Combination Therapy with HSP90 Inhibitor 17-DMAG
Reconditions the Tumor Microenvironment to Improve
Recruitment of Therapeutic T cells

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Abstract

Ineffective recognition of tumor cells by CD8+ T cells is a limitation of cancer immunotherapy. Therefore, treatment regimens that coordinate promote enhanced antitumor CD8+ T-cell activation, delivery, and target cell recognition should yield greater clinical benefit. Using an MCA205 sarcoma model, we show that in vitro treatment of tumor cells with the HSP90 inhibitor 17-DMAG results in the transient (proteasome-dependent) degradation of the HSP90 client protein EphA2 and the subsequent increased recognition of tumor cells by Type-1 anti-EphA2 CD8+ T cells. In vivo administration of 17-DMAG to tumor-bearing mice led to slowed tumor growth, enhanced/prolonged recognition of tumor cells by anti-EphA2 CD8+ T cells, reduced levels of myeloid-derived suppressor cells and regulatory T cells in the tumor microenvironment, and activation of tumor-associated vascular endothelial cells in association with elevated levels of Type-1 tumor-infiltrating lymphocytes. When combined with EphA2-specific active vaccination or the adoptive transfer of EphA2-specific CD8+ T cells, 17-DMAG cotreatment yielded a superior tumor therapeutic regimen that was capable of rendering animals free of disease. Taken together, our findings indicate that 17-DMAG functions as an immune adjuvant in the context of vaccines targeting EphA2.

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Introduction

Receptor tyrosine kinases (RTK) are an extended family of cell surface proteins (1) that bind growth factors and hormones and play important roles in cell survival, growth, migration, and differentiation (2). In neoplastic/cancerous tissues, RTK overexpression, mutation, and/or constitutive activation may result in uncontrolled proliferation and increased malignant phenotype (3).

EphA2 is an RTK that facilitates intercellular interactions via binding to its ligands ephrin-A1, -A3, -A4, and -A5 expressed on a proximal, opposing cell surface (3). EphA2 is expressed primarily in cells of epithelial origin in a broad range of adult tissues including lung, spleen, and kidney. In addition, EphA2 is expressed by activated endothelial cells and is associated with tissue neovascularization in adults (4–6). Numerous studies have described EphA2 overexpression in a variety of tumors including melanoma, renal cell carcinoma, and colon carcinoma, where the degree of overexpression of this RTK has been linked to poor prognosis and increased metastatic potential (7–9). As a consequence, EphA2 has become an attractive target for therapeutic intervention in patients with solid tumors (10).

Currently, there are several EphA2-centric therapeutic strategies contemplated for translation into clinical trials, including antibody-based strategies that antagonize the binding of EphA2 to its ligands or which block EphA2-mediated signal transduction (11–15). Such approaches would inherently negate intrinsic EphA2-associated protumor effects and provide a degree of (at least transient) therapeutic efficacy that is independent of the host immune system. However, because EphA2 protein levels are stabilized in tumor cells by HSP90 (16, 17), a more therapeutically desirable situation would occur if one were to drive EphA2 degradation via the proteasome, enhancing the likelihood for enhanced MHC class I presentation of derivative peptide epitopes and improved recognition of tumor cells by EphA2-specific CD8+ T cells (18). Because low-to-moderate avidity EphA2-specific CD8+ T cells have been detected in the peripheral blood of patients with renal cell carcinoma or prostate carcinoma (19, 20), levels of circulating CD8+ T cells could also be amplified by vaccination for improved immune targeting of EphA2+ tumor cells in vivo. We report that in vivo administration of the HSP90 inhibitor 17-DMAG enhances EphA2+ tumor cell recognition by specific CD8+ T cells for a period of several days, while concomitantly serving as: (i) a restrictor of myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) and (ii)

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an activator/normalizer of the blood vasculature in the TME. When applied in the context of active immunization or adoptive CD8⁺ T-cell therapy, 17-DMAG co-administration led to enriched frequencies of tumor-infiltrating Type-1 (anti-EphA2) CD8⁺ T cells and coordinately improved treatment outcomes.

**Materials and Methods**

**Mice**

Six- to 10-week-old female C57BL/6 (H-2b), and male and female B6;129S6- *EphA2<sup>tm1Jrui</sup>* (EphA2<sup>−/−</sup>; H-2<sup>b</sup>) mice were purchased from The Jackson Laboratory and maintained in the pathogen-free animal facility in the Biomedical Sciences Tower at the University of Pittsburgh. All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC).

**Tumor cell lines and tumor establishment**

The EphA2<sup>+</sup> MCA205 sarcoma and EphA2<sup>−/−</sup> B16 melanoma (H-2<sup>b</sup>) cell lines were purchased from the American Type Culture Collection (ATCC). Cell lines were cultured in complete media [CM; RPMI-1640 supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 10 mmol/L l-glutamine, and 10% heat-inactivated FBS (all from Life Technologies)] in a humidified incubator at 37°C and 5% CO₂. All cell lines were negative for known mouse pathogens, including mycoplasma. Tumors were established by injection of 5 × 10⁵ MCA205 or 1 × 10⁵ B16 tumor cells s.c. into the right flank of syngeneic mice, with tumor size (in mm²) assessed every 3 to 4 days thereafter. Mice were sacrificed when tumors became ulcerated or they reached a size of 400 mm², in accordance with IACUC guidelines.

**17-DMAG–based therapy**

HSP90 inhibitor 17-DMAG (NSC 707545) was obtained under a material transfer agreement from the Division of Cancer Treatment and Diagnosis at the National Cancer Institute (Bethesda, MD). For in vivo use, tumor-bearing mice were orally administered 17-DMAG or distilled water in a total volume of 50 µL on a daily basis (for up to 10 consecutive days), beginning approximately 18 days after tumor inoculation, when tumors were ~100 mm² in area.

**Isolation of tumor, tumor-draining lymph node, and spleen cells**

Single-cell suspensions were obtained from mechanically disrupted spleen and tumor-draining lymph node, and from enzymatically digested tumors, as previously described (21).

**Western blot**

MCA205 cell lines were grown to 80% to 90% confluence and then incubated with 17-DMAG (10–1,000 nmol/L) in CM for 24 to 48 hours. To assess the impact of proteasome function and endosomal acidification on EphA2 protein degradation promoted by 17-DMAG, MG-132 (50 µmol/L; Peptides International), and chloroquine (50 µmol/L; Sigma-Aldrich), respectively, were added to cells for 3 hours. After washing in PBS, cells were cultured in the presence of 17-DMAG (500 nmol/L) for an additional 24 hours. Harvested cells were then incubated with lysis buffer, and cell-free lysates were resolved by SDS-PAGE before electro-transfer onto polyvinylidene difluoride membranes as previously described (17), before probing with polyclonal anti-EphA2 antibody and horseradish peroxidase-conjugated goat anti-rabbit antibody reagents (both from Santa Cruz Biotechnology). Probed proteins were visualized by the Western Lighting Chemiluminescence Detection Kit (Perkin-Elmer) and exposed to X-Omat film (Eastman Kodak) for 5 to 7 minutes.

**Immunization of EphA2<sup>−/−</sup> mice to generate EphA2-specific CD8⁺ T effector cells**

EphA2<sup>−/−</sup> mice that are not tolerant to ‘self’ EphA2 protein were vaccinated with syngeneic dendritic cells (DC; transduced with recombinant adenovirus encoding mIL-12p70 as previously described to generate DC.II.12, ref. 22) alone or DC.II.12 pulsed with synthetic mEphA2<sub>671</sub>-<sub>679</sub> (FSHHNIRL; H-2Db class I-presented; ref. 23) and mEphA2<sub>682</sub>-<sub>690</sub> (VVSKYKPM; H-2Kb class I-presented; ref. 23) peptides on a weekly basis in the right flank. After 4 vaccinations, CD8⁺ splenic T cells (MACS<sup>™</sup>- selected; Miltenyi Biotec) were analyzed for specific reactivity using CD107 cytotoxicity and IFN-γ ELISA assays.

**CD107 cytotoxicity assay**

CD8⁺ T cells were cocultured with MCA205 tumor cells (either derived from culture or single-cell suspensions of excised tumors) for 6 hours in the presence of anti-CD107 antibody (BD Biosciences). Monensin (Sigma-Aldrich) was added to the culture to prevent the re-internalization of exocytosed CD107 after the first hour of incubation (final concentration = 10 µmol/L). Cultures were allowed to incubate at 37°C for an additional 5 hours before cell harvest and assessment of T-cell surface CD107 expression as monitored by flow cytometry.

**IFN-γ analyses**

For tumor recognition assays, splenic CD8⁺ T cells were cocultured with freshly irradiated (100 Gy at room temperature from a <sup>137</sup>Cs irradiat (GammaCell40, Atomic Energy of Canada Limited, at a dose rate of 0.87 Gy/min) tumor cells for 48 hours, after which, cell-free supernatants were harvested and assessed for mIFN-γ concentrations using a specific ELISA (BD Biosciences). The data are reported as mean ± SD of quadruplicate determinations. In some assays, where indicated, bulk tumor-infiltrating lymphocytes (TIL)/splenocytes were restimulated in vitro with irradiated (100 Gy) MCA205 cells for 5 days at a T-cell-to-tumor cell ratio of 10:1 in CM supplemented with 20 units/mL of recombinant human interleukin-2 (IL-2; PeproTech). Recovered T cells were then cultured in CM alone, with syngenic DCs alone, or DCs pulsed with EphA2 peptides at a 10:1 T-cell-to-DC ratio. In additional assays, CD8⁺ TIL from B16 tumor lesions or CD8⁺ T cells from the spleens of vaccinated EphA2<sup>−/−</sup> mice were cultured with flow-sorted CD31⁺ vascular endothelial cells (VEC) isolated from enzymatically digested B16 tumors or tumor-uninvolved kidneys harvested from untreated or treated animals, as previously described (24). T cells stimulated with 5 µg/mL
anti-CD3 (eBioscience) served as a positive stimulation control. In some assays, as indicated, 1 μg/well (final concentration of 5 μg/mL) of anti-K39/–D53 mAb or isotype control mAb (BD Biosciences) were added to assess the MHC class I–restricted nature of target cell recognition by T cells. For intracellular IFN-γ staining, T cells were assessed after a 6 hours culture using an intracellular cytokine staining kit (BD Biosciences), with stained cells screened using an LSR II flow cytometer (Beckman Coulter) and data analyzed using FlowJo software (Tree Star, Inc.). Levels of IFN-γ in culture supernatants were quantified by specific ELISA.

**Immunofluorescence staining and imaging**

Tumor tissue was processed and sectioned as previously reported (24), followed by immunofluorescence staining and microscopy. The following primary antibodies were used for staining sections: rat anti-mouse CD31 (BD Biosciences), rabbit anti-mouse EphA2 (Santa Cruz Biotechnology), rat anti-mouse VCAM-1, goat anti-mouse CXCL10 (R&D Systems). The following secondary antibodies were used: donkey anti-rat Alexa Fluor 488 (Molecular Probes), donkey anti-rat Cy3 (Jackson ImmunoResearch), donkey anti-rat Cy5 (Jackson ImmunoResearch), goat anti-rat Fab1 fragment Cy3 (Jackson ImmunoResearch), and goat anti-rat Alexa Fluor 488 (Molecular Probes). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining for detection of apoptotic cells was done using a cell death detection kit (Roche Diagnostics) per the manufacturer’s instructions. All tissue sections were briefly incubated with 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and then mounted. Images were captured using an Olympus Provis microscope (Olympus America). Isotype control and specific antibody images were taken using the same level of exposure on the channel settings. MetaMorph (Molecular Devices) software was used for cell quantification.

**Flow cytometry**

Before all cell stainings, Fc receptors were blocked with an anti-CD16/CD32 antibody (Becton Dickinson). Single-cell suspensions were stained using the following fluororescently labeled antibodies: APC- or FITC-conjugated anti-CD4 and –CD8 (eBioscience), FITC-conjugated anti-Gr-1, PE-conjugated anti-CD25, and FITC-conjugated anti-CD11c (all Becton Dickinson); FITC-conjugated anti-Class K39/D53, anti-Class I-Aβ, anti-CD107a and anti-CD107b, PE-conjugated anti-IFN-γ (all eBioscience) and APC-conjugated anti-CD11b and anti-Foxp3 (eBioscience); or matched, fluorochrome-labeled isotype control monoclonal antibody (mAb). For Foxp3 intracellular staining, CD4+ T cells were surface stained as described earlier and then further processed using an APC-conjugated anti-mouse/ rat Foxp3 Staining Kit (eBioscience) according to the manufacturer’s instructions. Fluorescently stained cells were assessed using an LSR II flow cytometer (Beckman Coulter), with data analyzed using FlowJo software (Tree Star, Inc.).

**Statistical analysis**

Comparisons between groups were done using a 2-tailed Student t test or 1-way ANOVA with post hoc analysis, as indicated. All data were analyzed using SigmaStat software, version 3.5 (Systat Software). Differences with a P < 0.05 were considered as significant.

**Results**

**17-DMAG affects tumor RTK expression and viability in a dose-dependent manner**

17-DMAG is an HSP90 inhibitor currently being evaluated in phase I/II clinical trials (25–29). In preliminary in vitro studies, we determined that treatment of tumor cells with 17-DMAG resulted in their loss of EphA2 protein expression, with a clear drug dose dependency (Fig. 1A). Expression of alternate tumor RTKs and known HSP90 client proteins, such as erbB2/Her2 and VEGFR2 (16, 17, 30), and p53 (31), was also inhibited by 17-DMAG treatment in a dose-dependent manner (data not shown). 17-DMAG–induced loss of EphA2 protein expression in MCA205 sarcoma cells was dependent on the proteolytic activity of the proteasome and was not related to the enzymatic action of endosomes/lysosomes. Hence, addition of the proteasomal inhibitor MG-132 to cultures prevented tumor cell EphA2 degradation induced by 17-DMAG treatment, whereas addition of the lysosomal inhibitor chloroquine to cultures had no discernable effect on 17-DMAG–associated EphA2 degradation (Fig. 1B). Treatment of MCA205 cell cultures with 17-DMAG did not modulate the expression of MHC class I molecules on the tumor cell surface (Fig. 1C) or tumor cell viability/apoptotic frequency (data not shown). Notably, EphA2-specific CD8+ T cells developed from EphA2–/– mice (Supplementary Fig. S1) showed increased in vitro recognition of EphA2+ MCA205 (but not EphA2neg B16) tumor cells pre-treated with 17-DMAG (Fig. 1D; Supplementary Fig. S1).

**17-DMAG promotes sarcoma regression in association with the altered immunophenotype of the MCA205 TME**

To determine how 17-DMAG would affect the growth and immunophenotype of well-established (~100 mm3, 18-day-old) tumors, the HSP90 inhibitor was administered orally at doses of 10, 15, and 25 mg/kg once a day for 2, 3, 5, 7, or 10 consecutive days. As shown in Fig. 2A, untreated tumors displayed rapidly progressive growth, whereas tumors in animals treated with 17-DMAG at 10 mg/kg grew more slowly. Tumors in mice treated with 17-DMAG doses ≥15 mg/kg regressed during the 10 days of active drug administration. To analyze the immunophenotype of the TME, treated animals were sacrificed 1 day after the last dose of drug, with enzymatically digested tumors analyzed for immune cell infiltrates and the ability of the freshly isolated tumor cells to be recognized by EphA2-specific CD8+ T cells in vitro. We observed that all doses of 17-DMAG were capable of transiently (maximal on day 5 postinitiation of treatment) increasing the level of tumor-infiltrating CD4+ (Foxp3neg, CD4eff) and CD8+ T effector cells, while reducing the levels of tumor-associated cells bearing a CD11b+ Gr1+ MDSC or CD4+Foxp3+ Treg suppressor cell phenotype (Fig. 2B). Interestingly, in vivo–treated tumor cells were better recognized by anti-EphA2 CD8+ T cells, particularly after 5 days of treatment with 15 mg/kg of 17-DMAG, based on both the CD107 translocation and IFN-γ production.
assays (Fig. 2C and D). Notably, treatment of animals for more than 5 consecutive days with 17-DMAG resulted in the gradual erosion of this optimal day 5 Type-1 immunophenotype in the TME. On the basis these results, all subsequent experiments used a standard 17-DMAG treatment regimen (i.e., 15 mg/kg provided orally for 5 days).

The beneficial effects of 17-DMAG administration persist even after discontinuation of therapy on day 5

To evaluate the durability of 17-DMAG–associated immunomodulation in vivo, MCA205 tumor-bearing mice were treated with 15 mg/kg of 17-DMAG for 5 days and then followed for up to 28 days. As shown in Fig. 3A, treatment with 17-DMAG promoted tumor regression through day 10 (5 days after drug discontinuation), after which time slow tumor growth was observed through day 28. Tumor expression of EphA2 protein in vivo was precipitously reduced during the drug treatment window and only began to return to control levels 10 to 15 days after the discontinuation of drug (Fig. 3B). The ability of anti-EphA2 CD8\(^+\) T cells to recognize in vivo–treated tumor cells remained significantly elevated through day 10 to 14 after treatment initiation (Fig. 3C) and the predominance of CD4\(^+\) and CD8\(^+\) effector cells (and CD11c\(^+\) DC) over regulatory (MDSC and Treg) cells within the treated TME persisted through day 28 in these experiments (Fig. 3D). We also observed that 17-DMAG–treated tumors displayed a prolonged, increased expression of both VCAM-1 and the CXCR3 ligand chemokine CXCL10 in situ, even after discontinuation of this monotherapy (Supplementary Fig. S2). Furthermore, TUNEL staining of tumor sections showed increased frequencies of apoptotic cell death within the TME of 17-DMAG–treated versus untreated MCA205 lesions at all time points through day 28 (Supplementary Fig. S2).

Combination vaccination + 17-DMAG immunotherapy yields superior antitumor efficacy

Given 17-DMAG’s ability to promote the enhanced recognition of treated tumor cells by anti-EphA2 CD8\(^+\) T cells in vivo, and a protective immunophenotype within the TME, we hypothesized that a combination therapy based on active vaccination against EphA2 protein along with 17-DMAG administration would provide superior efficacy against EphA2\(^+\) tumors. In such a paradigm, vaccine-induced, anti-EphA2 CD8\(^+\) T cells would be recruited into the TME based on the ability of 17-DMAG to activate tumor (VCAM-1\(^+\)) endothelial cells, to increase locoregional production of
CXCL10, and to improve the ability of anti-EphA2 Tc1 to recognize MCA205 tumor cells with reduced antagonism from suppressor cell populations in vivo. As shown in Fig. 4A, the combination of 17-DMAG administration plus active vaccination against EphA2 resulted in vastly superior anti-tumor efficacy when compared with treatment with either single modality. Indeed, this combination immunotherapy was the only treatment capable of rendering animals free of disease (80 of 10 cases; i.e., 80%), with "cured" animals competent to reject a corollary re-challenge with MCA205 tumor cells infiltrated by flow cytometry as described in Materials and Methods. Tumor cells isolated from enzymatic digests (per B) were also analyzed as target cells for anti-EphA2 CD8+ T effector cells generated from EphA2-/- mice (see Supplementary Fig. S1) as monitored using CD107 translocation (C) and IFN-γ secretion (D) assays as described in Materials and Methods. All data are representative of those obtained in 3 independent experiments. For B–D, *, P < 0.05; **, P < 0.01 (ANOVA) versus all other determinations.

Figure 2. Treatment of mice bearing established MCA205 tumors with oral 17-DMAG transiently promotes a therapeutically preferred immunophenotype in the TME and is optimally effective in a 5-day regimen. A, C57BL/6 mice bearing established MCA205 tumors (day 18; ~100 mm3 mean tumor size) were left untreated or they were administered 17-DMAG (10, 15, or 25 mg/kg/d for up to 10 days via oral gavage) and tumor size (mean ± SD, 5 animals per group) monitored longitudinally. *, P < 0.05; **, P < 0.01 (ANOVA) for 15 or 25 versus 10 mg/kg/d or untreated; not significant (ANOVA) for 15 versus 25 mg/kg/d. Tumors were excised on the indicated day after initiating treatment, and single-cell suspensions of enzymatic tumor digests analyzed for immune cell infiltrates by flow cytometry as described in Materials and Methods. Tumor cells isolated from enzymatic digests (per B) were also analyzed as target cells for anti-EphA2 CD8+ T effector cells generated from EphA2-/- mice (see Supplementary Fig. S1) as monitored using CD107 translocation (C) and IFN-γ secretion (D) assays as described in Materials and Methods. All data are representative of those obtained in 3 independent experiments. For B–D, *, P < 0.05; **, P < 0.01 (ANOVA) versus all other determinations.
VECs isolated from DMAG (± vaccine)-treated mice in an MHC class I-restricted manner (Fig. 5C), but these T cells failed to recognize tumor-uninvolved kidney-associated VECs or cultured B16 tumor cells (Fig. 5C and D). 

Preconditioning the cancer-bearing host with 17-DMAG enhances the therapeutic efficacy of adoptively transferred anti-EphA2 CD8\(^+\) T cells

On the basis of the recent clinical successes for adoptive T-cell transfer therapy in the cancer setting (32–34), we next examined whether 17-DMAG conditioning of the MCA205 TME would improve the delivery and antitumor effectiveness of EphA2-specific CD8\(^+\) T cells delivered via i.v. injection. EphA2-specific CD8\(^+\) T cells were isolated from the spleens of EphA2\(^{-/-}\) mice previously vaccinated with syngenic DC pulsed with EphA2 peptides (Supplementary Fig. S1). Splenic CD8\(^+\) T cells from EphA2\(^{-/-}\) mice vaccinated with syngenic DC alone (no peptide) served as controls. The optimum time-point for injection of the therapeutic Type-1 EphA2-specific T cells (i.e., day 4) was determined empirically by performing adoptive transfers at various time-points after initiating 17-DMAG treatment (Supplementary Fig. S3). We observed that adoptive transfer of EphA2-specific T cells 4 days after initiating a 5-day course of 17-DMAG (15 mg/kg/d) yielded superior antitumor protection when compared with all other treatment cohorts (Fig. 6A), in concert with improved levels of CD4eff and CD8\(^+\) TIL and reduced levels of tumor-associated regulatory cell populations (Fig. 6B), and the accumulation of anti-EphA2\(^+\) Tc1 in the MCA205 TME (Fig. 6C). We also noted that tumor core necrosis and ulceration occurred uniquely in animals treated with the combination of EphA2-immune T cells + 17-DMAG (necessitating the euthanasia of these regressing mice per the guidelines of our IACUC-approved protocol).

Discussion

The major finding in this report is that the HSP90 inhibitor 17-DMAG functions as an immune adjuvant in the context of vaccines targeting the HSP90 client protein, EphA2. It seems to perform this function in at least 3 ways, by (i) reducing suppressor cell populations such as MDSC and Treg within the TME; (ii) activating the tumor-associated vasculature and promoting locoregional production of chemokines (such as CXCL10) that recruit protective, Type-1 T effector cells, and (iii) enhancing the (proteasome-dependent) processing of tumor EphA2 protein and subsequent recognition of these tumor (and tumor-associated VEC) by anti-EphA2 CD8\(^+\) T cells elicited by specific vaccination or provided via adoptive transfer. These therapeutically beneficial effects of orally...
administered 17-DMAG occurred rapidly, were maximal by day 5 of drug provision, and were sustained for a prolonged period of 1 to 3 weeks (depending on the specific index), as long as treatment with the HSP90 inhibitor was discontinued after a 5-day course. Prolonged application of 17-DMAG for >5 days seems to result in the erosion of its potent adjuvant-like qualities by as early as day 7 in chronic treatment protocols. Why such immunologic silencing occurs upon extended 17-DMAG administration is currently unclear. However, previous studies have suggested the potential attenuating effects of high-dose, long-term dosing of 17-DMAG on the immune system (7, 35, 36). We plan to intensively investigate the mechanism(s) underlying the deleterious effects of more "chronic" 17-DMAG administration in future studies.

The capacity of this combination immunotherapy to target both EphA2+ tumor cells and/or VEC in the TME has important translational ramifications because EphA2+ cancer cells have been reported to be more migratory (greater metastatic potential; refs. 37–39) and the immune regulation of tumor-associated blood vessels reduces concerns for the immunophenotypic status of the tumor cell population (i.e., variation in MHC and antigen expression by heterogeneous populations of tumor cells in the TME). The ability of this treatment strategy to facilitate immune targeting of stromal cells provides the possibility of effectively treating MHC I- or antigen-loss (as modeled by EphA2neg B16) tumor variants.

Importantly, 17-DMAG administration combined with either active vaccination to induce anti-EphA2 Tc1 in vivo (Figs. 4A and 5A) or the adoptive transfer of anti-EphA2 CD8+ T cells (Fig. 6A) proved therapeutically superior to any single component modality. Both combination protocols resulted in the rapid regression of well-established (~day 18 tumors), with a high rate of complete responses in the vaccine setting. Evidence of a protective memory CD8+ T-cell response was evident, given the rejection of a subsequent tumor rechallenge in these mice. The only distinguishing clinical variable between the 2 immunotherapy approaches was the core necrosis observed only for tumors treated with the adoptive

Figure 4. 17-DMAG administration improves the immunogenicity and antitumor efficacy of an EphA2 peptide–based vaccine in the MCA205 tumor model. A, C57BL/6 mice bearing established EphA2+ MCA205 sarcomas (s.c. right flank) remained untreated or they were treated with DC-based vaccines (s.c., left flank on days 0 and 7 of the treatment regimen) that contained or lacked EphA2 peptide epitopes, alone or in combination with 17-DMAG (15 mg/kg/d on the first 5 days of the treatment regimen by oral gavage). Tumor size (mean ± SD) is reported in mm2. All the mice in the DC/EphA2 + 17-DMAG–treated group rendered tumor-free (80%) were rechallenged (s.c., right flank) on day 30 of the experiment (as indicated by arrow with R inset) and monitored through day 60 after treatment initiation. B, CD8+ TILs recovered from tumors on day 14 after treatment initiation were assessed for their ability to recognize syngenic control DCs pulsed with no peptide or DC pulsed with the EphA2(671–679) + EphA2(682–689) peptides. After 48-hour incubation, cell-free supernatants were analyzed for IFN-γ content by ELISA. Response to DC (no peptide) was <50 pg/mL in all instances. C, single-cell suspensions of enzymatically digested day 14 (posttreatment initiation) tumors were analyzed by flow cytometry for the indicated T-cell, DC, and MDSC phenotypes as described in Materials and Methods. Each filled circle represents data from an individual animal in a given control or treatment cohort, with the mean of data indicated by a gray bar for each cohort. All data are representative of those obtained in 3 independent experiments. *, P < 0.05; **, P < 0.01 (ANOVA) versus all other cohorts.
immunotherapy (AIT) approach. The simplest explanations for this biologic difference would reflect: (i) the comparative numbers of specific CD8+ T cells in infiltrating tumors at early time points (i.e., presumed to be greater in the AIT protocol), (ii) the higher functional avidity of the anti-EphA2 Tc1 generated from the EphA2/C0/C0 versus wild-type (self-tolerant) mice allowing these T effector cells to more efficiently recognize tumor cells or VEC expressing modest levels of MHC I-EphA2 peptide complexes on their cell surfaces in vivo, or (iii) possible variance in the polyfunctionality of anti-EphA2 T cells in these treatment cohorts. We are investigating each of these intriguing possibilities in on-going experiments.

HSP90 inhibitors, such as 17-DMAG (alvespimycin), have been investigated in multiple phase I/II clinical trials over the past several years. These drugs exhibited variable anti-tumor efficacy and toxicity when administered as single agents (26, 27, 40–42). In a phase I study of 17-DMAG administered i.v. to patients with advanced solid tumors, objective clinical responses (including 1 complete response) based on RECIST criteria were reported in a minority of patients with kidney or prostate carcinoma, melanoma or chondrosarcoma (26). Like many chemotherapeutic agents, HSP90 inhibitors fail to exert durable anticancer efficacy based on intrinsic disease resistance or the development of acquired resistance among treated populations of cancer cells (43–45). In the case of 17-DMAG, such acquired resistance could be because of the ability of this agent to upregulate expression of the cytoprotective HSP70 and/or Bcl-2 molecules (46, 47). Such clinical limitations reinforce the need to evolve more effective combinational therapeutic strategies.

Our data suggest that sustained therapeutic benefits can be obtained by combining a short (5 day) course of 17-DMAG treatment along with an immunotherapy promoting the CD8+ T-cell targeting of EphA2+ cells in the TME. Given the clinical experience suggesting only moderate efficacy for single-modality HSP90 inhibitors, as well as for antigen-based vaccination in the cancer setting (26, 27, 48–50), combination protocols predicated on these individual treatment modalities would be anticipated to provide superior clinical benefits to patients. Although our modeling has been based on combined vaccine/AIT + 17-DMAG approaches focusing on disease-associated

Figure 5. 17-DMAG improves the antitumor efficacy of an EphA2 peptide–based vaccine in the EphA2neg B16 melanoma model based on immune targeting of EphA2+ VEC. A, C57BL/6 mice bearing established s.c. B16 melanomas (right flank) were left untreated or treated as outlined, with tumor size (mean ± SD) reported in mm2 followed for up to 30 days. **, P < 0.001 (ANOVA) versus all other cohorts. B, day 14 (posttreatment initiation) tumors were harvested and tissue sections analyzed by immunofluorescence microscopy and MetaMorph quantitation for coexpression of CD31 (i.e., VEC) and EphA2 proteins as described in Materials and Methods. *, P < 0.05; **, P < 0.01 (ANOVA) versus all other cohorts. Anti-EphA2 CD8+ T cells isolated from the spleens of immune EphA2−/− mice (C; as outlined in Supplementary Fig. S1), or TIL from B16 tumor–bearing animals treated with combined DC/EphA2 peptide vaccination + 17-DMAG (D; per Supplementary Fig. S5A) were analyzed for reactivity against flow-sorted CD31+ VECs isolated from the tumors of B16-bearing animals left untreated or treated for 6 days with DC/EphA2 vaccine only, 17-DMAG or DC/EphA2 vaccine + 17-DMAG. CD31+ kidney VECs were also flow-sorted from animals treated for 6 days with DC/EphA2 vaccine + 17-DMAG to discern autoimmunity of T cells against tumor-uninvolved VECs. C, the MHC class I–restricted nature of VEC recognition by CD8+ T cells was assessed by inclusion of anti-class I or isotype control mAb per culture well, as described in Materials and Methods. C and D, *, P < 0.05; **, P < 0.01 (t test) versus control antibody treatment or untreated controls, respectively. All data are representative of those obtained in 3 independent experiments. HPF, high-power field.
EphA2 protein, one could also clearly envision similar therapeutic protocols predicated on the immune targeting of one or more alternate HSP90 client proteins that are commonly (over)expressed by tumor cells or tumor-associated stromal cells, such as beclin 1, cyclin B, EGFR, HER2/neu, IGF1-R, PDGFR, PIM-1, STAT3, survivin, TGFβR, VEGFR1, VEGFR2, among many others (16, 30).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: A. Rao, M. Kawabe, W.J. Storkus
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Figure 6. 17-DMAG improves the antitumor efficacy of adoptively transferred anti-EphA2 CD8+ T cells in a combination therapy. A, C57BL/6 mice bearing established s.c. MCA205 sarcomas (right flank) were left untreated or they were treated with 17-DMAG (15 mg/kg/d provided orally on the first 5 days of the treatment regimen) ± adoptive transfer (i.v. tail vein on day 4 of the treatment regimen) of 5 × 106 CD8+ T cells isolated from EphA2–/– mice previously vaccinated with syngenic DCs (control T cells) or DCs loaded with the EphA2671–679 + EphA2682–691 peptides. Tumor size was measured longitudinally and is reported (mean ± SD) in mm2 from 5 mice per group. All animals treated with combined anti-EphA2 (immune) T cell + DMAG therapy required euthanasia because of core necrosis on day 14 after treatment initiation. B, day 14 untreated or treated tumors underwent enzymatic digestion, with single cells analyzed by flow cytometry for the indicated T-cell, DC, and MDSC phenotypes as described in Materials and Methods. Each filled circle represents data from an individual animal/cohort with the data mean indicated by the gray bar. C, CD8+ TILs harvested from day 14 (posttreatment initiation) tumors were analyzed for IFN-γ secretion in response to EphA2 peptide-pulsed syngenic (control) DCs by ELISA as outlined in Materials and Methods. *, P < 0.05; **, P < 0.01 (ANOVA) versus all other cohorts. All data are representative of those obtained in 3 independent experiments.
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