Intratumor CD4 T-Cell Accumulation Requires Stronger Priming than for Expansion and Lymphokine Secretion

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Abstract

T cells need to migrate to and accumulate inside tumors before mediating rejection of the tumor. The number of specific T cells inside tumors may depend on the efficiency of priming in the draining lymph node (DLN), intratumor deletion, suppressive phenomena, or both. We used monoclonal anti-male antigen CD4 (Marilyn) T cells and tumor cell lines expressing or not the corresponding antigen (Dby) to analyze CD4 T-cell accumulation in tumors. Priming by MHC II- or MHC II+ male splenocytes or Dby+ tumor cells induced similar Marilyn T-cell expansion in the DLN and recirculation in other lymph nodes and capacity to produce IFN-γ. However, intratumor accumulation was different for each priming condition. In mice with Dby+ tumors, MHC II+ male splenocyte priming induced greater, although not statistically significant, Marilyn T-cell accumulation in the tumors than MHC II- male splenocyte priming. In mice with Dby- tumors, priming in the tumor DLN induced less Marilyn T-cell intratumor accumulation than priming by MHC II+ male splenocytes. We saw comparable differences for Marilyn T-cell accumulation in gut lamina propria, suggesting that priming affects effector T-cell accumulation in inflamed tissues. Mature dendritic cells were loaded with graded doses of Dby peptide to control for antigen-presenting cell characteristics during priming. We observed similar proliferation, with higher concentrations inducing higher intratumor accumulation. Thus, intratumor accumulation requires stronger stimulation than for proliferation or the capacity to secrete lymphokines. In this system, priming intensity alone can explain the number of intratumor T cells without having to call for intratumor deletion or suppression phenomena. (Cancer Res 2006; 66(10): 5443-51)

Introduction

As well as helping CD8 T cells to become efficient cytotoxic cells, CD4 T cells can also mediate the rejection of skin grafts (1), kidney grafts (2), or tumors (3–6). The migration of the specific T lymphocytes into the tumor bed is one of the first steps in tumor rejection by the immune system. First, naïve T cells in the tumor draining lymph node (DLN) must be activated by specific peptide-MHC complexes on professional antigen-presenting cells (APC) that have captured the antigen at the tumor site or in the tumor DLN (7–9). Then, the activated T cells either recirculate through the different lymphoid organs or migrate into nonlymphoid inflamed tissues (10–12). Activated T-cell migration into tissues relies on the modification of their chemokine receptors and integrin expression profiles (13–15). The survival of activated T cells implies they express Bcl-2, Bcl-xl, and survivin, and it has been shown that the involvement of costimulatory molecules during T-cell activation is an important variable that regulates this process (16, 17).

Antigenic stimulation strength is a function of the concentration and involvement of costimulatory molecules, the duration of antigen stimulation, and the number of peptide-MHC complexes present on each APC. Indeed, in vitro, when more peptide-MHC complexes are expressed per APC, activated T cells divide more and acquire a greater cytokine secretion capacity (18–22). In vivo, it is more difficult to control the amount of peptide-MHC complexes per APC. Therefore, its influence on T-cell migration and accumulation in nonlymphoid tissues is poorly understood, especially for tumors. Many studies have been carried out using replicating pathogens, peptides, or whole protein antigens in complete Freund’s adjuvant (CFA) (10, 11, 23). According to the progressive differentiation model, the T cells that divide the most are those that emigrate from secondary lymphoid organs to nonlymphoid tissues (12, 24–26).

We have shown previously that anti-male TCR transgenic Marilyn monoclonal (RAG2+/−) CD4 T cells, which recognize the Dby epitope in I-Ab molecules, can induce rejection of male skin grafts in the absence of direct antigen recognition on the graft cells (1). Male skin grafts with a nonrestricting MHC haplotype were rejected if the host displayed the restricting MHC haplotype. In another study using the same transgenic CD4 T cells and male heart grafts not bearing the restricting MHC haplotype, specific T cells primed through the indirect presentation pathway were shown to migrate to the donor organ (27). Altogether, these results highlight the role of the indirect presentation of antigens by the host APCs in inducing effector functions and graft rejection and suggest that the target for rejection is the vascular endothelium of the graft. Many tumors do not express MHC II, MHC I, or both on the cell surface. Therefore, T-cell antitumor responses should be generated through the indirect presentation of tumor antigens by host APCs. We wondered whether the results from skin or heart grafts could also apply to solid tumors. Indeed, it is difficult to assess whether poor tumor rejection observed in some antigen-expressing tumor experimental models is only due to inefficient intratumor T-cell migration and accumulation caused by a poor priming or whether it is due to intratumor phenomena, such as suppression or deletion or a nonpermissive tumor vascular endothelium.

Using the above-mentioned Marilyn TCR transgenic and a Dby-transfected MHC II- fibrosarcoma, we have shown previously that
Materials and Methods

Mice. The Marilyn TCR transgenic mice on a Rag-deficient background have been described elsewhere (28) and recognize the I-\(\text{A}^\text{b}\)-restricted male Dby peptide NAGFNSRNASSRS with a V\(\alpha\)1/V\(\gamma\)6 TCR. Marilyn mice were used as a source of monoclonal specific CD4 T cells. Female CD45.2 C57BL/6 (B6) mice from IflaCredo (Labresle, France) and CD45.1 B6 mice from the Centre de Distribution, Typage et Archivage, Centre National de la Recherche Scientifique (Orléans, France) were used as recipients. Cells from CD45.1 and CD45.2 Marilyn mice were used for adoptive transfer in CD45.2 and CD45.1 B6 mice, respectively. Male and female CD\(\text{C57BL/6}\) mice and male I-\(\text{A}^\text{b}\)/I-\(\text{A}^\text{d}\) mice (Centre de Distribution, Typage et Archivage, Centre National de la Recherche Scientifique) were used as a source of splenocytes for the footpad immunizations. Live animal experiments were carried out in accordance with the guidelines of the French Veterinary Department.

Cell lines. MCA-101 is a methylcholanthrene-induced fibrosarcoma that does not express the male HY antigen. MCA-101 was transfected with Dby cDNA as described previously (26). Cells were cultured in complete DMEM. For MCA-101-Dby, this was supplemented with 0.5 mg/mL neomycin (Invitrogen, Cergy Pontoise, France).

Mouse experiments. Tumor cells (2 \(\times\) 10\(^5\)) in a 100 \(\mu\)L volume of PBS-0.5% bovine serum albumin (BSA) were injected i.d. onto the left flank of normal CD45.1 or CD45.2 female B6 mice. After 5 days, 1 \(\times\) 10\(^{4}\) cells from Marilyn lymph nodes labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) as described previously (29) were injected i.v. into the tumor-bearing mice. The following day (day 0), 3 \(\times\) 10\(^5\) female or male CD\(\text{C57BL/6}\) and male I-\(\text{A}^\text{b}\)/I-\(\text{A}^\text{d}\) splenocytes in a 50 \(\mu\)L volume of PBS-0.5% BSA were injected into the hind footpad in half of the mice. After 5 or 15 days, the tumors and the lymph nodes were harvested and the number and phenotype of the mononuclear T cells were studied. When indicated, 2 \(\times\) 10\(^{4}\) bone marrow–derived dendritic cells (BMDC) from male B6 pulsed with Dby peptide were used as stimulatory cells at day 0. BMDCs were generated by culture in granulocyte-macrophage colony-stimulating factor–containing Iscove’s modified Dulbecco’s medium for 10 days; fluorescence-activated cell sorting analysis of the cell population revealed >75% CD11c\(^+\) cells. All BMDCs were matured by incubation with 10 \(\mu\)g/mL lipopolysaccharide (LPS; Sigma, Lyon, France) for 16 hours at 37°C in a 5% CO\(_2\) atmosphere. BMDCs from female B6 mice were pulsed with 2, 40, or 800 nmol/L or for 4 \(\mu\)mol/L Dby peptide for 3 hours at 37°C, 5% CO\(_2\).

Tumor-infiltrating lymphocyte isolation. Mouse tumors were harvested and stored in PBS-0.5% BSA. Tumors were then weighed, cut into small pieces, and resuspended in CO\(_2\)-independent medium (2.5 mL for 200 mg tissue) containing 20% FCS, 1 mg/mL collagenase D (Roche, Mannheim, Germany), and 25 \(\mu\)g/mL DNase I (Sigma). This was then agitated at 250 rpm for 90 minutes at 37°C with vigorous pipetting every 20 minutes. The cell suspension was then filtered through a 40-\(\mu\)m cell strainer (Becton Dickinson, Le Pont-De-Claux, France) and washed in PBS-0.5% BSA. Tumor-infiltrating lymphocytes (TIL) were isolated on a lympholyte M (Cedarlane, Hornby, Ontario, Canada) gradient (100% and 75%) in DMEM from 100% to 75% interface and washed in PBS-0.5% BSA.

Gut lamina propria lymphocytes isolation. Briefly, the gut was flushed with PBS to remove the intestinal content. After removing Peyers’s patches, the intestine was opened longitudinally and washed several times in PBS.
methods to assess the priming efficiency of Dby+ tumors. MCA-101 fibrosarcoma or its Dby-transfected relative was implanted into B6 mice, which were injected 5 days later with CFSE-labeled Marilyn T cells. The following day, some of the mice were immunized with MHC II+ or MHC II male or female splenocytes injected into the Dby+ tumor contralateral hind footpad (Fig. 2A). We then determined the number and phenotype of the Marilyn T cells in the different lymphoid organs and tumor 5 or 15 days after T-cell priming.

We observed a large proliferation of Marilyn T cells in the lymph nodes draining either MHC II+ or MHC II male splenocytes as well as in the lymph nodes draining Dby+ tumors (Fig. 2B). The number of divided T cells in the tumor DLN (mean ± SE, 0.1 ± 0.13 × 106) 5 days after T-cell priming was slightly lower than in the lymph nodes draining MHC II+ cells (imm-DLN; mean ± SE, 0.4 ± 0.14 × 106; P = 0.002; Fig. 2C). However, 15 days after priming, the number of divided Marilyn T cells was similar in the groups primed by Dby+ tumor and MHC II male splenocytes, whereas it was slightly lower after priming by MHC II male splenocytes (Fig. 2D). Thus, although the amount of antigen per cell was similar in all three groups (Fig. 1C and D), the growing Dby+ tumors induce only slightly less Marilyn T-cell proliferation than MHC II male splenocytes. Altogether, these results indicate that the induction of Marilyn T-cell proliferation by indirect antigen presentation after injection of tumor cells or MHC II splenocytes is almost as efficient as the induction by direct antigen presentation.

The three types of immunization enabled the stimulated Marilyn T cells recovered from the DLN 15 days after priming to secrete IFN-γ after specific restimulation in vitro (Fig. 2E), indicating that the Dby+ tumor cells, and the MHC II+ and MHC II male splenocytes were all equally efficient at inducing effector functions in Marilyn T cells.

Another measure of priming efficiency is the ability of activated T cells to recirculate to other lymph nodes (non-DLN) away from the immunization site. Before day 5 after priming, only activated Marilyn T cells that have undergone more than four cell divisions are able to reach the non-DLN. After day 5, most of the cells found in the non-DLN have divided more than six times, although a few that have divided less are also found (26). Here, we found similar numbers of divided Marilyn T cells in the non-DLN at both days 5 and 15 after priming irrespective of the cell type used for priming (Fig. 2F and G). These results show that 5 days after priming Dby+ tumor cells induce effector Marilyn T-cell recirculation in non-DLN as efficiently as MHC II+ and MHC II male splenocytes despite there being a slightly lower expansion of the cell population in tumor DLN.

**Intratumor T-cell accumulation varies depending on the priming method.** T cells have to migrate into tissues to mediate their specific effector functions. Therefore, we measured the number of Marilyn T cells and examined their CFSE phenotype in either Dby+ or Dby+ tumors 5 and 15 days after priming with either MHC II+ or MHC II male splenocytes (Fig. 3). In all priming conditions, we found only CFSE-negative cells in the tumors (Fig. 3A; data not shown), indicating that the activated Marilyn T cells had divided at least six times to be able to migrate into and/or survive in the tumor. As observed previously (26), the number of Marilyn T cells was similar in Dby+ (13.9 ± 1.4% at day 5 and 8.5 ± 2.8% at day 15) and Dby+ (8.2 ± 1.5% at day 5 and 11.3 ± 1.5% at day 15) tumors after priming with MHC II male splenocytes, whereas in Dby+ tumors the number of Marilyn

indicating similar amounts of antigen per stimulating APC for each group. These results show that Marilyn T cells display some antitumor activity and that MCA-101-Dby cells express enough antigen to be the target of rejection. Moreover, for a given number of antigen-expressing cells, direct and indirect antigen presentation result in a similar early and transitory CD69 expression.

**Both direct and indirect antigen presentation induce Marilyn CD4 T cells priming.** The absence of rejection of the Dby+ tumor in the absence of additional priming (Fig. 1A) suggests that the growing Dby+ tumor is not very effective at priming Marilyn T cells despite inducing a similar expression of CD69 on the transgenic T cells when the same number of male cells is injected (Fig. 1C and D). Efficient priming should induce proliferation, a capacity to produce lymphokines and the recirculation of T cells into secondary lymphoid organs and nonlymphoid peripheral tissues, including tumors. Therefore, we analyzed this using Marilyn T cells transferred into B6 adoptive hosts (that have normal lymphoid organs) and different priming conditions to assess the priming efficiency of Dby+ tumors. MCA-101 fibrosarcoma or its Dby-transfected relative was implanted into B6 mice, which were injected 5 days later with CFSE-labeled Marilyn T cells. The following day, some of the mice were immunized with MHC II+ or MHC II male or female splenocytes injected into the Dby+ tumor contralateral hind footpad (Fig. 2A). We then determined the number and phenotype of the Marilyn T cells in the different lymphoid organs and tumor 5 or 15 days after T-cell priming.

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T cells was lower in the absence of additional priming ($2.2 \pm 1.8\%$; $P = 0.018$ at day 5 and $1.5 \pm 2.5\%$; $P = 0.008$ at day 15). We also found that the number of Marilyn T cells in the Dby− tumor after priming with MHC II+ male splenocytes was low ($3.9 \pm 2.5\%$ at day 5 and $3.4 \pm 2.9\%$ at day 15) although not statistically different from the Dby− tumor after priming with MHC II+ male splenocytes. Thus, the poor intratumor accumulation of Marilyn T cells observed in Dby+ tumors may be due to the low priming capacity of indirect antigen presentation.

The lower number of Marilyn T cells observed in Dby+ tumors compared with Dby− tumors in mice immunized with MHC II+ male splenocytes is definitely not related to antigen-specific intratumor mechanisms, such as deletion or suppression, for two reasons. Firstly, the number of Marilyn T cells was similar in both Dby− or Dby+ tumors after MHC II+ male splenocyte priming (Fig. 3B). Secondly, we did an experiment in which Dby− and Dby+ tumors were grafted onto the same mouse and were immunized or not with MHC II+ male splenocytes (Fig. 4A). At day 12, without MHC II+ male immunization, $1.3 \pm 0.9\%$ of the TILs in the Dby− tumor and $0.8 \pm 0.4\%$ of the TILs in the Dby+ tumor were Marilyn T cells that had been primed by the Dby+ tumor. After MHC II+ male immunization, $25 \pm 5\%$ and $22 \pm 6.7\%$ of total TILs in Dby− and Dby+ tumors were Marilyn T cells, respectively (Fig. 4B). The similar numbers of Marilyn T cells present in Dby+ or Dby− tumors according to the priming methods dismiss any intratumor antigen-specific or suppressive mechanism to explain the low number of Marilyn T cells in Dby+ tumors observed in the absence of additional priming. However, we cannot exclude a non-antigen-specific suppression mediated by the tumor microenvironment. For instance, a nonpermissive vasculature could prevent an effective T-cell intratumor accumulation but could be overcome by the high priming capacity of MHC II+ male splenocytes.

Marilyn T-cell accumulation in the gut lamina propria after priming correlates with intratumor accumulation. We examined whether the lower numbers of activated Marilyn T cells...
observed in tumors after priming with MHC II\(^{-}\) male cells or Dby\(^{+}\) tumor cells was specific to the tumors or also applied to other tissues. To do this, we measured the number of Marilyn T cells in the skin, kidney, and gut lamina propria after priming with male splenocytes or Dby\(^{+}\) tumor cells. We found no Marilyn T cells in the kidney or skin irrespective of the stimulation, consistent with there being no inflammation in these tissues (data not shown). After MHC II\(^{+}\) male splenocyte immunization, we found significant numbers of Marilyn T cells in the gut lamina propria of mice bearing a Dby\(^{-}\) tumor (0.09% of total lymphoid cells) or no tumor at all (0.015%; Fig. 5A), consistent with the known location of activated cells (11, 23) and the weak inflammation of the gut lamina propria (10, 32) and thereby dismissing the hypothesis that tumors would prevent the migration of activated T cells into tissues independently of any antigen specificity. Despite the high proliferation (Fig. 2C and D) and recirculation of Marilyn T cells in non-DLN (Fig. 2F and G) and mesenteric lymph nodes (data not shown), we found almost none (0.0003%) in the gut lamina propria of mice with a Dby\(^{+}\) tumor, whereas some Marilyn T cells (0.09%) were found in the gut lamina propria in mice immunized with MHC II\(^{-}\) male splenocytes (Fig. 5A). These results indicate that indirect antigen presentation induced by Dby\(^{+}\) tumor priming results in less activated T cells accumulating in inflammatory tissues, such as tumors and the gut lamina propria.

**Level of antiapoptotic molecule expression correlates with intratumor accumulation.** As we could not detect any intratumor suppressive phenomena, we wondered whether indirect antigen presentation after priming with MHC II\(^{-}\) male splenocytes or Dby\(^{+}\) tumor cells may be related to a lower survival rate of the activated cells, thus resulting in their poor intratumor accumulation. Indeed, the antigenic stimulation strength influences the expression of antiapoptotic molecules, such as Bcl-2 and Bcl-xL, and survivin molecules (16, 17). It has also recently been shown that the overexpression of Bcl-2 in tumor-specific T cells used for adoptive immunotherapy leads to their improved survival (33). Therefore, we measured the intracellular expression of Bcl-2 and Bcl-xL in Marilyn T cells that had undergone at least four cell divisions in the imm-DLN or tumor DLN 5 days after priming. We found that both Bcl-2 and Bcl-xL expression were higher after MHC II\(^{-}\) male splenocyte priming (mean fluorescence intensity, 196 ± 23 and 150 ± 7, respectively) than after MHC II\(^{-}\) male splenocyte (154 ± 16 and 136 ± 8, respectively) or Dby\(^{+}\) tumor priming (116 ± 6 and 99 ± 7, respectively). The expression level of these molecules correlated with intratumor accumulation found in each group of mice (Figs. 3B and 5B) and the lower expression of Bcl-2 and Bcl-xL after Dby\(^{+}\) tumor priming was statistically significant. These results suggest that the cells that have divided at least four times and recirculate first 5 days after priming (26) and express different levels of antiapoptotic molecules depending on the priming method. We suggest that the expression level of these antiapoptotic molecules is a function of the stimulation strength and that, as the expression level becomes higher, the cells survive better and accumulate more in the tumor.

**Large amounts of peptide-MHC complexes on immunizing APCs is required for effective Marilyn T-cell intratumor accumulation.** The priming caused by both the Dby\(^{+}\) tumor and

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**Figure 3.** Intratumor T-cell accumulation varies according to the priming method. A, intratumor accumulation of Marilyn T cells was assessed at day 15 by flow cytometry on explanted tumor cell suspensions. Marilyn T cells were gated on CD45.1\(^{+}\) and \(\beta\)TCR\(^{+}\) cells (R1) and endogenous B6 T cells were gated on CD45.1\(^{-}\) and \(\beta\)TCR\(^{-}\) cells (R2). B, intratumor accumulation of Marilyn T cells at days 5 and 15 plotted as the percentage of Marilyn T cells (R1) among total TILs (R1 + R2) present in the tumor to take into account variations in tumor size and processing sample yield.

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\text{% Marilyn T cells among total TIL} = \frac{(R1)}{(R1 + R2)}
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MHC II- male splenocytes occurs through indirect antigen presentation. The ineffective intratumor accumulation of T cells after priming through indirect antigen presentation may be due to a low number of specific MHC-peptide complexes and costimulatory molecules on the host APCs. Indeed, the amount of endogenous antigen presented directly on MHC II+ male APCs may be higher than the one presented indirectly because of a dilution of the antigen. Therefore, we used a different stimulation method that kept the maturation state and number of APCs constant but varied the number of peptide-MHC complexes per APC used for the priming. We injected female LPS matured BMDCs pulsed with serial dilutions of Dby peptide (2, 40, and 80 nmol/L and 4 μmol/L) into the footpad of B6 mice bearing tumor and harboring Marilyn T cells. After 15 days, we observed no significant difference between the number of divided Marilyn T cells in each type of imm-DLN (Fig. 6A), although we found a clear quantitative relationship ($R^2 = 0.81; P < 0.001$) between the concentration of Dby peptide used to load the dendritic cells and the number of Marilyn T cells found inside the tumors (Fig. 6B). These results show that the number of Marilyn T cells in the tumor strongly depends on the number of peptide-MHC complexes on the priming APC. In our model, the number of Dby-MHC complexes on the host APCs in the Dby+ tumor DLN is probably not high enough to enable efficient intratumor accumulation compared with immunization with MHC II- male splenocytes.

Discussion

Here, we have analyzed the poor intratumor accumulation of specific T cells into tumors. Our experimental model allows us to distinguish intratumor mechanisms (such as deletion or suppressive) from poor priming. We have shown that CD4 T-cell migration and accumulation inside tumors depends strictly on the strength of antigenic stimulation in the lymph node draining the tumor cells or immunizing cells. Indeed, when all other variables remained constant, we found that the more specific MHC-peptide complexes that are present on the priming APC, the more the CD4 T cells accumulate inside the tumor (Fig. 6). We also showed that intratissue accumulation requires a more vigorous priming than for activation, proliferation, and recirculation to different lymphoid organs or for the ability to secrete IFN-γ. Finally, for Dby+ tumors, the intensity of priming can alone explain the magnitude of intratumor accumulation, although we cannot totally rule out a possible role for a non-antigen-specific local suppression by the tumor that would be overcome by a strong priming. In cancer vaccine trials, one has to look for a very strong antigen stimulation to induce intratumor T-cell accumulation and tumor rejection. To find increased numbers of and effector capacities by tumor antigen-specific T cells in the blood or lymphoid organs may not reflect effective capacity to accumulate in the tumors.

As seen in some in vitro studies (18–20), the intensity of antigenic stimulation is a function of the peptide-MHC complex
density, the number of APCs, the amount of costimulation, and the duration of stimulation. The more peptide-MHC complexes that are present on each APC, the more there is proliferation of specific T cells. In vivo, the priming strength can be estimated, with increasing specificity, from CD69 expression, proliferation of T cells in the imm-DLN, IFN-γ secretion after restimulation, and recirculation in the different lymphoid organs. In vivo, naive CD4 T cells need to divide several times before expressing perforin (34). For CD4 T cells, this is less clear. One study has shown that CD4 T cells can secrete effector lymphokines without dividing (35), whereas others have shown that lymphokine secretion only occurs after several cell divisions (24, 36). In our model, only those cells that have divided at least five times are able to secrete IFN-γ (data not shown; Fig. 2). One can hypothesize that migration and accumulation in inflamed tissues, such as tumors or the gut lamina propria, require a stronger stimulation, although this has not yet been shown. We have shown that when all other variables are constant (APC maturation state and APC number) the more specific peptide-MHC complexes that are present per priming APC, the more there is accumulation of the CD4 T cells inside the tumor (Fig. 6). T-cell accumulation inside tissues depends also on the ability of the T cells to survive. Our results show that the expression levels of the antia apoptotic effectors Bcl-2 and Bcl-xl were correlated to the efficiency of intratumor accumulation (Fig. 3B and 5B). The increased ability to survive may be correlated with the number of cell divisions; however, this could not be tested because CFSE staining does not allow more than six or seven cell division cycles to be quantified.

In our study, we observed similar levels of CD69 expression on Marilyn T cells in the DLN after injecting Dby+ tumor cells or MHC II+ or MHC II- male splenocytes in the footpad of mice. This showed that similar amounts of antigen reached the lymph node when the same amount of immunizing cells are injected. However, when T cells were transferred 5 days after a tumor challenge in the flank of the mice, the amount of antigen from the tumor present in the DLN was unknown. The source of antigen could be either whole protein (or cellular debris) released by the growing tumor into the intercellular space after cell death and processed by resident lymph node dendritic cells or apoptotic cells processed by the tissue dendritic cells, which would then migrate to the DLN. In both cases, the amount of and kinetics of antigen presentation is not known. This probably explains the slight differences in intratumor Marilyn T-cell accumulation after priming by MHC II+ male splenocytes and Dby- tumor cells (Fig. 3), as the tumor cells have been injected 5 days earlier and the antigen load may have been different.

From the CFSE dilution, the proliferation pattern of Marilyn T cells was similar irrespective of immunization method (Fig. 2B), with the number of dividing cells being slightly less after Dby+ tumor priming 5 days after T-cell transfer (Fig. 2C). However, we found a similar proliferation pattern and a similar IFN-γ secretion capacity after 15 days and recirculation pattern for all time points (Fig. 2E-G), suggesting only a small difference in priming intensity. By contrast, the number of CD4 T cells inside the tumors (either Dby+ or Dby-) was less after Dby+ tumor priming than after male cell priming (Fig. 3). As we observed no difference in the number of Marilyn CD4 T cells in Dby+ or Dby- tumors after priming with MHC II+ male splenocytes, even when the two tumors were in the same mice (Figs. 3 and 4), we can rule out antigen-specific suppression or deletion phenomena. Therefore, it is more likely due to a lower priming intensity by the Dby+ tumor. This is supported by the slightly lower efficiency of MHC II+ male splenocyte priming compared with MHC II- male splenocyte priming because indirect presentation probably results in a reduction in the number of the peptide-MHC complexes on the host APC.

Differences in the costimulatory molecules expressed on the APCs may also explain these results. Vicari et al. have suggested that tumors could down-regulate the expression of costimulatory molecules on the dendritic cells capturing the antigen (37). However, this is unlikely in our model because when we injected CpG, CFA, or LPS into either the Dby+ tumors or the s.c. skin region drained by the same lymph node, we found no increase in intratumor accumulation (data not shown). These experiments also exclude the possibility that the presenting dendritic cells express less costimulatory molecules because they are far from the point of trauma caused by the implantation of the tumor cells or splenocytes. Finally, when mature dendritic cells loaded with the Dby peptide were used for priming, we found that the number of Marilyn T cells in the tumors was proportional to the concentration of the peptide used for loading (Fig. 6). The number of Marilyn T cells found in the gut lamina propria were similar to those found in the Dby+ or Dby- tumors for each priming method, again suggesting that no specific tumor-related mechanisms are involved.

The Dby+ fibrosarcoma we used was not ignored by the immune system because (a) its growth was slowed down by male cell immunization of the Marilyn mice, (b) the Marilyn T cells proliferated in the tumor DLN in the adoptive transfer model, and (c) the Dby+ tumors were smaller than the Dby- tumors 15 days after priming (data not shown). The tumors we used are MHC II+ but their vascular network comes from the donor. Therefore, they could be targeted by rejection phenomena. This was described by Braun et al. for skin grafts that were rejected by Marilyn T cells even when they did not carry the restricting MHC haplotype (1). That the Dby+ tumor were not rejected in Marilyn mice may be due to the priming phase not being strong enough to generate enough effector cells or to there being not enough antigen in the Dby+ tumor to reactivate the effector cells in situ.

There is strong evidence that many T cells accumulate in tumors in an antigen-independent manner (6, 25, 26, 38). However, the
presence of antigen in the tumor bed induces CD4+ T cells to secrete lymphokines, which in turn recruit macrophages, natural killer cells, or eosinophils that mediate tissue destruction (3, 6, 39). The decrease in Dby′ tumor growth that we observed after tumor inoculation in previously immunized Marilyn mice (Fig. 1A) suggests that there is enough antigen in situ to lead to some antitumor response. Therefore, Dby′ tumor-challenged Marilyn mice should be studied to determine the best vaccination conditions to induce tumor rejection by CD4+ T cells.

In this study, we have analyzed intratumor migration in a transplantable tumor model expressing or not a MHC II-restricted antigen. For Dby+ tumors, it is unclear whether T-cell priming occurs by the host dendritic cells capturing apoptotic bodies or cellular debris when the tumor is implanted or whether antigen is continuously captured as the tumor grows. This will affect the number of specific peptide-MHC complexes and costimulation molecules expressed per APC in the DLN. Indeed, we can suppose that the longer the interval is between tumor challenge and T-cell transfer, the fewer specific peptide-MHC complexes and costimulation molecules will be expressed per APC. This would apply to most transplanted tumor models. Therefore, these models may not best represent the situation in humans in which a tumor grows slowly without causing much tissue damage or dendritic cell activation. In the present study, we have observed an appreciable priming of the specific T cells. The Dby+/A complexes are remarkably stable, having an in vivo life span of >2 weeks (40). Therefore, even if the T cells are injected 5 days after tumor implantation, they may still be stimulated by the antigen released when the tumor was implanted and not by the growing tumor. We are now constructing a model in which the Dby antigen expression in the tumors can be drug induced at a chosen time several days after tumor implantation. This would allow us to better assess whether antigens expressed by solid tumors are ignored or lead to suppression or to abrogate immune responses followed by deletion.

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Intratumor CD4 T-Cell Accumulation Requires Stronger Priming than for Expansion and Lymphokine Secretion


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