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 coordinately promote enhanced anti-tumor 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 (MDSC) and regulatory T cells (Treg) in the tumor microenvironment (TME), and activation of tumor-associated vascular endothelial cells in association with elevated levels of Type-1 tumor infiltrating lymphocytes (TIL). When combined with EphA2-specific active vaccination or the adoptive transfer of EphA2-specific CD8$^+$ T cells, 17-DMAG co-treatment 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.
INTRODUCTION

Receptor tyrosine kinases (RTKs) 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 pro-tumor effects and provide a degree of (at least transient) therapeutic efficacy that is independent of the host immune system. However, since 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). Since 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 MDSC and Treg, and ii.) 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-2b) mice were purchased from The Jackson Laboratory (Bar
Harbor, ME) 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; Manassas, VA). 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 fetal bovine serum (all from Life
Technologies, Grand Island, NY)] in a humidified incubator at 37°C and 5% CO<sub>2</sub>. All cell
lines were negative for known mouse pathogens, including mycoplasma. Tumors were
established by injection of 5 x 10<sup>5</sup> MCA205 or 1 x 10<sup>5</sup> B16 tumor cells s.c. into the right
flank of syngeneic mice, with tumor size (in mm<sup>2</sup>) assessed every 3 to 4 days thereafter.
Mice were sacrificed when tumors became ulcerated or they reached a size of 400 mm<sup>2</sup>,
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 mm<sup>2</sup> in area.
Isolation of tumor, tumor-draining lymph node (TDLN), and spleen cells. Single-cell suspensions were obtained from mechanically-disrupted spleen and TDLN, and from enzymatically-digested tumors, as previously described (21).

Western blot. MCA205 cell lines were grown to 80-90% confluence and then incubated with 17-DMAG (10-1,000 nmol/L) in CM for 24-48 h. 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, Louisville, KY) and chloroquine (50 μmol/L; Sigma-Aldrich), respectively, were added to cells for 3h. After washing in PBS, cells were cultured in the presence of 17-DMAG (500 nM) for an additional 24h. Harvested cells were then incubated with lysis buffer, and cell-free lysates were resolved by SDS-PAGE prior to electro-transfer onto polyvinylidene difluoride membranes as previously described (17), prior to probing with polyclonal anti-EphA2 antibody and horseradish peroxidase-conjugated goat anti-rabbit antibody reagents (both from Santa Cruz Biotechnology, San Diego, CA). Probed proteins were visualized by the Western Lighting chemiluminescence detection kit (Perkin-Elmer, Waltham, MA) and exposed to X-Omat film (Eastman Kodak, Rochester, NY) for 5-7 min.

Immunization of EphA2 -/- mice to generate EphA2-specific CD8+ T effector cells. EphA2 -/- mice that are not tolerant to “self” EphA2 protein were vaccinated with syngeneic DCs (transduced with recombinant adenovirus encoding mIL-12p70 as previously described to generate DC.IL12; ref. 22) alone or DC.IL12 pulsed with synthetic mEphA2671–679 (FSHHNIIRL; H-2Db class I-presented; ref. 23) and mEphA2682–689 (VVSKYKPM; H-2Kb class I-presented; ref. 23) peptides on a weekly basis in the right flank. After 4 vaccinations, CD8+ splenic T cells (MACSTM-selected; Miltenyi Biotec,
Auburn, CA) were analyzed for specific reactivity using CD107 cytotoxicity and IFN-γ ELISA assays.

**CD107 cytotoxicity assay.** CD8+ T cells were co-cultured 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, San Diego, CA). 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 μM). Cultures were allowed to incubate at 37°C for an additional 5h 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 co-cultured with freshly-irradiated (100 Gy at room temperature from a 137Cs irradiator (Gammacell40, Atomic Energy of Canada Limited, Mississauga, Ontario, Canada at a dose rate of 0.87 Gy/minute) 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 TILs/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, Rocky Hill, NJ). 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 -/- mice were cultured with flow-sorted CD31+ 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, San Diego, CA) served as a positive stimulation control. In some assays, as indicated, 1 μg/well (final concentration of 5 μg/ml) of anti-Kb/-Db 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 h 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, San Diego, CA), rat anti-mouse VCAM-1, goat anti-mouse CXCL10 (R&D Systems, Minneapolis, MN). The following secondary antibodies were used: donkey anti-rat Alexa Fluor 488 (Molecular Probes, Eugene, OR), donkey anti-goat Cy3 (Jackson ImmunoResearch, West Grove, PA), donkey anti-rat Cy3 (Jackson ImmunoResearch), goat anti-rat Fab1 fragment Cy3 (Jackson ImmunoResearch), and goat anti-rat Alexa Fluor 488 (Molecular Probes). TUNEL staining for detection of apoptotic cells was performed 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, Center Valley, PA). Isotype control and specific
antibody images were taken using the same level of exposure on the channel settings. Metamorph (Molecular Devices, Sunnyvale, CA) 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 fluorescently-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 K\(^b/D^b\), anti-Class I-A\(^b\), anti-CD107a and anti-CD107b, PE-conjugated anti-IFN-\(\gamma\) (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 above 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 performed using a two-tailed Student's t test or one-way Analysis of Variance (ANOVA) with post-hoc analysis, as indicated. All data were analyzed using SigmaStat software, version 3.5 (Systat Software, USA). Differences with a p-value < 0.05 were considered as significant.
RESULTS

17-DMAG affects tumor RTK expression and viability in a dose-dependant 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 dependant on the proteolytic activity of the proteasome and was not related to the enzymatic action of endosomes/lysosomes. Hence, addition of the proteosomal inhibitor MG132 to cultures prevented tumor cell EphA2 degradation induced by 17-DMAG treatment, while addition of the lysosomal inhibitor chloroquine (CLQ) 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 (Fig. S1) demonstrated increased in vitro recognition of EphA2+ MCA205 (but not EphA2neg B16) tumor cells pretreated with 17-DMAG (Fig. 1D and 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 mm², 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, while tumors in animals treated with 17-DMAG at 10 mg/kg
grew more slowly. Tumors in mice treated with 17-DMAG doses $\geq 15$ mg/kg regressed during the 10 days of active drug administration. To analyze the immunophenotype of the TME, treated animals were sacrificed one 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 \textit{in vitro}. We observed that all doses of 17-DMAG were capable of transiently (maximal on day 5 post-initiation of treatment) increasing the level of tumor infiltrating CD4$^+$ (Foxp3$^{neg}$; 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 (\textbf{Fig. 2B}). Interestingly, \textit{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-$\gamma$ production assays (\textbf{Fig. 2C and 2D}). 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. Based on these results, all subsequent experiments used a standard 17-DMAG treatment regimen (i.e. 15 mg/kg provided orally for 5 days).

\textbf{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 \textit{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 \textbf{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 \textit{in vivo} was precipitously reduced during the drug treatment window and only began to return to control levels 10-15 days after the discontinuation of drug (\textbf{Fig. 3B}). The ability of anti-EphA2 CD8$^+$ T cells to recognize \textit{in
in vivo treated tumor cells remained significantly elevated through day 10-14 after treatment initiation (Fig. 3C) and the predominance of CD4+ and CD8+ T 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, increase in expression of both VCAM-1 and the CXCR3 ligand chemokine CXCL10 in situ, even after discontinuation of this monotherapy (Fig. S2). Furthermore, TUNEL staining of tumor sections demonstrated increased frequencies of apoptotic cell death within the TME of 17-DMAG-treated versus untreated MCA205 lesions at all time points through day 28 (Fig. S2).

**Combination vaccination + 17-DMAG immunotherapy yields superior anti-tumor efficacy.** Given 17-DMAG’s ability to promote the enhanced recognition of treated tumor cells by anti-EphA2 CD8+ T cell 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 to treatment with either single modality. Indeed, this combination immunotherapy was the only treatment capable of rendering animals free of disease (in 8 of 10 cases; i.e. 80%), with “cured” animals competent to reject a corollary re-challenge with MCA205 tumor cells (Fig. 4A). An analysis of TIL harvested from the various treatment groups supports the superior
induction/recruitment of Type-1 anti-EphA2 CD8+ T cells (Fig. 4B) and improved population of tumor lesions by CD4+ and CD8+ T effector cells and CD11c+ DC over regulatory immune cell subsets (Fig. 4C) after vaccine + 17-DMAG combination therapy.

We have previously shown that EphA2 peptide-based vaccination was capable of slowing the growth of EphA2neg B16 melanoma progression in syngenic mice (based on the hypothesized CD8+ T cell targeting of EphA2+ vascular endothelial cells in the TME; ref. 24). As a consequence, we next chose to evaluate whether the combined EphA2-based vaccine + 17-DMAG therapy established in the MCA205 model would provide a superior level of protection against B16 progression. As shown in Fig. 5A, DC/EphA2 peptide vaccination or 17-DMAG alone served to slow B16 tumor growth, while the combination therapy led to disease stabilization for over 30 days after initiating treatment. Immunofluorescence microscopy and Metamorph quantitation of B16 tumor sections suggested fewer EphA2+CD31+ vascular endothelial cells (VEC) in animals treated with DC/EphA2 vaccine only, DMAG only and DC/EphA2 vaccine + DMAG, with the most striking reductions occurring in the DC/EphA2 vaccine + 17-DMAG cohort (Fig. 5B). To further investigate the therapeutic targeting of tumor-associated EphA2+ VEC in the B16 melanoma model, we isolated CD31+ VEC by flow-sorting from enzymatic digests of B16 tumors and/or tumor-uninvolved kidneys excised from untreated versus treated animals, and analyzed the ability of these target cells to be recognized by anti-EphA2 CD8+ T cells developed from EphA2 -/- mice (Fig. 5C) or by CD8+ TIL isolated from mice treated with the superior combined therapy (i.e. DC/EphA2 peptide vaccine + 17-DMAG) 6 days after the initiation of treatment (Fig. 5D). We observed that anti-EphA2 Tc1 populations preferentially recognized tumor-associated VEC 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 VEC or cultured B16 tumor cells (Fig. 5C, 5D).

**Pre-conditioning the cancer-bearing host with 17-DMAG enhances the therapeutic efficacy of adoptively transferred anti-EphA2 CD8\(^+\) T cells.** Based on 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 anti-tumor 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 (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 (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/day) yielded superior anti-tumor protection when compared to 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 appears 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 1-3 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 appeared 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 since 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 EphA2\textsuperscript{neg} B16) tumor variants.

Importantly, 17-DMAG administration combined with either active vaccination to induce anti-EphA2 Tc1 \textit{in vivo} (Fig. 4A, 5A) or the adoptive transfer of anti-EphA2 CD8\textsuperscript{+} 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\textsuperscript{+} T cell response was evident, given the rejection of a subsequent tumor re-challenge in these mice. The only distinguishing clinical variable between the two immunotherapy approaches was the core necrosis observed only for tumors treated with the adoptive immunotherapy (AIT) approach. The simplest explanations for this biologic difference would reflect: i.) the comparative numbers of specific CD8\textsuperscript{+} T cells 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 (-/-) 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 \textit{in vivo}, or iii.) possible variance in the poly-functionality 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 anti-cancer 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 due to 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 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).
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FIGURE LEGENDS

**Figure 1. 17-DMAG promotes proteasome-dependent EphA2 protein degradation in MCA205 sarcoma cells and the enhanced recognition of tumor cells by anti-EphA2 CD8⁺ T cells in vitro.**

**A,** MCA205 tumor cells were treated with various doses of 17-DMAG for 24h in vitro, then lysed, with EphA2 and control β-actin protein expression subsequently monitored by western blotting as described in Materials and Methods. NC; negative control lysate from EphA2neg B16 melanoma cells. In **B,** proteasome inhibitor (MG-132), but not lysosome inhibitor chloroquine (CLQ), blocks 17-DMAG (500 nM)-induced degradation of EphA2 protein in MCA205 tumor cells. In **C,** treatment of MCA205 cells with 17-DMAG at the indicated doses for 24h (or 48h, data not shown) did not affect MHC class I expression on tumor cells. In **D,** 17-DMAG-treated EphA2⁺ MCA205 cells were better recognized versus control, untreated tumor cells by anti-EphA2 CD8⁺ T cells (developed from EphA2 -/- mice, per Fig. S1 and Materials and Methods) in CD107 translocation assays as described in Materials and Methods. All data are representative of those obtained in 3 independent experiments.

**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 mm² mean tumor size) were left untreated or they were administered 17-DMAG (10, 15 or 25 mg/kg/day for up to 10 days via oral gavage) and tumor size (mean +/- SD, 5 animals/group) monitored longitudinally. *p < 0.05; **p < 0.01 (ANOVA) for 15 or 25 mg/kg/day versus 10 mg/kg/day or untreated; not significant (ANOVA) for 15 versus 25 mg/kg/day. In **B,** 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 Fig. 2B) were also analyzed as target cells for anti-EphA2 CD8⁺ T effector cells generated from EphA2 -/- mice (see 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 panels B-D, *p < 0.05; **p < 0.01 (ANOVA) versus all other determinations.

**Figure 3. The impact of 17-DMAG-based therapy for 5 days persists after discontinuation of drug delivery.** A, MCA205 tumor-bearing mice (5 mice/group) were left untreated or they were treated for 5 days with orally-administered 17-DMAG (15 mg/kg/day), with tumor growth then monitored over a 4 week period. B, EphA2 protein expression in tumors harvested from 17-DMAG-treated versus untreated mice was analyzed longitudinally by western blotting as outlined in Materials and Methods. C, Tumor cells from untreated or 17-DMAG-treated mice were analyzed at the indicated time points for their ability to be recognized by anti-EphA2 CD8⁺ T cells generated from EphA2 -/- mice (see Fig. S1) in CD107 translocation and IFN-γ secretion assays, as described in Materials and Methods. D, Single cell suspensions from harvested tumor digests were analyzed by flow cytometry for the indicated T cell, DC and MDSC phenotypes. All data are representative of those obtained in 3 independent experiments. *p < 0.05 (t-test) for treated versus untreated controls.

**Figure 4. 17-DMAG administration improves the immunogenicity and anti-tumor 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/day 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) with MCA205 tumor cells on day 30 of the experiment (as indicated by arrow with “R” inset) and monitored through day 60 after treatment initiation. B, CD8⁺ TIL recovered from tumors on day 14 after treatment initiation were assessed for their ability to recognize syngenic control DC pulsed with no peptide or DC pulsed with the EphA2⁶⁷¹-⁶⁷⁹ + EphA2⁶₈₂-⁶₈⁹ peptides. After 48h 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 (post-treatment 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.

**Figure 5. 17-DMAG improves the anti-tumor 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 in Fig. 5A, 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 (post-treatment initiation) tumors were harvested and tissue sections analyzed by immunofluorescence microscopy and Metamorph quantitation for co-expression of CD31 (i.e. VEC) and EphA2 proteins as described in Materials and Methods. *p < 0.05 (ANOVA) versus all other cohorts. Anti-EphA2 CD8⁺ T cells isolated from the spleens of
immune EphA2 -/- mice (panel C; as outlined in Fig. S1), or TIL from B16 tumor-bearing animals treated with combined DC/EphA2 peptide vaccination + 17-DMAG (panel D; per Fig. 5A) were analyzed for reactivity against flow-sorted CD31+ VEC 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 VEC 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 VEC. In 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. For panels C and D, *p < 0.05 (t-test) versus control antibody treatment or untreated controls, respectively. All data are representative of those obtained in 3 independent experiments.

**Figure 6. 17-DMAG improves the anti-tumor 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/day 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 x 10^6 CD8+ T cells isolated from EphA2 -/- mice previously vaccinated with syngenic DC (control T cells) or DC loaded with the EphA2_{671-679} + EphA2_{682-689} peptides. Tumor size was monitored longitudinally and is reported (mean +/- SD) in mm^2 from 5 mice/group. All animals treated with combined anti-EphA2 (immune) T cell + DMAG therapy required euthanasia due to core necrosis on day 14 after treatment initiation. In 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. In C, CD8⁺ TIL harvested from day 14 (post-treatment initiation) tumors were analyzed for IFN-γ secretion in response to EphA2 peptide-pulsed syngenic (control) DC 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.
Fig. 2 Rao et al.
Fig. 3 Rao et al.
Combination therapy with HSP90 inhibitor 17-DMAG reconditions the tumor microenvironment to improve recruitment of therapeutic T cells

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