Antigen-Experienced CD4+ T Cells Limit Naïve T-Cell Priming in Response to Therapeutic Vaccination \textit{In vivo}

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Abstract

CD4+ T cells play a central role in protective immunity. In a mouse tumor model, we previously found that tumor growth elicits natural CD4+ T-cell responses, but impedes therapeutic vaccination. We show here that inhibition of vaccine-mediated naïve T-cell priming is due to the presence of a minor but distinct population of tumor-reactive CD4+ T cells. These cells are generated in the tumor draining lymph nodes (LN), are capable of systemic redistribution, and act to limit the representation of antigen-bearing MHC II+ antigen-presenting cells (APC) in contralateral LNs or when transferred to tumor-free mice. Surgical tumor resection, which lowers the representation of tumor primed CD4+ T cells, restored to some extent vaccine-induced CD4+ T-cell activation. Likewise, vaccination with artificial APCs (latex beads) or higher numbers of dendritic cells allowed comparable CD4+ T-cell priming in tumor-free and tumor-bearing mice. Together, our results emphasize the ability of antigen-experienced CD4+ T lymphocytes to interfere with therapeutic vaccination and highlight the need for alternative strategies able to surmount limitations imposed by ongoing immune responses. Cancer Res; 70(15); 6161–70. ©2010 AACR.

Introduction

The presence of tumor antigen (Ag)–specific lymphocytes in cancer patients (1) is direct evidence for the ability of the immune system to recognize malignant cells. Immunotherapeutic approaches for the treatment of cancer have mostly targeted tumor Ag–specific CD8+ cytotoxic effector T lymphocytes (2). This is in spite of the critical role for CD4+ T cells in the development of an immune response (3) and in antitumor immunity (4–6). CD4+ T-cell help indeed favors CD8+ T-cell priming and memory T-cell activation (7–9), as well as recruitment of macrophages and eosinophils (4, 10) and CD8+ T cells within the tumor (11), and exerts indirect and direct antitumor effects through the release of IFN-\(\gamma\) (12–14). Likewise, in T-cell receptor (TCR) transfer settings, most potent antitumor responses are observed when gene-modified T cells are used at a CD4/CD8 ratio of 1:1 (15).

Data from our previous work also support a unique role for CD4+ T-cell responses. Indeed, vaccination with a class II-restricted peptide was sufficient to induce protective antitumor immunity in prophylactic settings (16, 17), and CD4-based adoptive cell therapy conferred resistance to tumor development (18). However, although CD4+ T cells contributed to the control of tumor growth, they were insufficient for rejection (19), and therapeutic vaccination was largely ineffective (16, 17). Furthermore, because of their plasticity, CD4+ T cells have reported opposing effects in tumor immunity (reviewed in ref. 6) and in adoptive T-cell therapy (20, 21). These data, together with independent evidence from other preclinical mouse models (4, 13, 19, 22–25), and ascribing to CD4+ T-cell anergy (26, 27) or the limitation of the development of a protective memory repertoire (16, 19, 28), support the notion that suboptimal T-cell help might limit protective immunity against tumors.

In this article, we investigate the immunologic causes for the failure of therapeutic vaccination. We report that the reduced immunogenicity of dendritic cell (DC)–based vaccines found in tumor-bearing mice is associated with the presence of a small but distinct population of tumor Ag–sensitized CD4+ T cells. These cells are primed in tumor-draining lymph nodes (LN) and are capable of limiting the representation of Ag-bearing MHC II+ antigen-presenting cells (APC) in contralateral LNs or when transferred to tumor-free mice. We also report that the use of artificial APCs or higher numbers of Ag-pulsed DCs restores CD4+ T-cell priming, paving the way to novel vaccine formulations able to overcome limitations imposed by natural antitumor immunity.

Materials and Methods

Mice and tumor cells

BALB/c and C57Bl/6 mice were obtained from Charles River. 16.25 transgenic (Tg) mice (29) were bred and housed in a...
specific pathogen-free institutional animal house and used as recipients of tumor or vaccination as detailed in each experiment. T lymphocytes derived from 16.2β mice, backcrossed for more than 10 generations on the BALB/c background, express a Tg TCRβ chain specific for the immunodominant I-A\(^d\)-restricted peptide derived from the Leishmania major-derived Ag LACK (FSPSLHPIVVSGSWD), coupled to the endogenous α-chain TCR repertoire. TS/A (30) and TSA-LACK (19) mammary adenocarcinoma tumor cells (of BALB/c origin) were grown in vitro at 37°C in complete medium (RPMI-10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 units/mL streptomycin; Life Technologies]. MHC class II+ TS/A-LACK cells express LACK as a cytosolic protein, which is representative of a tumor-associated Ag, against which a CD4\(^+\) T-cell response can be measured (19). In spite of gained antigenicity, TS/A and TS/A-LACK grow with comparable kinetics in both BALB/c and 16.2β mice (19). All the in vivo studies were approved by the Ethical Committee of the San Raffaele Scientific Institute and performed according to its guidelines.

**Immunization and treatments**

Mice were challenged s.c. in the right flank with 4 × 10\(^5\) exponentially growing TS/A or TS/A-LACK cells suspended in 100 μL of PBS. Typically, 5 days after tumor cell injection (unless otherwise indicated), mice were vaccinated s.c. in the left flank with 2 × 10\(^5\) or 1 × 10\(^5\) lipopolysaccharide (LPS)-matured syngeneic bone marrow-derived DCs loaded with the LACK immunodominant CD4 peptide (FSPSLHPIVVSGSWD; DC-LACK) as previously described (31). DC maturation and purity were routinely evaluated by flow cytometry after staining with monoclonal antibodies (mAb) recognizing CD11c, MHC class II, B7.1, B7.2, and CD40 molecules (all from BD Bioscience).

Where indicated, immunization was performed by injecting soluble LACK peptide (20 ng in 2 μL) directly into the inguinal LN (32) or using artificial APCs (aAPC; ref. 17). aAPCs were prepared by incubating 5-μm polystyrene-sulfate latex microparticles (Invitrogen) with the proteins of interest: I-A\(^d\)-LACK dimers (5 μg/mL) and anti-CD28 antibodies (clone 37.51; 2 μg/mL). In selected experiments, C57Bl/6 female mouse donors were presensitized against HY-encoded, non-tumor-bearing recipients.

**Cell labeling and in vivo cytotoxic assays**

To track DCs homing to peripheral LNs, bone marrow–described, LPS-matured DCs were labeled with 1 μmol/L CFSE [5-(and-6)-carboxyfluorescein dicacetate succinimidyl ester] or 10 μmol/L CMTMR (Molecular Probes, Invitrogen) according to the manufacturer’s instructions. For CMTMR labeling, cells were resuspended at 5 × 10\(^6\)/mL in complete medium. Equal numbers of CFSE- and CMTMR-labeled cells were injected s.c. at the time of sacrifice. DCs were recovered from LNs by digestion for 60 minutes at 37°C with 2.4 mg/mL collagenase D and 1 mg/mL DNase in magnesium- and calcium-free HBSS supplemented with 20% FBS.

In vivo cytotoxic assays were performed as previously described with some modifications (31). Briefly, splenocytes were labeled with CFSE at two different concentrations (0.125 and 1.25 μmol/L) and loaded with the OVA (ISQAV-HAAHAEINEAGR), the LACK (FSPSLHPIVVSGSWD), or the Dby (NAGFNSRANSSSS; 2 μmol/L) peptide as indicated in the experiments. Equal numbers of CFSE\(^{\text{high}}\) and CFSE\(^{\text{low}}\) cells were mixed and iv. injected in control or tumor-bearing mice. Forty-eight hours later, mice were sacrificed and representation of CFSE-labeled cells was determined by flow cytometry analyses. The specific cytolytic activity was calculated as follows: 100 – (percentage CFSE\(^{\text{high}}\) cells × 100/percentage CFSE\(^{\text{low}}\) cells). Naïve mice were used as controls to determine the relative representation/survival of CFSE\(^{\text{high}}\) and CFSE\(^{\text{low}}\) following in vivo infusion in nonimmunized, non–tumor-bearing recipients.

**Flow cytometry and ELISA assays**

I-A\(^d\)-LACK multimer staining was performed on single-cell suspensions of LN cells (axillary, brachial, and inguinal) as previously described (19). Cells were also stained with anti-CD4, anti-CD44, anti-CD11b, anti-B220, and anti-CD8a mAbs (Pharmingen) and TO-PRO-3 (1 nmol/L; Molecular Probes). CD8a, CD11b, B220, TOPRO\(^+\) cells were excluded by electronic gating during the acquisition. CD4\(^+\), I-A\(^d\)-LACK\(^+\) T cells (50,000–100,000) were generally collected (FACSCanto 2, Becton Dickinson) and analyzed using FlowJo software.

In some cases, CD4\(^+\) cells were purified from LN suspensions by negative selection using Dynabeads (Dynal, Invitrogen, Inc.) Purity was determined before culture by flow cytometry using anti-CD4 mAb and was ≥95%. Purified cells (3 × 10\(^5\)) were cultured with 6 × 10\(^5\) γ-irradiated splenocytes pulsed with the LACK peptide in 96-well round-bottomed plates at 37°C for 24 hours [for interleukin 2 (IL-2)] or 48 hours (for IFN-γ). IL-2 and IFN-γ were measured in the culture supernatants by capture ELISA according to the protocol provided by the manufacturer (BD Pharmingen). Intracellular IL-2 and IFN-γ release was performed as previously described (16).

**5-Bromo-2-deoxyuridine/5-ethyl-2′-deoxyuridine and immunofluorescence**

5-Bromo-2-deoxyuridine (BrdUrd) or 5-ethyl-2′-deoxyuridine (EdU; 50 μg/mL, 0.3 μL) was injected intranodally into the inguinal LN in the right flank. Fast Green was used to monitor correct delivery of BrdUrd/EdU solution in the LN. After 24 hours, inguinal LNs of both flanks were collected. For BrdUrd detection, LNs were fixed in optimum cutting temperature (OCT) compound and frozen, from which 10-μm cryostat tissue sections were obtained. Slides were washed in PBS, boiled in citrate buffer, and blocked in 0.2% bovine serum albumin, 10% FCS, and 0.1% Triton X-100. Thereafter, sections were stained with anti-CD3 primary antibody (Serotec), fixed with cold 4% PFA, and then treated with 2 N HCl (20 minutes, room temperature), equilibrated with borate buffer (pH 8.5), and incubated with secondary anti-rat Alexa-488 antibody (Invitrogen) and anti-BrdUrd antibody (BD Pharmingen), then detected with Zenon-546 kit (Invitrogen). For EdU detection, LNs were fixed with cold 4% PFA, rinsed in PBS, and fixed in OCT. Cryostat tissue sections (12 μm) were fixed in cold acetone for 5 minutes, rinsed...
in PBS, and processed for EdU detection according to the manufacturer’s instructions (Invitrogen). Thereafter, sections were incubated overnight with anti-CD4 mAb (BD Pharmingen) and revealed by anti-rat Alexa-488 antibody. Nuclei were detected by 4′,6-diamidino-2-phenylindole staining.

**Statistical analysis**

Results were analyzed using GraphPad Prism 4 software and statistical analyses were performed using unpaired two-tailed Student’s *t* test. Results were considered statistically significant when *P* < 0.05 (*), *P* < 0.01 (**), and *P* < 0.001 (***)..

**Results**

**LACK-expressing TS/A tumors impede DC-induced T-cell priming**

We previously found that depletion of CD4+ T cells in mice bearing TS/A tumors expressing the model Ag LACK (TS/A-LACK) results in faster tumor growth, but neither natural nor vaccine-induced immunity is sufficient for tumor rejection (16, 19). To investigate whether this is possibly due to suboptimal T-cell priming, we quantified CD4+ T-cell responses following therapeutic vaccination of 16.2β mice, in which both naïve (~0.5% of CD4+ cells) and memory polyclonal LACK-specific CD4+ T cells can be readily identified by fluorescent I-Aβ/LACK multimers (see Materials and Methods and ref. 29).

In response to vaccination by the s.c. route with DCs loaded with the MHC class II–restricted immunodominant LACK peptide (DC-LACK), I-Aβ/LACK+ CD4+ T cells rapidly accumulated in the DC-draining LNs of control and TS/A tumor-bearing 16.2β mice (Fig. 1A and C), and most of the I-Aβ/LACK+ CD4+ T cells acquired a CD44high, CD62Llow phenotype (Fig. 1B and D) and proved capable of IL-2 and IFN-γ secretion (16, 19). In contrast, most (>70%) of the I-Aβ/LACK+ CD4+ T cells derived from DC-draining LNs of TS/A-LACK tumor-bearing mice retained a CD44low, CD62Lhigh naïve phenotype and failed to secrete significant amounts of cytokines both at 5 days after vaccination (Fig. 1A) and at later times (16).

**Figure 1.** DC-LACK–induced immune responses are impaired in the presence of established TS/A-LACK tumors. 16.2β Tg mice were challenged s.c. with 0.4 × 10⁶ TS/A or TS/A-LACK tumor cells on the right lateral flank 5 d before (day −5) s.c. vaccination on the left lateral flank (day 0) with 0.2 × 10⁶ DCs pulsed with the immunodominant LACK peptide (DC-LACK). On day 5 after vaccination, the axillary, brachial, and inguinal LN cells draining the site of DC vaccination (DC-dLN) and the cervical LN cells distal to the site of vaccination and tumor growth were analyzed by flow cytometry after staining with anti-CD4, CD44 mAb, and I-Aβ/LACK multimers. A, events are depicted after gating on CD4+ TOPRO− CD8− B220− CD11b− cells. B, relative expression of CD44 and CD62L among CD4+, I-Aβ/LACK+ cells. C, columns, mean (n ≥ 7) obtained from two independent experiments; bars, SEM. D, mean distribution of naïve (CD44low, CD62Lhigh), central memory–like (CD44high, CD62Lhigh; TCM), and effector memory–like (CD44high, CD62Llow; TEM) CD4+, I-Aβ/LACK+ cells.

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To further dissect this limited responsiveness to vaccination, we analyzed LACK-specific CD4+ T cells at 30, 60, and 100 hours after immunization with vaccine-draining LNs (Fig. 2). In TS/A-bearing mice, DC-induced TCR triggering was evident at 30 hours after vaccination by the appearance of a small but detectable population of I-A\(^{d}\)/LACK\(^+\) CD4\(^+\) T cells expressing high levels of the early activation marker CD69 (Fig. 2A and B). This population was absent in mice with TS/A-LACK tumors and could not be detected at any later time points. Likewise, the LNs draining the site of DC injection in TS/A-LACK–bearing mice contained significantly fewer I-A\(^{d}\)/LACK\(^+\) CD4\(^+\) CD25\(^+\) (Fig. 2C) and I-A\(^{d}\)/LACK\(^+\) CD44\(^+\) CD4\(^+\) T cells (Fig. 2D) than the corresponding LNs in TS/A-bearing mice (Fig. 2A–C) at all time points analyzed. These data strongly suggest that in tumor-bearing mice, DC-induced LACK-specific CD4\(^+\) T-cell responses are impaired at the time of encounter with this tumor Ag.

Unavailability of Ag does not account for reduced vaccine immunogenicity

Having found that DC-mediated CD4\(^+\) T-cell priming is impaired in TS/A-LACK–bearing mice as early as 30 hours after vaccination, we analyzed whether this could be explained by defective DC migration to peripheral LNs. To this aim, we injected control and TS/A-LACK tumor–bearing mice with 1:1 mixtures of LACK peptide–pulsed DCs labeled with the fluorescent dye CFSE and DCs pulsed with an irrelevant MHC class II peptide and labeled with the dye CMTMR (Fig. 3A and B). CFSE\(^+\) and CMTMR\(^+\) DCs could be detected at similar frequencies in both tumor-free and TS/A-LACK tumor–bearing mice. Overall DC recovery seemed to be lower in tumor-bearing mice when compared with tumor-free controls, both at 40 hours (Fig. 3B) and at 60 to 72 hours after injection (not shown). However, although DCs had forward and side scatter compatible with dying cells as early as 40 hours after administration, this was not restricted to LACK-pulsed cells (Fig. 3B, and data not shown), hindering the assessment of DC persistence.

To further investigate whether impediment of access by Ag to the LN accounted for defective T-cell priming, we performed intranodal delivery of the LACK peptide. Also in this case, I-A\(^{d}\)/LACK\(^+\) CD4\(^+\) CD44\(^{high}\) T cells appeared within 24 hours of intranodal injection of Ag in tumor-free mice, but not in TS/A-LACK tumor–bearing mice (Fig. 3C and D). Thus, in spite of the Ag being readily available, LACK-specific T cells fail to accumulate in mice bearing TS/A-LACK tumors.

Tumor-primed CD4\(^+\) T lymphocytes hinder the persistence of I-A\(^{d}\)/LACK–bearing APCs

In a previous study, we had shown that impaired activation of LACK-specific CD4\(^+\) T cells seemed to be associated with the LACK Ag (16), implying the existence of LACK-specific suppression. As we previously failed to detect tumor Ag–primed LACK-specific CD4\(^+\) T cells in tumor-distal LNs

Figure 2. Kinetics of DC-induced LACK-specific CD4\(^+\) T-cell responses in the presence of TS/A or TS/A-LACK tumors. TS/A or TS/A-LACK tumor–bearing 16.2B Tg mice were vaccinated as described in Fig. 1. Mice were sacrificed 30, 60, or 100 h after DC-LACK vaccination and the LN cells draining the site of vaccination were stained with I-A\(^{d}\)/LACK multimers in combination with CD69 (A and B), CD25 (C), and CD44 (D). Naïve mice were used as controls. A, events are shown after gating on CD4\(^+\) TOPRO\(^-\) CD8\(^-\) B220\(^-\) CD11b\(^-\) cells. B to D, line graphs represent the total number of CD69\(^{high}\), CD25\(^+\), and CD44\(^{high}\) cells (\(\times 10^3\)) at the indicated times. Total numbers were obtained by multiplying the frequency obtained by flow cytometry by the total number of lymphocytes determined by viable trypan blue counts. Points, mean of four mice per group; bars, SEM. The experiment was independently repeated with comparable outcomes.
IL-2-secreting cells (Fig. 4B), but not for CD25^high, FoxP3^+ when compared with cells derived from control, 16.2β mice. I.N., intranodal.

The corresponding inguinal LN of tumor-free 16.2β mice was similarly injected. Mice were sacrificed 24 hours later and the injected and the contralateral inguinal LNs were analyzed by immunofluorescence confocal analysis and flow cytometry. By confocal analysis, CD^3^ + BrdUrd^+ cells were found in the injected LNs of both tumor-free and tumor-bearing mice (Fig. 4D), but only in the contralateral LNs of tumor-bearing mice (Fig. 4D; Supplementary Fig. S2A). Whereas the number of BrdUrd^+ CD^3^ cells per area in BrdUrd-injected LNs was comparable in tumor-free and tumor-bearing mice (Fig. 4D), their overall recovery was higher in the latter because of drastic LN enlargement (not shown). By flow cytometry analysis, we found that CD^4^ + T cells were preferentially labeled in tumor-draining LNs (Supplementary Fig. S2B and C). Furthermore, we found that CD3/EdU and CD4/EdU double-positive cells were found in both tumor-draining and nondraining LNs (Supplementary Fig. S2D–G). Thus, taken together, these data support the idea that a small but distinct population of CD^4^ + T cells primed in tumor-reactive LNs is capable of recirculation to contralateral nodal sites.

Several reports have shown that CD^4^ + T cells can exert direct cytotoxic action (34–38). To investigate whether tumor-derived CD^4^ + T cells can selectively remove LACK APCs, we performed an in vivo cytotoxic assay with differentially CFSE-labeled spleen cells pulsed with the LACK-derived peptide or with the irrelevant OVA peptide. Whereas MHC class II^+ (I-A^d^+) OVA-pulsed target cells were found in comparable frequencies in the LNs of tumor-free and TS/A-LACK tumor-bearing 16.2β mice (Fig. 5A), MHC class II^+ LACK-pulsed target cells were selectively decreased in both the tumor draining and the nondraining LNs of TS/A-LACK tumor-bearing 16.2β mice (Fig. 5A and B). Comparable results were obtained in nontransgenic settings, that is, in tumor-free and TS/A-LACK tumor-bearing immunocompetent or CD8^-/- mice (Fig. 5C), which have a normal repertoire of Ag-specific CD^4^ + T lymphocytes. Finally, the lower representation of Ag-bearing MHC class II^+ cells in mice previously sensitized to the given Ag was independent of IFN-γ, as it was also observed in IFN-γ^-/- mice (Supplementary Fig. S3A), and was not restricted to tumor-bearing mice or limited to LACK, but was also found in C57BL/6 females previously sensitized to male Ags (Supplementary Fig. S3B).

To show directly that CD^4^ + T cells were responsible for the reduced persistence of LACK-pulsed cells, CD^4^ + cells purified from the LNs draining the site of TS/A and TS/A-LACK tumor growth were injected into tumor-free BALB/c mice, and persistence of CFSE-labeled splenocytes pulsed with OVA or LACK peptide was measured. Whereas OVA or LACK-pulsed cells were equally represented in recipients of cells derived from TS/A tumor-bearing mice, I-A^d^ +, LACK-pulsed cells were reduced in frequency in mice infused with cells derived from TS/A-LACK-bearing mice (Fig. 5D). Thus, CD^4^ + T cells derived from tumor-bearing mice are able to reduce tumor peptide.}

**Figure 3.** DC trafficking and Ag presentation in the presence of TS/A-LACK tumors. A, mice with detectable (5 d) TS/A-LACK tumors were vaccinated in the flank opposite to the site of tumor growth with 1 × 10^6 CFSE-labeled LACK-pulsed and CMTMR-labeled OVA-pulsed DCs. Mice were sacrificed 40 h after DC-LACK vaccination and the percentage (A) and total number (B) of dye-positive cells in the LNs draining the site of vaccination were determined by flow cytometry. Representative plots from BALB/c mice are shown and similar results were obtained in 16.2β Tg mice in four independent determinations. C and D, LACK peptide (LACKp; 50 μmol/L) was delivered in the LNs contralateral to established TS/A-LACK tumors or into the LNs of tumor-free mice (see Materials and Methods). Five days later, the mice were sacrificed, and LACK-specific T cells quantified by flow cytometry. Three mice per group were independently analyzed. Three independent determinations gave similar outcomes. I.N., intranodal.

by flow cytometry (19), we chose to reexamine their presence by Ag-specific T-cell proliferation and cytokine release assays following CD^4^ + T-cell enrichment. Purified CD^4^ + T cells from TS/A-LACK tumor–distal LNs proliferated at similar extents when compared with cells derived from control, 16.2β tumor–free mice (Fig. 4A) and seemed to be enriched for IFN-γ^- and IL-2-secreting cells (Fig. 4B), but not for CD25^high, FoxP3^- cells (Fig. 4C; Supplementary Fig. S1). To identify the origin of this population, we traced the fate of individual tumor-primed T cells in vivo by intranodal labeling. TS/A-LACK tumor–bearing 16.2β mice received an intranodal injection of the thymidine analogue BrdUrd or EdU in the inguinal LN draining the site of tumor growth. The corresponding inguinal LN of tumor-free 16.2β mice was similarly injected. Mice were sacrificed 24 hours later and the injected and the contralateral inguinal LNs were analyzed by immunofluorescence confocal analysis and flow cytometry. By confocal analysis, CD^3^ + BrdUrd^+ cells were found in the injected LNs of both tumor-free and tumor-bearing mice (Fig. 4D), but only in the contralateral LNs of tumor-bearing mice (Fig. 4D; Supplementary Fig. S2A). Whereas the number of BrdUrd^+ CD^3^ cells per area in BrdUrd-injected LNs was comparable in tumor-free and tumor-bearing mice (Fig. 4D), their overall recovery was higher in the latter because of drastic LN enlargement (not shown). By flow cytometry analysis, we found that CD^4^ + T cells were preferentially labeled in tumor-draining LNs (Supplementary Fig. S2B and C). Furthermore, we found that CD3/EdU and CD4/EdU double-positive cells were found in both tumor-draining and nondraining LNs (Supplementary Fig. S2D–G). Thus, taken together, these data support the idea that a small but distinct population of CD^4^ + T cells primed in tumor-reactive LNs is capable of recirculation to contralateral nodal sites.

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Ag presentation by removing APCs in a manner independent of IFN-γ.

**Surgical tumor resection or vaccination with artificial APCs or high DC numbers restores T-cell priming in tumor-bearing mice**

Surgical resection of the tumor lowers the systemic representation of tumor-specific IFN-γ-producing CD4+ effector T cells (19). We thus tested whether this might improve vaccine immunogenicity. Resection of the tumor before vaccination rescued, at least to some extent, the appearance of I-A<sup>d</sup>/LACK<sup>+</sup> CD4<sup>+</sup> CD44<sup>high</sup> T cells (Fig. 6A), also capable of IL-2 and IFN-γ secretion (Fig. 6B).

We then investigated whether latex beads coated with recombinant peptide-linked I-A<sup>d</sup>/LACK multimers and anti-CD28 costimulatory mAb might overcome inhibition by LACK Ag–primed CD4<sup>+</sup> T cells (17). These artificial APCs would be resistant to killing and/or Ag stripping, likely responsible for the disappearance of I-A<sup>d</sup><sup>+</sup>/LACK-pulsed cells. Intravenous injection of LACK-aAPCs into tumor-free or tumor-bearing mice elicited priming of I-A<sup>d</sup>/LACK<sup>+</sup> CD4<sup>+</sup> CD44<sup>high</sup> T cells in LNs of tumor-free and TS/A-LACK tumor–bearing (draining and nondraining (ndLN)) mice. D, BrdUrd was delivered in the LNs draining established TS/A-LACK or into the LNs of tumor-free mouse (see Materials and Methods). Twenty-four hours later, the mice were sacrificed and BrdUrd<sup>+</sup> (red) and CD3<sup>+</sup> (green) identified by confocal microscopy (Supplementary Fig. S2). Independent sections of equivalent LN areas were blindly scored for the presence of CD3<sup>+</sup>, BrdUrd<sup>+</sup> cells. Representative results of the analysis of five independently analyzed mice. The experiment was repeated twice.

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**Figure 4. IFN-γ–secreting tumor Ag–primed T cells home to contralateral LNs.** A and B, CD4<sup>+</sup> T cells were purified from the tumor-distal LNs of 16.2β Tg mice bearing 5- or 14-d-old TS/A-LACK tumors or from tumor-free control mice and stimulated with irradiated BALB/c splenocytes in the presence of the depicted amount of LACK peptide (0-5 μmol/L). A, cells were also labeled with the CFSE fluorescent dye and analyzed by flow cytometry after 6 d of culture. In B, IL-2 and IFN-γ release were determined 24 and 48 h after start of culture, respectively. Purity of CD4<sup>+</sup> T cells was >95%. Columns, mean; bars, SEM. C, frequency of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> T cells in LNs of tumor-free and TS/A-LACK tumor–bearing (draining and nondraining (ndLN)) mice. D, BrdUrd was delivered in the LNs draining established TS/A-LACK or into the LNs of tumor-free mice (see Materials and Methods). Twenty-four hours later, the mice were sacrificed and BrdUrd<sup>+</sup> (red) and CD3<sup>+</sup> (green) identified by confocal microscopy (Supplementary Fig. S2). Independent sections of equivalent LN areas were blindly scored for the presence of CD3<sup>+</sup>, BrdUrd<sup>+</sup> cells. Representative results of the analysis of five independently analyzed mice. The experiment was repeated twice.
frequency of I-Ad/LACK+ CD4+ CD44high T cells (Fig. 6D) gradually increased whenever tumor-free mice received 0.2 × 10⁶ (2 μmol/L), 1 × 10⁶ (2 μmol/L), and 1 × 10⁶ (20 μmol/L) DC-LACK. Accordingly, I-Ad/LACK+ CD4+ CD44low CD62L high cells were gradually reduced (Supplementary Fig. S4). No further improvement of the response was observed when 10 × 10⁶ (2 μmol/L) DC-LACK were injected (Fig. 6D). Also in the case of TS/A-LACK tumor–bearing mice, increasing the number of DCs improved CD4+ T-cell priming (Fig. 6D). Most importantly, vaccination with 10 × 10⁶ LACK (2 μmol/L)–pulsed DCs elicited comparable T-cell responses in TS/A-LACK–bearing mice and tumor-free mice (Fig. 6D; Supplementary Fig. S4). Thus, the use of aAPC or high DC numbers can rescue, at least to some extent, vaccine-induced T-cell expansion in mice with active tumor disease.

**Discussion**

In this study, we analyzed the events hindering the immunogenic competence of CD4+ T-cell–targeted therapeutic vaccination. We report that vaccination fails to induce optimal CD4+ T-cell expansion in tumor-bearing mice because of reduced persistence of tumor Ag–bearing APCs. This is due to the presence of a small but distinct population of tumor-primed CD4+ T cells, which is generated in the tumor-proximal LN and capable of systemic recirculation.

In our model system, the mechanism impinging on the immunogenicity of peptide-pulsed DCs in tumor-bearing mice relies on Ag-specific events, cannot be attributed to CD4+ T-cell anergy or to the activity of myeloid-derived suppressor cells, and cannot be overcome by depletion of CD25+ and CD8+ cells (16) or by intranodal delivery of Ag. Instead, it seems to depend on the existence of a rare population of CD4+ T cells generated in response to the tumor and capable of limiting the persistence of tumor Ag–bearing MHC class II+ APCs. These tumor-primed CD4+ CD3+ T cells are induced to proliferate in tumor-proximal LNs, and although remaining undetectable by peptide/I-Ad fluorescent multimer analysis in tumor-distal LNs, they can be revealed by in vivo labeling and Ag-specific functional assays (IFN-γ release). Although we cannot exclude that other cells primed in response to the tumor also play a role, highly purified CD4+ cells derived from tumor-bearing mice prove able to reduce the number of LACK-pulsed MHC class II+ cells when adoptively transferred into tumor-free mice.

Although the ability of Ag-primed T cells to impede further T-cell priming was observed in other experimental conditions (41–47), it has not previously been linked to the impairment of vaccine efficacy. In previous studies, CD4+ T cells...
were shown to (a) cross-compete for access to specific Ag-MHC complexes (41, 42), (b) remove Ag-MHC complexes from the surface of APCs (43, 44), and (c) induce internalization and downmodulation of specific Ag-MHC complexes (45–47). Cytotoxic CD4+ T cells were described previously against viral Ags (36–38) and alloantigens (48, 49). While the manuscript was being reviewed, three articles supporting the contribution of cytotoxic CD4+ T cells became available. Amigorena and coworkers described a distinct population of tumor-primed CD4+ T cells expressing CD25 and FoxP3 capable of perforin-dependent DC cell death in tumor-draining LNs (50). Likewise, the groups led by Antony and Allison showed that adoptively transferred TCR Tg CD4+ T cells acquire granzyme-B–dependent cytotoxic activity resulting in eradication of large, established tumors (14, 51). In our model, persistence of peptide-MHC class II+ cells was similarly reduced in wild-type and IFN-γ−/− mice. As we do not find evidence for the enrichment of CD4+ CD25+ FoxP3+ cells, we speculate that the reduced persistence of LACK-pulsed, MHC class II+ cells in TS/A-LACK tumor–bearing mice might be dependent on Fas, tumor necrosis factor-related apoptosis-inducing ligand, perforin (50, 52, 53), or granzyme B (14, 51). Although at present we cannot exclude that Ag-MHC stripping or internalization, rather than direct killing of Ag-pulsed DCs or splenocytes, accounts for inefficacious T-cell priming, our data support the idea that growing tumors generate CD4+ T cells capable of inhibiting further T-cell priming by reducing the Ag load.

Of note, elective removal of Ag (LACK)-pulsed, MHC class II+ cells was not peculiar to the 16.2β Tg settings, but was also observed in BALB/c mice with TS/A-LACK tumors and was recapitulated in tumor-free mice recipients of adoptively transferred CD4+ cells purified from TS/A-LACK tumor–bearing mice. Furthermore, it was not restricted to tumor-bearing mice, nor to the LACK Ag, as the representation of HY (Dby)-pulsed MHC class II+ cells was reduced when compared with OVA-pulsed cells in female mice immunized against male (HY) Ags. This suggests that competition between Ag-primed T cells and naïve T cells might normally occur, possibly at times when the former are more abundant.
This might be the case during chronic exposure to Ag, such as in the presence of growing tumors or persistent viral infections, or shortly after vaccination. Consistent with this possibility is the finding that surgical resection of the tumor, which lowers the representation of tumor-primed CD4+ T cells (19), ameliorated vaccine-induced T-cell priming in our experimental setting. The generation of cytotoxic CD4+ T cells over the course of exposure to Ag might also represent a physiologic negative feedback mechanism preventing an overreaction by adaptive immunity. This might account for suboptimal memory T-cell expansion during the course of secondary responses, which is blunted when compared with that of naive T cells (54). Taken together, our data indicate that therapeutic vaccines should be administered in the absence of active immune responses to avoid possible interference by recently primed T cells. In our experimental setting, we found that killing-resistant latent bead–based APCs or provision of sufficient numbers of DCs overcame inhibitory mechanisms and elicited comparable CD4+ T-cell responses in tumor-free and tumor-bearing mice. Unfortunately, whereas the former approach failed to confer protection in a therapeutic setting, possibly because of suboptimal T-cell induction (17), the latter is not suitable for clinical purposes. Thus, alternative vaccination strategies able to overcome limitations imposed by Ag-experienced T cells, preexisting to the vaccination, will have to be considered for improving current immunotherapeutic protocols. As an alternative, the ex vivo expansion of tumor-reactive CD4+ T cells could be attempted (18), as adoptive cell therapy might represent a more potent therapeutic option.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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