph nodes with Abs. B, The skin of control and tumor-bearing mice was painted with FITC as described in Materials and Methods. After 24 h, single-cell suspensions from lymph nodes were prepared. The total number of leukocytes per lymph node was counted on a hemocytometer. DC were enriched on a metrizamide gradient, counted using morphologic criteria, and confirmed by staining with anti-B7-1, CD11c, and I-A<sup>d</sup> Abs. The numbers of cells per lymph node in five independently performed experiments is shown. *, Statistically significant differences (p < 0.05) from mice painted with vehicle alone. TB, tumor-bearing mice; FL1-H, FITC-positive cells; FL2-H, PE-positive cells.

We then studied how FITC application affected the number of LC in the skin from tumor-bearing mice. To our surprise, we found a significantly lower number of LC in the skin from tumor-bearing mice than in that from control animals before treatment with FITC in two tumor models (Fig. 5). Treatment with FITC decreased the number of LC by ~30% both in control and in tumor-bearing mice (data not shown). These data suggest that a major reason for defective LC migration to nodes in tumor-bearing mice may be a decreased LC density in the skin of tumor-bearing hosts.

The defective ability of DC from tumor-bearing mice to stimulate FITC-mediated T cell proliferation could be explained either by a reduced number of LCs carrying FITC to lymph nodes or by a reduced competence of FITC-positive LCs to stimulate T cell proliferation. To distinguish between these possibilities, FITC-positive cells from control and tumor-bearing mice were sorted using a flow cell sorter. These cells were used for stimulation of control T cells. As shown in Figure 6, despite a more than threefold decrease in the number of FITC-positive LC in tumor-bearing mice, sorted FITC-positive cells were fully competent in stimulating primary T cell responses. This suggests that if a LC can take up an Ag and transport it to the lymph node, then it is capable of stimulating a primary response. Tumor-bearing animals thus have
a decreased number of LCs capable of taking up FITC and transporting it to the lymph nodes, and this results in the observed decreased stimulatory capacity of nodal DC.

**Dynamics of changes in LC number and response in tumor-bearing mice**

One of the possible explanations for the decreased numbers of LC in the skin of tumor-bearing mice is that LCs migrate toward the tumor because of the effect of tumor-derived chemotactic factors. Because these stimuli are continuously present in tumor-bearing mice, this may result in fewer LC in skin. To test this possibility, we inoculated greater numbers of tumor cells (10 times higher than in previous experiments) to shorten the length of time required for the tumor to reach a given size. At this dose, tumors reached 220 to 280 mm² within 2 to 2.5 wk, twice as fast as in the previous experiments. If the described effects were solely the result of migration of LC out of skin toward the tumor, we would expect to see the same level of decrease in the number of FITC-positive DC in lymph nodes as in the previous experiments. If the described effects were solely the result of migration of LC out of skin toward the tumor, we would expect to see the same level of decrease in the number of FITC-positive DC in lymph nodes as in the previous experiments. However, when we evaluated the number of FITC-positive DC in draining lymph nodes from these animals, only a minimal decrease in the percentage of FITC-positive DC was detected in lymph nodes of tumor-bearing mice (data not shown). We then studied how further

**FIGURE 2.** FITC-positive DC are decreased in lymph nodes from tumor-bearing mice. Mice were painted with FITC in a site distant from the tumor as described in Materials and Methods. The DC-enriched fraction of lymph node cells were labeled with PE-conjugated B7-2 or I-A d Ab. FITC (FL1-H)- and PE (FL2-H)-positive cells represent the population of LC that carried FITC to the node from the skin. One typical experiment of five performed is shown. The top panel shows labeling with isotype control IgG. The cumulative data from five experiments demonstrated a statistically significant decrease in the fraction of FITC-positive LC in tumor-bearing mice (16.8 ± 3.2% in control and 5.8 ± 1.8% in tumor-bearing mice, p < 0.01).

**FIGURE 3.** Stimulation of FITC primary T cell responses by DC from control and tumor-bearing mice. Control and tumor-bearing mice were painted with FITC as described in Materials and Methods. DC were isolated from draining lymph nodes, irradiated, and incubated in triplicate with 5 × 10⁴ T cells from untreated control syngeneic mice. FITC, results with FITC painted mice; Vehicle, results with mice painted with vehicle alone. The number of DC was evaluated using morphologic criteria and labeling with anti-CD11c Ab. Typical results of one of three experiments are shown.
increased numbers of inoculated tumor cells affected LC number in skin during shorter exposures to tumor (12 days). Even a 50-fold increase in the number of injected tumor cells did not change the number of LC in the skin in both experimental models (data not shown). It is important that in all these experiments, the tumor was injected into the dorsal trunk and the skin was obtained from ventral trunk. These experiments demonstrated that decreased numbers of LCs in the skin and the decreased responses of these cells to additional stimulation with Ag require relatively long exposure to tumor. This suggests that the tumor may affect the process of LC production and maturation from precursors rather than directly affecting mature LC. Since the half-life of LC in skin is relatively long, the observed effect may require the prolonged presence of tumor to be observed.

**LC maturation in tumor-bearing bone marrow chimeric mice**

To distinguish newly produced LC from preexisting ones, bone marrow chimera experiments were performed. Tumor-bearing BALB/c mice (haplotype d/d; tumor size, 50–70 mm²) were lethally irradiated, and bone marrow from A/J mice (haplotype d/k) was used for reconstitution. The bone marrow from the donor was depleted for lymphocytes, macrophages, and DC. After 3 weeks, mice were still free from visible signs of graft-vs-host disease. When tumors reached 120–150 mm², mice were killed, and the number of donor-derived LC (I-Ak cells) were counted in the skin as described in Materials and Methods. The same experiments were performed in parallel in tumor-free mice. As a control, bone marrow cells were also injected into nonirradiated control mice. As
Two tumor models (D459 and MethA sarcoma) were used. Each experiment was performed in duplicate. Accumulated results of four experiments are shown. B. Photomicrograph of LC in the skin of control and tumor-bearing mice. Top, control mouse; bottom, tumor-bearing mouse. Magnification, ×400.

**FIGURE 5.** Effect of tumor on number of LC in skin. A, The number of LC in the skin was counted as described in Materials and Methods in control and tumor-bearing mice (6 wk after tumor injection; tumor size, 240–280 mm²). Two tumor models (D459 and MethA sarcoma) were used. Each experiment was performed in duplicate. Accumulated results of four experiments are shown. B. Photomicrograph of LC in the skin of control and tumor-bearing mice. Top, control mouse; bottom, tumor-bearing mouse. Magnification, ×400.

shown in Figure 7, large numbers of I-Ak⁺ cells were detected in the skin of control irradiated mice (Fig. 7B). No I-Ak⁺-positive cells were detected in the skin of nonirradiated mice (data not shown). The number of LC in the skin of tumor-bearing mice was significantly lower than those observed in control animals (Fig. 7A).

**Effect of neutralizing anti-VEGF Ab on LC function in vivo**

The data shown above indicate that tumor cells may affect the process of maturation from bone marrow progenitors to mature LC in skin. Several factors could potentially contribute to this process. Recently, we have shown that VEGF, which is produced by most tumors can directly affect DC maturation in vitro (16). We thus hypothesized that blockade of VEGF with a neutralizing Ab may improve the function of LC in tumor-bearing hosts. D459 cells produce VEGF in vitro (our unpublished observations) and in mice bearing 220- to 280-mm² tumor, the serum level of VEGF was 120 to 180 pg/ml as was determined by ELISA (R&D Systems). Since direct antitumor effects of VEGF blockade are possible and tumor regression by any means may improve LC function, we tested whether anti-VEGF Ab was able to affect the growth of established, poorly immunogenic D459 tumors. Mice were injected with D459 cells, and when tumors reached 5 to 6 mm in diameter, treatment with anti-VEGF Ab was initiated. We tested three doses of Ab (2, 5, and 10 μg/mouse i.p. twice a week during 4 wk). These doses were chosen on the basis of the in vitro neutralizing activity of the Ab. As controls, mice were treated with the same concentration of goat Ig. In six independently performed experiments, anti-VEGF Ab did not affect tumor growth (4 mice/group in each experiment, data not shown). No differences were found between mice treated with goat Ig and untreated animals. Then, we asked whether anti-VEGF Abs had any effect on the ability of LC to migrate to draining lymph nodes. Tumor-bearing mice were treated with 10 μg/mouse of anti-VEGF Ab for 4 wk. After that time, they were painted with FITC as described above. Figure 8A shows the result of one of these experiments demonstrating that treatment with anti-VEGF Ab increased the percentage of FITC-positive DC in LN. This was associated with an improved ability of these cells to stimulate primary FITC-specific T cell responses (Fig. 8B). Treatment with anti-VEGF Ab also reversed the tumor-associated decrease in the number of LC in the skin (Fig. 8C).

**Discussion**

LC are bone marrow derived from B cells that belong to the family of DC, the professional APCs. They play an important role in induction of immunity against foreign Ags, including tumor-specific Ags (2). They are crucial for the effectiveness of recent efforts to immunize with the cutaneous application of tumor specific Ags or genes encoding those Ags. Until now, no information was available about their function in tumor-bearing hosts, except for a few reports describing considerable changes in the number and morphology of LC in the skin at the tumor site. Bergfelt at al showed a statistically significant reduction in the number of LC near the tumor in basal cell carcinoma (17). They also found that LC had a reduced number of dendrites, and these were often short and had few branches. Essentially the same findings were reported by Townsend et al. (18) for neoplastic ovine skin and by Toriyama et al. (19) in patients with melanoma. They also demonstrated that decreases in LC number in the skin adjacent to the tumor was not due to cell migration toward the tumor (19). We and others have recently reported the defective function of DC from tumor-bearing mice, from the peripheral blood of cancer patients, and in tumor-associated DC (4–7, 10, 20, 21). These observations suggest that defects in DC function may be an important part of tumor escape from immune system control.

However, it was not clear whether the described defects were localized or systemic. Also not clear was whether LC in tumor-bearing animals had a decreased ability to take up Ag and migrate to the site of lymphoid stimulation (mostly lymph nodes), the major function of LC. These questions are important not only for our understanding of the immunopathogenesis of cancer but also for devising better strategies for the immunotherapy of cancer and cannot be addressed with in vitro models. Therefore, we adopted an in vivo model of LC migration in response to the topical application of FITC. As has been shown before, after application of FITC, LCs migrate to draining lymph nodes and stimulate primary FITC-specific T cell responses (8, 9). Transfer of these cells into naive mice led to development of FITC hypersensitivity (22). We use this model to investigate the possible effect of tumors on LC function. The two tumor models used in our study have been described elsewhere (10, 23, 24). In most experiments, we used the poorly immunogenic D459 tumor. This tumor reaches 15 mm in diameter (220–280 mm²) ~6 wk after the s.c. injection of 2 × 10⁵
cells. After that time, tumors quickly exceed the 2 cm in diameter often associated with ulceration of the skin above the tumor. Therefore, we chose 6 wk as an end point in this study. Painting of the skin with FITC resulted in significant increases in the number of lymphocytes and DC in the lymph nodes of control animals, a usual finding after the application of contact sensizers. However, the total number of DC and the number of FITC-positive DC in lymph nodes was significantly lower in tumor-bearing mice. To our surprise, the same results were obtained when mice were painted at the tumor site (dorsal trunk) or at a site distant from the tumor (ventral trunk).

These data indicate that there is a systemic defect in LC function in mice bearing tumors. The mechanism of this effect is most likely via soluble tumor-derived factors. We first tested the hypothesis that these factors affected the ability of LC to migrate to lymph nodes and stimulate T cells via the down-regulation of LC surface receptors known to play a major role in DC function. We asked whether tumor cells induce down-regulation of those molecules in FITC-positive DC in draining lymph nodes and whether the total number of DC in the nodes of tumor-bearing mice was decreased. Our data demonstrated only a slight decrease in the number of FITC-negative DC in lymph nodes from tumor-bearing mice compared with control animals (~15%), whereas the number of FITC-positive DC was decreased almost threefold. This implies defective Ag uptake and transport in these animals. On the surface of DC from tumor-bearing mice, we found a reduced expression of MHC class II and B7-2 molecules compared with DC from control animals. This decrease was seen on the surface of FITC-negative DC and, to a lesser extent, on FITC-positive cells. However, in tumor-bearing mice sorted FITC-positive LC that migrated from the skin to the lymph nodes had the same ability to stimulate naive T cells as these cells from control mice. This indicates that defects we observed in the ability of FITC-positive cells from tumor-bearing mice to stimulate primary T cell responses were not due to decreased expression of the surface molecules on DC, but rather due to reduced numbers of LC taking up FITC and migrating from the skin. We then examined the number of LC in skin. Significantly lower numbers of LC were found in tumor-bearing mice than in control animals. The same decrease was observed in mice bearing another syngeneic tumor (MethA sarcoma). These data indicate that the substantial decrease in the number of FITC-positive cells in lymph nodes was not due to defects in the function of LC capable of conveying Ag to the nodes, but rather a consequence of a decreased pool of LC in the skin.

There are two possible explanations for a generalized decrease in the number of LC in the skin of tumor-bearing animals. First, tumor-derived factors may work as chemokines to attract LC to the site of the tumor, depleting LC from the skin in spite of normal rates of LC production. Increased numbers of LCs at the site of some (but not all) types of human tumors have been described and are correlated with a good prognosis (reviewed in Ref. 25), consistent with this hypothesis. The other possible explanation is that tumor-derived factors may also prevent replenishment of LC in the skin by interfering with their production and maturation from precursors. The half-life of LC has been reported to be around several weeks (26, 27), so it is possible that a 6-wk exposure to a tumor is sufficient to cause decreased numbers in the skin due to decreased

![FIGURE 6. Sorted FITC-positive (FL1-H) LC from tumor-bearing mice are competent in the stimulation of primary T cell responses. Control and tumor-bearing mice were painted with FITC as described above. After 24 h, lymph node DC were isolated and sorted using a FACStar flow cell sorter. The gates were set around FITC-positive cells (A). Top, cells from control untreated mice; middle, control FITC-painted mice; bottom, FITC-painted tumor-bearing mice. Sorted cells were counted, irradiated, and cultured in triplicate with T cells from control untreated mice as described in Figure 3 at a DC:T cell ratio of 1:20 (B). TB mice, tumor-bearing mice; No FITC, unsorted DC from control untreated mice. FL2-H, PE-positive cells.](http://www.jimmunol.org/)

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generation of LC from progenitors. The histologic analyses of tumors so far reported in the literature cannot distinguish between these hypotheses.

To address these questions, we established tumors using increased numbers of tumor cells, thus shortening the time the tumor takes to achieve a given size. We determined the dose that resulted in the same tumor size used before (220–280 mm$^2$) but achieved this size in half the time (2–2.5 wk). If the migration of LC toward the tumor was the only reason for the decreased LC number in the skin and their decreased migration to lymph nodes after FITC painting, we should see roughly the same effect after this short exposure to the tumor. However, these effects were much smaller in mice exposed to tumor for 2 to 2.5 wk than in mice exposed to the same size tumor for 6 wk. A 12-day exposure to a 50-fold increased number of tumor cells did not affect the number of LC in the skin distant from the tumor at all. These data strongly suggest that defective replenishment of LCs in the skin after naturally occurring LC turnover or migration induced by tumor-derived factors is the major cause for defective LC function in tumor-bearing animals. These findings were also supported by our experiments with radiation chimeras, as donor bone marrow cells transplanted into tumor-bearing mice showed considerably reduced generation of donor LC in the skin of tumor-bearing mice.

The observed systemic effects of tumors can best be explained by soluble factors, perhaps directly produced by tumors. Tumors are known to produce factors that may affect maturation of LC from progenitors. For example, numerous studies of the inhibition of LC function by IL-10 have been described (28–35). Elevated levels of IL-10 in the sera of cancer patients have been reported by several groups (36, 37). Expression of IL-10 mRNA was detected in renal carcinoma cells (38). It is possible that IL-10 or other cytokines or tumor-derived factors may affect the maturation of LC in vivo. However, it is important that IL-10 affects the latest stages of in vitro maturation of LC from a relatively immature state (freshly isolated LC) to a fully mature state. Since even these relatively immature LCs still express MHC class II, they would be detected immunohistochemically by the methods used in this study. Therefore, decreases in the number of LC observed in our study are more likely to be due to effects of tumor-derived factors on earlier stages of LC maturation.

Previously, we have reported on the possible involvement of VEGF in defective DC maturation in vitro (16). This effect could be mediated by the blockade of TNF-α-inducible activation of the transcription factor NF-κB in hemopoietic progenitor cells (39). VEGF is produced by almost all tumors and was found in the sera from the tumor-bearing mice in this study. We thus asked whether blockade of VEGF would improve LC function in this system. This assessment is complicated by the fact that VEGF directly promotes tumor neovascularization (reviewed in Ref. 40), and anti-human VEGF inhibits the growth of human tumors in nude mice (41–44). The effect of anti-VEGF Ab is most pronounced in the inhibition of the growth of small tumors and micrometastases, so we tested whether anti-mouse VEGF Ab affected the growth of the established tumors used in our study. In six independent experiments (four mice per group per experiment) we did not find any effect of anti-VEGF Abs at doses as high as 10 μg per mouse treated twice a week for 4 wk. We did not escalate this dose further. It is possible that antitumor effects could be seen at higher doses of the Ab than were tested here. It is important to emphasize that we used anti-mouse VEGF Ab, not the anti-human one used in previous studies. Despite the lack of antitumor effects at these doses, we detected a marked improvement in LC function after 4 wk of treatment with anti-VEGF Ab in tumor-bearing mice. The

![FIGURE 7. Donor-derived LC are reduced in the skin of radiated tumor-bearing chimeras. A, Three independent experiments were performed as described in Materials and Methods. Mice were injected with 2 × 10$^5$ D459 cells. When tumors reached 50 to 70 mm$^2$, mice were lethally irradiated and reconstituted with bone marrow from normal A/J mice. Three weeks later, the number of I-A$^k$ cells in the skin was analyzed. Each experiment was performed in duplicate. *, statistically significant differences between groups ($p < 0.05$). B, LC in the skin of control and tumor-bearing mice. Top, cells stained only with streptavidin-peroxidase (nonspecific staining); middle, control mouse (cells labeled with anti-I-A$^k$ Ab); bottom, tumor-bearing mouse (cells labeled with anti-I-A$^k$ Ab). FITC (FL1-H, FITC-positive cells; FL2-H, PE-positive cells. Magnification, ×400.](http://www.jimmunol.org/)

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number of LC in the skin was also increased compared with tumor-bearing mice treated with goat Ig. Thus, anti-VEGF Ab was able to improve LC function in tumor-bearing mice, consistent with our previously reported data that VEGF might be one of the factors responsible for defective Ag presentation function in cancer. Despite the effects of this Ab on LC function, we did not observe significant spontaneous induction of antitumor immunity. D459 cells are very poorly immunogenic and have not been observed to induce spontaneous immune responses or spontaneous rejection under any experimental conditions. Apparently, low expression of tumor-specific Ags does not induce an immune response even when DC function is improved. However, when these mice are immunized with tumor-specific Ag, anti-VEGF Ab markedly increases the effectiveness of immunotherapy (manuscript in preparation).

In conclusion, we describe for the first time that tumors, via soluble tumor-derived factors (including VEGF), considerably affect the function of LC even at sites distant from the tumor. This effect requires relatively prolonged exposure to the tumor and probably works through inhibition of LC differentiation. This phenomenon may represent one of the possible mechanisms of tumor escape from immune system control. Anti-VEGF Ab may provide a new tool for the improvement of DC function in cancer and therefore could be potentially used in immunotherapy of cancer.

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References


