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# Altered Immune Function during Long-Term Host-Tumor Interactions Can Be Modulated to Retard Autochthonous Neoplastic Growth<sup>1</sup>

Trina J. Stewart and Scott I. Abrams<sup>2</sup>

Ag-specific and generalized forms of immunosuppression have been documented in animal tumor models. However, much of our knowledge on tumor-induced immunosuppression was acquired using tumor implant models, which do not reiterate the protracted nature of host-tumor interactions. Therefore, a transgenic mouse model of autochthonous mammary tumor development and progression was chosen to investigate the long-term consequences of neoplastic growth on the immune system. *In vitro* proliferation of unfractionated splenocytes from tumor-bearing mice, as assessed by [<sup>3</sup>H]thymidine uptake, was inhibited by the presence of suppressor cells within these splenocyte preparations, because purifying the T cells restored their biological activity. However, the level of inhibition did not correlate with either tumor load or the percentage of myeloid-derived CD11b<sup>+</sup>Gr1<sup>+</sup> cells. To evaluate tumor-specific immune dysfunction, transgenic mice were challenged with autologous tumor cells. Mice with extensive, but not minimal autochthonous tumor burdens demonstrated a significantly enhanced rate of autologous tumor growth compared with age-matched controls. In contrast, an allogeneic tumor challenge was efficiently rejected from both groups of transgenic mice. It was also noted that allogeneic tumor challenge of mice with minimal disease significantly inhibited autochthonous primary tumor growth. We therefore demonstrated that 1) a generalized form of immunosuppression occurred, but not as a result of permanent alterations to T cell function, because purified T cell subsets retained normal biological activity following polyclonal or allostimulation; and 2) tumor-specific immunosuppression emerged as a consequence of tumor progression, but could be modulated to enhance antitumor responses against autochthonous primary neoplastic growth. *The Journal of Immunology*, 2007, 179: 2851–2859.

The phenomenon of immune suppression may explain, at least in part, a central paradox in tumor immunology whereby tumors develop and progress even in the presence of tumor-specific immune effector cells. Immune dysfunction is also an important consideration in cancer immunotherapy, which by its very nature requires the patient to be immunocompetent. Despite the discovery of numerous tumor-associated-Ags (1, 2) and the development of a multitude of cancer vaccines (3, 4), there is still no consistently successful immunotherapy for cancer patients (3, 5). This may in part be due to the fact that current immunotherapeutic strategies attempt to modulate an immune system that is deficient or tolerized and unable to overcome the many tumor-induced immunoregulatory barriers (6). A tumor-specific immune response is thought to be important in attaining antitumor activity. However, a generalized systemic immune suppression may also affect the Ag-specific immune cells, which comprise just a small subset within the broader immune response. Furthermore, systemic dysfunction is also likely to impact innate immunity, which can play an important role in regulating the adaptive T cell response induced by many immunotherapies (7, 8).

Immune suppression or dysfunction has long been held to occur in cancer patients and there is mounting evidence for the presence of immunosuppressive mechanisms, particularly with respect to tumor-specific responses (9, 10). However, data that support the presence of a systemic or more generalized immune dysfunction that could inhibit the efficacy of immunotherapeutic strategies requires further investigation (11, 12). Changes in host-tumor interactions as tumors grow will probably result in adjustments of the mechanisms of immune evasion and immunosuppression that occurs during the time course of tumor progression (6). An understanding of the nature, timing, and mechanisms of immune dysfunction will likely lead to a more informed design and application of immunotherapeutic strategies that take into account these inhibitory elements to maximize clinical outcomes.

Mouse models have become an important preclinical tool in evaluating the fundamental principles that guide the analysis and design of cancer therapeutics for use in the clinic. However, studies on the mechanisms of immune dysfunction have largely been conducted in tumor implant models, which do not necessarily recapitulate the more protracted or chronic nature of immune cell-tumor cell interactions that occur in human disease development and progression. Emerging and established tumors continue to implement mechanisms of escape from the host immune system, with the balance of host-tumor interactions changing over time and with disease progression (13, 14). In tumor transplant models, tumor growth is usually rapid with experimental durations lasting only a few weeks following tumor implantation (15–17). This type of “assault” on the host and in particular the immune system may create aphysiologic consequences unrelated to the natural process of tumor development, growth, and progression (18, 19). Therefore, data supporting the existence of systemic and/or Ag-specific T cell dysfunction in such models needs verification in patients or

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in more relevant preclinical models. This has led us to investigate these concepts further in a transgenic mouse model of autochthonous mammary carcinoma, termed MTAG, which should more accurately represent human disease progression.

The MTAG (MMTV-PyMT/B6) mouse was originally developed by using the MMTV-LTR promoter to specifically target polyoma virus middle T Ag expression to mammary gland tissue (20). This strategy resulted in the generation of transgenic mice that developed autochthonous mammary carcinomas, with eventual metastatic spread to the lungs that occurs over an approximate 6-mo life span. It has previously been demonstrated in this transgenic model that changes in tumors during disease progression mimic the alterations seen in human breast tumors at both morphological (disease stage) and molecular levels (21). These studies have validated the MTAG mouse as an important preclinical model to investigate the consequences of cancer development and progression on the host immune response. Although other studies have reported on the use of various transgenic mouse models of cancer (22, 23), there has been limited detailed examination or characterization of their endogenous immunologic properties and whether systemic or tumor-specific immune dysfunction was an important consequence of neoplastic growth and progression (24).

## Materials and Methods

### Mice

Female C57BL/6, BALB/c, and athymic (*nu/nu*) mice were obtained from the National Cancer Institute-Frederick facility (Frederick, MD). The MTAG (MMTV-PyMT/B6)-transgenic mouse expresses the polyoma virus middle T Ag controlled through the MMTV-LTR promoter (20). These transgenic mice were originally derived in FVB mice (20) and were backcrossed on a C57BL/6 (H-2<sup>b</sup>) background (25) and provided by S. Gendler (Mayo Clinic, Scottsdale, AZ). Polyoma virus middle T oncogene expression results in the generation of multifocal mammary carcinomas and progression to pulmonary metastases. Only female MTAG mice were used in experiments and were obtained by breeding transgenic male MTAG mice with wild-type C57BL/6 female mice. Progeny were monitored for transgene (Tg)<sup>3</sup> expression by PCR, with 100% of Tg<sup>+</sup> mice developing mammary carcinoma. Mice designated as "aged" were routinely >160 days of age and harbored extensive tumor burden. Young mice were used between the ages of 42 and 80 days and did not possess palpable tumors. Tg<sup>-</sup> littermates were used as age/gender-matched controls. Development of pulmonary metastases occurred in >95% of mice that were >160 days of age. Mice were housed in a specific pathogen-free environment, and experiments were conducted in accordance with institutional guidelines for animal care and use. In accordance with these regulations, no single tumor mass was allowed to exceed 2 cm<sup>3</sup>, although mice may develop up to 10 discrete tumors.

### Establishment of an autologous MTAG-derived tumor cell line

A tumor cell line, termed AT-3, was established from the cells of the primary mammary gland carcinoma of a MTAG mouse. A single-cell preparation was made by disruption of a resected primary tumor through a 100- $\mu$ m cell strainer (BD Biosciences). Cells were washed and resuspended in HBSS at  $5 \times 10^7$  cells/ml. Female athymic mice were s.c. injected on the flank with  $5 \times 10^6$  cells of the primary tumor cell preparation. A tumor mass was allowed to establish in these athymic mice to an approximate volume of 1 cm<sup>3</sup> before being resected and disrupted through a 100- $\mu$ m cell strainer to form a single-cell suspension. These cells were then placed in a tissue culture flask and grown on DMEM-based medium (see components below). The flask was incubated horizontally for 2 days before nonadherent cells were removed and fresh medium was added. Adherent cells were allowed to grow until confluency, followed by culture at 1:3 splits for 2 wk. The AT-3 tumor cell line was then routinely maintained in tissue culture by splitting twice weekly.

### Cell line culture conditions

RMA, EL4, P815, and MC38 tumor cell lines were maintained on complete RPMI 1640. AT-3 tumor cells were maintained in T-75 flasks and grown

in complete DMEM. Lymphocyte cultures were grown and assayed in complete RPMI 1640 medium. Complete RPMI 1640 (Invitrogen Life Technologies) or DMEM (Invitrogen Life Technologies) contained 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 15 mM HEPES, 100 U/ml penicillin/100  $\mu$ g/ml streptomycin solution, 50  $\mu$ M 2-ME, and 10% heat-inactivated FBS (Gemini).

### Preparation of lymph node cells (LNCs) and adoptive transfer

Cell preparations used for adoptive transfer experiments were obtained by culturing LNCs from young female MTAG mice or from age/gender-matched Tg<sup>-</sup> control mice. A pool of draining lymph nodes (LNs) were taken from each donor including cervical, axillary, brachial, inguinal, mesenteric, periaortic, and popliteal. The LNs were disrupted into a single-cell suspension through a 70- $\mu$ m cell strainer. Briefly,  $2 \times 10^7$  cells were cultured in 40 ml of complete DMEM with 200  $\mu$ l/flask of mouse CD3/CD28 expander beads (DynaL Biotec) and recombinant mouse IL-15 (PeproTech) at 10 ng/ml for 5 days in upright T-75 flasks. Recombinant human IL-2 was added at a final concentration of 10 U/ml for another 24 h before cells were centrifuged over Lymphocyte Separation Medium (MP Biomedicals). The cells recovered from the interface were washed and resuspended in HBSS before adoptive transfer. Cells were transferred i.v. into tumor-bearing mice at  $10 \times 10^6$  cells/mouse in 100  $\mu$ l of HBSS.

### Cell enrichment

Lymphocyte populations were purified from single-cell preparations of mouse splenocytes. Negatively selected T cells were isolated using either CD4 (no.130-090-860) or CD8 (no.130-090-859) magnetic bead isolation kits from Miltenyi Biotec as per the manufacturer's protocols. Positively selected cells were purified using the following Miltenyi Biotec immunoseparation magnetic beads: CD4<sup>+</sup> T cells (no.130-049-201), CD8<sup>+</sup> T cells (no.130-049-401), and CD45R<sup>+</sup> cells (no.130-049-501) as described in the manufacturer's protocols. Selections were performed using an AutoMACS separation system (Miltenyi Biotec). The purity of the selected cells was determined by flow cytometry following each separation and was routinely found to be >95%.

### Flow cytometry

Cells were incubated with directly conjugated mAb in 100  $\mu$ l of buffer (PBS plus 2% FBS) for 30–60 min and washed three times. At least 10,000 gated events were collected on a FACSCalibur flow cytometer (BD Biosciences) and analyzed by CellQuest software (BD Biosciences). The following mAbs were used for cell labeling: PE-conjugated anti-CD4, anti-CD8, anti-NK1.1, anti-Gr1 (Ly6C<sup>+</sup>Ly6G); PE-Cy5-conjugated anti-CD3; FITC-conjugated anti-TCR, anti-CD45R, anti-CD4, anti-CD8 (all the above mAbs were obtained from BD Biosciences Pharmingen); and anti-CD11b (Southern Biotechnology Associates). All samples were run with appropriate isotype controls.

### Proliferation assay

Bulk splenocytes ( $2 \times 10^5$  cells/well) or purified lymphocyte populations ( $1 \times 10^5$  cells/well) were plated, in triplicate, in round-bottom 96-well plates in the presence of  $5 \times 10^5$  irradiated autologous splenocytes as accessory cells and either Con A (Sigma C-0412) or LPS (Sigma L-6143) at various concentrations. After 2 days, 1  $\mu$ Ci of [<sup>3</sup>H]thymidine was added to each well and the cells were incubated for an additional 24 h. Cells were harvested using an automated plate harvester (Tomtec) and the extent of proliferation was determined by measuring [<sup>3</sup>H]thymidine incorporation by liquid scintillation spectroscopy.

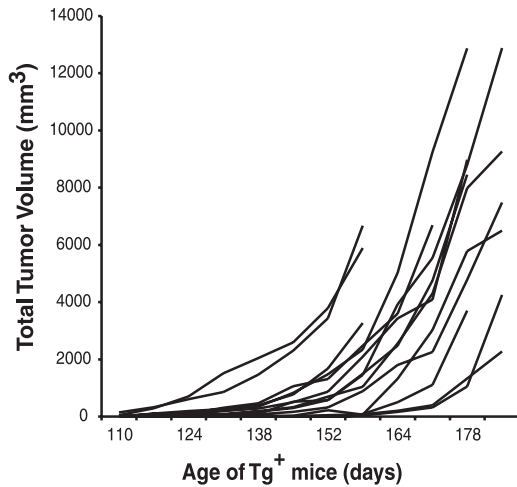
### Cytokine analysis

Purified lymphocyte preparations were cultured for 48 h in 24-well plates at  $5 \times 10^5$  cells/ml per well and stimulated with anti-CD3/CD28 (at 1  $\mu$ g/ml each Ab). Supernatants were collected and centrifuged to remove any debris before freezing at  $-20^\circ\text{C}$  until assayed. Samples were analyzed for cytokine concentration using a BD Cytometric Bead Array kit (BD Biosciences). The Th1/Th2 kit detects IL-2, IL-4, IL-5, IFN- $\gamma$ , and TNF- $\alpha$ . Assays were performed as outlined by the manufacturer's protocols with dual laser acquisition. The fluorescent intensity was measured by flow cytometry on a FACSCalibur (BD Biosciences) before calculation of analyte concentrations using manufacturer software.

### Allogeneic MLC and cytotoxicity assays

Cytotoxic T cell activity following allogeneic Ag stimulation was measured using a standard 4-h <sup>51</sup>Cr release assay. Bulk splenocytes from Tg<sup>+</sup> and Tg<sup>-</sup> mice (H-2<sup>b</sup>) were coincubated with irradiated allogeneic stimulator BALB/c (H-2<sup>d</sup>) splenocytes at a 1:1 ratio. Cultures were set up in 30

<sup>3</sup> Abbreviations used in this paper: Tg, transgene; DC, dendritic cell; LNC, lymph node cell; MDSC, myeloid-derived suppressor cell; LN, lymph node.



**FIGURE 1.** Autochthonous mammary tumor growth over time. The growth of autochthonous tumor in individual MTAG mice was measured over time ( $n = 15$ ). Individual primary mammary tumors were measured weekly as they developed and progressed. Mice were euthanized when at least one of the tumors reached the 2-cm<sup>3</sup> ethical limit. Total tumor volume was calculated from the sum of all discrete mammary tumor volumes in a single mouse (up to 10 individual tumors per mouse).

ml of complete RPMI 1640 (at  $2.5 \times 10^6$  responder cells/ml) in upright T-75 flasks and incubated for 6 days at 37°C with 5% CO<sub>2</sub>. Before assaying, effector cells were recovered over a density gradient. These effector cell preparations were found to be composed of ~80% CD8<sup>+</sup> and 20% CD4<sup>+</sup> cells. Appropriate target cells were labeled with Na<sup>51</sup>CrO<sub>4</sub> (0.25 mCi) for 1 h at 37°C in OPTI-MEM I medium (Invitrogen Life Technologies). Target cells were washed three times to remove excess <sup>51</sup>Cr before plating in triplicate with effector cells at the indicated E:T ratios in 96-well round-bottom plates. After 4 h, the supernatants were harvested using the Supernatant Collection System (Skatron), and the level of isotope released was quantitated. The maximum release of isotope was determined by treating target cells with 5% SDS and the spontaneous release was measured from wells containing labeled target cells incubated with medium alone. The percentage of specific lysis was calculated using the following formula: percent specific lysis = [(experimental release - spontaneous release)/(maximal release - spontaneous release)] × 100.

#### Statistical analysis

Tumor volumes were calculated using the formula:  $W^2 \times L/2$ , where  $W$  = width and  $L$  = length of tumor mass, measured in millimeters. Total or overall tumor volume equals the sum of individual tumor volumes, which in the case of MTAG mice could be up to 10 individual tumors. To compare mouse mammary tumor growth over time between experimental and control groups, the Wilcoxon rank sum test was used to compare sizes between prespecified groups, at each time point. For comparisons where results suggested potentially consistent differences, a global evaluation of the differences over time was done using the method of O'Brien (26). All other statistical analyses were based on Student's  $t$  test. All  $p$  values were two tailed and a value <0.05 was considered to be significant.

## Results

### Variability in the rate and overall amount of autochthonous neoplastic growth

One aspect of cancer in humans is the variability in the timing and progression of disease (27, 28). To investigate this aspect of disease in the MTAG mammary carcinoma model, tumor development and progression was monitored (Fig. 1). Total tumor volume was calculated as the sum of each distinct tumor volume within an individual mouse. Discrete tumors were seen to develop at any and up to all 10 mammary glands in individual mice. The number of tumors and the age at which they arose varied considerably between individual mice, with each discrete tumor growing at a rate that appeared independent of other tumor growth rates within the

same mouse. These mice could also develop different tumor types between mice and within the same mouse. Tumors were primarily composed of solid, glandular, and acinar forms, with many also having cystic areas. The progression of disease in this model was, therefore, unpredictable and mirrored what occurs in cancer patients, which supports the utility of this MTAG transgenic mouse as a preclinical model of long-term disease progression.

### Systemic immunosuppression is variable among individual mice

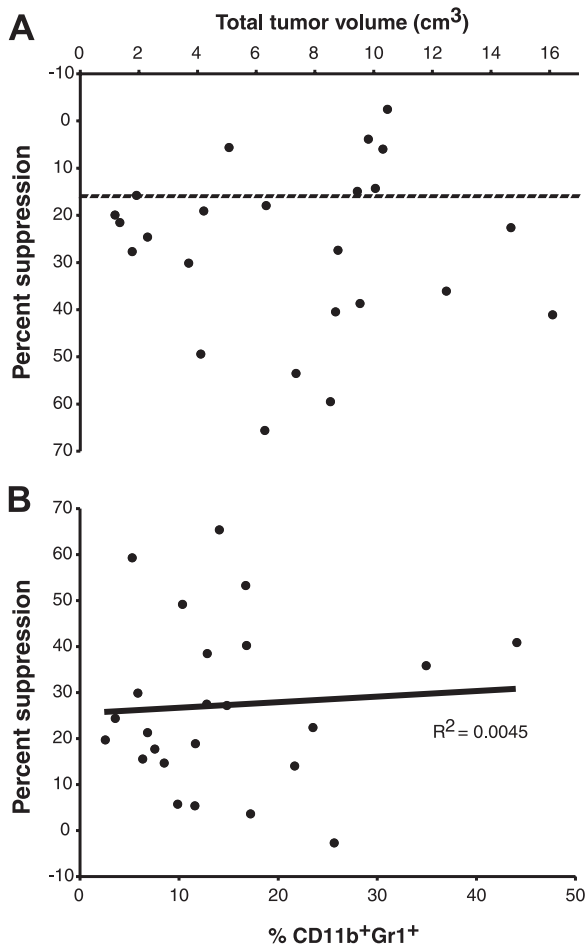
The status of the systemic immune response in MTAG mice was first analyzed by testing unfractionated splenocyte preparations from mice with a range of tumor burdens in lymphoproliferation assays using the polyclonal T cell activator Con A. To control for age and interassay differences, proliferative responses were compared with littermate Tg<sup>-</sup> mice (Fig. 2A). The majority of tumor-bearing mice, when compared with their corresponding control, exhibited a suppressed proliferative response to mitogen stimulation (18 of 24 tested). The variability in the percentage of suppression among mice with varying tumor loads indicates that the amount of proliferative suppression did not necessarily correlate with the overall amount of tumor burden. A difference in the level of proliferative suppression was observed in some mice with similar splenocyte compositions (determined by flow cytometry; data not shown), suggesting that the reduced response was not simply a result of quantitative differences in T cell numbers within the well. Earlier experiments that used anti-CD3 mAb to trigger T cell proliferation yielded comparable patterns to those of Con A (data not shown). The unpredictability of these proliferative responses mirrored what has been seen in clinical settings (29, 30) and indicates that tumor load alone does not necessarily determine the immunocompetency of these mice. Furthermore, an analysis of the presence of CD11b<sup>+</sup>Gr1<sup>+</sup> cells, which are commonly referred to as myeloid-derived suppressor cells (MDSC), revealed no correlation between the percentages of these cells within the splenocyte preparations and the level of reduced proliferation (Fig. 2B).

### No qualitative differences in purified T cells from MTAG mice with extensive disease

To examine in further detail whether this altered lymphocyte response (Fig. 2) was due to qualitative differences in the T cells, T cell subsets were purified to remove potentially non-T cell inhibitory host cells before being tested for proliferation and cytokine production. To normalize the data for comparison between assays, a representative ratio or index was obtained by dividing the results of Tg<sup>+</sup> mice by the responses of matched Tg<sup>-</sup> mice. An index value of less than one indicated a decrease in response from cells of Tg<sup>+</sup> mice relative to their Tg<sup>-</sup> controls, while an index value greater than one denoted an enhanced response. Purified CD4<sup>+</sup> or CD8<sup>+</sup> T cells from the spleens of MTAG mice harboring a range of tumor burdens demonstrated no reduction in Con A-induced proliferative responses when compared with their gender/age-matched controls (Fig. 3A).

When purified CD4<sup>+</sup> or CD8<sup>+</sup> T cells were stimulated by anti-CD3/CD28, there was also no reduction in the ability of these cells to produce either IFN- $\gamma$  or TNF- $\alpha$ , regardless of the level of tumor burden (Fig. 3B). Therefore, proliferation and cytokine production from purified splenic T cells (both CD4<sup>+</sup> and CD8<sup>+</sup> subsets) of mice harboring a spectrum of tumor loads was found to be equivalent to, or greater than, that of T cell subpopulations isolated from matched Tg<sup>-</sup> control mice. Comparable results were achieved when either positively or negatively selected T cells were used (data not shown). When B cell-enriched populations were stimulated with LPS, there was also no reduction in the proliferative ability of these cells compared with Tg<sup>-</sup> controls (Fig. 3C). These





**FIGURE 2.** Level of suppression in splenocyte proliferation. Splenocyte preparations from mice with varying tumor loads were placed in a standard proliferation assay with Con A stimulation and compared with the response from an age/gender-matched (in most cases littermate) control mouse. cpm values of [<sup>3</sup>H]thymidine incorporation from triplicate wells at a final concentration of 2  $\mu$ g/ml Con A were chosen as representative of a Con A dose curve. A, Percent suppression was calculated by  $[(1 - (\text{average cpm Tg}^+ / \text{average cpm Tg}^-)) \times 100]$ . A value of 15% suppression was taken as the cutoff for significant suppression, based on the significance calculated by comparing cpm readings. cpm values of unstimulated control cells from the 24 Tg<sup>-</sup> mice tested ranged from 892–21,598 (mean  $\pm$  SEM = 6,053  $\pm$  1,491) across all experiments. cpm values of Con A (2  $\mu$ g/ml)-stimulated cells from those same mice ranged from 87,994–205,659 (mean  $\pm$  SEM = 139,592  $\pm$  8,953). The highest value observed for both unstimulated and stimulated cells is from the same mouse. The value for total tumor volume was calculated by adding the tumor volumes from all discrete mammary tumors within a single mouse (up to 10 per mouse). However, mice were euthanized when at least one of the tumor masses reached the 2-cm<sup>3</sup> ethical limit. B, Flow cytometric analysis for CD11b<sup>+</sup>Gr1<sup>+</sup> cells in each of the splenocyte preparations from the individual tumor-bearing MTAG mice depicted in A was performed. The graph represents the percentage of CD11b<sup>+</sup>Gr1<sup>+</sup> cells vs the percentage of proliferation suppression. The linear trend line is represented.

data suggest that there was no qualitative decrease in the ability of either T or B cells from mice with extensive tumor burden to respond to polyclonal stimulation.

Systemic Ag-specific T cell function was further studied by examining the ability of MTAG mice to generate an allogeneic CTL response. A similar level of allogeneic-specific CTL activity was demonstrated by unfractionated splenocyte preparations from either young or aged Tg<sup>+</sup> or Tg<sup>-</sup> mice following allogeneic stimulation *in vitro* (Fig. 3D). T cells expanded to an equivalent num-

ber in all of the groups and splenocyte compositions appeared comparable following *in vitro* stimulation as determined by flow cytometry (data not shown). Therefore, there was no diminished functional priming of, or reduction in, the cytolytic ability of T cells from MTAG mice in response to *in vitro* stimulation by allogeneic Ags.

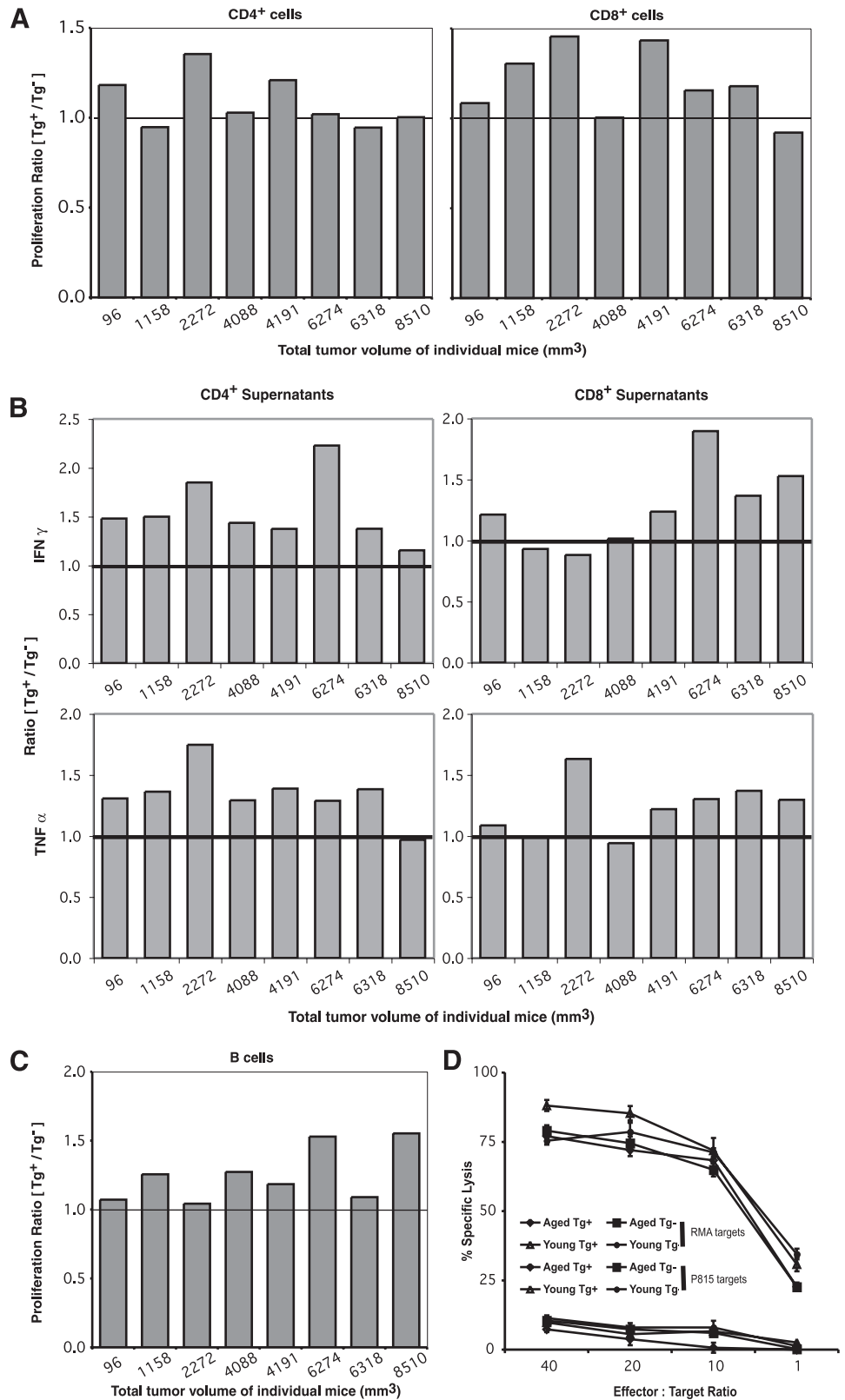
#### Analysis of MTAG mice for tumor-specific immune dysfunction

An important consideration for cancer immunotherapies is whether tumor-specific T cells exist and are functional in tumor-bearing individuals. Since, to date, no tumor rejection Ag or epitopes for CD4<sup>+</sup> or CD8<sup>+</sup> T cells have been defined in the B6-MTAG model, an alternative approach was used to determine whether tumor-specific T cells were present and not completely centrally deleted in these mice. An adoptive immunotherapy model was established where the cells from the LNs of young Tg<sup>+</sup> mice with little to no primary tumor growth were compared with LNCs from the tumor-draining LNs of aged Tg<sup>+</sup> mice with extensive disease. LNCs were isolated and used as effector cells, following *in vitro* stimulation, for adoptive therapy against an autologous tumor implant (AT-3) in naive syngeneic hosts. The autologous tumor cell line, termed AT-3, was derived previously from primary mammary gland carcinoma of MTAG mice.

Syngeneic wild-type B6 mice were injected s.c. with AT-3 tumor cells, followed 4 days later by the adoptive transfer of anti-CD3/CD28-stimulated and expanded LNCs from Tg<sup>+</sup> mice with either nonpalpable or extensive tumor loads, as well as the appropriate Tg<sup>-</sup> control preparations. The adoptive transfer of LNC preparations from both groups of Tg<sup>+</sup> mice were found to significantly inhibit AT-3 tumor growth when compared with the transfer of LNC preparations from their respective Tg<sup>-</sup> control mice (Fig. 4A). Flow cytometric analysis of the transferred populations revealed that >99.5% of these cells were CD3<sup>+</sup>, of which 78–90% were CD8<sup>+</sup> and 10–22% were CD4<sup>+</sup>. In a subsequent experiment, the adoptive transfer of purified LN-derived CD8<sup>+</sup> T cells of Tg<sup>+</sup> mice with minimal disease led to a similar and significant pattern of inhibition of tumor growth (Fig. 4B;  $p = 0.00071$  at day 35), which implicated CD8<sup>+</sup> T cells as a relevant effector cell. Interestingly, there was a statistically significant difference in the ability of LNCs of mice with minimal disease vs those with extensive disease to mediate rejection of AT-3 tumor cells (Fig. 4A;  $p = 0.0017$  at day 21). This suggested a progressive alteration in the functionality of the donor T cells as a consequence of continued exposure to autochthonous tumor growth. This inhibitory effect on s.c. tumor growth was specific to an AT-3 autologous tumor because no effect was observed in the growth of an irrelevant, but syngeneic tumor, such as RMA (Fig. 4C). Importantly, the observation that AT-3 tumor growth was significantly inhibited following transfer of LNCs from both groups of Tg<sup>+</sup> mice, but not Tg<sup>-</sup> LNCs, provided evidence for the existence of tumor-specific lymphocytes that had been primed to tumor Ag in MTAG mice and that could be stimulated or expanded *in vitro* to exhibit antitumor activity.

To evaluate evidence for tumor-specific immune dysfunction, we challenged young or aged Tg<sup>+</sup> mice with AT-3 and monitored the rate and size of implanted tumor growth compared with tumor growth in age-matched Tg<sup>-</sup> control mice (Fig. 5). Aged Tg<sup>+</sup> mice with extensive preexisting tumor burden, when injected with AT-3 at a secondary site distal from primary mammary gland carcinoma, developed s.c. tumors with a significantly elevated growth rate ( $p = 8.4 \times 10^{-4}$ ) compared with AT-3 growth in age-matched Tg<sup>-</sup> littermate control mice. In contrast, the rate of autologous

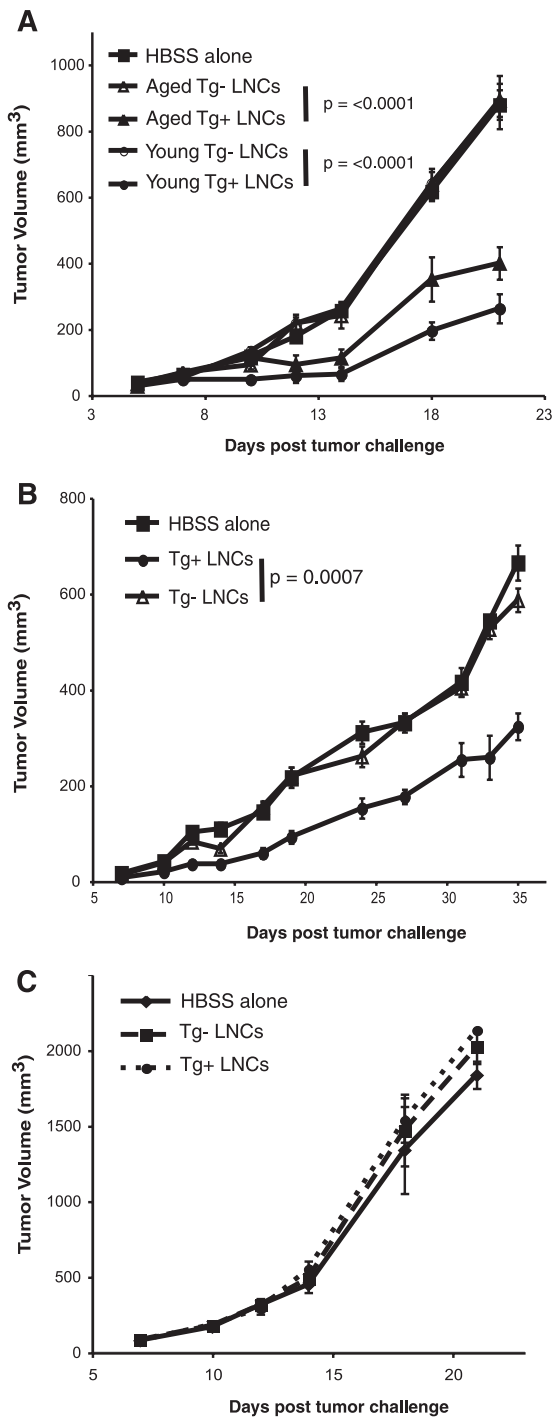
**FIGURE 3.** Proliferation and cytokine production by purified T cell populations, and splenocyte allogeneic CTL function. To adjust for interassay variability, the difference in responses between the purified cell populations of a Tg<sup>+</sup> tumor-bearing mouse and its Tg<sup>-</sup> control was determined using a ratio. For either proliferation (A) or cytokine production (B), this index was calculated by dividing the values (average of triplicate wells) from the cells of a Tg<sup>+</sup> mouse by the average value from its matched Tg<sup>-</sup> control. A value of less than one indicates a reduced response of cells from Tg<sup>+</sup> mice compared with their control, and an index equal to or greater than one demonstrates stable to enhanced proliferation respectively. A, Purified CD4<sup>+</sup> or CD8<sup>+</sup> populations from the spleens of individual mice with varying tumor loads were placed in a standard proliferation assay against a titration of Con A and compared with the responses from age/gender-matched control mice. Results shown are at a concentration of 2 μg/ml Con A and are representative of a Con A dose curve. B, CD4<sup>+</sup> and CD8<sup>+</sup> cells from these mice were also placed in culture with anti-CD3 (1 μg/ml immobilized) and anti-CD28 (10 μg/ml soluble) and stimulated for 48 h before harvesting culture supernatants for cytokine analysis by cytometric bead array. C, Enriched B cell populations were used in proliferation assays against a titration of LPS. Results from a 5-μg/ml final concentration of LPS were used to calculate the index value and were representative of a LPS titration curve. D, Splenocytes from the spleens of MTAG Tg<sup>+</sup> with little-to-none (young) and extensive tumor loads (aged) and their matched Tg<sup>-</sup> control mice (H-2<sup>b</sup>) were coinoculated with irradiated allogeneic BALB/c (H-2<sup>d</sup>) splenocytes for 6 days. The resulting effector cell populations were set up in a standard chromium release assay against syngeneic (RMA) or allogeneic (P815) target cells. Results are representative of two separate experiments.



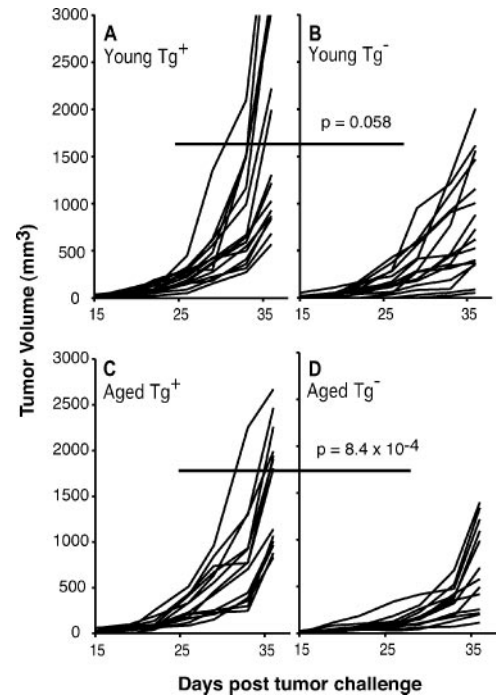
AT-3 growth in young Tg<sup>+</sup> mice with minimal preexisting disease displayed only a tendency toward statistical significance ( $p = 0.058$ ) when compared with controls.

Next, we challenged Tg<sup>+</sup> mice with either minimal or extensive disease with allogeneic tumor cells to assess for a more generalized or systemic form of immune suppression. In contrast to the results with AT-3, all Tg<sup>+</sup> and Tg<sup>-</sup> mice, regardless of their level

of preexisting disease, efficiently rejected a CMS4 (H-2<sup>d</sup>) allogeneic tumor challenge (data not shown). These observations were consistent with our earlier results that showed a lack of an impaired allogeneic CTL response in vitro (Fig. 3D) and suggested that these mice were not severely immune compromised. These data therefore provide evidence for tumor-specific immune dysfunction as a consequence of autochthonous tumor growth.



**FIGURE 4.** Tumor-specific activity of adoptively transferred MTAG LNCs. Cell preparations used for adoptive transfer experiments were obtained by culturing pooled LNCs from MTAG mice without palpable tumor (young) and mice with extensive tumor load (aged) or from their age/gender-matched Tg<sup>-</sup> controls. Cells were cultured *in vitro* with CD3/CD28 expander beads and recombinant mouse IL-15 (10 ng/ml) for 5 days. Recombinant human IL-2 (10 U/ml) was added for a further 24 h before harvesting and adoptive transfer. **A**, Groups of five B6 mice were challenged s.c. with  $5 \times 10^5$  syngeneic AT-3 tumor cells and allowed to establish for 4 days before the i.v. adoptive transfer of  $10 \times 10^6$  cultured LNCs. The efficacy of antitumor activity mediated by LNCs derived from transgenic mice with minimal vs extensive disease was significant ( $p = 0.0017$ ). **B**, B6 mice (five mice per group) were challenged with AT-3 tumor cells (as in **A**) before the adoptive transfer of  $10 \times 10^6$  LN-derived CD8<sup>+</sup> T cells purified from Tg<sup>+</sup> mice with minimal disease, which led to significant ( $p = 0.00071$ ) antitumor effects. **C**, Groups of five B6 mice

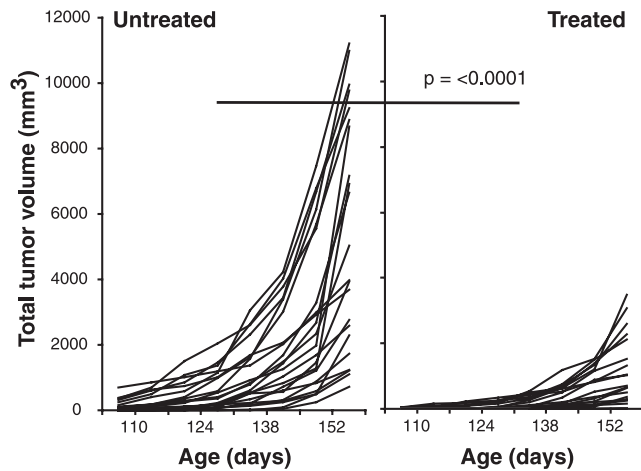


**FIGURE 5.** Ag-specific T cell function in transgenic mice with or without extensive tumor loads. MTAG mice were challenged s.c. with  $5 \times 10^5$  AT-3 autologous tumor cells in the flank region distal to the mammary tract. Four groups of mice were studied: young Tg<sup>+</sup> mice with none-to-minimal tumor burden (**A**) and their Tg<sup>-</sup> age-matched controls (**B**), aged Tg<sup>+</sup> with existing tumor load (**C**) and their Tg<sup>-</sup> age-matched controls (**D**). Tumor growth data consisted of seven evaluations from days 15 to 36 after AT-3 challenge. There was a mixture of normally and non-normally distributed tumor sizes; therefore, a Wilcoxon rank sum test was used to compare sizes between prespecified groups at each of the seven time points. A global evaluation of the differences was done using the method of O'Brien (26), which resulted in a strongly significant difference between groups in **C** and **D** ( $p = 8.4 \times 10^{-4}$ ) and a trend toward significance between groups in **A** and **B** ( $p = 0.058$ ). Results are a collation of three separate experiments with a total of  $n = 13$ –16 mice/group.

#### *Inhibition of autochthonous tumor growth by treatment with allogeneic tumor cells*

In the studies on systemic immune responses to allogeneic tumor cell challenge, in mice with minimal preexisting disease, we originally observed a reduction in the incidence and rate of autochthonous tumor growth following a single allogeneic tumor challenge. We therefore went on to investigate in further detail whether allogeneic tumor cells could be used to enhance the antitumor immune response against autochthonous mammary tumor growth. In these experiments, we opted against the use of mice with advanced disease because their limited life expectancy would preclude an assessment of the long-term impact of this approach. MTAG mice that initially harbored little-to-no tumor burden were challenged twice with CMS4 tumor cells, given 1 mo apart. Autochthonous tumor load in treated mice was measured weekly and compared with tumor growth in a group of untreated MTAG mice

were challenged s.c. with  $2 \times 10^5$  syngeneic but irrelevant RMA tumor cells and allowed to establish 4 days before the i.v. adoptive transfer of  $10 \times 10^6$  cultured LNCs from young MTAG or control mice. Tumor growth was monitored thrice weekly until termination of the experiment due to ethical considerations. Results represent one of two similar experiments. Groups were compared for statistical significance at the last tumor measurement using the Student *t* test. Error bars, SD values.



**FIGURE 6.** Modulation of autochthonous tumor growth by allogeneic tumor cell treatment. Two groups of young Tg<sup>+</sup> mice with no palpable primary tumor were used starting at 45–70 days of age. One group was treated by challenging s.c. with  $5 \times 10^5$  allogeneic CMS4-met tumor cells on the flank distal to the mammary tract. Another allogeneic tumor cell challenge was conducted 1 mo later. Primary tumor growth of individual tumors was measured weekly from their development until at least one of the tumors reached ethical limits. Total tumor volumes were calculated from the sum of all discrete tumor volumes. Results represent eight time point measurements of autochthonous tumor growth in mice of ages 110–159 days, collated from across three separate experiments for a total of  $n = 22$ /group. A global evaluation of the differences was done using the O'Brien method (26) and resulted in a  $p$  value of  $1.3 \times 10^{-5}$ .

to determine whether an allogeneic tumor cell challenge would confer enhanced protection (Fig. 6). Weekly tumor growth monitoring elicited eight tumor volume evaluations that could be compared between the two groups of mice ( $n = 22$ /group). There was a statistically significant difference between the treated and non-treated groups of MTAG mice at all of the weekly points of evaluation. A global analysis of the data also revealed a highly significant difference of  $p = 1.3 \times 10^{-5}$  between the two groups of mice. No obvious deleterious effects of allogeneic tumor challenge, including signs of autoimmunity, were observed. Taken together, the results suggested that preexisting antitumor responses could be modulated to retard autochthonous primary tumor growth.

## Discussion

A transgenic mouse model of autochthonous mammary tumor development and progression was used to investigate the consequences of tumor development and progression on the host immune system. This MTAG model is more representative of what occurs in a human cancer setting than many of the current tumor implant models that do not necessarily recapitulate the protracted nature of host-tumor cell interactions. The following major conclusions were drawn regarding the impact of long-term tumor progression on generalized/systemic and tumor-specific immune responses. First, systemic T cell dysfunction existed at some level, likely due to the presence of inhibitory host non-T cells within lymphoid compartments (i.e., spleens) of tumor-bearing MTAG mice, because purified CD4<sup>+</sup> or CD8<sup>+</sup> T cell subsets showed no inhibition of function. Interestingly, the level of this generalized immunosuppression did not correlate with the overall amount of tumor burden or changes in the frequencies of myeloid-derived CD11b<sup>+</sup>Gr1<sup>+</sup> cells. Along with data revealing the induction of potent allogeneic-specific CTL activity, this indicated that tumor growth did not lead to an irreversible functional defect in the T

cells themselves. Second, progressive tumor growth was accompanied by a decline in tumor-specific immune function *in vivo*. This suggested that tumor-induced peripheral mechanisms of suppression affected the behavior of tumor-specific T cells. Third, treatment of MTAG mice with allogeneic tumor cells resulted in reduced autochthonous tumor growth. Together, these data argue that a generalized immune suppression can occur and that tumor-specific immune cells were present, although retarded in function, during long-term host-tumor interactions. These immune responses however, could be modulated to enhance antitumor activity against primary mammary carcinoma, which indicates no irreversible impairment in immune function.

In the majority of MTAG mice tested, suppressed T cell function was observed based on reduced splenocyte proliferation to polyclonal stimulation. However, this suppression did not correlate with the overall amount of tumor load in individual mice or the cellular composition of their spleen. Given the variability of tumor growth and the presence of different tumor types within individual mice, it was not surprising that there was unpredictability in the observed immune responses from different mice. Indeed, some mice with extensive disease did not exhibit a suppressed response, whereas other mice with much lower tumor load did. This variability in the model reinforces the notion that Tg expression is not the single driving force behind tumor growth or progression and that other unidentified factor(s) influence both tumor development and host immune responses.

Despite evidence of immune dysfunction in unfractionated splenocytes from tumor-bearing MTAG mice, the lack of suppressed responses (proliferation or cytokine production) from purified T and B cells suggests that the altered responses were not due to a qualitative defect in these lymphocytes. The observation that T cells exhibited diminished function only in the presence of other splenic cells indicated the presence of a cell type, within the mixed splenocyte preparation, that was responsible for the reduced responses, such as myeloid-derived suppressor cells (31, 32), regulatory T cells (33), immature IDO-producing dendritic cells (DCs) (34), or NKT cells (35), rather than an inherent, irreversible defect in the T cells themselves. It is unlikely that CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells contributed to this *in vitro* suppression, because a reduction in proliferation was not observed in assays using purified, total CD4<sup>+</sup> T cells. Furthermore, an analysis of the presence of myeloid-derived CD11b<sup>+</sup>Gr1<sup>+</sup> cells in the splenocyte preparations from tumor-bearing mice revealed no correlation with the extent of reduced proliferation. This may be due to alterations in the biology of these emerging populations, as well as with interactions with other cellular networks. For example, if MDSC are involved, suppression may better reflect qualitative aspects of these cells in how they functionally mediate suppression, which incidentally may vary among individual mice. Furthermore, such MDSC may act alone or, perhaps, in combination with other potential suppressor cell types, such as IDO-producing DCs, or the factors that they secrete. Therefore, the mechanisms underlying the suppression of proliferation in this system are likely complex and require further investigation.

Given the unresolved issues on whether systemic T cell suppression occurs in tumor-bearing hosts (24, 36–38), our data support the view that a deficiency in systemic immune function does exist. These data therefore support several studies that demonstrated systemic immunosuppression in tumor implant models (15, 39). Importantly, our data extend those findings to a Tg model of long-term tumor progression, and indicate that under these conditions altered immune function may occur on a host-to-host basis, appears to be a rapidly reversible event (as demonstrated *in vitro*



using purified cell populations), and is likely due to cell-cell interactions rather than an inherent defect in the T cells themselves, such as impaired TCR-associated events (40, 41).

No apparent reduction in the cytolytic capacity of T cells from MTAG mice either with minimal or advanced disease was observed following *in vitro* priming of an allogeneic response. This result strengthens the conclusion that there is no irreversible functional defect in the T cells of this Tg mouse model. Although several studies in implantable tumor models have shown that *in vitro*-primed, splenic-derived CTL responses were diminished in tumor-bearing animals (36, 42), it is likely that such differences in CTL function reflect the nature of the tumor model. Thus, this autochthonous tumor model offers new insights into how a chronic, rather than acute, tumorigenic process impacts the functional integrity of host defense and immunosurveillance mechanisms.

Because no tumor rejection Ags or epitopes for CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been identified in the B6-MTAG model, we used alternative *in vivo* approaches to study alterations in the tumor-specific immune responses during autochthonous tumor growth. The effective inhibition of autologous AT-3 tumor growth following adoptive transfer of LNCs from Tg<sup>+</sup> mice, either derived from mice with minimal or advanced disease, demonstrated that primed tumor-specific lymphocytes existed in this MTAG model. Thus, in this model of autochthonous tumor development, central tolerance cannot be solely responsible for the lack of tumor growth control. These observations are also consistent with the identification of tumor-specific T cells in other Tg mouse models of spontaneous tumor development, such as the Her-2/*neu* mammary carcinoma model (43, 44) or the TRAMP model of prostate carcinoma (45).

Furthermore, because the transfer of purified CD8<sup>+</sup> T cells mediated similar tumor growth inhibition, this implicated the CD8<sup>+</sup> subset as a relevant effector cell. Interestingly, the extent of antitumor activity seen with purified CD8<sup>+</sup> T cells was no greater than that observed with unfractionated LNCs. These data suggested that under these *in vivo* conditions, the percentage of CD8<sup>+</sup> T cells in the unfractionated LN population (78–90%) was still sufficient to achieve comparable levels of antitumor activity. There may be a role for other immune cells, which would require further detailed studies. However, it appears clear that the transferred CD8<sup>+</sup> T cells are integral to the observed antitumor response. Interestingly, we observed a significant difference in the functional efficacy of LNCs from mice with extensive disease compared with LNCs from mice with minimal disease. The fact that the *ex vivo* expansion was performed under T cell-specific stimulation conditions (using anti-CD3 mAb) supports the notion that the observed differences in tumor rejection efficiency between these two LNC populations reflected a progressive alteration in the functionality of the transferred T cells influenced by autochthonous tumor growth.

MTAG mice with either minimal or extensive disease were also challenged with AT-3 tumor cells and monitored for their response to this autologous antigenic challenge. Following tumor challenge, Tg<sup>+</sup> mice with extensive tumor burden showed a significantly reduced ability to affect *s.c.* AT-3 tumor growth. In contrast, Tg<sup>+</sup> mice with minimal preexisting disease demonstrated only a trend toward a significant difference in *s.c.* tumor growth compared with AT-3 growth in Tg<sup>-</sup> control mice. This suggested that a tumor-specific T cell response was required to inhibit growth of an autologous tumor challenge and that a milder form of tumor-specific T cell suppression occurs in mice with early stage disease compared with mice with more advanced disease. Use of these approaches to study tumor-specific responses has the advantage of assessing a broad range of antitumor responses, which may be found *in vivo* and is not necessarily limited to the response of a

single epitope or Ag. Therefore, tumor-specific immune dysfunction appeared to emerge gradually as a consequence of progressive tumor growth, which is distinct from the rapid kinetics of tumor-specific immunosuppression development that has been reported in implantable tumor models (46–48). Together with our LNC adoptive transfer experiments, these findings are consistent with the hypothesis that endogenous antitumor responses were regulated, at least in part, by peripheral mechanisms of tumor-induced tolerance or suppression.

In contrast to an autologous tumor challenge, an allogeneic tumor challenge was strongly rejected in Tg<sup>+</sup> mice with either minimal or extensive disease, suggesting that an allogeneic tumor challenge generates a potent inflammatory response that is unaffected or at least capable of overcoming certain types of tumor-induced suppressive mechanisms that affect various adaptive immune responses. Although allogeneic tumor challenge was initially chosen to assess for a more generalized or systemic form of immune suppression, we also observed during the course of these experiments that such an approach significantly retarded autochthonous tumor growth. Together with the observation that *in vitro* allogeneic stimulation resulted in no reduction in the ability of T cells from MTAG mice to expand and lyse allogeneic-specific tumor cell targets, it appears that a suppressed response can be overcome or modulated if the immune system is given a strong enough stimulus. Indeed, allogeneic-based vaccine therapies have been used by other groups to enhance tumor immunogenicity or vaccine-mediated immune responses (49–53) and, therefore, has potential clinical value. The mechanisms accounting for such antitumor effects are complex, but may involve an enhanced induction of tumor-specific T cell responses, perhaps through modulating the functionality of APCs; the production of a type 1 cytokine environment; or the reversal of a tolerogenic immune state, which can all lead to improved innate and adaptive immunity, particularly to shared tumor Ags (49–53). In our model, the mechanisms that underlie the enhanced autochthonous tumor latency following allogeneic tumor cell challenge are likely to be similarly complex and await further investigation, such as a detailed analysis of changes in tumor-specific T cell precursor frequencies, as well as potential qualitative alterations affecting functional properties of the resultant T cell responses. From a translational standpoint, future studies are also warranted to explore the development and efficacy of other cancer vaccine strategies, such as the use of tumor Ag-pulsed DC.

Understanding changes in immune responses that occur as a result of tumor progression is important in the development or improvement of therapeutic strategies. Although we have shown that certain forms of immune dysfunction or suppression occur during disease development and progression, we have also demonstrated that they can be modulated, at least early in cancer progression, to allow enhancement of antitumor responses. This is promising for combination immunotherapies if their design can incorporate strategies that suppress the suppressive mechanisms responsible for the immune dysfunction induced in tumor-bearing hosts.

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## Disclosures

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