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Surgical Removal of Primary Tumor Reverses Tumor-Induced Immunosuppression Despite the Presence of Metastatic Disease

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ABSTRACT

Immunotherapy is a promising approach for the management of malignancies. It may be particularly useful for tumors that do not respond to conventional therapies, such as many metastatic cancers. The efficacy of immunotherapy will depend on many factors, one of which is the immunocompetence of the host. Patients with large primary tumors frequently are immunosuppressed, making them poor candidates for immunotherapy. Although a few studies have reported that surgical removal of primary tumor reverses immunosuppression, it is not known whether metastatic disease in postsurgery patients inhibits this recovery. To determine the role of metastatic disease, we examined tumor-free mice *versus* mice with primary tumor and metastatic disease *versus* mice whose primary tumors were removed surgically but who had metastatic disease. We have used the mouse 4T1 mammary carcinoma, a BALB/c-derived transplantable tumor that shares many characteristics with human breast cancer and is an established model for spontaneous, metastatic cancer. Cell-mediated and humoral adaptive immunity, as measured by rejection of allogeneic tumor, antigen-specific T-cell proliferation, and antigen-specific antibody responses, was suppressed in 4T1-bearing nonsurgery mice relative to tumor-free mice. Surgical removal of primary tumor resulted in rebounding of antibody and cell-mediated responses, even in mice with metastatic disease. Macrophage activity, as measured by lipopolysaccharide responsiveness, and dendritic cell function, as measured by nominal and alloantigen presentation, were not suppressed in tumor-bearing mice. Therefore, the presence of primary tumor suppresses T-cell and antibody responses; however, surgical removal of primary tumor restores immunocompetence even when disseminated metastatic disease is present.

INTRODUCTION

For >20 years immunologists have noted that tumor-bearing patients often are immunosuppressed and unable to respond to their tumors (1). For some patients, the suppression is limited to responses to their resident tumor cells (2), whereas others are unable to respond to a variety of tumors (3), and still others are suppressed globally and unable to respond to nominal antigens (4). In many of the earlier studies, cells were identified as the "suppressor" elements (5–7). In more recent studies, a range of additional mechanisms has been identified that decrease tumor immunity in tumor-bearing persons. These mechanisms include (*a*) immune tolerance of the host to tumor antigens (8); (*b*) genetic changes in tumor cells that render the tumor cells "immune" to the host's immune system (9–11); (*c*) "ignorance" or lack of activation to tumor antigens (12); (*d*) dysfunction of potentially tumor-reactive lymphocytes rendering them unresponsive to antigen (13); and (*e*) immune suppression mediated by tumor cell secretion of inhibitor factors and/or activation of systemically immunosuppressive cells (14, 15). Many of these mechanisms have been documented in a variety of animal models of cancer and in cancer patients, and T-cell (13), B-cell (16), and antigen-presenting cell (14, 17, 18) deficits have been reported.

Immunotherapy has been proposed as a novel therapy for cancers that do not respond to conventional therapies. However, if cancer patients are immunosuppressed, then immunotherapy may be less effective. Studies with experimental animals have led to the conclusion that cancer immunotherapy efficacy is inversely proportional to tumor burden. This conclusion is supported by the paucity of studies in the literature demonstrating effective immunotherapy against large, established tumors (1). The relationship between tumor burden and immunosuppression raises the important question of whether tumor-induced immunosuppression is reversible by surgical removal of the primary tumor. Only a few studies have assessed immunosuppression after primary tumor removal in either mice (19, 20) or humans (21, 22). Although these authors observed different levels of immunosuppression, most report at least partial recovery of immune function following tumor resection.

Although these immunosuppression studies are important to understand the role of primary tumor in inducing immune suppression, they do not address the important question of immune suppression in postsurgery patients with metastatic disease. Surgical removal of primary, solid tumors can be curative. However, if metastatic disease is present at surgery and if the metastases do not respond to conventional therapies, then the cancer can be lethal. Therefore, metastatic cancer is a major target for immunotherapy, and immunotherapy is likely to be used in a postsurgery setting. Because immunotherapy will be most effective for patients who are maximally immunocompetent, it is important to determine whether patients whose primary tumors have been removed, but who have established metastatic disease, are immunosuppressed.

To address this question, we used the 4T1 mouse mammary carcinoma. This poorly immunogenic, BALB/c-derived transplantable tumor shares many characteristics with human breast tumors and is an established model for metastatic cancer (23–25). Using this model, we compared immune responses in mice without tumor ("tumor-free") *versus* mice with intact primary tumors ("nonsurgery") *versus* mice whose primary tumors have been removed but who have established, spontaneous metastatic disease ("postsurgery"). Our studies demonstrate that although tumor-bearing animals have reduced B- and T-cell responses, the immunosuppression is reversed following primary tumor removal even when metastatic disease is present. Therefore, immunotherapy may be useful for postsurgery patients with metastatic disease and for whom conventional therapies are not effective.

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MATERIALS AND METHODS

Mice

Female BALB/c, C57BL/6, and C3H/HeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and/or bred at the University of Maryland Baltimore County animal facility. All of the mice used were between 6 weeks and 6 months of age. Mice were housed and bred according to the NIH guidelines for the humane treatment of laboratory animals, and the University

of Maryland Baltimore County Institutional Animal Care and Use Committee approved all of the procedures.

Tumor Cell Lines, 4T1 Inoculations, and Metastasis Assays

4T1 and B16 melF10 cells were grown *in vitro* as described previously (23, 26). BALB/c mice were challenged s.c. in the abdominal mammary gland with 7×10^3 4T1 tumor cells/50 μl serum-free Iscove's modified Dulbecco's medium or RPMI. Primary tumor growth and spontaneous lung metastases were measured as described previously (23). Briefly, mean primary tumor diameter (TD) was calculated as the square root of the product of two perpendicular diameters. Lung metastases were quantified using the clonogenic assay by plating dissociated lung cells in medium containing 6-thioguanine and counting foci of 6-thioguanine-resistant 4T1 tumor cells (23, 24).

Surgery

Mice were anesthetized, and tumors were resected as described previously (24, 27). Wounds were closed with Nexaband liquid (Henry Schein, Melville, NY). Mice underwent autopsy at the time of death to confirm the presence of lung metastases and to check for recurrence of the primary tumor.

Immunizations and Bleeds for Antibody Studies

Mice were injected i.p. with 200 μg hen egg white lysozyme (HEL; Sigma-Aldrich, St. Louis, MO) in 100 μl RIBI adjuvant (monophosphoryl lipid A, synthetic trehalose dicorynomycolate, and mycobacteria cell wall skeleton; Sigma-Aldrich) prepared according to the manufacturer's instructions. Briefly, RIBI adjuvant was prepared by warming the vial to 45°C, injecting 1 ml PBS into the vial, and vortexing vigorously for 3 min. Equal volumes of RIBI adjuvant and 4 mg/ml HEL in PBS were mixed by vortexing. Following immunization, mice were bled from the tail vein or heart at selected intervals. For studies of primary antibody responses, results of two experiments were pooled. In the first primary response experiment, 4T1 was inoculated on day -21; primary tumors were surgically removed on day 0; and mice were immunized with HEL on day 2 and bled on days 2 (prebleed) and 17–18 (final bleed). In the second experiment, 4T1 was inoculated on day -26; primary tumors were removed on day 0; and mice were immunized with HEL on day 3 and bled on days 3 (prebleed) and 16 (final bleed). Lung metastases were quantified on day 16.

ELISA for Anti-HEL Antibody

Anti-HEL antibody (total immunoglobulin) in serum was quantified by ELISA. Flat-bottomed 96-well plates (Nalge Nunc International, Rochester, NY) were coated overnight with 5 μg/ml HEL in PBS or 5 μg/ml BSA (Sigma-Aldrich) in PBS. Excess protein was removed by washing with PBS, 0.2% Tween, and 0.05 M Tris using an ELISA plate washer (Tecan, Research Triangle Park, NC) set for six passes of 300 μl/well/cycle. All of the subsequent washes used the same solution and same number of wash cycles. The wells were blocked with 0.02% horse hemoglobin containing 0.01% thimerosal in PBS for 1 h and then washed. Diluted serum samples were added, and after an overnight incubation, the plates were washed. One hundred μl of affinity-purified biotinylated antimouse IgG (whole molecule H and L chains; Cappel/ICN, Irvine, CA; in PBS, 0.02% hemoglobin, and 0.01% thimerosal) were added to each well, and after a 1-h incubation, excess antibody was removed by washing. One hundred μl of a 0.156 μg/ml solution of streptavidin-horseradish peroxidase (Zymed, San Francisco, CA; in PBS, 0.02% hemoglobin, and 0.01% thimerosal) then were added to each well and incubated for 30 min, followed by washing. Tetramethylbenzidine substrate then was added (100 μl/well for 5–15 min; Dako, Carpinteria, CA), and by adding 100 μl 0.18 M H_2SO_4 per well, the enzymatic reaction was stopped. Plates were read at 450 nm using a Microplate 311 Autoreader (Bio-Tek Instruments, Winooski, VT). To assay levels of anti-HEL IgG or IgM in serum, the aforementioned procedure was followed, substituting affinity-purified biotinylated monoclonal antibody to mouse IgG (γ chain; 0.6 μg/ml; Zymed) or mouse IgM (μ chain; Cappel/ICN), respectively. The positive control HyHEL7 antibody (28) was prepared as described previously (29). Positive control purified mouse IgM was obtained from Zymed.

Absorbance values were converted to μg/ml of anti-HEL antibody using a standard curve. Final values for anti-HEL antibody concentrations were calculated as follows:

$$\textit{Anti-HEL Ab} = (\textit{dilution factor}) \times \{[(\mu g/ml\ \textit{HEL Ab on Day } x) - (\mu g/ml\ \textit{BSA control for Day } x)] - [(\mu g/ml\ \textit{HEL Ab on Day } 0) - (\mu g/ml\ \textit{BSA control for Day } 0)]\}$$

B16 Inoculations

B16 melF10 cells (1×10^6 or 5×10^5/100 μl serum-free Iscove's modified Dulbecco's medium or RPMI) were inoculated s.c. in the flank of BALB/c, C57BL/6, or 4T1 tumor-bearing BALB/c mice. Tumor growth was assessed as described previously (23). Mice were followed for B16 growth until 4T1 tumor-bearing mice died. Because BALB/c mice die ~42 days after 4T1 inoculation (23), mice that had 4-week-old 4T1 tumors at the time of B16 inoculation died relatively soon after B16 was administered compared with mice with 3-week-old 4T1 tumors. As a result, B16 TDs for mice in the 4-week group were smaller than for mice in the 3-week group.

T-Cell Proliferation Assay and Immunizations

Mice were immunized with HEL in PBS (1:1 v/v emulsion with complete Freund's adjuvant; Sigma) s.c. at the base of the tail (25 μg/50 μl) and in each hind footpad (12.5 μg/25 μl per footpad). Nine days later, spleens were removed; splenocytes were depleted for RBC as described previously, washed twice with serum-free Iscove's modified Dulbecco's medium, and cultured in flat-bottomed 96-well plates at 5×10^5 cells/210 μl/well in serum-free HL1 medium (Bio-Whittaker, Walkersville, MD) containing 1% penicillin, 1% streptomycin, 1% Glutamax (Life Technologies, Rockville, MD) 5×10^{-5} M β-mercaptoethanol, and 10 μg HEL/well (30). Each splenocyte sample also was cocultured with concanavalin A (0.2 μg/ml; Sigma) to ensure cell viability. Each well was pulsed with 1 μCi [^{3}H]thymidine (ICN Biochemicals, Costa Mesa, CA) on day 4, and cells were harvested 16 h later onto glass fiber filter mats using a Packard Micromate 196 cell harvester (Packard, Downers Grove, IL). Filter mats were sealed into plastic bags with 4 ml of Betaplate scintillation fluid (Perkin-Elmer, Gaitherburg, MD), and radioactivity was assessed using a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer). The HEL-specific T-cell proliferative response is reported as:

$$\Delta CPM = (\textit{CPM for HEL-stimulated splenocytes}) - (\textit{CPM for splenocytes without HEL}).$$

Values are the average of five replicate wells per point.

Lipopolysaccharide Injections

Mice were injected i.p. with 50 or 100 μg of lipopolysaccharide (LPS; Sigma)/200 μl PBS and weighed daily. Percent weight change was calculated as follows:

$$\%\ \textit{weight change} = 100\% \times [(\textit{weight on day } x \textit{ after injection})/(\textit{weight before injection})].$$

Dendritic Cell (DC) Assays

DC Isolation. Splenic DCs were isolated by a modification of the procedure described previously (31). Briefly, spleens were injected with 0.5 ml of a 1-mg/ml solution of collagenase D (Boehringer Mannheim, Indianapolis, IN) in HEPES Hanks' solution, chopped into small pieces, incubated at 37°C for 45–60 min, and filtered through a 70-μM cell strainer (Falcon, BD, Franklin Lakes, NJ). Resulting cells were depleted for RBC, washed twice with DC wash buffer (PBS containing 0.5% BSA and 2 mM EDTA), and resuspended to 400 μl in DC wash buffer. One hundred μl of CD11c magnetic microbeads (Miltenyi Biotech, Auburn, CA) were added per up to 10^8 splenocytes; the mixture was incubated on ice for 15 min and washed once with DC wash buffer; and the resulting cells were resuspended to 3 ml. Beaded cells were applied to LS columns (Miltenyi Biotech), eluted in 5 ml of DC wash buffer, reapplied to a second LS column, and eluted with 2.5 ml of wash buffer.

Resulting cells were >60% CD11c positive as measured by flow cytometry using a CD11c-FITC monoclonal antibody (PharMingen, San Diego, CA). DC enrichment produced ~2.9–5.2 $\times$ 10^6 cells per spleen.

DC Presentation of Ovalbumin. To isolate DO11.10 T cells, up to 4 $\times$ 10^8 splenocytes from D011.10 transgenic mice were depleted of RBC, resuspended in wash buffer (HEPES Hanks' solution and 2% calf serum), and adhered to plastic to remove macrophages and DCs (1 spleen, 10 ml wash buffer, and T75 flask; 37°C for 90 min; Ref. 32). Cells then were washed once, resuspended in 2 ml of a 1:20 dilution of B220 culture supernatant, incubated at 4°C for 1 h, washed once, resuspended in 2–4 ml of Lowtox M rabbit complement (Accurate, Westbury, NY), and incubated at 37°C for 30 min. Resulting DO11.10 T cells were >46% $CD4^+DO11.10^+$, <4% $CD8^+DO11.10^+$, and <4% $B220^+$ as measured by flow cytometry. Purified D011.10 T cells were washed once and resuspended in assay medium (RPMI, 10% FCS, 1% penicillin/streptomycin, 1% gentamicin sulfate, 1% Glutamax, and 5 $\times$ 10^{-5} M β-mercaptoethanol). Antigen presentation assays were performed in 96-well flat-bottomed plates with 5 $\times$ 10^4 DO11.10 T cells, the indicated number of DCs, and 10 μg ovalbumin (Sigma) or 1 μg ova peptide 323–339 (synthesized in the University of Maryland Biopolymer facility) per 200 μl assay medium per well. Cells were incubated at 37°C for 48–72 h, and the supernatants were harvested and assayed by ELISA for IFN-γ using matched pairs of antibodies according to the manufacturer's directions (Pierce-Endogen, Rockford, IL). Values are the average of triplicates.

Allogeneic DC Assay. Splenocytes from C3H/J mice were harvested, depleted of RBC, mixed with 1500 rad irradiated (Gammator B; Kewaunee Scientific, Statesville, NC) BALB/c DCs (2 $\times$ 10^5 responders plus 1.4 $\times$ 10^5 DCs in 200 μl assay medium/well in 96-well round-bottomed plates), and incubated at 37°C for 5 days. Cultures were pulsed with 1 μCi/well [^{3}H]thymidine and harvested and counted as per the T-cell activation assays. Values are the average of triplicates.

Statistical Analyses

Student's *t* test for unequal variances was performed using Microsoft Excel 2000 (Redmond, WA). Statistical analyses were performed for all of the experiments for which there were sufficient data points.

RESULTS

Antigen-Specific Antibody Responses Are Suppressed in Tumor-Bearing Mice but Return to Normal Following Primary Tumor Removal Despite the Presence of Metastatic Disease. Antibody production in response to immunization is a fundamental element of adaptive immunity. Thus, we examined B-cell activity in tumor-bearing mice by assaying antibody production in response to the foreign antigen HEL. To compare primary antibody responses in mice without tumors ("tumor-free"), mice with intact primary tumors ("nonsurgery"), and mice whose primary tumors have been surgically removed but who retain established, spontaneous metastatic disease ("postsurgery"), BALB/c mice were inoculated with 4T1 tumor cells (nonsurgery and postsurgery groups only), and primary tumors were removed surgically from mice in the postsurgery group after 21 or 26 days (day 0 is the day of surgery; see the timeline in Fig. 1*A*). Mice in the nonsurgery and postsurgery groups were matched for primary TD on the day of surgery (nonsurgery TD, 5.59 $\pm$ 1.93 mm; postsurgery TD, 5.52 $\pm$ 1.82 mm). On day 2 or 3 after surgery, all of the mice were bled (prebleed) and then immunized with HEL in RIBI adjuvant. On days 16–18, mice were bled again, and serum samples were assayed using ELISA for primary antibody responses to HEL.

To assay total HEL-specific immunoglobulin by ELISA, a biotinylated antibody to whole-molecule mouse IgG was used. Because this antibody reacts with light chains and heavy chains, it detects all of the isotypes of HEL-specific antibody. To control for the binding of non-HEL-specific serum immunoglobulin to ELISA plates, each sample was tested in wells coated with HEL and in wells coated with BSA. After converting absorbance values to g/ml of antibody, antibody levels from BSA wells were subtracted from the HEL antibody value for each sample, and HEL antibody levels from day 0 prebleeds were subtracted from postimmunization values. Because the immunization time course varied slightly between experiments, HEL-specific antibody values from each experiment were normalized by dividing the antibody concentration for each mouse by the antibody concentration of the highest responder in the experiment.

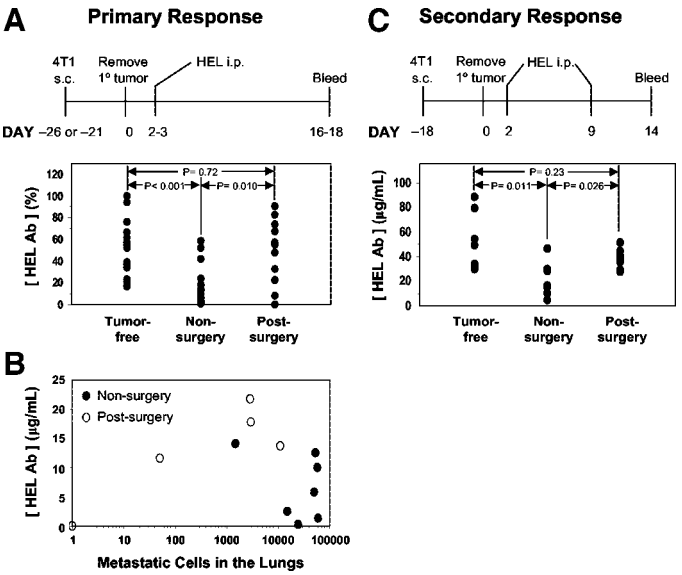

Fig. 1. Antibody production is reduced in 4T1 tumor-bearing mice but recovers following primary tumor removal despite the presence of metastatic disease. BALB/c mice without tumors ("tumor-free"), with a primary tumor in place ("nonsurgery"), or with their primary tumors surgically removed ("postsurgery") were immunized with hen egg white lysozyme (*HEL*) and bled as indicated in the timelines. Sera were tested using ELISA for total HEL-specific antibodies. *A*, primary antibody responses; pooled results of two independent experiments. HEL-specific antibody concentrations were normalized as described in the text. *B*, lungs of some of the mice from *A* were harvested, and metastases were quantified using the clonogenic assay. *C*, secondary antibody responses. Each dot represents antibody levels or number of metastatic cells in an individual mouse.

As shown in Fig. 1*A*, following HEL immunization, serum levels of total HEL-specific antibody were significantly lower in nonsurgery mice than in tumor-free mice. However, postsurgery mice had HEL-specific antibody levels that were significantly higher than nonsurgery mice and comparable with tumor-free mice. Interestingly, serum levels of HEL-specific IgM were not significantly different between tumor-free, nonsurgery, and postsurgery mice (data not shown). Therefore, production of total antibody, but not of IgM, is suppressed in mice with 4T1 tumors but returns to normal following primary tumor removal.

We have established previously that mice inoculated with 4T1 develop disseminated metastases within 10–21 days of 4T1 inoculation (23). To confirm that mice in the present experiment had metastatic disease, lung metastases were quantified using the clonogenic assay. As shown in Fig. 1*B*, mice in the nonsurgery and postsurgery groups developed metastatic disease. However, there appears to be no relationship between the number of lung metastases and the ability to mount a primary antibody response. Thus, the observed recovery of primary antibody responses in postsurgery mice occurred despite the presence of extensive, established metastatic disease.

To determine whether secondary B-cell responses also recover following primary tumor removal, we compared secondary antibody responses in tumor-free, nonsurgery, and postsurgery mice. BALB/c mice were inoculated with 4T1 tumor cells (nonsurgery and postsurgery groups only), and primary tumors were removed surgically from mice in the postsurgery group after 18 days (day 0 is the day of surgery; see the timeline in Fig. 1*C*). Mice in the nonsurgery and

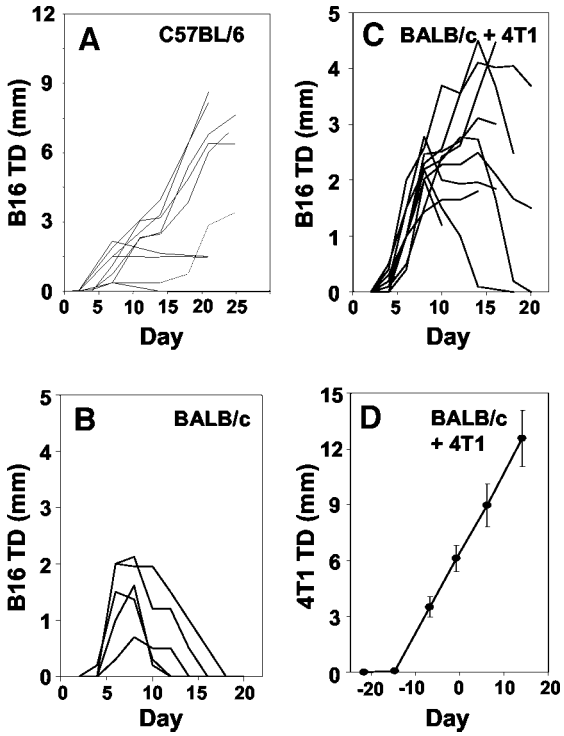

Fig. 2. 4T1 tumor-bearing mice do not efficiently reject allogeneic tumor. B16 tumor growth in C57BL/6 (*A*) or BALB/c (*B*) mice inoculated s.c. in the flank on day 0 with 1×10^6 B16 tumor cells (*H-2*^{b}). *C*, B16 tumor growth in BALB/c mice inoculated s.c. in the mammary gland on day −22 with 7×10^3 4T1 cells and additionally inoculated on day 0 with 1×10^6 B16 tumor cells. Each line represents B16 tumor growth in an individual mouse. Termination of a line indicates mouse death. *D*, average growth of 4T1 tumor in the 10 mice shown in *C*. Data are from one of two independent experiments, except for *A*, which is pooled from two separate experiments.

postsurgery groups were matched for primary tumor diameter on the day of surgery (nonsurgery TD, 3.82 ± 0.68 mm; postsurgery TD, 4.05 ± 1.13 mm). All of the mice were bled on day 2 after surgery and immunized with HEL in RIBI adjuvant on days 2 and 9. On day 14, mice were bled, and serum samples were assayed using ELISA for secondary antibody responses to HEL.

As shown in Fig. 1*C*, in response to two immunizations with HEL, serum levels of HEL-specific antibody were lower in nonsurgery, tumor-bearing mice than in tumor-free mice. However, HEL-specific antibody levels in postsurgery mice were significantly higher than in nonsurgery mice and comparable with tumor-free mice. Therefore, like primary responses, secondary antibody responses are reduced in mice with 4T1 tumors but recover following primary tumor removal despite the presence of established metastatic disease.

Rejection of Allogeneic Tumor Is Impaired in Mice with 4T1 Tumors. Many experimental cancer immunotherapeutic strategies focus on the activation of T lymphocytes. Because rejection of allogeneic tumor is mediated by T cells, we used the growth of the C57BL/6-derived (*H-2*^{b}) B16 melF10 melanoma to measure T-cell activity in tumor-free *versus* nonsurgery BALB/c (*H-2*^{d}) mice (33). The numbers of B16 cells inoculated were based on previous studies in which B16 melF10 tumors grew progressively in syngeneic C57BL/6 mice.[1] Tumor-free BALB/c or BALB/c mice that had been inoculated 22 days earlier with 4T1 cells were inoculated with 1×10^6 B16 cells (contralateral side for 4T1-bearing mice). C57BL/6 mice also were inoculated with 1×10^6 B16 cells to monitor tumor progression in the syngeneic host. Growth of B16 tumors was tracked for 20 days or until mice became moribund or died. As expected, B16 tumors grew progressively in most C57BL/6 mice (Fig. 2*A*) but were rejected by BALB/c mice within 20 days of B16 inoculation (Fig. 2*B*). In contrast, B16 tumors were not rejected by 60% of 4T1 tumor-bearing BALB/c mice within the same period (Fig. 2*C*). 4T1 tumors in these mice (Fig. 2*D*) grew at a rate comparable with growth in mice without B16 tumors (data not shown). Therefore, allogeneic tumor rejection is impaired in mice carrying 4T1 tumors.

In a second experiment, C57BL/6, tumor-free BALB/c, or BALB/c mice that had been inoculated 3 weeks earlier with 4T1 cells (4T1 TD, 5.21 ± 1.61 mm) were inoculated with 5×10^5 B16 cells (contralateral side for 4T1-bearing mice). Growth of B16 tumors was tracked for 26 days or until mice became moribund or died. As expected, palpable B16 tumors were present in 100% of C57BL/6 mice (Fig. 3*A*). When compared with BALB/c mice without 4T1, mice with 3-week established 4T1 tumors were more likely to develop palpable B16 tumors (Fig. 3*A*), and the tumors that developed grew to a significantly larger maximum TD before regression or mouse moribundity (Fig. 3*B*). In a similar experiment, B16 growth was tracked in C57BL/6, tumor-free BALB/c, and BALB/c mice that were inoculated with 5×10^5 B16 cells 4 weeks after 4T1 inoculation (4T1 TD, 8.41 ± 1.85 mm). Tumor growth was tracked until 16 days post-B16 inoculation or until mice became moribund or died. In this experiment, palpable B16 tumors developed in 100% of C57BL/6 mice. B16 tumor incidence and maximum TD were higher in mice with 4-week established 4T1 tumors than in mice without 4T1. Therefore, allogeneic B16 tumors grew larger and were more likely to develop in 4T1 tumor-bearing BALB/c mice than in mice without 4T1 tumors. These results collectively demonstrate that mice with bulky, primary tumor are less able to immunologically reject allogeneic tumor, suggesting that their cellular immunity is compromised.

T-Cell Responses to HEL Are Impaired in Mice with 4T1 Tumors but Recover Following Surgery Despite the Presence of Disseminated Metastatic Disease. To assess antigen-specific T-cell-mediated immunity, tumor-free, nonsurgery, and 9-day postsurgery mice with metastatic disease were immunized with HEL. Mice in the nonsurgery and postsurgery groups were matched for primary TD on the day of surgery (4.93 ± 0.83 mm and 4.71 ± 1.2 mm, respectively). Splenocytes were harvested 9 days after immunization, restimulated in culture with HEL, and T-cell proliferation was measured

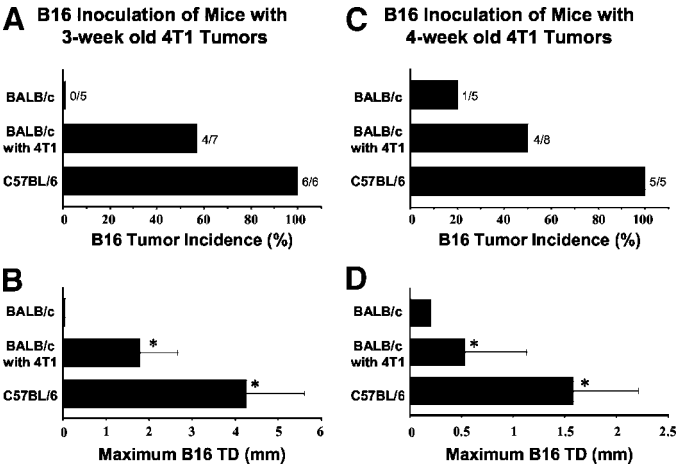

Fig. 3. Incidence and growth of allogeneic tumor are greater in 4T1 tumor-bearing mice than in tumor-free mice. BALB/c, C57BL/6, or 4T1 tumor-bearing nonsurgery BALB/c mice were inoculated s.c. with 5×10^5 B16 cells and followed for incidence of B16 tumor growth (*A* and *C*) and maximum B16 tumor diameter (*B* and *D*). BALB/c mice were inoculated s.c. with 7×10^3 4T1 cells 3 weeks (*A* and *B*) or 4 weeks (*C* and *D*) before B16 inoculation. Fractions indicate number of mice that developed palpable B16 tumors/number of mice inoculated with B16. *, A significant difference between groups ($P < 0.05$). Data are from one of three independent experiments.

[1] Unpublished observations.

by [^{3}H]thymidine incorporation. As shown in Fig. 4*A*, HEL-specific proliferative responses were reduced significantly in nonsurgery mice compared with tumor-free mice. However, surgical removal of primary tumor returned HEL-proliferative responses to levels comparable with that of tumor-free mice.

To ascertain that the HEL-immunized mice had metastatic disease, the lungs of the nonsurgery and postsurgery groups were removed at the time of splenocyte removal and assayed using the clonogenic assay for metastatic tumor cells. As shown in Fig. 4*B*, both groups have metastatic cells in their lungs. Therefore, although the presence of bulky primary tumor significantly inhibits antigen-specific T-cell responses, surgical resection of primary tumor reverses this inhibition even when metastatic disease is present.

Macrophage Activity Is Unimpaired in Mice with 4T1 Tumors. As professional antigen-presenting cells, macrophages play an important role in adaptive immune responses. The endotoxin LPS induces toxic shock and cachexia in mice via a macrophage-dependent mechanism, leading to severe weight loss (34). Thus, we have examined responses of mice to LPS as a measure of macrophage function, with the degree of weight loss corresponding to macrophage activity. To compare responsiveness to LPS in tumor-free mice and nonsurgery mice, BALB/c mice were inoculated with 4T1 cells (tumor-bearing group only) and 2 or 4 weeks later inoculated with LPS. 4T1 TDs for mice with 2-week and 4-week established 4T1 tumors were 2.68 ± 1.47 mm and 7.87 ± 1.81 mm, respectively. Weight change was tracked for 3 days or until mice became moribund or died. Tumor-free (Fig. 5*A*) and nonsurgery (Fig. 5*B*) mice experienced significant weight loss within 1 day of LPS inoculation. Percent weight change did not vary significantly between tumor-free and nonsurgery mice.

To determine whether 4T1 tumor burden affected LPS-induced weight loss, tumor-free and nonsurgery mice with either 2-week or 4-week established 4T1 tumors were inoculated with PBS (Fig. 5*C*) or LPS (Fig. 5*D*) and followed for weight changes. TD does not impact percent weight change. Therefore, macrophage activity is not altered in mice carrying 4T1 tumors, suggesting that macrophage function is not suppressed by the presence of bulky, primary tumor.

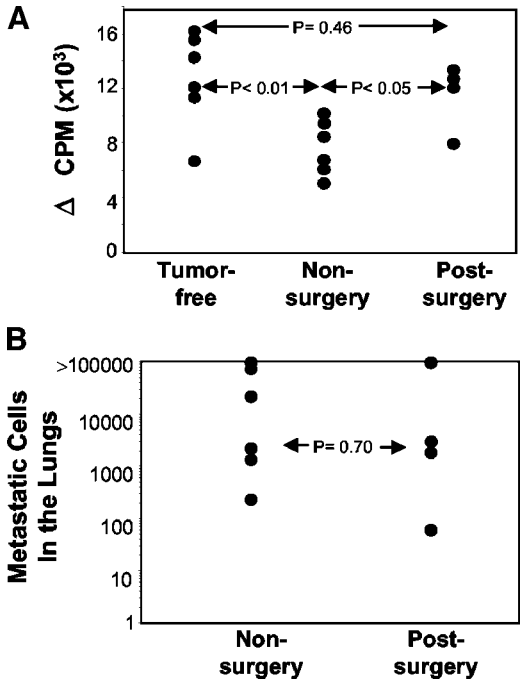

Fig. 4. Hen egg white lysozyme (HEL)-specific T-cell responses are reduced in 4T1 tumor-bearing mice but recover following primary tumor removal despite the presence of metastatic disease. *A*, tumor-free, nonsurgery, and postsurgery BALB/c mice were immunized with HEL. Nine days later, their spleens were removed and boosted *in vitro* with HEL, and T-cell proliferation was measured by incorporation of [^{3}H]thymidine. *B*, lungs of some of the mice from *A* were harvested, and metastases were quantified using the clonogenic assay. Each symbol represents the number of metastatic tumor cells in the lungs of an individual mouse. Data are pooled from three independent experiments.

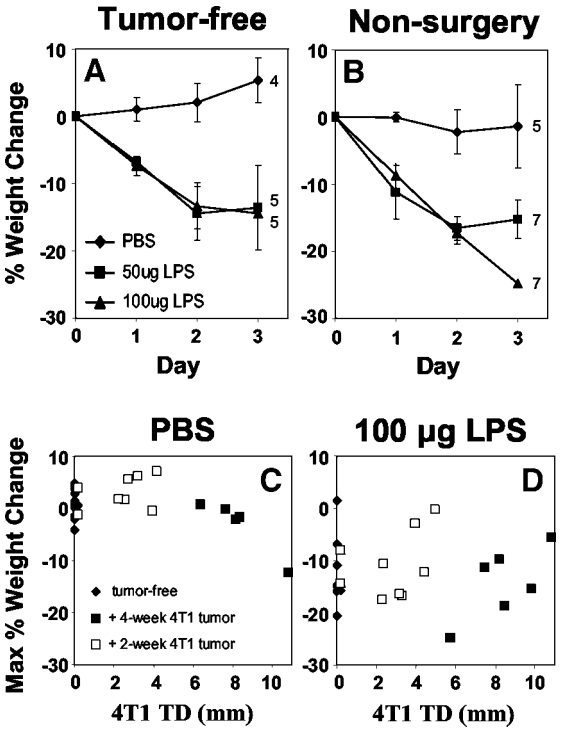

Fig. 5. 4T1 tumor burden does not affect macrophage activity. *A* and *B*, tumor-free BALB/c mice (*A*) and BALB/c mice inoculated s.c. with 4T1 on day −28 (nonsurgery; *B*) were inoculated with lipopolysaccharide (*LPS*) or with PBS on day 0. Weight loss was tracked daily or until mice died. At day 1, there were statistically significant differences between PBS-injected and LPS-inoculated tumor-free mice (50 μg LPS, $P = 0.001$; 100 μg LPS, $P < 0.001$) and between PBS-injected and LPS-inoculated tumor-bearing mice (50 μg LPS, $P = 0.012$; 100 μg LPS, $P < 0.001$). The number of mice in each group is indicated at the end of each line. Nine nonsurgery mice and five tumor-free mice died within 3 days of LPS inoculation. Data are from one of three independent experiments, in which mice with 2-week or 4-week 4T1 tumors were used. *C* and *D*, tumor-free BALB/c mice and BALB/c mice with 2-week or 4-week established 4T1 tumors were inoculated with PBS (*C*) or LPS (*D*) on day 0 and weighed daily for 3 days or until they became moribund or died. 4T1 tumor diameter (*TD*) was measured on the day of LPS inoculation. Each symbol represents the weight of an individual mouse. Five tumor-free mice, five mice with 4-week 4T1 tumors, and six mice with 2-week 4T1 tumors died within 3 days of LPS inoculation. Data are pooled from three independent experiments.

DC Activity Is Not Suppressed in Tumor-Bearing or Postsurgery Mice. Impaired DC activity has been reported in patients with bulky, primary tumors (31, 35). To determine whether DC activity is decreased in 4T1 tumor-bearing mice and/or is affected by surgery, splenic DCs were purified from tumor-free, nonsurgery, and 10–15-day postsurgery mice with metastatic disease using Miltenyi magnetic bead sorting for CD11c$^+$ cells. Mice in the nonsurgery and postsurgery groups were matched for primary TD on the day of surgery (7.7 ± 1.6 mm and 7.6 ± 1.6 mm, respectively). Recovery of splenic CD11c$^+$ cells from the three groups ranged from 3–5.2 × 10^6 per spleen, and there were no significant differences in yield between the treatment groups. Purified cells were double stained for CD11c plus CD40, CD80, or I-A^d to ascertain phenotype. More than 60% of the recovered cells were CD11c$^+$, and the CD11c$^+$ cells from the three groups did not differ in cell surface expression of MHC class II, CD40, or CD80 as measured by immunofluorescence and flow cytometry (data not shown). Functional activity of the CD11c$^+$ cells from the three treatment groups was measured by (*a*) activation of allogeneic (C3H/HeJ) T cells, (*b*) presentation of exogenous ovalbu-

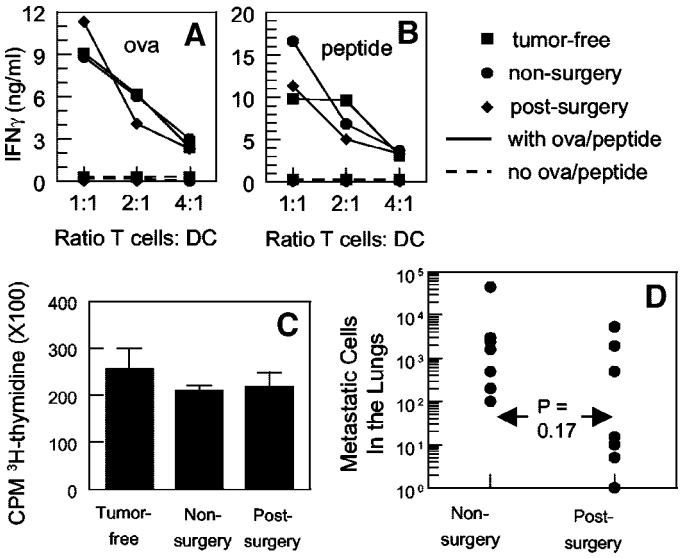

Fig. 6. Dendritic cell (*DC*) activity is not suppressed in tumor-bearing mice. Splenic DCs were purified from tumor-free, nonsurgery, and 10-day postsurgery BALB/c mice. DCs were pulsed with ovalbumin protein (*A*) or ovalbumin peptide 323–339 (*B*) and cocultured at varying ratios with I-A^d-restricted $ova_{323–339}$-specific DO11.10 T cells, and supernatants were assayed for IFN-γ. *C*, irradiated DCs were cocultured with allogeneic C3H splenocytes, and T-cell proliferation was measured by [^{3}H]thymidine uptake. The cpm for DC and C3H splenocytes cultured separately were <8% of the allo response. Each graph in *A* through *C* represents splenocytes from one or two mice per treatment group and is representative of three to five independent experiments. *D*, lungs of mice from the same inoculation cohort of *A* and *B* were harvested, and metastases were quantified using the clonogenic assay. Each symbol represents the number of metastatic tumor cells in the lungs of an individual mouse. Data are pooled from two independent experiments.

min to I-A^d-restricted, $ovalbumin_{323–339}$-specific $CD4^+$ DO11.10 transgenic T cells (32), and (*c*) presentation of ovalbumin peptide 323–339 to DO11.10 T cells. $CD11c^+$ cells from tumor-free, nonsurgery, and postsurgery mice are approximately equivalent in their ability to present ovalbumin protein (Fig. 6*A*) and ovalbumin peptide (Fig. 6*B*). Similarly, $CD11c^+$ cells from all of the three treatment groups are equal in their ability to activate allogeneic T cells (Fig. 6*C*). To confirm that mice in the nonsurgery and postsurgery groups have metastatic disease, lungs were harvested and assayed by the clonogenic assay for metastatic tumor cells (Fig. 6*D*). These results collectively demonstrate that splenic DCs from 4T1 tumor-bearing mice and from postsurgery mice are not impaired in their ability to process and present antigen and to activate T cells.

DISCUSSION

Immunotherapy offers a promising approach for the management of metastatic cancers; however, the development of effective strategies is complicated by the ability of tumors to evade host immunity. Although there are a few studies in which tumor-induced immunosuppression has not been noted (36), most investigators have reported reduced immune functions in tumor-bearing individuals (1), establishing tumor-induced immunosuppression as a fundamental mechanism allowing tumors to escape immune destruction. Because immunotherapy becomes less effective as tumor mass increases, it is thought that immunosuppression intensifies with increasing tumor burden (1). Despite the apparent critical role of tumor-induced immune suppression, few studies have evaluated immunocompetence following the reduction of tumor burden via primary tumor resection (19–22). There is a particular shortage of information regarding the clinically relevant question of whether tumor-induced immunosuppression can be reversed by primary tumor resection even when metastatic disease is present. Because many metastatic cancers are not responsive to conventional therapies, postsurgery patients with established metastatic disease may benefit from novel treatments such as immunotherapy. Therefore, a better understanding of the immunocompetence of patients whose primary tumors have been removed surgically, but who have metastatic disease, is essential to evaluate whether immunotherapy will be a useful treatment strategy.

The studies reported here indicate deficits in cell-mediated and humoral immune responses in mice with bulky primary tumors, relative to tumor-free mice. $CD8^+$ and $CD4^+$ T-cell responses are suppressed based on inadequate rejection of allogeneic tumor, inability to switch from antigen-specific IgM to IgG isotypes following immunization, and reduced antigen-specific T-cell activation following immunization. Whether the decreased antigen-specific IgG responses of tumor-bearing mice demonstrate direct suppression of B lymphocytes or are the result of T-cell dysfunction is unclear. Because immunized tumor-bearing mice make normal levels of antigen-specific IgM but produce significantly reduced levels of total antibody, the tumor-induced deficit in antibody production may reflect a problem with $CD4^+$ T-helper cell-mediated immunoglobulin class switching rather than an inherent B-cell defect. Regardless of the mechanisms responsible for the reduced immunocompetence, our studies agree with previous reports documenting T-cell (13) and B-cell deficiencies (16, 37) in tumor-bearing patients. Surprisingly, we do not find deficiencies in macrophage or DC activity, although previous reports have documented such defects (31, 35, 38).

Tumor cells are known to synthesize and secrete several immunosuppressive factors. For example, transforming growth factor β inhibits $CD8^+$ effector T cells and Th1 $CD4^+$ T cells, thereby suppressing T-cell-mediated antitumor immunity (39). Vascular endothelial growth factor also is an effective immunosuppressive agent. It blocks normal myeloid cell differentiation and causes a buildup of immature myeloid cells, known as myeloid suppressor cells, that inhibit the activity of $CD4^+$ and $CD8^+$ T cells (14, 17, 21, 40). Similar to many tumors, the 4T1 mammary carcinoma used in this study produces both of these cytokines.[1] Other immunosuppressive factors secreted by tumor cells include interleukin 10, which when present in high levels is hypothesized to skew the immune response toward a type 2 response, thereby minimizing an effective Th1 response (41). The activity of these cytokines is thought to be roughly proportional to their *in vivo* level, and this level correlates directly with tumor burden because the cytokines are synthesized and secreted by the tumor cells. Therefore, it is likely that surgery reverses immune suppression because it reduces the quantity of immunosuppressive factors, thereby allowing the immune response to recover in the absence of the inhibitory cytokines. If this is the case, then immunosuppression may recur as metastatic lesions grow and inhibitory cytokine levels increase, becoming more severe as metastatic tumor burden increases. However, after surgery there clearly is a "window" during which relatively large quantities of metastatic cells are present, but immune suppression is not active. This window of immunocompetence may be the result of less efficient cytokine production by metastatic tumor cells *versus* primary tumor cells, or alternatively, there may be qualitative differences in cytokine production by primary tumor cells *versus* metastatic tumor cells. Such differences could result from distinct cytokine secretion profiles for primary tumor *versus* metastatic tumor cells or from a requirement for a large focus of tumor cells in a common location to induce immunosuppression. Either of these mechanisms would result in the recurrence of immune suppression as metastatic tumor burden increases.

The reversal of tumor-induced immunosuppression in patients with metastatic disease following primary tumor resection has important implications for cancer immunotherapy. Because patients will be most

responsive to immunotherapy when they are maximally immunocompetent, it is imperative that tumor-induced immune suppression is considered when planning immunotherapy regimens. Our studies indicate that although patients with bulky primary tumors are profoundly immunosuppressed, primary tumor removal reverses immune suppression even in the presence of extensive metastatic disease. Thus, for maximal efficacy, immunotherapy should be administered only after tumor burden is reduced, either by surgery or by other conventional therapies. Because conventional treatments such as radiation therapy, chemotherapy, and surgery also can reduce host immunocompetence, the ultimate timing of an immunotherapy regimen must consider all of these conditions (42).

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