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Research Article

Immune Rejection of Mouse Tumors Expressing Mutated Self

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Abstract

How the immune system recognizes and responds to mutations expressed by cancer cells is a critical issue for cancer immunology. Mutated self-polypeptides are particularly strong tumor-specific rejection antigens for natural tumor immunity, but we know remarkably little about T-cell responses to mutated self during tumor growth *in vivo*, including levels of response, kinetics, and correlates that predict tumor rejection. To address these questions, a mutated self-antigen, designated tyrosinase-related protein 1 (Tyrp1)-WM, derived from Tyrp1 was expressed in the poorly immunogenic, spontaneously arising B16 melanoma and the immunogenic, chemically induced LiHa fibrosarcoma. Syngeneic mice challenged with LiHa fibrosarcoma cells expressing Tyrp1-WM, but not native Tyrp1, induced specific $CD8^+$ and $CD4^+$ T-cell responses against defined mutated epitopes in tumor-draining lymph nodes and in tumors. Subsequently, specific $CD8^+$ T-cell responses contracted as a minority of tumors progressed. B16 melanomas expressing Tyrp1-WM induced minimal T-cell responses, and no tumor immunity was detected. Treatment with an agonist monoclonal antibody against glucocorticoid-induced tumor necrosis factor receptor family–related gene (GITR) increased the level of $CD8^+$ T cells recognizing a peptide derived from the Tyrp1-WM sequence and the proportion of mice rejecting tumors. These results show that B16 tumors expressing mutations that generate strongly immunogenic epitopes naturally induce T-cell responses, which are insufficient to reject tumors. Immune modulation, such as inducing GITR signaling, is required to enhance $CD8^+$ T-cell responses to specific mutations and to lead to tumor rejection. [Cancer Res 2009;69(8):3545–53]

Introduction

Cancer is a genetic disease characterized by accumulation of somatic mutations and other genetic alterations during tumor progression. Mutations include point mutations, nucleotide insertions and deletions, gene deletions, and translocations. Large-scale genomic mutational analyses have been initiated to explore the extent of mutations in human cancers. An analysis of mutations in 518 genes encoding protein kinases in 210 tumor specimens identified >1,000 mutations (1). Sequencing of protein-coding exons of 13,023 genes in 11 breast and 11 colorectal cancer tumors revealed at least 90 mutations per specimen (2). In a survey of matched samples looking at single-nucleotide substitutions, tumors had a 200-fold increase in mutations compared with autologous normal tissues (3). Together, these observations support the accumulation of large numbers of mutations in established tumors (estimated to be $\sim 10^{12}$ mutations in small tumors composed of 10^9 cells; ref. 4).

Mutations are attractive targets for immunotherapy because of their potential immunogenicity and cancer specificity. Importantly, in contrast to the robust immunogenicity of chemically induced tumors, spontaneous tumors are weakly immunogenic (5). When immunity is detected in spontaneous tumors, it is directed against poorly immunogenic, shared self-antigens (6). Molecular studies have revealed that tumor-specific antigens in mice can be generated from mutated self-molecules (7–11). In studies of humans with cancer, mutated self-molecules can also be recognized by $CD8^+$ and $CD4^+$ T-cell lines and clones (generated through extended stimulation *in vitro*). As of October 2007, a review of the published literature revealed that processed and presented epitopes from 44 different mutated genes were recognized by T cells from cancer patients (12), although such responses have uncommonly been linked to clinical outcomes, such as unexpectedly prolonged survival (13–15). Point mutations in self-genes are, by far, the most prevalent genetic lesions recognized by T cells. Immunogenic point mutations most frequently have been shown to increase binding to MHC molecules for presentation to T-cell receptors but can also enhance avidity for T-cell receptors and increase antigen processing and presentation (16–19). A recent study indicates that each breast and colorectal cancer contains $\sim$10 and 7 novel and unique HLA-A*0201 epitopes, respectively (20).

We have studied T-cell responses to defined immunogenic mutated self-epitopes during tumor growth *in vivo* using an immunogenic, chemically induced tumor (LiHa fibrosarcoma) and a poorly immunogenic, spontaneously arising tumor (B16F10 melanoma).

Materials and Methods

Mice. C57BL/6 and NOD/SCID mice (6- to 8-week-old females) were acquired from The Jackson Laboratory. Mice were maintained in a pathogen-free vivarium according to NIH Animal Care guidelines. Experiments were done under the governance of an institutional protocol approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

Cell lines. B16F10 (hereafter called B16) is a cell line derived from a spontaneously arising mouse melanoma of C57BL/6 origin (provided by I. Fidler, M. D. Anderson Cancer Center). LiHa is a mouse fibrosarcoma, which was induced by injecting dimethylbenzanthrene in the skin of an *INK4a*$^{-/-}$ mouse on a C57BL/6 background (N15; ref. 21). EL4, a C57BL/6

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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mouse lymphoma cell line, was used as antigen-presenting cells in T-cell assays. Cell lines were cultured in RPMI 1640 supplemented with 7.5% FCS.

Peptides. Peptides were synthesized by Genemed Synthesis. Peptides included H2-D^b-restricted wild-type tyrosinase-related protein 1 (Tyrp1)$_{455\text{-}463}$ (TAPDNLGY*A*) and its A463M mutant, WM$_{455\text{-}463}$ (TAPDNL-GY*M*; ref. 19), I-A^b-restricted peptides WM$_{521\text{-}535}$ (RYA*Y*DYEELPNPNHS) and its wild-type counterpart Tyrp1$_{521\text{-}535}$ (RYA*E*DYEELPNPNHS), and pigeon cytochrome *c* PCC$_{43\text{-}58}$ (AEGFSYTDANKNKGIT).

Expression vectors. The cDNA encoding wild-type mouse Tyrp1 and mutated mouse Tyrp1 variant WM ("white magic" for its ability to induce vitiligo, originally designated Tyrp1ee; ref. 19) have been reported previously (16, 19, 22, 23). The cDNAs were cloned into pMG-IRES-Lyt2 for expression in tumor cells. The pMG-IRES-Lyt2 vector contains the mouse CD8 extracellular domain as a marker and does not contain a drug resistance gene (eliminating immunogenicity of products of drug resistance genes and avoiding transcription of *neo* or other gene products that generate amino 3′-glycosyl phosphotransferase, which may alter cell phosphorylation status). Tyrp1-WM455 and Tyrp1-WM455/521 were generated from native mouse Tyrp1 cDNA. A schematic of native Tyrp1 and mutant variants is shown in Fig. 1*A*.

Transfection. Cells were transfected with the cationic lipid-based FuGENE 6 reagent (Roche Diagnostics) or by electroporation with the Nucleofector device and solution V (Amaxa) using conditions described by the manufacturers. Between 24 and 48 h after transfection, stable transfectants were selected by flow cytometric cell sorting after staining with phycoerythrin anti-CD8 antibody by the Memorial Sloan-Kettering Cancer Center Flow Cytometry Core Facility. Stable transfectants were sorted every 2 to 4 weeks for at least 2 months before use in experiments. Multiple independent lines and clones were used for tumor regression/progression experiments to rule out variability in transfectants.

Treatment with antibodies for immune modulation. To deplete CD4$^+$ or CD8$^+$ T cells, anti-CD4 monoclonal antibody (mAb; clone GK1.5, rat IgG2b) or anti-CD8 mAb (clone 2.43, rat IgG2b) was injected intraperitoneally weekly, starting 2 days before tumor challenge and every 5 to 7 days thereafter (250 μg for the first three injections and 500 μg thereafter). To induce agonist signaling through glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), 1 mg anti-GITR agonistic mAb (DTA-1, mouse IgG2a) was injected intraperitoneally on day 4 following tumor challenge. Purified rat IgG (Sigma-Aldrich) was used as a control.

***I-A^b Optimizer* program to predict and optimize I-A^b-restricted epitopes.** *I-A^b Optimizer* code was written in HTML. This application ranks all the possible I-A^b-restricted peptide epitopes within an inquiry polypeptide sequence according to an embedded interchangeable scoring matrix. For an inquiry polypeptide with *N* amino acids, the *I-A^b Optimizer* breaks the sequence into N-8 nine-mer peptides and calculates the scores one by one as well as the maximum increase and maximum score obtainable with one amino acid substitution.

ELISPOT assays. The ELISPOT assay has been described previously (24). Briefly, MultiScreen-IP plates (Millipore) were coated with 100 μL anti-mouse IFN-γ antibody at a concentration of 100 μg/mL (10 mg/mL; clone AN18; Mabtech). Purified CD4$^+$ or CD8$^+$ T cells (>95% enriched) from tumor, spleen, or pooled inguinal lymph nodes, isolated using anti-CD4 or anti-CD8 MACS magnetic beads (Miltenyi Biotec), were plated at a concentration of 1×10^5 per well or as indicated. To assess CD8$^+$ T-cell

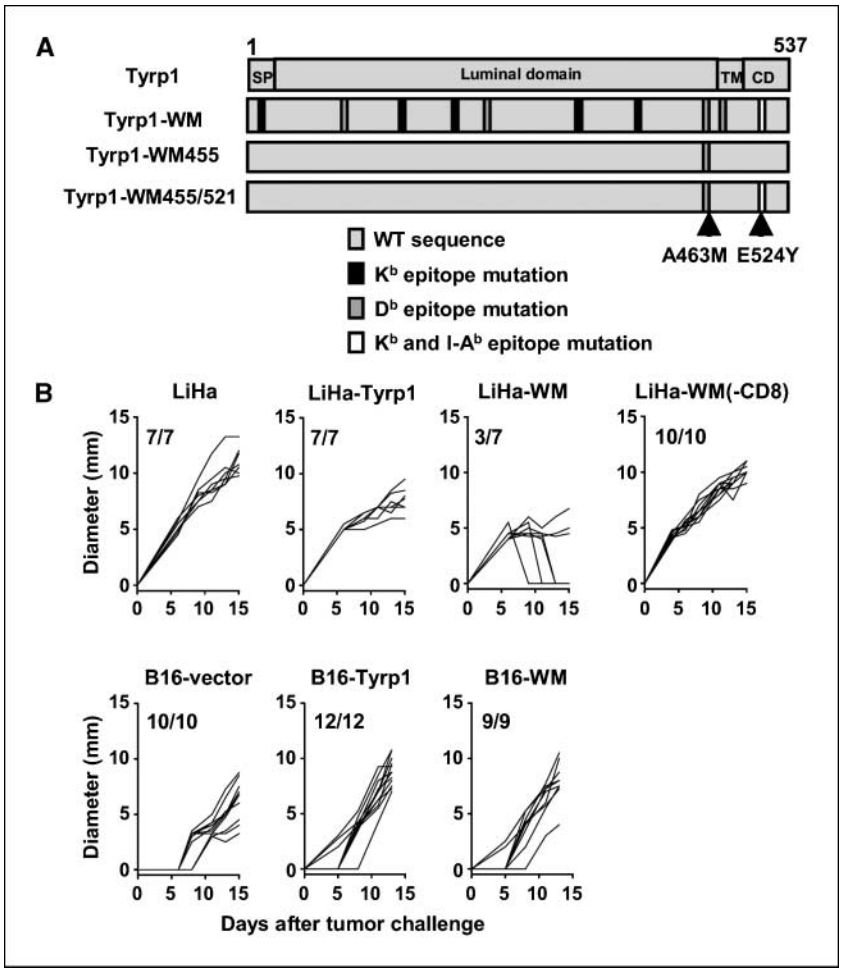

Figure 1. LiHa fibrosarcoma expressing WM regresses. *A,* schematic of mouse Tyrp1 and WM mutants. Mutations (*vertical bars*) were designed to improve binding to MHC I molecules. *SP,* signal peptide; *TM,* transmembrane domain; *CD,* cytoplasmic domain. *B,* 25,000 LiHa cells were used to challenge C57BL/6 mice. 25,000 LiHa-WM cells were also used to challenge CD8-depleted C57BL/6 mice (-CD8). 100,000 B16 cells were used to challenge C57BL/6 mice. Tumor growth was followed three times per week for >60 d. Ratios equal the number of mice with tumors over the total number of mice.

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responses, $CD8^+$ cells were incubated at 37°C for 20 h with irradiated (10,000 rads) antigen-presenting cells using either 1×10^4 EL-4 lymphoma cells pulsed with 1 μg/mL peptide or B16 cells. For $CD4^+$ T-cell assays, $CD4^+$ cells were incubated for 40 h with 10^5 irradiated (3,000 rads) naive syngeneic splenocyte antigen-presenting cells pulsed with 10 μg/mL peptide. Wells were then incubated with 100 μL/well biotinylated antibody against mouse IFN-γ at a concentration of 4 μg/mL (2 mg/mL; clone R4-6A2; Mabtech). Spots were developed with streptavidin-conjugated horseradish peroxidase and counted with an automated ELISPOT reader system with KS 4.3 software (Carl Zeiss MicroImaging).

Tumor challenge and measurement. For tumor challenge, indicated numbers of B16 or LiHa cells with a viability >95% were used. Tumor cells were challenged intradermally into the lower right flank of mice. Tumor diameters were measured by calipers every 2 to 3 days. Mice were sacrificed when tumors ulcerated or reached a maximum diameter of 10 mm or mice showed any sign of discomfort.

Flow cytometry. Fluorochrome-labeled anti-mouse mAbs recognizing mouse CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), Ly-6G and Ly-6C (Gr-1; RB6-11 8C5), NK1.1 (PK136), CD11b (M1/70), CD25 (3C7), and IFN-γ (XMG1.2) were used from BD Pharmingen. Phycoerythrin-labeled anti-mouse Foxp3 (FJK-16s) mAb from eBiosciences was used in our experiments. Cells were assessed on a FACSCalibur or FACScan flow cytometer (Becton Dickinson) and results were analyzed using FlowJo (TreeStar). For tetramer assays, phycoerythrin-conjugated $D^b/WM_{455-463}$ tetramer, containing the H2-D^b-restricted mutant Tyrp1 epitope $WM_{455-463}$ (19), was made by Beckman Coulter. One million cells were incubated with 1 μL tetramer for 30 min at 37°C and then incubated with fluorochrome-labeled anti-CD3, anti-CD4, and anti-CD8 antibodies (BD Pharmingen) for 20 min at 4°C. Percentage of tetramer-positive cells was calculated within the $CD3^+CD4^-CD8^+$ gate or as indicated. For intracellular IFN-γ assays, lymphocytes were stimulated either with or without 0.1 to 1 μg/mL peptide. Brefeldin A (Sigma-Aldrich) was added after 1 h at a concentration of 10 μg/mL. Following incubation for 12 to 16 h at 37°C, cells were stained for surface markers, fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), and stained for intracellular IFN-γ using phycoerythrin-conjugated anti-mouse IFN-γ (BD Pharmingen).

Statistical analysis. Differences in tumor-free survival were analyzed by log-rank analysis of Kaplan-Meier curves (comparisons pooled over strata using SPSS 10.0 software for Windows). Statistical differences and T-cell responses between groups of mice were determined by two-tailed, nonparametric Mann Whitney test (GraphPad Prism 4.0).

Results

Generation of tumor cells expressing wild-type and mutant Tyrp1. To investigate natural tumor immunity elicited by immunogenic point mutations in mutant self-proteins, mutant self-genes encoding defined immunogenic point mutations were expressed in inherently immunogenic LiHa tumor cells and poorly immunogenic B16 tumor cells (6). Native and a mutated version of mouse Tyrp1 were selected for this study based on extensive characterization of the biology and immunogenicity of these proteins (19, 22, 25–30). We have reported previously the mutant form of mouse Tyrp1, Tyrp1-WM, which contains point mutations to create heteroclitic epitopes with increased binding to K^b and D^b MHC I molecules (ref. 19; Fig. 1*A*). Immunization with plasmid expressing Tyrp1-WM induces strong $CD8^+$ T-cell responses, protects almost all mice from challenge with supralethal doses of B16 melanoma, and prolongs progression-free survival in therapeutic B16 models (19). Mutant Tyrp1-WM contains a dominant mutated MHC I-restricted epitope at amino acid positions 455 to 463 and two subdominant mutated MHC I epitopes (481-489 and 522-529; ref. 19).

Wild-type Tyrp1 and mutant Tyrp1-WM cDNA were subcloned into a bicistronic vector, pMG-IRES-Lyt2 (see Materials and Methods), which encodes the extracellular and transmembrane domains of mouse CD8 (Lyt2; for selection of transfected cells). Wild-type Tyrp1 and mutant Tyrp1-WM were expressed in the poorly immunogenic B16 melanoma and in the chemically induced, immunogenic LiHa fibrosarcoma. B16 melanoma is not immunogenic when measured either by concomitant immunity or by immunization with up to 50 million irradiated tumor cells, whereas LiHa fibrosarcoma induces both concomitant immunity and tumor immunity following immunization with irradiated LiHa cells (6). Multiple transfectants were generated for each cell line as described in Materials and Methods.

LiHa-WM tumors are regressors and B16-WM tumors are progressors. Syngeneic C57BL/6 mice were challenged cutaneously with supralethal numbers of tumor cells. For LiHa experiments, mice were challenged with 25,000 cells (LD_{50} for parental LiHa is 1,000 cells). In three separate experiments, parental LiHa, LiHa transfected with empty vector (data not shown), and LiHa-Tyrp1 tumors grew aggressively in all mice and were lethal within 3 weeks (Fig. 1*B*). In marked contrast, LiHa-WM initially formed tumors in all mice but tumors (~5-6 mm diameter) spontaneously regressed in the majority of mice after day 6 (a representative experiment of three is presented in Fig. 1*B*). Following regression, mice remained tumor-free for >60 days. Taking the results of three experiments together, parental LiHa and LiHa-vector (two independently transfected lines) each formed progressor tumors in 22 of 22 mice, LiHa-Tyrp1 (three different lines) in 17 of 17 mice, and LiHa-WM (three distinct lines) in only 6 of 17 mice. Rejection of LiHa-WM tumors was not due to intrinsic differences in tumorigenicity or growth but rather was CD8-dependent, as LiHa-WM tumors grew in CD8-depleted mice as aggressively as parental LiHa tumors (Fig. 1*B*).

Following challenge of syngeneic C57BL/6 mice with 100,000 B16 cells (LD_{50} for B16 is 2,500 cells), progressor tumors were observed in most mice, including B16 transfected with empty vectors (8 different lines), B16-Tyrp1 (8 lines), and B16-WM (9 different lines; representative experiment in Fig. 1*B*). Overall, B16-vector grew in 169 of 208 (81%) mice, B16-Tyrp1 in 79 of 85 (93%) mice, and B16-WM in 85 of 100 (85%) mice. These results show that expression of immunogenic point mutations is sufficient to lead to natural CD8-dependent tumor regression of LiHa fibrosarcoma but not of the poorly immunogenic B16 melanoma.

LiHa expressing WM induces $CD8^+$ T-cell responses against the dominant mutant $WM_{455-463}$ epitope. To characterize $CD8^+$ T-cell responses elicited by tumors expressing immunogenic mutations, mice were challenged with parental or mutant LiHa and B16 tumor cells. Nine days after tumor challenge, individual tumors were analyzed for infiltration of $CD8^+$ T cells. LiHa-Tyrp1 and parental LiHa tumors contained little infiltrate (<0.5%; Fig. 2*A*, *top row*). All B16 tumors contained comparable infiltration of $CD8^+$ T cells (1.5-1.8%; data not shown for parental B16). No increases in $CD8^+$ T-cell infiltrates were observed in B16-WM tumors. In contrast, LiHa-WM tumors elicited a marked infiltration of $CD8^+$ T cells, increased 81- and 14-fold compared with LiHa and LiHa-Tyrp1 tumors, respectively (Fig. 2*A*, *top row*). These results reveal preferential recruitment and/or expansion of host $CD8^+$ T cells in LiHa-WM (but not B16-WM) tumors.

$CD8^+$ T cells against the dominant D^b-restricted mutant epitope $WM_{455-463}$ (A463M mutation in a D^b-binding anchor residue) were analyzed in tumors (Fig. 2*A*, *middle row*) and lymph nodes (Fig. 2*B*, *top row*) using $D^b/WM_{455-463}$ tetramers. Remarkably,

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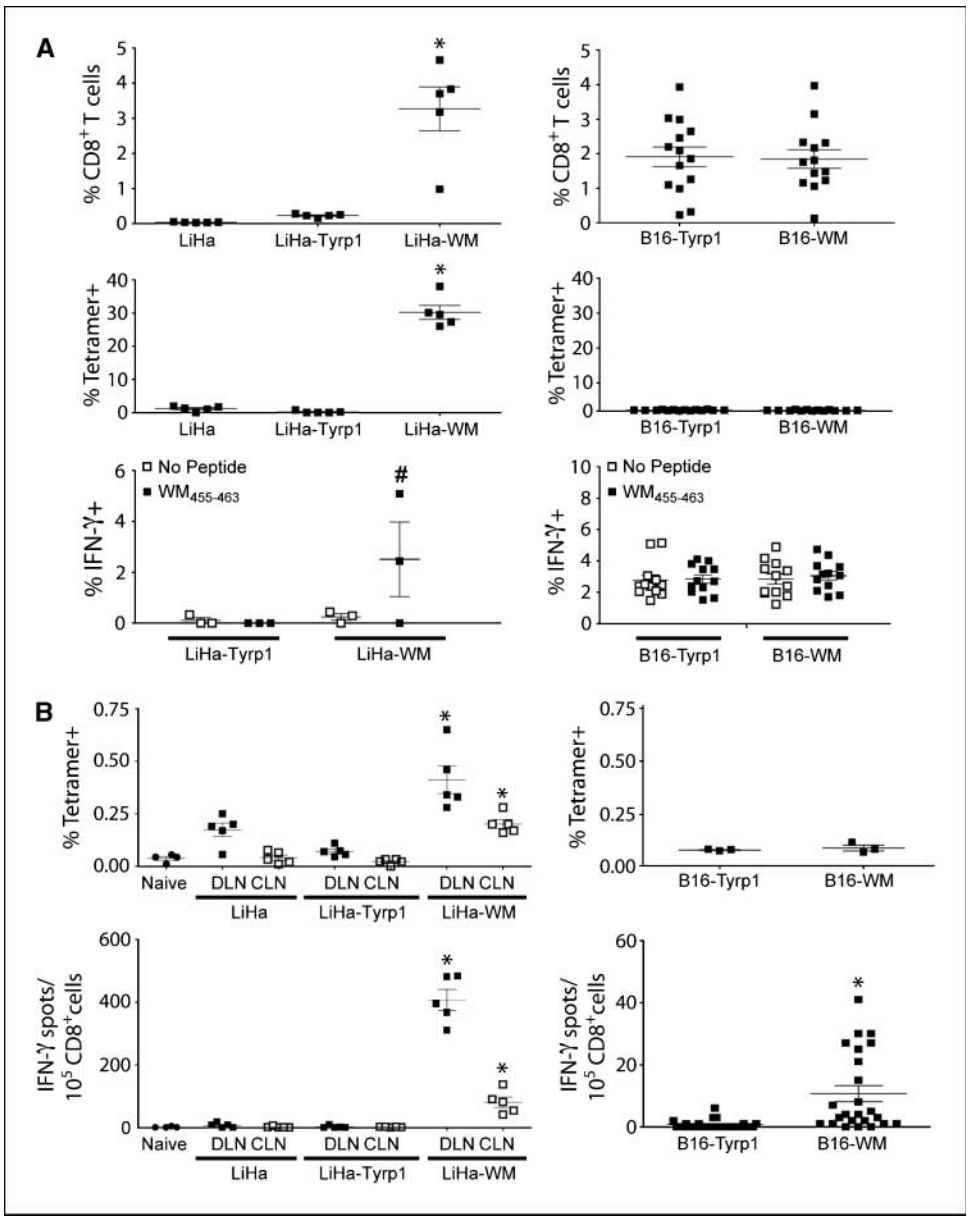

Figure 2. Tumor cells expressing WM but not wild-type Tyrp1 induce $CD8^+$ T-cell responses. Mice were injected intradermally with 50,000 LiHa cells or 100,000 B16 cells. Each symbol represents one mouse. *A,* proportion of $CD8^+$ T cells to total viable cells in tumors (*top row*); proportion of tetramer-positive cells within the $CD3^+CD8^+$ gate of tumor-infiltrating cells (*middle row*); proportion of IFN-γ–positive cells in the $CD3^+CD8^+$ gate after single-cell suspension from tumors was stimulated with 1 μg/mL $WM_{455-463}$ peptide overnight (*bottom row*). *, $P = 0.008$; #, $P = 0.03$, compared with LiHa-Tyrp1. *B, top row,* proportions of tetramer-positive cells within the $CD3^+CD8^+$ gate in individual lymph nodes; *bottom row,* frequencies of functional $CD8^+$ T cells by ELISPOT assay. *LN,* lymph node; *DLN,* tumor-draining lymph node; *CLN,* contralateral lymph node. *Horizontal lines,* means for individual mice. *Bars,* SE. *, $P = 0.008$, compared with LiHa-Tyrp1 or B16-Tyrp1.

tetramer-positive $CD8^+$ T cells comprised 30% of $CD8^+$ T cells infiltrating LiHa-WM tumors but were essentially undetectable in parental LiHa, LiHa-Tyrp1, B16-Tyrp1, or B16-WM tumors (Fig. 2*A*, *middle row*). Tetramer-positive $CD8^+$ T cells were also increased in draining and contralateral nodes in hosts with LiHa-WM but not B16-WM tumors (Fig. 2*B*, *top row*). These results suggest a major contribution of $CD8^+$ T cells recognizing the $WM_{455-463}$ epitope for mediating regression of LiHa-WM tumors.

Specific $CD8^+$ T cells secreting IFN-γ in response to $WM_{455-463}$ could be detected in LiHa-WM but not B16-WM tumors (Fig. 2*A*, *bottom row*). Although these responses were detectable in B16-WM-draining lymph nodes by sensitive ELISPOT assays, the responses were found only in 8 of 24 (33%) mice and were very weak (14-42 spots/100,000 $CD8^+$ cells), whereas the same responses were markedly elevated in draining (407 ± 75) and nondraining lymph nodes (81 ± 37) of mice bearing LiHa-WM tumors (Fig. 2*B*, *bottom row*). Thus, LiHa-WM tumor progression naturally elicited functional $CD8^+$ T-cell responses against the dominant $WM_{455-463}$ epitope.

LiHa expressing WM induces $CD4^+$ T-cell responses against the mutant $WM_{521-535}$ epitope. LiHa-WM, but not B16-WM, tumors had increased infiltration of $CD4^+$ T cells (Fig. 3*A*). Although point mutations in Tyrp1-WM were originally created to generate heteroclitic peptides that bind to MHC I (19), mutant (or wild-type) Tyrp1 peptides might also bind to MHC II molecules. To search for candidate peptides with potential for binding to the I-A^b MHC II molecule, we created an algorithm called *I-A^b Optimizer* using Position Specific Scoring Matrixes (31, 32) to rank candidate peptides. The four peptides with the highest scores were identified and synthesized.

To determine whether any of these four peptides were epitopes that could be processed and presented to $CD4^+$ T cells, mice were immunized with plasmid DNA expressing full-length Tyrp1-WM and $CD4^+$ T cells were purified from spleens for ELISPOT assays.

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Strong $CD4^+$ T-cell responses were detected against the mutant peptide $WM_{521-535}$ (E524Y) but not against the corresponding nonmutated $Tyrp1_{521-535}$ peptide or the three other candidate peptides (Supplementary Fig. S1; data not shown). Responding $CD4^+$ T cells against $WM_{521-535}$ primarily produced IFN-γ, although IL-2 production was detectable at a low level (Supplementary Fig. S1). Neither IL-4-producing nor IL-10-producing $CD4^+$ T cells could be detected (data not shown). These data are consistent with Th1-type responses of $CD4^+$ T cells against the mutant $WM_{521-535}$ epitope.

We next asked whether MHC II-negative B16 or LiHa tumors expressing Tyrp1-WM or Tyrp1 naturally primed $CD4^+$ T-cell responses to this mutant epitope during tumor progression. Around day 10 after tumor challenge, LiHa-WM tumors had ~5-fold increased $CD4^+$ T-cell infiltration, compared with LiHa-Tyrp1 tumors, but no increase was observed for B16-WM tumors compared with B16-Tyrp1 (Fig. 3*A*). Mice with LiHa-WM tumors, but not LiHa, LiHa-Tyrp1, or B16 tumors, had $CD4^+$ T-cell responses to $WM_{521-535}$ mutant epitope in tumor-draining lymph nodes (Fig. 3*B*). These results show the priming of Th1-type $CD4^+$ T-cell responses against the mutant $WM_{521-535}$ epitope by MHC II-negative LiHa-WM tumors presumably through cross-priming by antigen-presenting cells.

These observations suggested that combinations of cytotoxic $CD8^+$ T-cell responses and $CD4^+$ Th1 helper responses against mutant self-peptides within the same protein act synergistically to induce tumor immunity. To address this hypothesis, we created a cDNA encoding Tyrp1-WM455, which contains only the D^b-restricted mutant $WM_{455-463}$ epitope through insertion of a single mutation at amino acid 463 (A463M). A second cDNA,

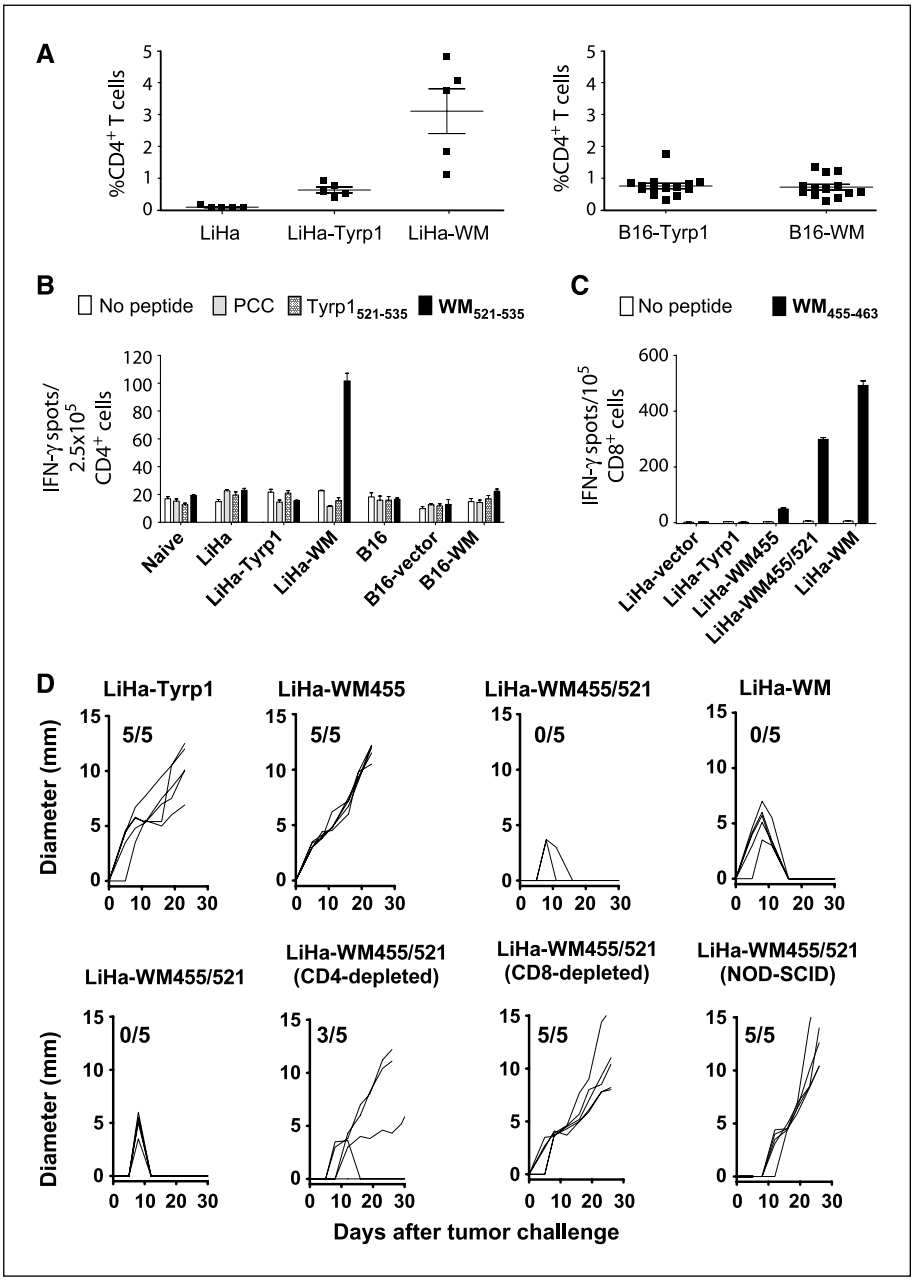

Figure 3. Tumor cells expressing WM induce strong $CD4^+$ T-cell responses to a novel epitope. *A,* mice were challenged intradermally with 50,000 LiHa cells or 100,000 B16 cells. Percentages of $CD4^+$ T cells in individual tumors. *B* and *C,* mice were challenged with either 50,000 LiHa cells or 100,000 B16 cells. IFN-γ ELISPOT assays with $CD4^+$ cells (*B*) or $CD8^+$ cells (*C*) from tumor-draining lymph node 10 d after tumor challenge. *D,* mice were challenged with 50,000 LiHa cells. 50,000 LiHa-WM455/521 cells were also used to challenge CD4-depleted mice, CD8-depleted mice, or NOD/SCID mice. Tumor growth was followed three times per week for >60 d. *Bars,* SE. Ratios equal the number of mice with tumors over the total number of mice.

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Tyrp1-WM455/521, was created, which contains the MHC I-restricted mutant $WM_{455-463}$ epitope plus a second mutation (E524Y), inserting the I-A^b-restricted $WM_{521-535}$ helper epitope (Fig. 1*A*). Both constructs were expressed in LiHa cells.

Challenge with LiHa-WM455 induced modest $CD8^+$ T-cell responses against the $WM_{455-463}$ epitope in tumor-draining lymph nodes, whereas $CD8^+$ T-cell responses against this epitope were 3-fold higher following challenge with LiHa-WM455/521 (Fig. 3*C*), confirming the role for T-cell help in generating the $CD8^+$ T-cell responses. Importantly, all LiHa-WM455/521 tumors regressed, whereas all LiHa-WM455 tumors progressed (Fig. 3*D*). Regression of LiHa-WM455/521 was dependent on $CD8^+$ T cells, and partially dependent on $CD4^+$ T cells, based on progression of LiHa-WM455/521 tumors in hosts depleted of $CD8^+$ and $CD4^+$ cells and in NOD/SCID mice (Fig. 3*D*). These results show that a single mutation encoding a strongly immunogenic mutant MHC I-restricted epitope was insufficient to trigger regression. However, a combination of mutant MHC I- and II-restricted epitopes was sufficient to induce a regressor phenotype mediated through $CD8^+$ T cells and dependent on $CD4^+$ T-cell help.

Kinetics of $CD8^+$ T-cell responses and tumor infiltration by regulatory cells: predictors of progression versus regression. Studies of concomitant tumor immunity have shown that, following tumor challenge, a critical window exists between days 6 and 12, during which time T-cell responses are induced (6, 33). Events in this window likely determine regression versus progression. Growth of LiHa-WM tumors plateaus between days 6 and 12 (Figs. 1B and 4A) followed by regression or progression. To better characterize this window, mice (10 per group) were challenged with 5,000, 50,000, or 500,000 LiHa-WM cells. Tumor growth plateaued between days 6 and 12 in all groups (Fig. 4A; data not shown), although higher numbers of tumor cells generated higher plateau levels (plateaus at 2, 5, and 7 mm mean diameters, respectively; Fig. 4*A*; data not shown).

Specific $CD8^+$ T-cell responses against mutant $WM_{455-463}$ were evaluated between days 7 and 21. $CD8^+$ T-cell responses peaked between days 7 and 14 in blood (Fig. 4*A*), spleen (Fig. 4*B*), and lymph nodes (data not shown), corresponding to the growth plateau of LiHa-WM tumors, followed by contraction by day 21 (whether or not tumors progressed). Higher doses of LiHa-WM cells did not induce higher $CD8^+$ T-cell responses in blood (data not shown), suggesting that the magnitude of systemic $CD8^+$ T-cell responses is not dependent on antigen dose. Furthermore, $CD8^+$ T-cell responses, measured individually in the blood of 10 mice over time (days 7, 14, and 21 following LiHa-WM challenge), revealed no relationship between levels of T-cell response and tumor regression (data not shown).

To address the question of how the immunogenicity of a tumor affects cellular infiltration, we examined infiltrating cells in LiHa-WM and LiHa-Tyrp1 tumors sequentially between days 7 and 21 (Fig. 4*C*, representative results of two experiments; Supplementary Fig. S2 for gating strategies). On day 10, at the peak of $CD8^+$ T-cell

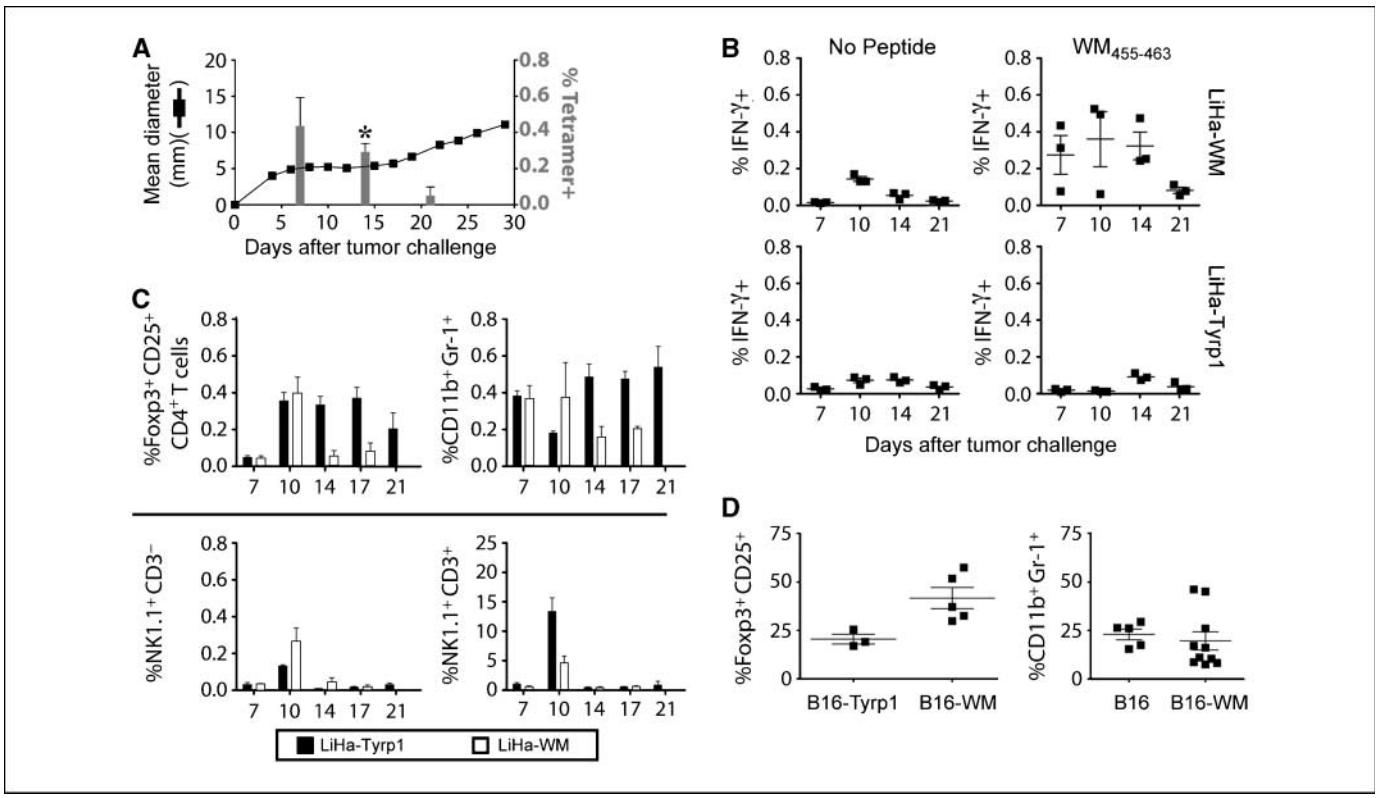

Figure 4. Kinetics of T-cell responses and tumor infiltration. *A,* mice were challenged with 50,000 LiHa-WM cells. *Line,* growth curve of tumors; *squares,* mean tumor diameters ($n = 10$). *Bars,* proportions of tetramer-positive cells in blood. *Left Y axis,* tumor sizes; *right Y axis,* proportions of tetramer-positive cells. *, $P < 0.05$, compared with naive mice. *B,* C57BL/6 mice were challenged with 100,000 LiHa cells. At indicated days after tumor challenge, splenocytes were used for intracellular IFN-γ assays. *C,* two groups of mice were challenged intradermally with LiHa-Tyrp1 (*filled columns*) or LiHa-WM cells (*open columns*). At indicated days after tumor challenge, tumors were analyzed by flow cytometry. Proportions of $Foxp3^+CD25^+$ (*top left*), $CD11b^+Gr\text{-}1^+$ cells (*top right*), $NK1.1^+CD3^-$ (*bottom left*), and $NK1.1^+CD3^+$ (*bottom right*). *D,* proportions of $Foxp3^+CD25^+CD4^+$ T cells (*left*) and $CD11b^+Gr\text{-}1^+$ cells (*right*) in B16 tumors. *Bars,* SE of individual mice.

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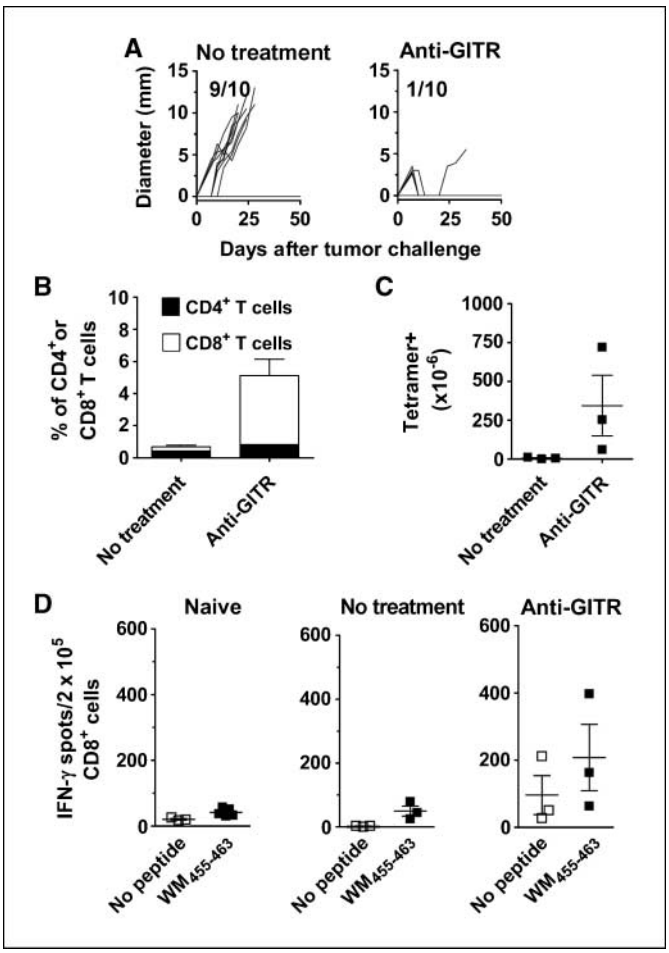

Figure 5. Anti-GITR agonistic antibody treatment augments $CD8^+$ T-cell responses and tumor immunity. *A* to *D,* 100,000 B16-WM cells were injected intradermally in nontreated or anti-GITR–treated mice. *A,* mice were followed for tumor growth three times weekly for >100 d. *B,* frequencies of $CD4^+$ (*filled*)/$CD8^+$ (*open*) T cells in tumors. *C,* frequencies of tetramer-positive cells in tumors were calculated and plotted. *D,* on day 11 after tumor challenge, tumor-draining lymph nodes were used for IFN-γ ELISPOT assays. *Bars,* SE of three mice.

responses to the $WM_{455\text{-}463}$ epitope in draining lymph nodes, spleen, and blood, both LiHa-WM and LiHa-Tyrp1 tumors were infiltrated by $Foxp3^+CD25^+CD4^+$ regulatory T cells (Treg; Fig. 4*C*, *top left*). There was no significant difference in the proportion of Treg cells in LiHa-WM versus LiHa-Tyrp1 tumors on day 10. However, on day 14, the proportion of Tregs decreased significantly in LiHa-WM tumors but remained elevated in progressor LiHa-Tyrp1 tumors out to day 21. B16-WM and B16-Tyrp1 tumors were both infiltrated with Tregs on day 14, unexpectedly with somewhat higher levels in B16-WM tumors (Fig. 4*D*, *left*). These results show that strong T-cell responses against mutant self-peptides do not prevent initial infiltration of Tregs into tumors but rather lead to a dramatic drop in Tregs at the end of the critical window of $CD8^+$ T-cell induction.

Continued infiltration of Tregs beyond the day 6 to 12 window predicted progression; however, tumors develop complex microenvironments, including multiple myeloid, lymphoid, and other bone marrow-derived cell types. A prominent population in mouse and human tumors is made up of myeloid-derived cells, with a $CD11b^+Gr\text{-}1^+$ phenotype and suppressor function (34, 35). Myeloid cells with the $CD11b^+Gr\text{-}1^+$ phenotype were equivalent in both LiHa-WM and LiHa-Tyrp1 tumors on day 7 (Fig. 4*C, top right*; data not shown) but similar to the kinetics of Tregs; this population subsequently decreased >2-fold in LiHa-WM tumors relative to LiHa-Tyrp1 tumors. No differences between B16-WM and B16-Tyrp1 tumors were observed on day 14 (Fig. 4*D*, *right*). Thus, infiltration of myeloid cells precedes Treg infiltration, but the declines in proportions of Tregs and myeloid-derived cells occur synchronously.

We examined two other lymphoid cell types, natural killer (NK) and natural killer-T (NKT) cells, implicated in regulating tumor immunity. Recruitment of both NK and NKT cells in LiHa tumors was prominent on day 10 after tumor challenge but not at other time points (Fig. 4*C*, *bottom*). Notably, LiHa-WM tumors had a higher proportion of NK cells (NK/NKT ratio 2: 1) compared with a higher proportion of NKT cells in LiHa-Tyrp1 tumors (NK/NKT ratio, 1:3.5; Fig. 4*C*, reproduced in two separate experiments). Thus, the earliest event that predicted the regressor phenotype of LiHa-WM tumors was a NK/NKT cell ratio >1 on day 10 followed by declines in Treg and myeloid cell infiltrates.

Immune modulation treatment directed at Treg cells. Abrogation of immunogenicity for B16 tumors in concomitant tumor immunity experiments is determined by $Foxp3^+CD25^+CD4^+$ Tregs (6). Therefore, infiltration of B16-WM tumors by $Foxp3^+CD25^+CD4^+$ Treg cells likely mediates tolerance (Fig. 4*D*). This central role of Treg cells led us to investigate an agonist mAb against GITR (6, 36–39), which has been reported to overcome Treg-dependent tolerance.

The anti-GITR mAb DTA-1 induces signaling through GITR on Tregs to disable suppressor functions (39–42). Mice challenged with B16-WM cells were treated with intraperitoneal injection of anti-GITR mAb DTA-1 on day 4 when tumors were palpable or were left untreated. B16-WM tumors regressed in 15 of 20 DTA-1-treated mice, without recurrence for >100 days, whereas B16-WM tumors progressed in 19 of 20 nontreated mice (results of one of two experiments are shown in Fig. 5*A*). DTA-1 treatment produced a marked infiltration of $CD8^+$ T cells in tumors (Fig. 5*B*) accompanied by an elevated frequency of $CD8^+$ T cells against the mutant $WM_{455\text{-}463}$ epitope in tumors, draining lymph nodes, and blood (Fig. 5*C* and *D*; data not shown).

Tumors are composed of self-molecules and mutated self-molecules, with presumably limited or no adjuvanticity for activation of antigen-presenting cells. We examined the survival of mice challenged with B16-WM and LiHa-WM tumor cells coinjected with ligands for the following innate immune receptors: TLR1 to TLR7, TLR9, Nod2, and the adapter molecule IPS-1 (Supplementary Fig. S3). The TLR9 ligand CpG ODN2395 induced rejection of B16-WM tumors as well as enhanced $CD8^+$ T-cell responses (Supplementary Fig. S4). None of the other ligands had clear effects on tumor progression. Thus, progressor B16-WM tumors were converted to regressor tumors by GITR or TLR9 signaling.

Discussion

The results described in this study reveal the *in vivo* kinetics of immune responses to a dominant mutated MHC class I-restricted epitope, expressed in an altered self-protein by both naturally immunogenic LiHa fibrosarcoma and poorly immunogenic B16 melanoma. The mutated self-antigen used is strongly immunogenic when delivered in vaccines incorporating immune adjuvants

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(e.g., bacterial CpGs). Remarkably, expression of these strongly immunogenic mutations in LiHa fibrosarcoma led to spontaneous regression of established tumors (5-6 mm in diameter) when 5,000 to 25,000 LiHa-WM cells were used for challenge (LD_{50} for parental LiHa is 1,000 cells), whereas expression of the same mutations in poorly immunogenic B16 melanoma was insufficient to induce tumor rejection. A major question emerging from these results is the reason for the difference between immunogenicity and tumor protection induced by these two tumor types. One possibility stems from the fact that the LiHa tumor has acquired other mutations due to chemical induction. These other mutations could theoretically have created helper epitopes, which enhance immunologic potency. Thus, the natural history of an individual tumor, including the relative accumulation of mutations, can have important ramifications on its immunogenicity. We are currently investigating differential expression of growth factors, chemokines, or cytokines in B16 or LiHa cells or tumors by quantitative PCR analysis to determine if this represents an alternative explanation for the difference between the two cell lines.

Treatment targeting Treg cells converts tumors expressing immunogenic mutations into regressors. We have attempted to augment $CD8^+$ T-cell responses and tumor immunity by targeting Treg cells using DTA-1 (6, 36–39). GITR is expressed constitutively at high levels on Tregs ($CD4^+CD25^+$ T cells) and expressed at low levels on resting $CD4^+$ and $CD8^+$ T cells, with up-regulation following T-cell activation. It has been shown that DTA-1 treatment can abrogate suppressive activity of $CD4^+CD25^+$ Tregs *in vitro* (39–42) as well as provide costimulatory signals to effector $CD4^+$ and $CD8^+$ T cells (43–45). In other studies, DTA-1 treatment is efficacious in rejecting not only chemically induced, immunogenic cell lines (38) but also the poorly immunogenic B16 melanoma in a concomitant immunity model (6). Recently, studies from our laboratory indicate that anti-GITR agonistic antibody treatment in B16 models may act through a costimulatory effect on $CD8^+$ T cells (36, 37). Thus, the effects of agonist anti-GITR mAb may involve both Treg suppression and costimulation of previously activated T cells. This combination of inhibiting suppressor cells and stimulating helper and effector T cells by a single agent provides an attractive strategy for cancer therapy.

Relevance to cancer immunology. How the immune system recognizes and responds to mutations expressed by cancer cells is a critical but underappreciated question for the field of cancer immunology. Many reports have rationalized the use of foreign epitopes of microbial pathogens (e.g., from influenza or SV40 viruses) or xenogeneic proteins (e.g., ovalbumin) as mimics of mutated self-proteins. However, most mutations in cancer cells that are recognized by the immune system are point mutations, which convert individual amino acids. Although, in some cases, these point mutations can lead to increased binding to T-cell receptors, in the large majority of cases, point mutations recognized by T cells of both mice and humans produce peptides that have enhanced binding to MHC molecules. For these mutant peptides, the amino acid residues that are bound by T-cell receptors are often left unchanged. For this reason, point mutations in the context of self-amino acid sequences, within the backbone of unaltered self-molecules, are almost certainly not mimicked by foreign polypeptides, such as hen ovalbumin or influenza hemagglutinin.

This work represents the first report characterizing, in detail, spontaneous immune responses induced by tumors expressing immunogenic mutated self-epitopes, restricted by both MHC I and II molecules. The demonstration of regression of LiHa-WM tumors and the progression of B16-WM tumors despite expression of strongly immunogenic mutations emphasizes that the major challenge in generating effective cancer immunity is not simply to understand immune responses to mutated self. It has become apparent that the barriers to effective cancer immunity are erected at the level of the tumor, but we have much to learn about the tumor microenvironment, especially the relationship between and the function of different bone marrow-derived cell types infiltrating tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Immune Rejection of Mouse Tumors Expressing Mutated Self

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