Extra-Lymphatic Solid Tumor Growth Is Not Immunologically Ignored and Results in Early Induction of Antigen-Specific T-Cell Anergy: Dominant Role of Cross-Tolerance to Tumor Antigens

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ABSTRACT

A better understanding of how solid malignancies arise in an immunocompetent host, avoid immune recognition, and ultimately progress to widely disseminated cancer is essential to effectively harness the immune system against solid tumors. Because of their extra-lymphatic localization, it has been proposed that solid malignancies are just ignored by the immune system, thereby allowing their uncontrolled growth and dissemination. Alternatively, as most of the solid tumors are unable to express costimulatory molecules, the “signal one without signal two” model of tolerance induction has been frequently evoked to account for the failure of the immune system to reject antigenic tumors in vivo. In this study, we showed, however, that the extra-lymphatic growth of solid tumors is not immunologically ignored by the lymphoid compartment, resulting instead in the early induction of antigen-specific CD4⁺ T-cell tolerance. Furthermore, analysis of parent-into-F1 bone marrow (BM) chimeras demonstrates that presentation of tumor antigens by BM-derived antigen-presenting cells represents the dominant mechanism in solid tumor-induced CD4⁺ T-cell tolerance. Our findings of early development of antigen-specific T-cell unresponsiveness mediated by BM-derived antigen-presenting cells, not only provides a plausible explanation for the failure of the immune system to reject antigenic solid tumors in vivo, but more importantly, they have identified a barrier that, if appropriately manipulated, may lead to approaches to effectively harness the immune system against solid malignancies.

INTRODUCTION

Solid tumors, the most common form of malignancies in humans, remain largely incurable with the treatment modalities currently available. In recent years, a significant effort has been devoted to the development of novel therapeutic strategies against these malignancies (1–3). The demonstration that immune cells are able to destroy chemotherapy-resistant tumor cells (4, 5), together with the better understanding of the mechanisms regulating immune responses (6, 7), has led to a renewed interest in immunotherapy as a potential noncross-resistant treatment modality for solid malignancies. However, before we can effectively harness the immune system against solid tumors, it is critical to better understand first how it is that these tumors arise in an immunocompetent host, outmaneuver immune recognition, and ultimately progress to widely disseminated cancer.

In recent years, several explanations have been proposed to account for the inability of the immune system to recognize and reject a malignant tumor. Among them, the immune ignorance and the immune tolerance mechanisms have gained particular attention. Given that most solid tumors form outside of the lymphoid organs, it has been proposed that the lack of tumor immunity is just the result of tumor cells being ignored by tumor-specific T cells residing in the lymphoid compartment (8, 9). Alternatively, it has been argued that a successful tumor growth in an otherwise immunocompetent host relates to the immune system “seeing” tumors more as self than as foreign, leading to the induction of unresponsiveness to tumor antigens in a fashion similar to the induction of peripheral tolerance to self-antigens (10, 11). Recent studies in mice in which T cells specific for tumor-associated antigens could be followed in vivo have indeed provided experimental evidence that the natural response of the immune system to tumor antigens seems to be the induction of T-cell tolerance rather than T-cell activation (12, 13).

Despite the demonstrated ability of solid malignancies to induce antigen-specific T-cell tolerance in vivo, none of the mechanisms proposed, to date, can completely and adequately explain how it is that these tumors may induce this state of unresponsiveness. For many years it has been postulated that tolerance to tumor antigens results from a direct encounter of antigen-specific T cells with tumor cells that are ill-equipped to provide the necessary signals for T-cell activation. Solid tumor cells, it is argued, can provide an antigen-specific signal through engagement of the T-cell receptor (TCR) (signal one) but lack the full complement of costimulatory signaling (signal two) required to yield a fully activated and functional T-cell response. According to this model therefore, in the absence of signal two, the direct encounter of solid tumor cells with antigen-specific T cells would result in the induction of T-cell anergy rather than T-cell activation (14, 15). Unfortunately, such an encounter in the case of nonlymphatic tumors would require that naïve T cells traffic beyond the circulatory and lymphatic vessels, a scenario quite unlikely to occur in vivo, because only activated T cells venture into the peripheral tissues.

In this study, we evaluated the relative contribution of either mechanism (immune ignorance versus immune tolerance) in influencing the fate of antigen-specific CD4⁺ T cells during the extra-lymphatic growth of solid tumors. The results of this analysis provided the following conclusions: (a) extra-lymphatic solid tumor growth is not immunologically ignored by the lymphoid compartment because antigen-specific CD4⁺ T cells isolated from the lymphoid organs of tumor-bearing mice lost their naïve phenotype and undergo clonal expansion in vivo; (b) despite lack of tumor invasion to the lymphoid organs—and therefore lack of a direct tumor-T-cell interaction—antigen-specific T cells are rendered tolerant early during tumor progression; (c) analysis of parent-into-F1 bone marrow (BM) chimeras demonstrates that solid tumor-induced T-cell tolerance requires presentation of tumor antigen by BM-derived antigen-presenting cells (APC) (cross-tolerance); and (d) the development of antigen-specific
T-cell tolerance mediated by BM-derived APCs imposes a significant barrier to therapeutic vaccination against solid malignancies.

**MATERIALS AND METHODS**

**Mice.** Six to 8-week-old male BALB/c, C57BL/6, or BALB/cxC57BL/6 F1 mice were obtained from the NIH (Frederick, MD). Male BALB/c severe combined immunodeficiency (SCID) or C57BL/6 SCID, ages 6–8 weeks, were purchased from The Jackson Laboratory (Bar Harbor, ME). TCR-transgenic mice expressing an αβ TCR specific for amino acids 110–120 from influenza hemagglutinin presented by I-Eκ were a generous gift of Harold von Boehmer (16). The transgenic mice used in these experiments were heterozygous for the transgene. TCR-transgenic mice (OT-II) expressing an αβ TCR specific for peptide 323–339 from ovalbumin (OVA) presented by MHC class II, I-A^b^ (17) were provided by Dr. William Heath (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). All of the experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committees of the University of South Florida College of Medicine and Johns Hopkins University School of Medicine.

**Tumor Cells.** Renal cell carcinoma cells (Renca) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in vitro in RPMI 1640, supplemented with 10% FCS, penicillin/streptomycin (50 units/ml), 1-glutamine (2 mM), and 2-mercaptoethanol (50 μM; complete media), and were grown as an adherent population at 37°C, 5% CO₂, DA3 mammary adenocarcinoma cells were kindly provided by Dr. Diana M. Lopez (University of Miami School of Medicine). These cells grow as an adherent population in DMEM supplemented with 10% FCS, 50 units/ml penicillin/streptomycin, and Oxalacetaate, Pyruvate, Insulin supplement (Sigma, St. Louis, MO). RencaHA was generated by calcium phosphate-mediated plasmid transfection with the pCMV plHA, which encodes the HA molecule of the influenza virus A/PR/8/34 (H1N1; Ref. 18). Transfection of DA3 tumor cells with the construct pHA was achieved using the Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA). RencaHA and DA3 tumor-expressing HA (DA3HA) were selected and grown in their respective complete media supplemented with the neomycin analogue G418 (400 μg/ml). All tumor cell lines are regularly checked for Mycoplasma and endotoxin levels, and they have shown to be free of contamination. HA expression by transfected tumor cells was determined by staining with the anti-HA-biotinylated antibody H-18 as described previously (18). Expression of MHC class I molecules by transfected and nontransfected tumor cells was determined by staining with a FITC-conjugated anti-H-2K^b^{+} antibody (BD PharMingen, San Diego, CA). The expression of class II molecules was determined by staining with the biotinylated monoclonal antibody (MAb) 14.4.4 followed by phycoerythrin (PE)-conjugated streptavidin (BD PharMingen, CA) and expression of B7.1 costimulatory molecules by staining with a FITC-conjugated anti-CD80 antibody (BD PharMingen). Ten thousand gated events were collected on a FACScan (Becton Dickinson, San Jose, CA) and analyzed using Flow-Jo software.

B16 melanoma cells (H-2b) transfected with Ovalbumin (B16-OVA) were kindly provided by Dr. Paolo Dellabona (Instituto San Rafaelle, Milan, Italy). These cells are routinely cultured in complete media supplemented with hygromycin (Sigma).

**In Vivo Tumor Challenge.** Transfected tumor cells and their wild-type counterparts were detached from the culture flasks with trypsin (Sigma) and suspended in complete media. Tumor cells were counted, and viability was assessed by trypan blue exclusion. If viability was 100%, cells were washed three times in sterile HBSS and injected either i.v. (Renca tumors) or s.c. (DA3 and B16 tumors) in a total volume of 0.2 ml, 1 × 10^6 tumor cells/mouse. Tumor-free survival was determined by twice weekly inspection, and mice were euthanized after s.c. tumor development (DA3 mammary tumor and B16 melanoma). Renca-bearing mice were euthanized after the first sign of increased respiratory rate or decreased motion. These animals had the presence of tumors (lung nodules) confirmed at autopsy.

**Adaptive Transfer of Antigen-Specific T Cells.** Single-cell suspensions were made from peripheral lymph nodes and spleen collected from TCR-transgenic donors. The percentage of lymphocytes double positive for CD4 and the TCR was determined by flow cytometry. Briefly, the percentage of anti-HA-transgenic CD4^+ T cells was assessed by double staining with FITC-conjugated goat antimouse CD4 (Caltag) and biotinylated rat anti-CD8 ( PharMingen, anti-Vv-PE, and anti-Vβ5FITC antibodies (BD PharMingen). Cells were washed three times in sterile HBSS and injected into the tail vein of male recipients such that a total of 2.5 × 10^6 CD4^+ anti-HA TCR^+ T cells or anti-OVA OT-II-transgenic CD4^+ T cells was transferred to each animal.

**Resolation of Transgenic T Cells after in Vivo Transfer.** Transgenic CD4^+ T cells injected into tumor-free mice or tumor-bearing mice were resolated from either the draining lymph nodes or the spleen of these animals on the days specified under each experimental design. In all experiments, unless otherwise noted, 3 mice/group were used. Each mouse was given a unique identification number so that the various measurements of T-cell function was correlated within an individual, as well as between mice in the same group or between groups. Briefly, to analyze the fate and function of antigen-specific transgenic CD4^+ T cells present in the draining lymph nodes, the peritracheal, peribronchial, and mediastinal lymph nodes were harvested from tumor-free as well as from RencaWT- or RencaHA-bearing mice. Lymph nodes from 3 animals/group were pooled, and cell suspensions were made by passage over nylon mesh and centrifugation on a Ficoll gradient (Ficoll-Paque; Pharmacia Biotech, Uppsala, Sweden). Between 2 and 3 × 10^7 lymph node cells were obtained from the pooled samples of tumor-bearing mice and between 0.5 and 1 × 10^6 cells from tumor-free animals. T cells from the spleen of tumor-free or tumor-bearing mice were purified by passage through nylon mesh and centrifugation on a Ficoll gradient followed by passage through nylon wool. Optimization of this technique has allowed us to obtain at least 5 × 10^6 highly purified T cells/spleen.

Flow Cytometric Analysis. Purified T cells were stained with FITC-conjugated goat antimouse CD4 (Caltag) and biotinylated rat anti-CD8 TCR antibody MAb 6.5 followed by phycocerythrin-conjugated streptavidin (Caltag). For this analysis, 50,000 gated events were collected on a FACScan and analyzed using Flow-Jo software. Data represent the mean ± SE of the percentage of cells expressing the clonotypic TCR. Background staining of splenocytes or lymph node cells from naive BALB/c mice was usually <0.1%. Expression of activation markers (CD44 and CD45RB) on clonotype-positive cells resolated from the spleen and draining lymph nodes was determined by three-color flow cytometric analysis. Cells were stained with Cy-chrome-labeled anti-CD4 (BD PharMingen), biotinylated anti-TCR clonotype MAb 6.5 followed by phycocerythrin-labeled streptavidin, and either FITC-conjugated antimouse CD44 (BD PharMingen) or FITC-conjugated antimouse CD45RB (BD PharMingen). Live gating on CD4^+ T cells was set and 100,000 events collected/sample.

**Antigen-Specific Proliferation.** Purified T cells (4 × 10^5/well) from the different experimental groups were mixed with fresh splenocytes (8 × 10^4/well) from syngeneic mice to which either 12.5 μg/ml synthetic HA peptide (amino acids 110–120, SFRFEEFFKPE) or 3 μg of synthetic OVA-peptide (amino acids 323–329, ISQAVHAAHAEINEAGR) were or not added. Cells were pulsed with [3H]thymidine (1 μCi/well; Amersham, Arlington Heights, IL) after 3 days in culture. Cells were harvested 18 h later with a Packard Micromate cell harvester. Thymidine incorporation into DNA was measured as cpm on a Packard Matrix 96 direct β counter. Data are calculated as cpm in the peptide-pulsed group minus cpm from cells cultured in medium alone divided by the number of clonotype-positive cells in the well as determined by fluorescence-activated cell sorting (FACS). Values (cpm) for T cells cultured in media alone (no peptide group) are usually <10% relative to the peptide-pulsed group. Values are displayed as the mean ± SE cpm/100 clonotype^+ T cells/well.

**Cytokine Release.** T cells purified and plated as above were cultured with media alone or cognate peptide (either HA-peptide or OVA-peptide) plus fresh syngeneic splenocytes. Forty-eight h later, supernatants are collected and stored at −70°C until assayed for interleukin (IL)-2 or IFN-γ by ELISA (R&D Systems, Minneapolis, MN). Values for T cells cultured in media alone are
usually <10% of the values for HA-stimulated T cells. Data represent pg/ml of the specific cytokine/100 clonotype-positive T cells/well.

Response to Vaccinia Antigens. Normal BALB/c spleenocytes were infected with wild-type vaccinia virus (3 plaque-forming units/cell) for 4 h. Infected cells were washed three times and then cultured with purified T cells from the different experimental groups at a stimulator/responder ratio of 2:1. [3H]Thymidine incorporation was determined after 3 days in culture. Values represent mean ± SE of triplicate cultures. From a parallel plate, supernatants were collected after 48 h of T-cell stimulation with vaccinia antigens and assayed for IFN-γ by ELISA.

In Vivo Immunization with Recombinant Vaccinia Construct Encoding-HA. Vaccinia-HA was prepared as described previously (19). On the days indicated for each particular experiment, mice were immunized by s.c. inoculation with 1 × 10^7 plaque-forming units of recombinant vaccinia encoding HA suspended in 0.1 ml of HBSS.

PCR of Lymph Nodes. DNA was extracted from the lymph nodes of tumor-free and RencaHA-bearing mice by using the Qiagen Tissue kit (Qiagen, Chatsworth, CA). HA-specific PCR was performed using the primers F and R. Isolated DNA was mixed with these primers, Taq enzyme, 1% Triton, and water. A total of 25 μl of this solution was mixed with 25 μl of MjBo Easy Start solution. Cycling conditions were 95°C for 5 min, 95°C for 1 min, 62°C for 2 min, and 72°C for 2 min. Positive control DNA was extracted from RencaHA tumor cells. As negative control, we used lymph nodes from tumor-free animals or RencaWT-bearing mice. Sequence for the F primer is GGATCTCGAGGCTAAAGCTGCAAGCAGGGGAAAATAGCAACCC and for the R primer is CTGCAGAATTCCGCGATGCTATTTTCTCGACTGCA. The PCR product is ~1.8 Kb. The sensitivity of this technique was determined by serial dilutions of RencaHA tumor cells with either 5 × 10^5 RencaWT cells or normal spleenocytes, and it was able to detect one RencaHA tumor cell in 1 × 10^7 cells.

Construction of BM-Chimeric Mice. The femurs and tibiae from either BALB/c SCID mice or C57B6/SCID mice were obtained, and BM cells were harvested by flushing the bones with RPMI at 4°C. Single-cell suspensions were obtained by passing BM cells through a cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells were washed three times in sterile HBSS, and 4 × 10^6 BM cells from either BALB/c SCID mice (H-2^s) or C57BL/6 SCID mice (H-2^b) were injected into the tail vein of irradiated (1000 rads) BALB/c × C57BL/6 F1 (H-2^ab) recipients. Three months after BM transplant, one mouse from each group was sacrificed to assess donor chimerism by flow cytometry as described previously (20). Briefly, spleenocytes were stained for I-E^d and I-A^d using the MAb 14.4.4 and MAb Y3P, respectively, followed by FITC-goat-antimouse IgG2a secondary antibody (data not shown).

For the adoptive transfers of anti-HA-specific CD4+ T cells into the chimeric mice, we mixed the BALB/c TCR transgenes to C57B6/6 F1 (H-2^ab) recipients. Three months after BM transplant, one mouse from each group was sacrificed to assess donor chimerism by flow cytometry as described previously (20). Briefly, spleenocytes were stained for I-E^d and I-A^d using the MAb 14.4.4 and MAb Y3P, respectively, followed by FITC-goat-antimouse IgG2a secondary antibody (data not shown).

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To confirm the expression of RencaHA tumor cells in the lymphoid organs of tumor-bearing mice, we used HA-specific primers for PCR analysis of lymph nodes isolated from these animals. As shown in Fig. 2, no HA-signal was detected in the thoracic lymph nodes from RencaHA-bearing mice (Tum LN 1 and 2). Similarly, absence of RencaHA tumor cells in the spleen of these animals was confirmed by PCR analysis (data not shown). Lack of detection of RencaHA cells in lymphoid organs was not caused by a complete loss of HA expression by tumor cells because FACS analysis of RencaHA cells isolated from the lung nodules of these animals shows lower but still detectable levels of HA-expression (data not shown).

Extra-Lymphatic Growth of RencaHA Tumor. In previous studies, we have demonstrated that i.v. injection of RencaWT or RencaHA cells into BALB/c mice resulted in growth of multiple pulmonary malignant nodules, but no obvious metastasis were found in the lymph nodes or the spleen of tumor-bearing mice (19). To further confirm the absence of RencaHA tumor cells in the lymphoid organs of tumor-bearing mice, we used HA-specific primers for PCR analysis of lymph nodes isolated from these animals. As shown in Fig. 2, no HA-signal was detected in the thoracic lymph nodes from RencaHA-bearing mice (Tum LN 1 and 2). Similarly, absence of RencaHA cells in the spleen of these animals was confirmed by PCR analysis (data not shown). Lack of detection of RencaHA cells in lymphoid organs was not caused by a complete loss of HA expression by tumor cells because FACS analysis of RencaHA cells isolated from the lung nodules of these animals shows lower but still detectable levels of HA-expression (data not shown).

Extra-Lymphatic RencaHA Growth Is Not Immunologically Ignored by Tumor-Specific CD4+ T Cells Residing in Draining Lymph Nodes. The availability of tumor cells expressing a model antigen (HA) and an identifiable population of anti-HA/Eα-transgenic CD4+ T cell enabled us, therefore, to assess the fate and function of antigen-specific T cells during the extra-lymphatic growth
of a solid malignancy in vivo. On the basis of the adoptive transfer system reported by Kearny et al. (21), anti-HA/E4-transgenic CD4+ T cells were transferred into tumor-free BALB/c mice or mice with established RencaWT or RencaHA tumors. Eight days after T-cell transfer, mice were sacrificed, and the phenotypic and functional characteristic of clonotype-positive CD4+ T cells reisolated from the thoracic lymph nodes of these mice was determined. As seen in Fig. 3A, a significant expansion of clonotype-positive CD4+ T cells was found only in the draining lymph nodes of RencaHA-bearing mice relative to mice bearing RencaWT or tumor-free mice (4, 0.85, and 0.80%, respectively). After the first week, this percentage declined, and by 22 days after T-cell transfer, the percentage of clonotype-positive cells in the draining lymph nodes of RencaHA-bearing mice approached the baseline level present in the other groups (data not shown). Although the percentage of clonotype-positive T cells in mice bearing RencaHA declined after an initial expansion, their complete elimination was never observed, even at later time points in the face of an extensive tumor burden.

Three-color flow cytometric analysis of clonotype-positive CD4+ T cells was performed to address whether phenotypic changes associated with antigen recognition occur in the draining lymph nodes of RencaHA-bearing mice. An increase in the expression of CD44 was observed on HA-specific transgenic T cells in RencaHA-bearing mice (mean fluorescence: 300) relative to RencaWT (mean fluorescence: 119) and nontumor-bearing mice (mean fluorescence: 90; Fig. 3B). A FACS profile displaying this increased CD44 expression on antigen-specific CD4+ T cells reisolated from RencaHA-bearing mice, compared with antigen-specific CD4+ T cells from RencaWT, is shown in Fig. 3C. Clonotype-positive T cells isolated from RencaHA-bearing mice also display a decreased expression of CD45RB, indicative that these T cells have encountered HA-antigen in vivo and have lost their naïve phenotype (Fig. 3C). It should be noted that these phenotypic changes in antigen-specific T cells from RencaHA-bearing mice occurred in the absence of tumor metastasis to the lymphoid organs as determined by PCR evaluation.

Despite this initial expansion of HA-specific CD4+ T cells in RencaHA-bearing mice and loss of the naïve phenotype, T cells from this group are functionally impaired, as determined by their diminished antigen-specific proliferation (Fig. 3D) and IL-2 production (Fig. 3E), as compared with the functional responses of T cells from either tumor-free or RencaWT-bearing mice.

Taken together, the extra-lymphatic growth of RencaHA tumors is not immunologically ignored and results in a loss of the naïve phenotype and functional impairment of antigen-specific CD4+ T cells residing in the draining lymph nodes of tumor-bearing mice.

Induction of T-Cell Unresponsiveness in Vivo Is Not a Unique Characteristic of Renca Tumors. To assess whether the induction of T-cell unresponsiveness is a common feature of solid malignancies and not a unique characteristic of Renca tumors, we analyzed the fate and function of antigen-specific CD4+ T cells during the growth of DA3 mammary tumor and B16-melanoma tumors. DA3 is a well-characterized tumor cell line derived from the D1-7,12-dimethylbenz(a)anthracene-3 mammary tumor syngeneic to BALB/c mice (22, 23). DA3HA was generated by Lipofectamine transfection of wild-type mammary tumor cells. Similar to our findings in the RencaHA tumor model, the s.c. growth of DA3HA mammary tumor is not ignored by the immune system and also led to the induction of antigen-specific T-cell unresponsiveness. As seen in Fig. 4A, growth of DA3HA tumor cells resulted in increased percentage of antigen-specific CD4+ T cells in the spleen of tumor-bearing mice. Such an effect, indicative of tumor antigen recognition in vivo, occurred in the absence of DA3HA tumor metastasis to the spleen as determined by PCR analysis using HA-specific primers (data not shown). Despite this expansion however, antigen-specific T cells from DA3HA-bearing mice were found to be unresponsive as ascertained by their blunted proliferative response to HA-peptide in vitro (Fig. 4B).

The induction of antigen-specific CD4 T-cell unresponsiveness is not restricted to solid tumors expressing HA as a model tumor antigen. Indeed, CD4+ T cells expressing an αβ TCR specific for OVA-
C57BL/6 mice were given 2.5 × 10^6 anti-HA CD4^+ transgenic T cells i.v. Ten days later, mice were challenged with either 1 × 10^6 DA3-WT or DA3-HA tumor cells given s.c. or received no tumor. Twelve days later, mice were sacrificed, and T cells were isolated from their spleen as indicated in “Materials and Methods.” A, purified T cells were analyzed by two-color flow cytometry staining for CD4 versus anti-HA TCR clonotype as in Fig. 3. Values represent mean ± SE of the percentage of double positive T cells. Shown is a representative experiment of three independent experiments with similar results. B, HA-specific proliferation was determined as in Fig. 3. Data represent mean ± SE cpm/100 clonotype-positive T cells/well. C, C57BL/6 mice were given 2.5 × 10^6 anti-ovalbumin (OVA)-transgenic CD4^+ T cells i.v. Seven days later, mice were challenged s.c. with either 1 × 10^6 B16-WT or B16-OVA-expressing tumor cells or received no tumor. On day +7 after tumor challenge, all of the animals were sacrificed, and T cells were isolated from their spleen. Then, 5 × 10^4 purified T cells were cultured with fresh splenocytes from C57BL/6 mice in the presence or not of 3 μg/ml ovalbumin-peptide 323–339. Forty-eight h later, supernatants were collected and assayed for IL-2 by ELISA. Data represent mean ± SE of triplicate wells and is representative of two independent experiments.

Fig. 4. Changes in antigen-specific CD4^+ T cells isolated from the spleen of mammary tumor- or melanoma-bearing mice. BALB/c mice were given 2.5 × 10^6 anti-HA CD4^+ transgenic T cells i.v. Ten days later, mice were challenged with either 1 × 10^6 DA3-WT or DA3-HA tumor cells given s.c. or received no tumor. Twelve days later, mice were sacrificed, and T cells were isolated from their spleen as indicated in “Materials and Methods.” A, purified T cells were analyzed by two-color flow cytometry staining for CD4 versus anti-HA TCR clonotype as in Fig. 3. Values represent mean ± SE of the percentage of double positive T cells. Shown is a representative experiment of three independent experiments with similar results. B, HA-specific proliferation was determined as in Fig. 3. Data represent mean ± SE cpm/100 clonotype-positive T cells/well. C, C57BL/6 mice were given 2.5 × 10^6 anti-ovalbumin (OVA)-transgenic CD4^+ T cells i.v. Seven days later, mice were challenged s.c. with either 1 × 10^6 B16-WT or B16-OVA-expressing tumor cells or received no tumor. On day +7 after tumor challenge, all of the animals were sacrificed, and T cells were isolated from their spleen. Then, 5 × 10^4 purified T cells were cultured with fresh splenocytes from C57BL/6 mice in the presence or not of 3 μg/ml ovalbumin-peptide 323–339. Forty-eight h later, supernatants were collected and assayed for IL-2 by ELISA. Data represent mean ± SE of triplicate wells and is representative of two independent experiments.

Therefore, a common feature of solid tumor growth is the induction of CD4^+ T-cell unresponsiveness, which is antigen specific, occurs in the absence of tumor invasion to the lymphoid organs, and it is established early during the growth of a variety of solid malignancies.

Cross-Presentation of Tumor Antigens by BM-Derived APCs Is Responsible for the Induction of T-Cell Tolerance during Solid Tumor Growth. The above findings of induction of CD4^+ T-cell unresponsiveness in the absence of metastasis to the lymphoid organs suggest that cells other than tumor cells themselves may carry the tumor antigens from the extra-lymphatic site to the lymphoid organs and be responsible for tolerance induction. Given the increasing evidence supporting a central role for host BM-derived APCs in the induction of peripheral tolerance to self-antigens (24–27), we evaluated next whether these cells may also be responsible for tolerance to tumor antigens expressed by solid malignancies. To assess the relative contribution of BM-derived APCs, we compared T-cell recognition of a model tumor antigen (HA) in two sets of BM chimeras. In the first set (H2^d SCID → H2^dxb^d), the host’s APCs express the restricting element (I-E^d) required for presentation of the model antigen to TCR-transgenic T cells, whereas in the second set (H2^d SCID → H2^dxb^b), they do not. After immune reconstitution, anti-HA TCR-transgenic CD4^+ T cells from H2^dxb^b F1 donors were transferred to both sets of chimeras, which were then challenged—or not—with Renca-HA tumor. With this experimental design, if tolerance induction requires tumor-antigen presentation by BM-derived cells, T-cell unresponsiveness would only be seen in chimeras in which the APCs express the required restricting element (H2^d SCID → H2^dxb^b). As shown in Figs. 5 and 6, tumor antigen processing and presentation by host’s APCs is absolutely required for the development of antigen-specific CD4^+ T-cell tolerance. Indeed, a significant clonal expansion of HA-specific T cells was observed in the spleen of H2^d → H2^dxb^b tumor-bearing mice relative to their tumor-free counterparts (4.36 versus 1.15%, respec-
tively; Fig. 5). This clonal expansion was even more dramatic in the draining lymph nodes of H2<sup>a</sup> → H2<sup>ab</sup> tumor-bearing chimera as compared with tumor-free chimera (13.7% HA-specific T cells versus 0.14%, respectively; Fig. 5, bottom panel). In sharp contrast, no change in the percentage of clonotypic CD4<sup>+</sup> T cells was observed in the spleen of H2<sup>a</sup> → H2<sup>ab</sup> tumor-bearing chimeras (where host's APCs cannot present antigen) relative to tumor-free H2<sup>a</sup> → H2<sup>ab</sup> chimeras (1.09 versus 1.08% respectively; Fig. 5, top panel). Similarly, no change in the percentage of clonotypic T cells was observed in the thoracic lymph nodes of H2<sup>b</sup> → H2<sup>ab</sup> tumor-bearing chimeras (data not shown).

FACS analysis of activation/memory markers on clonotype<sup>+</sup> CD4<sup>+</sup> T cells revealed that T cells from the spleen of H2<sup>a</sup> → H2<sup>ab</sup> tumor-bearing chimeras had decreased expression of CD45R<sub>B</sub> as compared with T cells from tumor-free chimeras, indicative of having encountered HA-antigen in vivo. (Fig. 6A, left panel). In sharp contrast, T cells from H2<sup>a</sup> → H2<sup>ab</sup> tumor-bearing chimeras displayed similar expression of CD45R<sub>B</sub> as compared with T cells from tumor-free mice (Fig. 6A, right panel).

Despite the significant expansion of HA-specific CD4<sup>+</sup> T cells in H2<sup>a</sup> → H2<sup>ab</sup> tumor-bearing chimeras (Fig. 5), T cells from this group had a decreased proliferation (Fig. 6B, left panel) and are unable to produce IL-2 (Fig. 6C, left panel) in response to HA-peptide. Therefore, as previously observed in a syngeneic BALB/c system (12), antigen-specific CD4<sup>+</sup> T cells from H2<sup>a</sup> → H2<sup>ab</sup> tumor-bearing chimeras were rendered unresponsive during RencaHA progression. In sharp contrast, CD4<sup>+</sup> HA-specific T cells remained responsive in H2<sup>a</sup> → H2<sup>ab</sup> tumor-bearing chimeras. In fact, the persistence of their naïve phenotype (Fig. 6A, right panel) together with their normal proliferative response (Fig. 6B, right panel) and IL-2 production (Fig. 6C, right panel) after HA-stimulation in vitro is consistent with CD4<sup>+</sup> HA-specific T cells never having encountered antigen in vivo.

Therefore, because T-cell tolerance is only seen in the H2<sup>a</sup> → H2<sup>ab</sup> chimeras but not in the H2<sup>a</sup> → H2<sup>ab</sup> chimeras (which lack the appropriate APC population), the induction of this state of unresponsiveness requires APCs that uptake antigens shed from the extra-lymphatic solid tumor, followed by migration of these cells to the lymphoid organs for presentation of processed antigenic peptides to antigen-specific T cells.

**Induction of Tolerance to Tumor Antigens by BM-Derived APCs Limits the Efficacy of Therapeutic Vaccination.** Tumor antigen-specific CD4<sup>+</sup> T cells are central in orchestrating an effective and sustained antitumor response (13, 28–30). Our findings that BM-derived APCs can induce unresponsiveness of these critical T cells may have sobering implications for the continued development of effective cancer immunotherapy. Indeed, as shown in Fig. 7, antigen-specific CD4<sup>+</sup> T cells from RencaHA-bearing mice were found to be unresponsive to a potent immunogen such as a recombinant vaccinia-encoding influenza hemagglutinin (vacc-HA). Briefly, tumor-free animals or RencaHA-bearing mice were immunized at two different intervals after T-cell transfer (early vaccination: day +2; late vaccination: day +16). Six days after either immunization (days +8 and +22, respectively), animals were sacrificed, and the percentage and function of CD4<sup>+</sup> T cells were determined. Immunization of tumor-free mice resulted in a significant expansion of clonotype-positive CD4<sup>+</sup> T cells in the spleen of these animals (Fig. 7A, no tumor). Reminiscent of our findings in the draining lymph nodes of RencaHA-bearing mice (Fig. 3), the percentage of clonotype-positive T cells was also increased in the spleen of immunized tumor-bearing mice (Fig. 7A: day +8). Strikingly, immunization of RencaHA-bearing mice with vacc-HA failed to induce an additional expansion of HA-specific T cells (Fig. 7A: day +8, cross-hatched bar).

T cells from tumor-free mice primed with vacc-HA in vivo release IFN-γ upon in vitro culture with HA-peptide (Fig. 7B). This response was almost absent in mice bearing RencaHA, even when immunization with vacc-HA occurred as early as 2 days after T-cell transfer and assayed 6 days later (Fig. 7B: RencaHA, day +8). Although incubation with HA peptide resulted in measurable IL-2 release even in the absence of in vivo priming with vacc-HA, this response was also decreased in the RencaHA-bearing mice (Fig. 7C: day +8).

As expected, analysis of the response of antigen-specific T cells to
late vaccination (day +16 after T-cell transfer) reveals that although clonotype-positive T cells are still present in the spleen of tumor-bearing mice, they are now fully unresponsive to immunization (Fig. 7: day +22). Indeed, clonotype-positive T cells failed to expand in response to vaccinia-HA immunization (Fig. 7A) and display a blunted IFN-γ (Fig. 7B) and IL-2 production (Fig. 7C) upon restimulation with HA-peptide in vitro. This unresponsive state was associated with a lack of antitumor response to vaccination because RencaHA grew with similar kinetics in untreated as well as vaccinated tumor-bearing mice (data not shown).

This lack of response to therapeutic immunization was not the result of a global immunosuppression induced by solid tumor growth because at the time that clonotype-positive T-cells in RencaHA-bearing mice were already anergic, the other elements of the T-cell repertoire were still fully responsive to vaccinia antigens (Table 1). Indeed, although the T-cell proliferative response to HA peptide was again diminished in RencaHA-bearing mice relative to tumor-free mice (33,540 versus 64,797 cpm, respectively), these same mice had a proliferative response to vaccinia antigens that was equivalent to nontumor-bearing mice primed with vacc-HA (103,297 versus 106,101 cpm, respectively). Furthermore, at the time that clonotype-positive T cells from RencaHA were profoundly impaired in their ability to produce IFN-γ (170 versus 1603 pg/ml in tumor-free mice), the other elements of the T-cell repertoire were still responsive to immunization with vaccinia antigens, as determined by their production of IFN-γ in similar amounts to T cells from tumor-free mice (4757 ± 805 and 6348 ± 540 pg/ml, respectively).

**DISCUSSION**

These findings demonstrate that the extra-lymphatic growth of solid tumors is not ignored by the immune system. Instead, active induction of antigen-specific CD4+ T-cell tolerance develops in the lymphoid compartment of tumor-bearing hosts. The induction of this state of unresponsiveness requires BM-derived APCs that take up antigens from the extra-lymphatic solid tumor, followed by migration of these cells to the lymphoid organs for presentation of processed antigenic peptides to antigen-specific T cells (cross-tolerance).

Solid malignancies, being tumors derived from epithelial or mesenchymal cells, are mainly localized, at least early during their development, outside the secondary lymphoid organs. This extra-lymphatic localization of solid tumors, beyond the reach of the adaptive immune system, has been recently offered as a potential explanation for their successful growth in an otherwise immunocompetent host. In the absence of tumor invasion to lymphoid organs, it is argued that tumor-specific T cells remain largely ignorant to the extra-lymphatic presence of the tumor (8, 9, 31). In this study, however, we have demonstrated that in the absence of any detectable metastases to lymphoid organs, antigen-specific CD4+ T cells nonetheless lose their naïve phenotype and are functionally impaired in tumor-bearing mice. This lack of immune ignorance to tumor antigens is reminiscent of previous studies showing that extra-lymphatic growth of a panel of murine solid tumors is also associated with loss of the naïve phenotype and functional changes in antigen-specific CD8+ T cells residing...
in the draining lymph nodes of tumor-bearing mice (32–36). Notably, the functional outcome of these antigen-specific CD8⁺ T cells was, however, quite different to the outcome observed in our studies with antigen-specific CD4⁺ T cells. Although presentation of tumor antigens to antigen-specific CD8⁺ T cells resulted in productive responses (35, 37), similar presentation of tumor antigens to CD4⁺ T cells led instead to tolerance induction (Fig. 7). Therefore, early during solid tumor development and long before tumor invasion to the lymphoid organs occurs, antigen-specific T cells are either activated (CD8⁺ T cells) or rendered anergic (CD4⁺ T cells), but they definitively do not remain ignorant to the extra-lymphatic growth of solid malignancies.

In the immune response to tumors, multiple immune effector mechanisms are recruited to participate in tumor rejection. However, it is the T-cell arm of the response that achieves tumor specificity and mechanisms are recruited to participate in tumor rejection. In the successful invasion and establishment of tumor cells in secondary lymphoid organs, antigen-specific T cells may represent the initial step in a cascade of immunological events associated with tumor invasion to lymphoid organs. Indeed, Nelson et al. (38) have recently shown that tumor progression occurs despite efficient tumor antigen cross-presentation and activation of tumor antigen-specific CTLs residing in draining lymph nodes of tumor-bearing mice. Similarly, in a tumor model of spontaneously arising insulinosomas expressing a defined tumor-associated antigen (Rat Insulin Promoter-tag 2 model), the presence of nontolerized tumor-specific CD8⁺ T cells at a significantly high frequency was not sufficient to prevent tumor development and progression (36). Conversion from a nonproductive to effective antitumor CD8⁺ T-cell responses could be achieved, however, only when sufficient help was provided to antigen-specific CD8⁺ T cells by activated antigen-specific CD4⁺ T cells (13). Unfortunately, such help would be either absent or provided in insufficient amounts by antigen-specific CD4⁺ T cells that are being or have been already rendered unresponsive at early stages of solid tumor development (Figs. 3 and 4). It is plausible, therefore, that this absence of functional tumor-antigen-specific CD4⁺ T cells may represent the initial step in a cascade of immunological events associated with tumor invasion to lymphoid organs. In the absence of “help,” other elements of the antitumor immune responses may not be recruited and/or be fully activated, allowing for the successful invasion and establishment of tumor cells in secondary lymphoid organs.

That CD4⁺ T cells become rapidly nonresponsive to antigens expressed by solid tumors is an important observation, but it is equally important to determine how these T cells encounter antigen in the first place. As previously mentioned, one hypothesis proposes that T cells first encounter tumor antigens when malignant cells metastasize to the lymphoid organs. Given the inability of most solid tumors to provide the full complement of costimulatory signaling (signal two), such a direct tumor–T-cell encounter has been postulated to result in the induction of T-cell tolerance rather than T-cell activation (14, 15). However, as unambiguously shown in this study, both the encounter of tumor antigens and the induction of antigen-specific CD4⁺ T-cell tolerance occurs in the absence of obvious tumor metastasis to the lymphoid organs, pointing to cells other than tumor cells themselves as being responsible for tumor antigen presentation and tolerance induction in tumor-bearing mice. Our results using parent-into-F1 BM chimeras provide indeed an alternative explanation for the induction of antigen-specific T-cell tolerance during the growth of solid malignancies. Reminiscent of previous studies highlighting the critical role of BM-derived APCs in the induction of tolerance to self-antigens (24–27), we have demonstrated that tolerance to antigens expressed by solid malignancies requires processing and presentation of tumor antigens by BM-derived APCs. The recent demonstration that BM-derived APCs are also critical in the induction of tolerance to tumor antigens expressed by B-cell lymphomas (20) indicates that the intrinsic APC capacity of tumor cells has little influence over T-cell priming versus tolerance, a critical decision that is regulated at the level of BM-derived APCs.

It is now well established that BM-derived APCs play an important role in initiating productive T-cell responses against malignancies (34, 39, 40). Our demonstration, however, that these same cells are also required for the induction of antigen-specific T-cell tolerance to tumor antigens places APCs at the crossroads of immune activation versus immune tolerance. A potential explanation for this dual function of APCs is that perhaps a specialized subset of APCs could preferentially induce tolerance (26, 41, 42) while a different subpopulation may be responsible for T-cell priming. Alternatively, it is plausible that the differentiation and/or activation state of the APC population at the time of antigen presentation may represent the central determinant of T-cell priming versus tolerance (27, 43–45). APCs encountering the antigen in the context of inflammation or tissue destructive process, such as those that occur during viral or bacterial infection, most likely will be fully activated and therefore capable of triggering productive antigen-specific T-cell responses. Conversely, in the steady state (absence of inflammation), a host’s APCs may capture antigens in the periphery and then migrate to the lymphoid organs for presentation of the antigen to antigen-specific T cells in a tolerogenic fashion. Although this APC-T-cell encounter resulted in increased number of antigen-specific T cells, (Figs. 3A and 5), these cells, on a per cell basis, are significantly impaired in their ability to produce IL-2 (Figs. 3E and 6C). That such “partial activation” state is ultimately followed by the development of T-cell unresponsiveness (Fig. 7) suggests that in the absence of inflammatory signals capable of fully activate APCs and/or able of sustain an ongoing T-cell response, the normal default of a T-cell encounter with tumor antigen presented by APC is tolerance induction rather than T-cell activation.

From a therapeutic perspective, the induction of antigen-specific CD4⁺ T-cell tolerance mediated by BM-derived APCs (cross-tolerance) imposes a significant barrier to the current generation of immune-enhancing strategies against solid malignancies. Indeed, therapeautic immunization of RencaHA-bearing mice with a potent immunogen-encoding HA (vaccinia-HA) not only failed to break the unresponsive state of antigen-specific CD4⁺ T cells (Fig. 7) but also did not result in any rejection and/or delay of tumor growth (data not shown). Despite these sobering findings, however, the induction of tolerance to tumor antigens mediated by APCs seems not to be an insurmountable obstacle, as recently demonstrated by strategies manipulating specific signaling pathways in these cells (19, 46–48). Integration of these novel approaches into the current generation of immune-enhancing therapies may ultimately lead to strategies to more effectively harness the immune system against solid malignancies.

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Extra-Lymphatic Solid Tumor Growth Is Not Immunologically Ignored and Results in Early Induction of Antigen-Specific T-Cell Anergy: Dominant Role of Cross-Tolerance to Tumor Antigens

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