INTEGRATION OF HSP90 INHIBITION IN COMBINATIONAL IMMUNOTHERAPIES TARGETING RECEPTOR TYROSINE KINASE EPHA2

by

Aparna Rao

B.S. Biology, University of Mumbai, 2004
M.S. Applied Biology, University of Mumbai, 2007

Submitted to the Graduate Faculty of
School of Medicine in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

University of Pittsburgh
2012

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Aparna Rao

It was defended on

July 18, 2012

and approved by

Robert J. Binder Ph.D, Assistant Professor, Department of Immunology

Jeffrey L. Brodsky Ph.D, Professor, Department of Biological Sciences

Robert L. Ferris M.D, Ph.D, Professor, Department of Surgery

Soldano Ferrone Ph.D, Professor, Department of Surgery

Theresa Whiteside Ph.D, Professor, Department of Pathology

Dissertation Advisor: Walter J. Storkus Ph.D, Professor, Department of Immunology

ABSTRACT

INTEGRATION OF HSP90 INHIBITION IN COMBINATIONAL IMMUNOTHERAPIES TARGETING RECEPTOR TYROSINE KINASE EPHA2

Limitations in CD8+ T cell recognition of tumor cells due to defects in their antigen processing machinery or the selection of variants expressing low or absent levels of cognate tumor antigens have been previously identified as impediments to effective cancer immunotherapy. Hence, treatment regimens that coordinately promote enhanced activation of anti-tumor CD8+ T cells, improved delivery of such effector cells into tumor sites, and augmented recognition of tumor or tumor-associated stromal cells by therapeutic CD8+ T cells, would be expected to yield greater clinical benefit. Using an MCA205 sarcoma model, I 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: i.) slowed tumor growth; ii.) enhanced/prolonged recognition of tumor cells by anti-EphA2 CD8+ T cells; iii.) reduced levels of myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) in the tumor microenvironment (TME); and iv.) 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 cotreatment yielded a superior tumor therapeutic regimen that was capable of rendering animals free of disease.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

I would first like to thank my mentor, Dr. Walter J. Storkus. I feel extremely fortunate and honored to have spent my graduate study under the guidance of such an exceptional scientist and human being. He provided me with extraordinary scientific guidance, and managed to keep me motivated and enthusiastic about my project at all times. I would next like to thank my Thesis Committee for their suggestions and guidance throughout my study here. I would also like to thank all the people who have been a part of the Storkus Lab. I could not have asked for better lab mates who doubled up as great friends, and with whom I have shared many great memories.

Finally, I would like to extend a special thank you to my family for their unconditional support and love throughout my graduate study. I greatly appreciate their understanding when I decided to pack my bags and move half way across the globe to pursue a PhD. Finally, I would like to thank my fiancé Sushant for standing by me at every step along the way. I dedicate this thesis to all of you with love.

CHAPTER 1. INTRODUCTION

Receptor tyrosine kinases (RTKs) are broadly over-expressed on the surface of tumor cells, effectively contributing to the metastatic and proliferative potential of these cells. While increased activation has been associated with carcinogenesis, it is also clear that decreased deactivation and subsequent reduced internalization of the receptor plays a role in tumor progression [1]. For this purpose, antibody agonists and inhibitors of small molecules designed to block signaling mediated by RTKs have been developed [2]. However, intervention by single agents has demonstrated modest efficacy, at best. A desirable setting from the immunological point of view would be to have reduced expression of RTKs on the tumor cell surface, while simultaneously increasing their presentation as a cognate antigen on MHC class I molecules. This would have a two-fold advantage of reducing oncogenicity as well as increasing immunogenicity. Thus immunotherapy combining increased presentation of RTKs along with administration of RTK-specific T cells would be a highly effective tool for anti-tumor therapy. HSP90 is a molecular chaperone protein that facilitates the sustained overexpression of its client proteins, including RTKs, in tumors [3,4]. Therefore, HSP90 inhibitors have gained increasing importance due to their ability to facilitate the selective degradation of RTKs. 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) is the newest generation geldanamycin analogue available, which has been tested to have reduced toxicity. It also has an added advantage of increased bioavailability, due to its water-soluble nature [5,6]. Previous studies in our lab have looked at the effect of 17-DMAG on increasing RTK presentation on *human* tumor cells *in vitro.* These studies have shown that HSP90 inhibition in *human* tumor cells conditions them to be more effectively targeted by RTK-specific T cells [7]. *In vivo* studies involving 17-DMAG have focused on its toxicity and anti-tumor activity in mice, but no studies have looked at the potential of the drug to increase RTK presentation. Therefore, in order to assess the clinical significance of 17-DMAG treatment for enhancing anti-tumor immunotherapy with RTK-specific T cells, there was a need to recapitulate our studies in the mouse model. We hypothesized that increasing RTK presentation on mouse tumor cells *in vivo* by 17-DMAG treatment will enhance anti-tumor immunotherapy. For our studies, we focused on EphA2, an RTK that is commonly over-expressed in most cancers, and plays an

important role in their growth and metastasis [8]. We observed 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: i.) slowed tumor growth; ii.) enhanced/prolonged recognition of tumor cells by anti-EphA2 CD8+ T cells; iii.) reduced levels of myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) in the tumor microenvironment (TME); and iv.) 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 cotreatment yielded a superior tumor therapeutic regimen that was capable of rendering animals free of disease.

We believe these studies will advance the use of 17-DMAG in concert with anti-EphA2 immunotherapy in the clinical setting for cancer therapy.

1.1. Receptor tyrosine kinases – function and life cycle

Receptor tyrosine kinases (RTKs) are receptors for important growth factors and hormones. About 20 families of RTKs have been identified – and they include receptors of the EGFR, PDGFR and Eph family [9]. In a normal cell in steady state, RTKs mediate various signaling processes that play a role in cell growth, differentiation and survival [10]. Usually, ligand binding to an RTK leads to activation and dimerization of an RTK (either heterodimerization or homodimerization), and phosphorylation of tyrosine kinase residues in the cytoplasmic domain of the RTK [11-13]. Subsequently, phosphorylation sites act as docking sites for various signaling adaptor proteins like SH2, thus triggering binding of other signaling molecules and initiating an entire signaling cascade that mediate essential cell survival and growth processes [14]. Once signaling has been

initiated, RTKs are targeted for ubiquitination and the RTK-ligand complex is endocytosed in clathrin-coated vesicles [15]. After endocytosis, the RTK and the ligands are separated in sorting endosomes. Here, one of two scenarios may occur – i.) the RTK and ligand may progress through the endocytic pathway into lysosomes and where they are degraded, thus ending the signaling reaction ii.) in another scenario, the RTK and ligand are separated into different vesicles in the sorting endosomes, and the ligand progresses for degradation in the lysosome, while the RTK is recycled and sent back to the cell surface where it can initiate another round of signaling [16]. Thus, in a normal cell, RTK signaling is a tightly regulated process. This process is defective in cancerous cells, mostly due to mutations in the phosphorylation sites (leading to a sustained state of phosphorylation/activation) or in the ubiquitin binding sites (preventing endocytosis of the active RTK), thus causing sustained expression and signaling through the RTKs, and leading to uncontrolled cell growth, differentiation and survival – thereby sustaining tumorigenesis [13,17-21].

1.2. Receptor tyrosine kinase EphA2

1.2.1. Eph Receptor and Ephrin Ligands

The Eph family of receptors is comprised of 16 members, making it the largest known group of receptor tyrosine kinases (for nomenclature, refer to http://ephnomenclature.med.harvard.edu/cell_letter.html) [22]. They have been divided into classes- EphA and EphB, depending on sequence homology. Correspondingly, two classes of interacting ligands – ephrin-A and ephrin-B, have been defined. The EphA receptor family consists of 10 members (EphA1-10), and these have been observed to promiscuously bind 6 ephrin-A ligands (ephrinA1-ephrinA6). The EphB receptor family consists of 6 members (EphB1-EphB6) that bind ephrinB transmembrane ligands (ephrinB1-ephrinB3) [8,23]. However, dedicated binding of EphA and EphB receptors to ephrinA and ephrinB ligands, respectively, doesn't seem to be a steadfast rule. As an

example, Ephrin-B molecules bind with low affinity to EphA4, whereas ephrin-A5 is known to interact with EphB2 [24-26].

1.2.2. Eph receptors and Ephrins – structure and life cycle

Eph receptors are transmembrane glycoproteins with an extracellular domain (that plays a role in ligand binding) and a cytoplasmic domain that possesses tyrosine kinase activity. The extracellular domain is unique to the Eph receptor family and consists of a ligand binding globular domain, a cysteine-rich region and two fibronectin type III repeats that mediate receptor dimerization. The cytoplasmic domain consists of a transmembrane region with two autophosphorylation sites, a kinase domain, a PDZ-binding motif and a SAM domain that plays a role in receptor dimerization [8], Figure 1.

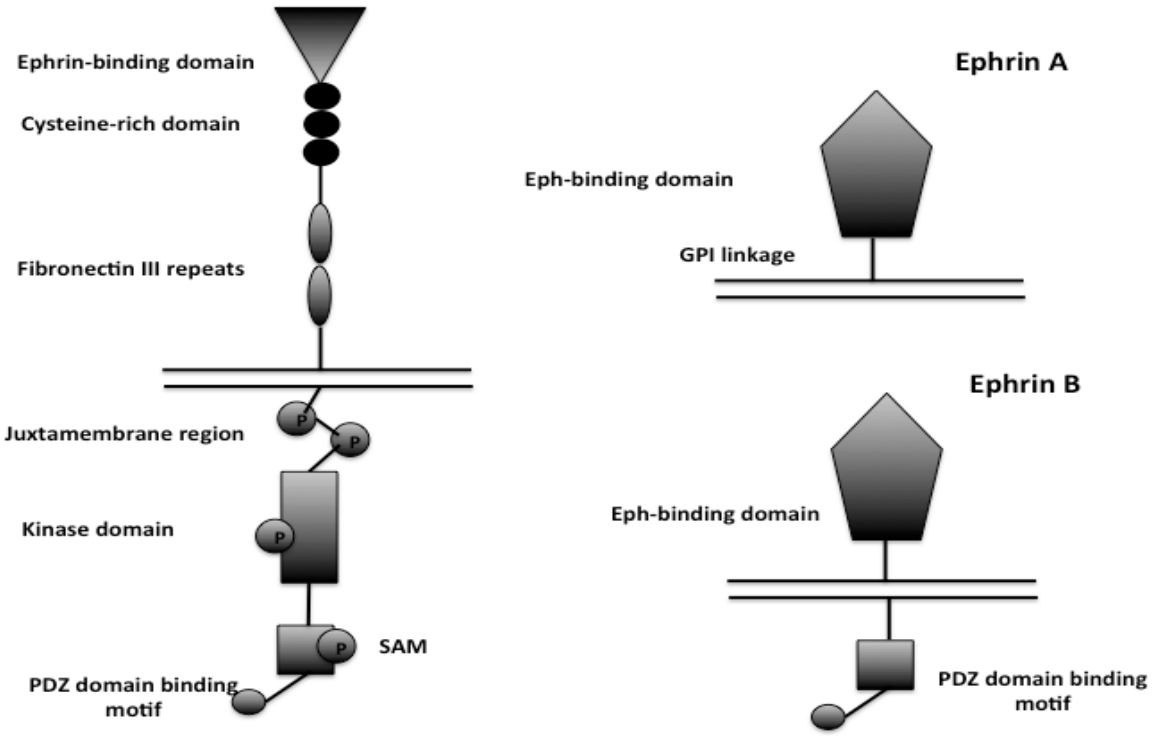


Figure 1: Structure of Eph receptors (left) and Ephrin A and B ligands (right)

Binding of Eph receptors to their ligands leads to their homo/heterodimerization with another receptor-ligand complex to form tetrameric complexes, causing receptor activation and autophosphorylation. This initiates a series of signaling events that are specific for the receptor and the type of cells they are expressed in. Generally, Eph receptors and their ligands are expressed abundantly during fetal development and at lower levels in normal adult somatic tissues [8].

Eph receptors were originally observed to be involved in a number of processes during embryonic development viz. neural crest cell migration, developing tissue boundaries and axon guidance and development [27-30]. Recently, however, they have also been implicated in adult tissues where signaling through the receptor modulates the attachment, shape and motility of the cells by affecting expression of integrins and adhesion molecules on the cell surface [31-38]. In addition, Eph receptors (especially EphA2) have also been implicated in neo-angiogenesis in adult tissues as well as tumors [23,39,40].

1.3. EphA2

The EphA2 gene is located on human chromosome 1, and encodes a 130 kDa, 976 amino acid Type-1 glycoprotein. EphA2 binds to ephrin-A1, -A3, -A4, -A5, but does not require ligand binding for its enzymatic activity [39]. Along with other Eph/ephrin family members, EphA2 is involved in the organization of the nervous system and vasculature during embryonic development, and has been observed to be expressed on a subset of stem cells as well [41-43]. In normal adult tissues, EphA2 is expressed at low levels on a broad range of epithelial tissues, where it is principally localized to sites of cell-to-cell contact, and may play a role in contact inhibition of cell growth/migration that is critical for the organization and formation of epithelial layers in EphA2+ tissues [44,45]. EphA2 is also expressed by endothelial cells where it contributes to normal tissue (and tumor) neovascularization in the adult. In addition, EphA2 expression on Langerhans- and

interstitial-type dendritic cells (DC) has been reported, suggesting the possible role(s) of EphA2 in the localization and networking functions of Langerhans cells in the epithelium, as well as their ability to traffic and stimulate T cells under the appropriate activating conditions [46-48].

1.3.1. Regulation of EphA2 Expression

1.3.1.1. EphA2 Expression and Life Cycle in Non-transformed Tissues

In normal adult tissues, EphA2 is expressed at low levels on a diverse array of epithelial tissues, where it may stably bind to its ligand, ephrin-A1 present on the surface of neighboring cells [49,50]. In contrast, malignant cells express high levels of EphA2 protein, but these molecules only poorly bind to relevant ligands [51]. The EphA2 gene is expressed most highly in tissues that contain a high proportion of epithelial cells (viz. skin, small intestine, and lung). However, lower expression levels have been detected in kidney, brain, and spleen. Very low EphA2 protein levels have been observed in heart, skeletal muscle, liver, testes, and thymus [44] .

Upon ligand binding, EphA2 seems to follow a similar internalization/degradation pathway as seen with other RTKs (as described earlier). Using ephrinA1-Fc fusion protein or EphA2-specific agonistic antibody, it has been shown that once ligand binding takes place, the EphA2 receptor interacts with c-Cbl protein and gets targeted for degradation through the proteasome. Moreover, these studies suggest that cellular levels of EphA2 protein are regulated by various proteins (Cbl, PTPs, etc.) and are dependent on ligand-based stimulation [52-55]. One interesting observation is that active EphA2 may be found in a non-phosphorylated state. Therefore, in the absence of ligand binding signals, EphA2 exhibits constitutively active enzyme activity, and yet this does not appear to accelerate the turnover of EphA2 protein. Such deregulation may result in the

overexpression of the EphA2 protein and a progressive accumulation of EphA2 signal strength (supporting tumorigenesis) [52].

1.3.1.2. Genetic Regulation of EphA2

EphA2 is generally present in low levels in adult tissues, while the levels increase 10-100 fold in tumors. Therefore, a number of studies have focused on elucidating the mechanisms that regulate EphA2 expression in normal cells but not tumor cells. The murine EphA2 promoter contains binding sites for Hoxa1 and Hoxb1 homeobox transcription factors, which appear to upregulate EphA2 expression during brain development [53]. Induced expression of E-cadherin has also been observed to increase EphA2 expression. In addition, several members of the p53 family have also been observed to regulate EphA2 expression, and p53 has a binding site in the EphA2 promoter region where it can bind in response to DNA damage [56].

EphA2 transcription is also enhanced by Ras-Raf-MAPK kinase pathway activation. Transforming mammary epithelial cells by Ras overexpression also induced EphA2 overexpression [38]. Raf activation has also been known to directly induce EphA2 mRNA and protein expression [57]. In addition, various stimuli/stressors that invoke the Ras-Raf-MAPK signaling cascade are potent inducers of EphA2 expression e.g. Interleukin (IL)-1ß, IL-2, and EGF. Deoxycholic acid, a well-known constituent of bile acid and cancer promoter, upregulates EphA2 expression, at least partially via activation of the MAPK pathway [58,59]. In addition, systemic injection of lipopolysaccharide (LPS; a MAPK activator via TLR2/4 stimulation) increased EphA2 mRNA expression in the liver [60]. Thus, although a number of factors have been identified in EphA2 regulation, further studies will be required to gain a comprehensive picture of EphA2 gene regulation.

1.3.2. Role of EphA2 in Cancer

1.3.2.1. Overexpression of EphA2 in Cancer

EphA2 overexpression has been reported in a variety of cancers, with differences being more pronounced in metastatic tumors versus non-invasive cancer cells. Metastatic prostate cancer cells express 10-100 times greater levels of EphA2 protein expression versus prostate epithelial cells [61]. Similar observations noted for malignant mammary tumor cells and pancreatic adenocarcinoma cells (when compared to benign mammary epithelial cells and non-invasive pancreatic cells respectively) [62,63]. Herrem *et al.* reported that EphA2 is expressed in metastatic RCC cell lines at levels significantly higher than primary RCC cell lines, an observation replicated in freshly resected clinical samples. In addition, we compared RCC and normal adjacent kidney (NAK) tissues and reported that higher levels of EphA2 were present in significantly advanced and more vascularized tumors [64].

Based on these reports, it could be argued that EphA2 protein levels can be used to predict prognosis and clinical outcome in patients with EphA2+ tumors. To support this hypothesis, our group has shown that the degree of EphA2 overexpression by RCC tissues (versus normal autologous kidney tissue) is predictive of short-term (<1 year) versus longer-term (1 year or more) disease-free status, as well as, overall survival. Also, patients that had tumors with low EphA2 levels were more likely to remain disease-free for a longer period of time, while those with tumors expressing high levels of EphA2 relapsed quickly and survived for shorter periods of time [64].

1.3.2.2. Mechanisms of EphA2 Overexpression in Cancer

Mechanisms by which EphA2 is consistently over-expressed in tumor cells have not been completely elucidated, and hence is the subject of much investigation. Possible

mechanisms could include EphA2 gene amplification, decreased rates of protein degradation, or a combination of both. Another reported mechanism proposes that miR-26b may act as a tumor suppressor in glioma and it directly regulates EphA2 expression. EphA2 is a direct target of miR-26b, and the down-regulation of EphA2 mediated by miR-26b may be dependent on the binding of miR-26b to a specific response element of microRNA in the 3'UTR region of EphA2 mRNA [65]. An attractive hypothesis with EphA2 over-expression by tumor cells involves the disruption of EphA2 homeostatic protein degradation. For EphA2 to be properly degraded, it needs to get phosphorylated, and internalized. Any defect in this process may result in the accumulation of cell surface EphA2 molecules [38,51,66]. Of particular interest in this regard is LMW-PTP, a phosphatase which preferentially dephosphorylates pEphA2, and which is overexpressed in several form of cancer. Notably, overexpression of LMW-PTP by tumor cells is associated with decreased levels of tumor pEphA2, and enhanced levels of EphA2 on the tumor cell surface [66-68].

Additional/alternative mechanism(s) linked to elevated EphA2 expression may involve E-cadherin expression on tumors. E-cadherin is a major protein involved in cellular adhesion, and is localized in adherent junctions formed between adjoining cells in tissues, the same sites as which EphA2 is expressed on tumors. In order to mediate signaling, EphA2 needs to bind its corresponding ligand (i.e. ephrin-A1) on adjacent cells, and in the case of tumor cells, this ability appears to be affected due to unstable cell-cell contact [69,70]. Hess *et al.* reported that E-cadherin and EphA2 seem to be co-localized in cell adhesion junctions and that E-cadherin regulates EphA2 expression by modulating its ability to interact with, ephrinA1 [71]. Thus, E-cadherin serves as a *regulator* EphA2 expression at the cell surface. In fact, an inverse correlation has been reported between E-cadherin and EphA2 expression in bladder carcinoma [72].

Furthermore, as discussed earlier, deficiencies in p53 and estrogen receptor levels may contribute to tumor cell overexpression of EphA2. An analysis of ovarian cell lines and human ovarian cancers showed that EphA2 overexpression occurred in 91% of tumors

with p53 deficiency versus 68% in tumors with wild type p53, suggesting the possible role of p53 in EphA2 accumulation in cancer cells [73].

An interesting observation is that in normal cells, EphA2 is expressed at low levels, and is present in a tyrosine phosphorylated state at points of cell contact. However, in neoplastic epithelial cells, it is present abundantly in a non-tyrosine phosphorylated state, localized at the leading edge of the invasive cells [52,74]. There may be several reasons associated with this anomaly. EphA2 phosphorylation and localization is observed to be dependent on E-cadherin expression, and loss of E-cadherin expression and decrease in cell adhesion is a characteristic often displayed by neoplastic cells [38]. The explanation could also be low levels of EphrinA1 (leading to low EphA2 phosphorylation) or rapid action by protein tyrosine phosphatases (PTPs) like LMW-PTP that may rapidly associate with EphA2 immediately after receptor phosphorylation [67]. Either way, EphA2 is a unique RTK since its tyrosine kinase activity doesn't seem to be entirely dependent on ligand binding and tyrosine residue phosphorylation.

1.3.2.3. Role of EphA2 in Tumorigenesis

A study performed by Zelinski *et al.*, showed that overexpression of EphA2 (via transfection of specific cDNA) in mammary epithelial cells was sufficient to promote cellular transformation, allowing these cells to form invasive tumors/metastases in nude (athymic) mice [51]. EphA2 cDNA transduced cells exhibited defects in adhesion, subcellular distribution, and decreased EphA2 p-Tyr content, all of which are characteristics of metastatic breast cancer cells. The prominent difference observed in cells overexpressing EphA2 was the inability of transgenic EphA2 to interact with its natural ligand, ephrinA1. However, artificial stimulation of EphA2 with an agonistic antibody was able to reverse the growth and invasiveness of EphA2-transformed cells in this model [38].

In addition, enforced overexpression of the EphA2-associated PTP, LMW-PTP, was found to be sufficient to transform normal epithelial cells *in vitro*, with LMW-PTP acting as an inducer of tumor onset and progression in animal models [67]. This oncogenic potential associated with LMW-PTP, may be attributed to its ability to dephosphorylate EphA2, leading to EphA2 accumulation in tumors. This is consistent with the constantly activated and non-phosphorylated form of EphA2 that's over-expressed in the tumor [51,54,66]. This form of EphA2 may be associated with poor cell adhesion, inhibition of MAPK pathway (important for cellular responses to growth factors), enhanced growth and metastatic potential of the tumors. It may also be possible that cellular overexpression/ decreased phosphorylation of EphA2 results in an abnormal distribution of this RTK, disruption of cell-to-cell contacts, and an enhancement in cell-to-extracellular matrix (ECM) attachment, giving rise to increased cell motility and metastasis [37,63,71]. However, further studies are needed to elucidate in further detail the underlying mechanisms of EphA2 in tumorigenesis. Pathways of EphA2-mediated tumorigenesis are highlighted in Figure 2.

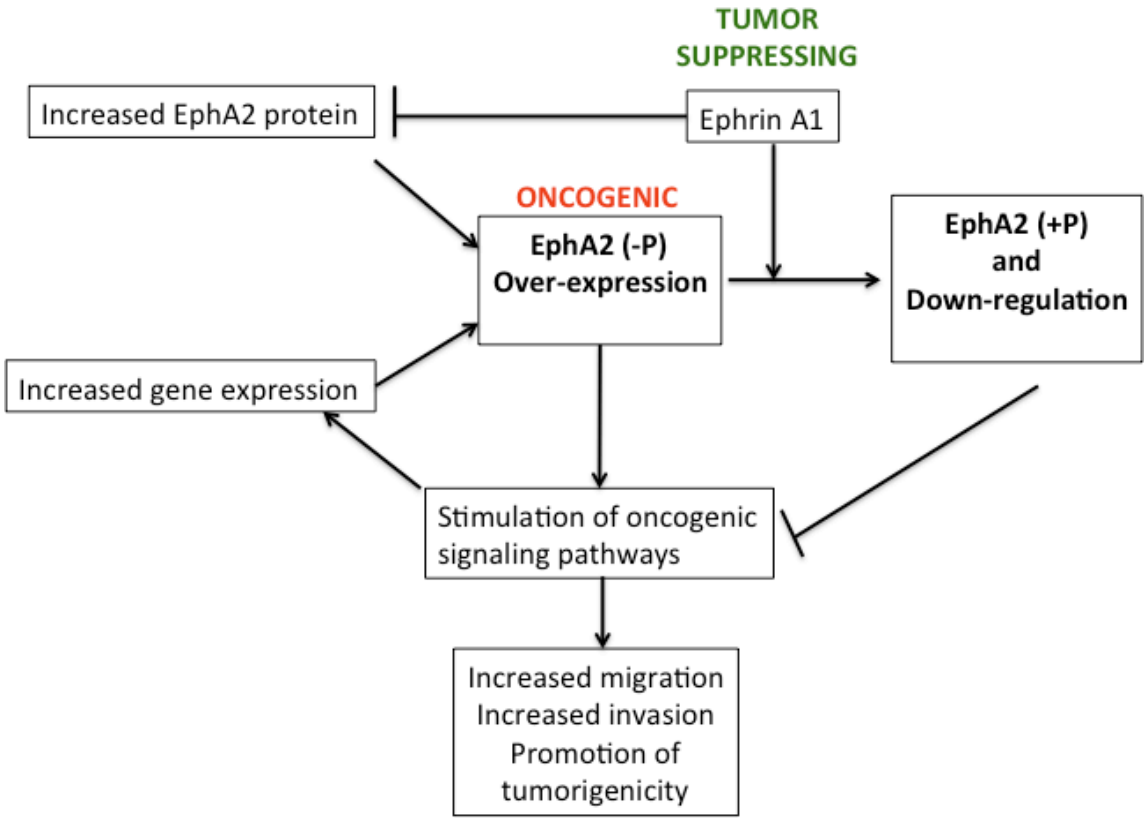


Figure 2: Proposed role of the EphA2/ephrinA1 system in solid tumor cells

EphA2 becomes overexpressed possibly due to increased gene expression or a lack of ephrinA1-induced receptor down-regulation. Overexpressed EphA2 is nonphosphorylated and stimulates oncogenic processes. EphrinA1 causes receptor phosphorylation and subsequent down-regulation, both of which likely contribute to the tumor-suppressing effects of the ligand in tumor cells. (−P), nonphosphorylated; (+P), phosphorylated.

1.3.2.4. EphA2 in angiogenesis

Apart from tumorigenesis, EphA2 and its interaction with EphrinA1 is also known to play an important role in angiogenesis and tumor neovascularization [75]. Ogawa *et al.* first reported the presence of EphA2 and ephrinA1 in blood vessels of breast cancer and Kaposi's sarcoma tissues, and it was subsequently proved that ephrinA1 could stimulate

EphA2-expressing endothelial cell migration and survival [76]. Several studies blocking EphA2 have demonstrated the demonstrated the disruption of angiogenesis in several tumor models as well as in other diseases, suggesting interaction between EphA2 in the endothelial cell with ephrinA1 in tumors or endothelial cells to mediate angiogenesis [77-80]. In addition, EphA2-positive mouse breast cancer cells, when implanted into EphA2-deficient mice, were deficient in tumor volume and failed to form solid tumors, thus underscoring the importance of EphA2 in tumor angiogenesis [81].

While ephrin-A1 is expressed in both tumor and normal vascular endothelial cells, EphA2 appears to be differentially expressed by tumor-associated vascular endothelial cells [80]. Interestingly, EphA2 overexpression in both tumor cells and tumor-associated endothelial cells has been linked to increased vascularity/angiogenesis and poor clinical outcome in renal and ovarian carcinomas [82,83]. Thus, therapeutic agents designed to antagonize the expression/function of EphA2 have two potential clinically meaningful target cell types; EphA2+ tumor cells themselves and EphA2+ tumor-associated neovessels.

1.4. Therapeutic Approaches Targeting EphA2

1.4.1. Anti-EphA2 Antibodies

Carles-Kinch *et al.* generated monoclonal antibodies to extracellular antibodies to EphA2 and observed that a subset of antibodies induced EphA2 phosphorylation and internalization, followed by degradation, leading to reduced levels of EphA2 expression in tumor cell [54]. Interestingly, these antibodies recognized a distinct conformation of EphA2 specific for tumors, thus sparing normal EphA2-expressing blood vessels. These mAbs effectively inhibited tumor growth in human xenograft models, promoted increased tumor cell apoptosis, and decreased EphA2 protein levels in treated tumor

lesions [84,85]. Since these mAbs were specific for the tumor-specific form of EphA2, potential toxicity issues towards normal tissues could be excluded, thus providing significant clinical benefits to these therapeutic agents. In addition, combinational therapies implementing EphA2 agonistic antibodies and chemotherapeutic drugs (such as paclitaxel or tamoxifen) has helped overcome sensitivity of tumors to these drugs, thus increasing the anti-tumor efficacy, compared to groups receiving either therapeutic agent alone [86,87].

1.4.2. Peptide Mimetics

There have been reports defining 2 peptides that selectively bind the extracellular domains of EphA2 and prevent ephrin binding. These peptides serve as agonists and stimulate EphA2 phosphorylation and internalization, and have also been noted for agonist anti-EphA2 mAbs. When linked to exterior surfaces, these peptides target the delivery of phage particles to EphA2+ cells, suggesting potential therapeutic value in selectively delivering therapeutic agents into EphA2+ tumor sites [88].

1.4.3. Interventions Targeting EphA2 Ligands

Soluble EphA2-Fc, a chimeric receptor of EphA2 fused with an IgG Fc fragment, inhibits signaling through the EphA2 receptor, and has been observed to inhibit VEGF-mediated and ephrin-A1-mediated angiogenesis [82]. Administration of soluble EphA2-Fc inhibits tumor angiogenesis, growth and even metastasis *in vivo* in murine tumor models. VEGF induces ephrin-A1 expression, which in turn activates EphA2-dependent angiogenesis. Hence, using this soluble EphA2-Fc would suppress tumor-associated VEGF-induced angiogenesis. With regard to safety concerns, no untoward toxicity on normal EphA2+

tissues has been observed, while inhibition of angiogenesis was seen specifically in neoplastic tissues [77,78,80].

1.4.4. Gene Silencing by siRNA

EphA2 gene silencing has recently gained interest as an attractive therapeutic approach to target EphA2 in tumors. Recent studies suggest that application of EphA2 siRNA suppresses EphA2 protein expression, tumor growth and inhibits metastasis *in vivo* via the induction of tumor cell apoptosis [89-91].

1.4.5. EphA2-based Vaccines

Several immunogenic EphA2 peptides have been identified by our group and by Alves *et al.* These peptides are recognized by CD8+ or CD4+ T cells generated from normal donors or cancer patients, and by CD8+ T cells developed in HLA-A*0201-transgenic HHD mice [92,93]. In all cases, T cell lines and clones produced using EphA2 peptides as a stimulus also recognized EphA2+, HLA-matched tumor cell lines, including RCC. This supports the natural processing and MHC presentation of these epitopes on the tumor cells, allowing for effector T cell reactivity. Such EphA2-specific T cells have been identified in the peripheral blood of patients with RCC, prostate cancer or glioma, suggesting that these responses may be naturally primed during cancer progression [92,94,95].

Therapeutic/protective EphA2+ cancer vaccines need to stimulate, polarize (i.e. Type-1) and protect anti-EphA2 T cells against the tumor. In this regard, active vaccination against EphA2, in order to elicit and sustain specific T cells would be anticipated to provide clinical benefit in EphA2+ cancer patients. Indeed, Hatano *et al.* demonstrated that DC pulsed with murine EphA2 peptide epitopes effectively elicit specific CTL

responses *in vivo* that are capable of inhibiting syngeneic tumor progression in C57BL/6 mice in an EphA2+ as well as an EphA2- tumor models. While there remains a theoretical concern that vaccination with EphA2-derived peptides may induce pathologic autoimmune reactions in normal EphA2+ tissues (i.e. lung, spleen, kidney and liver), these organs were not infiltrated by T cells, nor was tissue pathology observed in vaccinated animals [96]. This may reflect greater densities of EphA2 epitopes presented on the surface of tumor cells versus normal tissues, with T cells exhibiting moderate avidity able to functionally respond to tumor cells. Under such conditions, while flirting with potential autoimmune toxicities that warrant further scrutiny, this type of vaccine may ultimately prove both safe and clinical effective.

1.4.6. Other therapeutic approaches

Like EphA2-Fc, Ephrin-A1 Fc is a dimerized version of ephrin-A1 fused to human immunoglobulin G (IgG) Fc. *In vitro* experiments suggest that EphA2 ligation by ephrin A1-Fc results in EphA2 phosphorylation, and consequent degradation of this RTK in concert with reduced tumor growth [63,97]. To investigate the impact of sustained ephrin-A1 delivery on tumor cells *in vivo*, adenoviruses encoding secreted forms of ephrin-A1 Fc have also been investigated. Noblitt *et al.* showed that adenoviral delivery of ephrin-A1 Fc (i.e. rAd.ephrin-A1) into breast cancer cells increases the degree of EphA2 activation and degradation, along with inhibited tumor growth *in vitro*. Furthermore, they demonstrated that intra-tumoral injection of rAd.ephrin-A1 limited human tumor growth in xenograft models [98,99]. With regard to their potential clinical utility, one concern in using ephrin-A1 Fc (protein or gene constructs) as a therapeutic agent is the fact that ephrin-A1 serves as a ligand for multiple Eph receptors (i.e. EphA4, EphA5, EphA6 and EphA7 in addition to EphA2), which may increase chances of unanticipated toxicities. While no gross toxicities were noted in the reported xenograft model, further safety studies prior to clinical translation of this agent would be necessary [98].

In addition to targeting the EphA2 gene, one may consider targeting PTPs linked to EphA2 expression/function, i.e. LMW-PTP, SHP2. By reducing the overexpressed levels of these PTPs in tumor cells, one might anticipate the normalization of pEphA2 levels and consequent EphA2 protein degradation. Our own data demonstrated that LMW-PTP silencing with siRNA reduced EphA2 protein expression of metastatic RCC cells, suggesting the possibility for an alternative therapeutic method (Wesa et al, unpublished data).

1.5. MHC class I antigen presentation pathway

Cell-surface-expressed MHC class I molecules present antigenic peptides on the cell surface so that they can be specifically recognized by cytotoxic T lymphocytes (CTLs). The generation of these peptides requires the degradation of proteins into peptide fragments of precise size. The protease responsible for the degradation of polyubiquitylated proteins is the 26S proteasome, which is composed of the 20S proteasome, representing the catalytic core, and two 19S regulator complexes that regulate the binding and unfolding of ubiquitylated substrates. The hydrolysing activities of the 20S core are conferred by three of the seven β subunits located in both of the inner heptameric β-rings, whereas the 19S regulator complexes (composed of six ATPase subunits and 9–10 non-ATPase subunits) attach to the outer heptameric α rings of the 20S core [100,101]. A constant supply of functional Hsp90 is needed to maintain the tertiary structure of the proteasome [102,103].

Although attachment of ubiquitin to proteins (ubiquitination) was initially identified as a signal that leads to proteasome degradation of the target protein, it has since become clear that attachment of ubiquitin can lead to different outcomes depending on the type of this attachment. All lysine residues of the ubiquitin molecules can be used for isopeptide bond formation. In addition, the pattern of post-translational modification dictates the fate of

the modified protein. Diversification is conferred by whether one ubiquitin molecule or a chain of ubiquitins is attached.

Mono-ubiquitination is a signal involved in receptor endocytosis and lysosomal sorting. Many receptor tyrosine kinases (RTKs) undergo ligand-induced mono-ubiquitination. As mentioned in section 1.1, process ligand-induced phosphorylation of the receptor gives the signal for receptor ubiquitination. E3 ligase cbl facilitates receptor ubiquitination and is the major E3 ligase for this purpose. Ubiquitinated receptors interact with ubiquitin-binding proteins of the endocytic pathway and are escorted through clathrin-coated pits to clathrin-coated vesicles, endosomes and finally lysosomes. Mono-ubiquitination in multiple receptor sites (multiple mono-ubiquitination) has also been found to play a role in receptor endocytosis. Cbl E3 ligase also mediates multiple mono-ubiquitination. Multiple mono-ubiquitination is believed to stabilize interaction of receptors with ubiquitin receptors in order to enhance their transfer to lysosomes. Some ubiquitin receptors may also recognize only multi-ubiquitinated RTKs through multiple domain interactions ([104]).

A chain of at least four ubiquitin molecules linked through lysine 48 is the signal for recognition of a target protein by the proteasome complex in order to be degraded ([105]). Proteasome degradation after lysine 63 poly-ubiquitination has been described in to occur sometimes ([106]), but most often, lysine 63 poly-ubiquitination leads to proteolysis through autophagy-associated mechanisms ([107]).

Major histocompatibility complex (MHC) class I molecules are constitutively expressed by virtually all somatic cells and they present peptides of 8 to approximately 12 amino acids in length to $CD8^+$ T cells. Essential components for the formation of peptide-MHC class I complex (pMHC) are called as MHC class I antigen-presenting machinery (APM), including the proteasome, ERP1/ERAAP, transporter associated with antigen presentation complex (TAP, heterodimer of TAP1 and TAP2), general ER chaperones and tapasin [108,109]. There are two distinct pathways for presentation of peptides on MHC class I molecules.

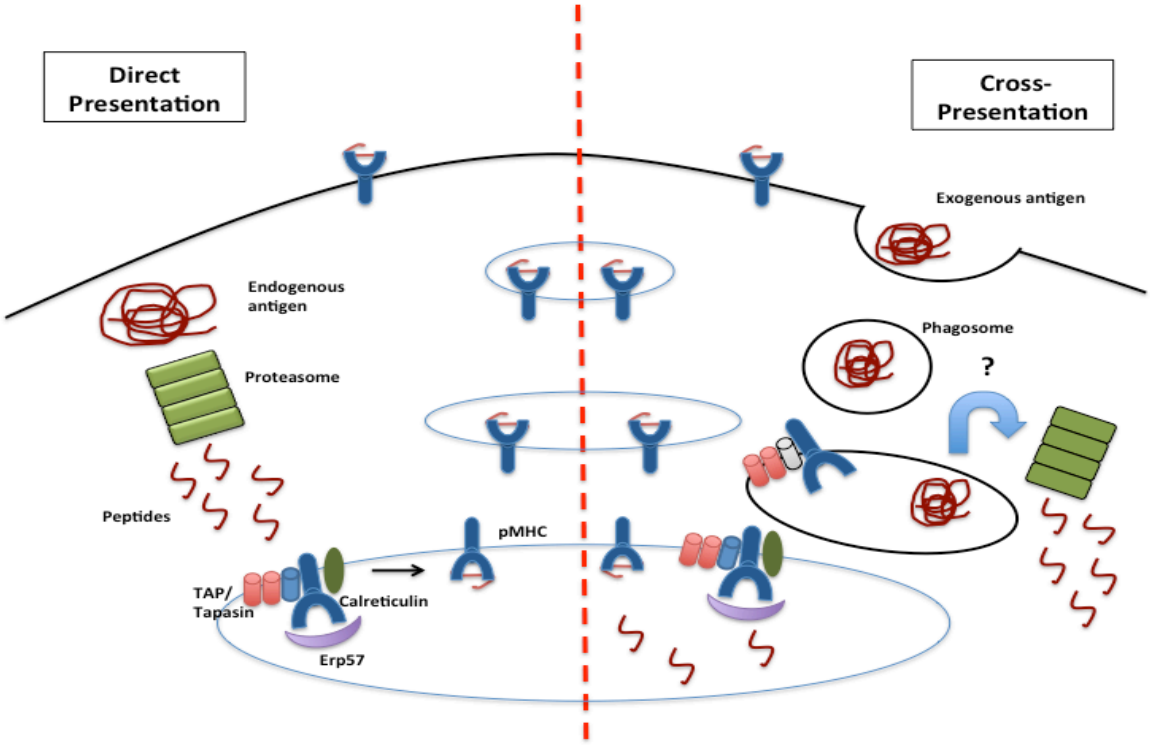


Figure 3: MHC class I presentation pathways

In the direct presentation pathway, endogenous proteins are degraded by the proteasome into peptides within the cell cytosol, which are then transported by the TAP complex into the endoplasmic reticulum (ER) for loading into newly synthesized MHC class I molecules. Fully assembled (mature) class I molecular complexes (consisting of the MHC class I heavy chain, non-covalently bound **β**2-microglobulin and peptide) are then transported through the Golgi to the cell surface. In the cross-presentation pathway, exogenous antigens are first phagocytosed, endocytosed (via specific or scavenger-type receptors), pinocytosed, or macropinocytosed by APC. During the formation of the phagocytic cup, the ER may fuse with the nascent phagosome to form early phagosomes that contain ER proteins, including all the components required for MHC class I antigen presentation (e.g. TAP, MHC-I), ubiquitin-conjugating enzymes and the translocon. Subsequently, internalized antigens in early phagosomes may transferred to the cytosol for degradation through the proteasome by as yet less understood mechanisms (blue ?) or remain in the phagosome, whereas antigenic peptides generated by proteolysis in these compartments may be loaded into nascent/recycled MHC class I complexes that are transported to the cell surface.

Most somatic cells have the capacity to present endogenous peptides in the context of MHC class I molecules. Endogenous proteins are degraded by the proteasome into peptides in the cytosol, which may then be transported by the TAP complex into the endoplasmic reticulum (ER) for loading into newly synthesized MHC class I molecules. Peptides may be trimmed by an ER-associated aminopeptidase (ERAAP or ERAP1) to a preferred loading length of 8–10 amino acids. Essential molecules for optimal peptide loading into MHC class I complexes include TAP, tapasin, calreticulin and ERp57. Fully-assembled class I molecules are then transported through the Golgi to the cell surface [110-112].

The degradation mechanism of newly-synthesized and mis-folded proteins (known as the ER-associated degradation (ERAD) pathway), is currently under intense investigation as a major conduit through which endogenous proteins may be delivered back into the cytosol to serve as a source of MHC-presented peptides. The current ERAD paradigm suggests that proteins that fail to achieve their native conformations may be ubiquitinylated and retrotranslocated from the ER back into to the cytoplasm, where they face degradation by the proteasome after retro-translocation mediated via proteins such as Sec61 [109,113].

This retrotranslocation pathway has also recently been reported to play a role in the ability of DC to cross-present antigenic peptides to responder $CD8^+$ T cells [114,115]. Exogenous antigens are first internalized by a variety of mechanisms (phagocytosed, endocytosed (via specific or scavenger-type receptors), pinocytosed, or macropinocytosed) by APC. From here the antigens are transferred to the cytosol by the translocon, ubiquitinated and processed by the proteasome, in a mechanism resembling ERAD. The degraded peptides are then transported to the ER by TAP and loaded into nascent MHC-I complexes [115].

Both the direct- and cross-presentation pathways rely on the cleavage of polypeptides by the proteasome [116-118]. The subunit composition of the constitutive proteasome varies in different tissues [119]. In addition to the constitutive proteasome, professional APCs and most cells exposed to IFNγ express the immunoproteasome, which contains three different catalytic domains. Due to this change in multicatalytic specificity (versus the conventional proteasome), immunoproteasomes exhibit an altered cleavage site preference as well as a different cleavage rate. The immunoproteasome generally favors the production of MHC-binding peptides [120-122], Figure 3.

To initiate a protective CTL response toward tumors, the antigens derived from tumor cells must be processed and presented by professional antigen presenting cells (APC) in the context of MHC class I molecules via cross-presentation pathway, since tumor cells are generally considered to be poor APCs due to defects in MHC molecule expression and/or a skewed balance towards co-inhibitory over co-stimulator molecule expression. On the other hand, to exert effector function, tumor specific CTLs need to recognize tumor cells in the form of (endogenously synthesized) tumor peptides presented by MHC class I through the direct presentation pathway [123].

1.6. Approaches to Increase RTK-derived Epitope Presentation in Tumor Cell MHC Class I Complexes

Our lab has been interested in the identification of treatment strategies that allow for biased improvement in tumor cell (MHC class I) presentation of RTK-derived peptide epitopes, leading to the evaluation of RTKs agonists, PTP inhibitors and HSP90 inhibitors. The first 2 modalities manipulate RTK internalization and subsequent proteasomal degradation, while the 3rd modality is based on the prevention of RTK folding/maturation by inhibiting chaperone function, leading to the re-routing of such

mis-folded proteins into the proteasome pathway as a clearance mechanism. In all cases, the derivative proteasome-generated peptides may serve as an enriched source of epitopes for MHC class I presentation to CD8+ T cells [7,124].

1.6.1. RTK Agonists and PTP Inhibition

As described earlier, upon ligand binding, RTKs may become phosphorylated, ubiquitinated and internalized within "sorting" endosomes. Ubiquitinated RTKs are subsequently targeted towards a lysosomal compartment for proteolytic degradation, while dephosphorylated and/or non-ubiquitinated receptors may be recycled to the cell surface. Recent studies, however, demonstrate that polyubiquitinated RTK may also be delivered to the proteasome for degradation [125].

In this context, reagents that promote RTK activation/internalization in tumor cells have the potential to facilitate the degradation of RTKs by enhancing (the normal life cycle of) RTK destruction by the proteasome. The net impact would be expected to be a conditional enhancement of RTK-derived peptide presentation within MHC class I complexes (i.e. by selectively driving RTK processing via the proteasome, the stochastic level of a given RTK peptide would be increased versus peptide derived from alternate source proteins) and improved recognition by low-moderate avidity anti-RTK CD8+ T cells. For example, RTK agonists (antibodies or ligand-Fc fusion protein) or PTP inhibitors would fall into this category and promote RTK internalization through direct activation of RTK or through inhibition of RTK dephosphorylation, respectively. Consequent proteasome activity could render treated tumor cells more sensitive to anti-RTK specific T cells. Indeed, recent studies have reported that anti-Her2/neu antibody (Herceptin) treatment of Her2/neu+ tumor cells promotes enhanced sensitivity to Her2/neu-specific CTLs *in vitro* [126-128].

1.6.2. HSP90 Inhibition

A rational approach to increase the proteasomal degradation of tumor cell-expressed RTKs would be through the inhibition of heat shock protein (HSP)90, a chaperone required in the decision of whether a misfolded "client" protein is recycled or degraded in cells. HSP90 is a constitutively expressed molecule that directs the normal folding and proteolytic turnover of its client proteins. HSP90 has an ever-expanding list of client proteins (see http://www.picard.ch/downloads/HSP90interactors.pdf), and various oncoproteins (including overexpressed RTKs) are a part of the list [4,129-131]. Furthermore, HSP90 is overexpressed manifold by tumor cells and may play a role in mediation of stabilization/ proper folding of mutant/mis-folded client proteins, thus permitting tumor cells to better endure imbalanced signaling pathways [4,132-134]. In fact, HSP90 has been deemed central to the 'Six Hallmarks of Cancer' i.e. six characteristics possessed by a cell to turn tumorigenic [3]. Therefore we hypothesized that when HSP90 function is inhibited, overexpressed and misfolded proteins would be delivered to the proteasome, degraded, and presented by MHC class I through the direct presentation pathway.

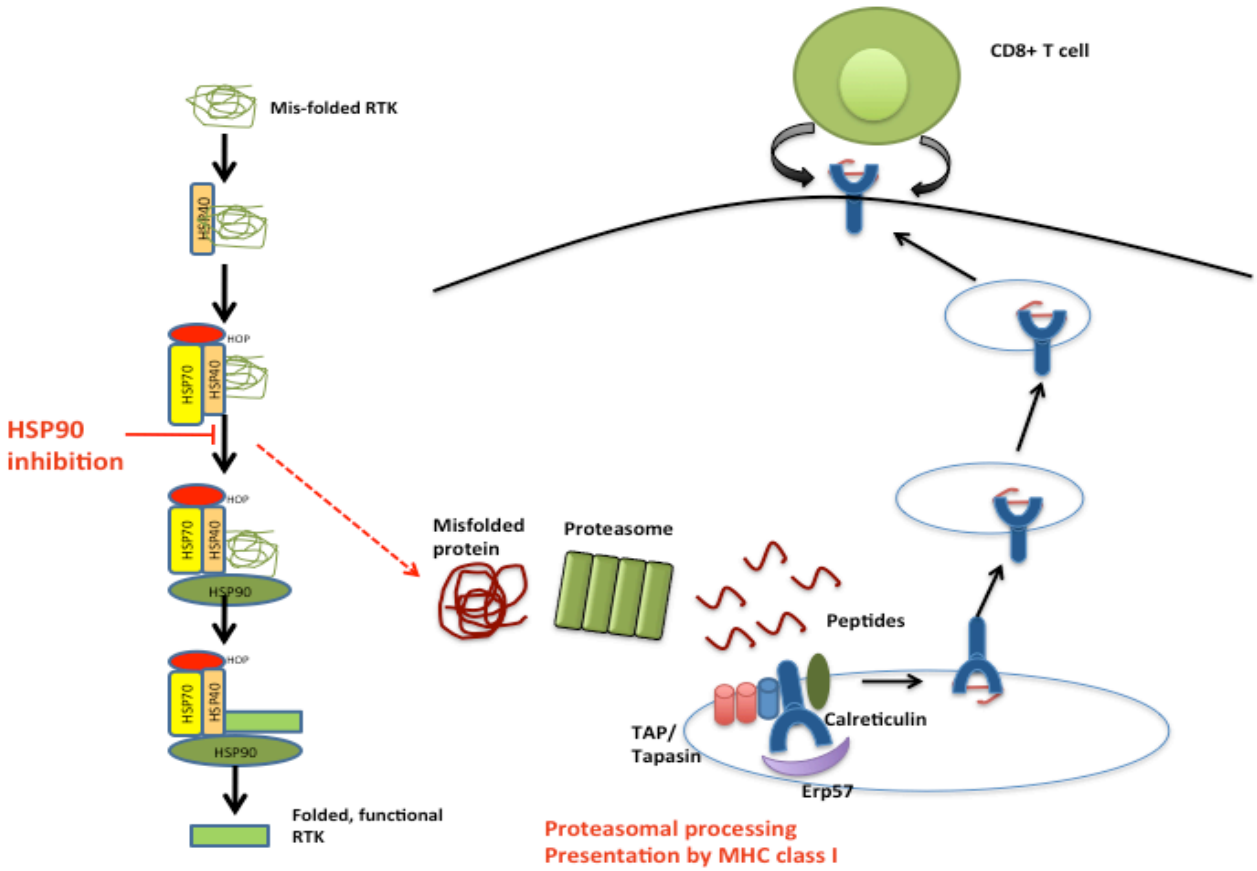


Figure 4: A paradigm for using HSP90 inhibitors to increase tumor and stromal cell presentation of RTK-derived peptides

Overexpression of WT/mutated RTKs by cells in the tumor microenvironment occurs in part due to the stabilizing influence of the HSP90 chaperone complex. Pharmacologic inhibition of HSP90 leads to the inability to salvage mis/un-folded RTKs, leading to their proteasome-dependent processing, TAP-transport and loading into, and presentation by MHC class I molecules expressed on the cell surface.

1.6.3. HSP90

1.6.3.1. HSP90 structure

The HSP90 is a constitutively expressed cellular protein that constitutes 1–2% of the total protein load [135,136]. Five HSP90 isoforms have been identified to date, including

cytoplasmic HSP90α- and β-isoforms, endoplasmic reticulum localized glucose regulated protein 94 (GRP94), mitochondrial tumor necrosis factor receptor-associated protein 1 (TRAP1) and membrane-associated HSP90N [137]. It is a flexible homodimer where the monomers consist of three domain -- an N-terminal, ATP-binding domain, a middle (M), ATP-hydrolysis-regulating domain and a C-terminal dimerization domain [138-140]. The C-terminus also regulates ATPase activity and recruits co-chaperones through a conserved EEVD motif). Co-chaperones, such as HOP, CDC37, p23, Aha1 and PPIase, play an important role in client protein maturation and modulation of ATPase activity. Co-chaperones also recruit specific client proteins to HSP90 and/or stabilize HSP90 in an ATP-bound state to prolong the half-life of the mature multi-chaperone complex [141]. Adenosine triphosphate hydrolysis alters HSP90 structure and promotes its chaperone function. Therefore, in cells, HSP90 generally exists in 2 conformations – an active "open" conformation that is an ATP bound state during client protein binding, and an inactive "closed" conformation [142].

1.6.3.2. HSP90 mechanism of action

The HSP90 protein contains three functional domains, the ATP-binding, protein-binding, and dimerizing domain, each of which play a crucial role in the function of the protein.

ATP binding

The region of the protein near the N-terminus has a high-affinity ATP-binding site. The ATP binds to a sizable cleft in the side of HSP90 protein, that has a high affinity for ATP, and in the presence of a suitable protein substrate, HSP90 hydrolyzes ATP. Direct inhibitors of ATP binding or allosteric inhibitors of either ATP binding or ATPase activity can block HSP90 function [143]. Another interesting feature of the ATP-binding region of HSP90 is that it has a "lid" that is open during the ADP-bound state and closed in the ATP-bound state. In the open conformation, the lid has no intraprotein interaction, and when closed comes into contact with several residues [131]. The ATPase-binding region of HSP90 is currently under intense study, because it is the principal binding site of HSP90-inhibiting drugs [144].

Protein binding

The protein-binding region of HSP90 is located toward the C-terminus of the amino sequence. As mentioned earlier, the HSP90 protein can adopt two major conformational states. The first is an open ATP-bound state and the second is a closed ADP-bound state. Thus, ATP hydrolysis drives what is commonly referred to as a "pincer-type" conformational change in the protein binding site [145].

HSP90, while in the open conformation, leaves some hydrophobic residues exposed, to which unfolded and misfolded proteins that have unusual hydrophobic regions exposed may be recruited with high affinity [146]. When a bound substrate is in place, ATP hydrolysis by the ATPase located near the N-terminus of the HSP90 protein forces conformational changes that ensnares the client protein [147]. The ability of HSP90 to physically "capture" proteins allows it perform several functions including assisting folding, preventing aggregation, and facilitating transport.

1.6.3.3. HSP90 function in normal cells

HSP90 is one of the most abundant molecular chaperones that regulate folding, maturation and stabilization of proteins. HSP90 interacts with a set of proteins, called client proteins (see http://www.picard.ch/downloads/HSP90interactors.pdf) [148]. In unstressed cells, HSP90 plays a number of important roles, which include assisting folding, intracellular transport, maintenance, and degradation of proteins as well as facilitating cell signaling [102,103,149].

HSP90 is known to associate with the non-native structures of many proteins, which has led to the proposal that HSP90 is involved in protein (re)folding in general. In addition,

eukaryotic proteins that are no longer needed or are misfolded / damaged are usually marked for destruction by the polyubiquitation pathway. These ubiquitinated proteins are recognized and degraded by the 26S proteasome. Hence the 26S proteasome is an integral part of the cell's mechanism to degrade proteins. Furthermore a constant supply of functional HSP90 needed to maintain the tertiary structure of the proteasome [102,103,149]. A lesser studied function of HSP90 is its role in signaling. The glucocorticoid receptor (GR) is the most thoroughly studied example of a steroid receptor whose function is crucially dependent on interactions with HSP90 [150]. In the absence of the steroid hormone cortisol, GR resides in the cytosol complexed with several chaperone proteins including HSP90. These chaperones maintain the GR in a state capable of binding hormone [151,152]. Another role of HSP90 is to bind immunophilins (e.g., FKBP52) that attach the GR complex to the dynein protein trafficking pathway, which translocates the activated receptor from the cytoplasm into the nucleus. Once in the nucleus, the GR dimerizes and binds to specific sequences of DNA and thereby upregulates the expression of GR responsive genes [151,152]. HSP90 is also required for the proper functioning of several other steroid receptors, including those responsible for the binding of androgen, estrogen, and progesterone [153-156]. In addition, Udono *et al.* demonstrated that HSP90 facilitates MHC class I antigen processing through epitope production in a complex of the 26 S proteasome, with inhibition of HSP90 *in vivo* using geldanamycin partially disrupting the 26 S proteasome structure, limiting the efficiency of MHC class I biosynthesis, leading to down-regulated MHC class I expression ([157]).

1.6.3.4. HSP90 function in tumors/ tumorigenesis

Cancerous cells over express a number of proteins, including growth factor receptors, such as VEGFR and EGFR [158,159], or signal transduction proteins such as PI3K and AKT. HSP90 plays a role in folding and stabilization of these proteins in the tumors. In addition, HSP90 also stabilizes mutant proteins such as v-Src, the fusion oncogene

Bcr/Abl, and mutant forms of p53 that appear during cell transformation. HSP90 is also required for induction of vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS) [160]. Both are important for *de novo* angiogenesis that is required for tumor growth. It also promotes metastasis by assisting the matrix metalloproteinase MMP2, which modulates cell adhesion and promotes cell migration. Thus, HSP90 plays a role in stabilizing tumor overexpressed client proteins, many of which serve to foster tumor growth and dissemination. HSP90-mediated “life support” for tumors also allows these cells to better tolerate genetic instability based on the accumulation of client proteins associated with DNA repair [161,162].

HSP90 plays multiple roles in the cell, where it is essential for the creation, maintenance, and destruction of proteins. Its normal function is critical to maintaining the health of cells, whereas its deregulation may contribute to carcinogenesis. Indeed in tumors, most HSP90 clients are overexpressed and the stability provided by HSP90 helps mediate acquisition and maintenance of the properties necessary for transformation of a normal cell into a cancer cell; ability to evade apoptosis, ability to be self-sufficient for growth, ability to invade surrounding tissue and to metastasize to distant sites, ability to undergo limitless replication, ability to promote neoangiogenesis, and ability to ignore antigrowth signals. Therefore, the use of HSP90 inhibitors in cancer treatment highlights HSP90's importance as a therapeutic target. [3,4,161,163]

1.6.4. HSP90 Inhibitors

Many chemicals have been developed to inhibit HSP90 function. These can be categorized into two groups depending on the sites of the HSP90 molecule that are targeted; 1) those impacting the N-terminal ATP/ADP pocket of HSP90 and 2) those affecting the C-terminal domain of HSP90 [4,164].

The concept of HSP90 inhibition was greeted with much initial skepticism because of the severe toxicity issues involved in targeting a ubiquitous housekeeping protein. This

misperception has since been dismissed and HSP90 inhibitors are one of the most actively studied pharmacologic agents, with 17 of them having entered clinical trials [165]. So far, impressive clinical activity has been achieved with several HSP90 inhibitors in multiple tumor types, showing great promise for these inhibitors as therapeutic agents [166].

1.6.4.1. Geldanamycin and its derivatives

Initially, researchers established the ability to target HSP90 using the natural products radicicol and geldanamycin. These products were isolated in 1953 and 1970, but their ability to interact with HSP90 was only determined years later [167]. Another breakthrough was the discovery that geldanamycin and radicicol mimic the relatively unusual structure that ATP adopts in the deep, N-terminal, nucleotide-binding pocket of HSP90, thereby leading to potent and selective inhibition of ATP binding and hydrolysis [168], and thus leading to depletion of the oncogenic client proteins and mediating their proteasomal degradation [169,170].

Geldanamycin and radicicol have provided invaluable insights on HSP90 structure, function and its value as an anti-cancer target. Although geldanamycin and radicicol proved too toxic and unstable/reactive for clinical use, they each provided the chemical basis for subsequent drugs that entered the clinic [171]. The first HSP90 inhibitor to progress to clinical trials was the better-tolerated geldanamycin analog 17-allylamino-17-demethoxygeldanamycin (17-AAG, KOS-953, tanespimycin). In phase I studies with 17-AAG, researchers successfully demonstrated HSP90 inhibition using a validated pharmacodynamic biomarker signature of client protein depletion and HSF1-dependent HSP70 induction [172,173]. 17-AAG also showed impressive results in clinical trials in phase I and II studies in HER2+, trastuzumab-refractory breast cancer where objective Response Evaluation Criteria in Solid Tumors (RECIST) responses were seen on a

weekly schedule of 450 mg/m^2 [174,175]. Both GA and 17-AAG have also been observed to affect other HSP90 isoforms like gp96 (Grp94) ([176]).

Although prolonged disease stabilization was achieved in phase I studies of 17-AAG in various tumor types, no complete or partial tumor responses were seen [177]. This limited activity could be due to suboptimal inhibition of the target client proteins, most likely due to insufficient drug dose or frequency of administration. Consistent with the clinical data, animal model studies in relevant tumors (e.g., ovarian, colon, breast and melanoma) show a similar pattern of growth inhibition or cytostatic arrest rather than tumor regression in response to 17-AAG alone [159,173,177-180]. Another (major) limitation of 17-AAG was its requirement to be dissolved in DMSO, which significantly increased the toxicity of the drug and had a dose-limiting effect, which ultimately reflected the mediocre performance of the drug in clinical trials [173,181].

1.6.4.2. 17-DMAG

Another geldanamycin analog, alvespimycin (17-dimethylaminoethylamino-17-demethoxygeldanamycin, 17-DMAG) has improved formulation and pharmacokinetic properties. The biggest advantage that 17-DMAG offers over its predecessors is its ability to be dissolved in water, and hence it can be given orally making it highly bioavailable. Egorin et al looked at the pharmacokinetics and tissue distribution of 17-DMAG in CDF21 mice and Fischer rats and observed that a dose as high as 75 mg/Kg did not seem to cause any discernible weight loss or other toxicity symptoms. In addition, they observed that giving the drug orally and i.v. provided more bioavailability than i.p. administration [5]. In a study comparing 17-AAG with 17-DMAG on 64 different tumor cell lines, 17-DMAG was found to be more effective at inducing tumor apoptosis and inhibiting tumor cell proliferation when compared to 17-AAG. In addition, a lower dose of 17-DMAG was required to induce these effects versus 17-AAG [182]. These

observations were also replicated *in vivo* in a number of tumor models viz. breast cancer, cervical cancer and ovarian cancer [6,183].

Phase I studies of 17-DMAG have recently concluded, where the drug was found to be well-tolerated and effective. In a study carried out on patients with advanced cancer, a dose of 21mg/m2/d given i.v. twice weekly, was observed to be well-tolerated with minor side-effects like nausea and low grade fever. About half the patients had stable disease. Similar finding were observed in a phase I clinical trial involving advanced acute myeloid leukemia patients [184]. More recently, studies by Ramanathan *et al.* and Pacey *et al.* have concluded that a 17-DMAG dose of 80mg/m2 (either given as a single bolus or a staggered dose) once a week for 3 weeks was well tolerated by patients with advanced tumors. They observed complete remission in patients with prostate cancer, partial response in melanoma patients while patients with sarcomas and renal cell carcinoma had stable disease [185-187]. Thus, in spite of encouraging results observed in Phase I trials, it is evident that a safe dose of 17-DMAG is insufficient to mediate complete tumor regression in patients. Therefore, a safe and effective combination therapy involving 17-DMAG could represent a superior anti-tumor therapeutic approach.

1.6.4.3. 17-DMAG combinational studies

17-DMAG has been used in combination with chemotherapy or irradiation to improve anti-tumor efficacy of either modality. Combination of 17-DMAG with irradiation has been used in a number of tumors *in vitro* as well as *in vivo*. 17-DMAG caused abrogation of the G2 and S phase of the cell cycle and interference in DNA damage response in tumor cells [182,188,189]. Ultimately, combination of 17-DMAG with irradiation have significantly higher anti-tumor efficacy compared to either modality in a number of tumors including lung and breast cancers *in vitro* as well as *in vivo* [182,189]. Combination of 17-DMAG with chemotherapy has been evaluated mostly in non-solid tumors. Studies combining 17-DMAG with an AKT inhibitor in multiple myeloma (MM)

showed increased susceptibility of MM cells to apoptosis and cell cycle arrest. This combination also induced apoptosis of endothelial cells and inhibited angiogenesis *in vitro* [190]. In mantle cell lymphoma, combination of 17-DMAG with a histone-deacetylase inhibitor Vorinostat induced cell cycle arrest in disparate cell cycle phases, and increased apoptosis of tumor cells was seen in combination therapy cohorts [191]. Apart from increasing anti-tumor efficacy of single-modality treatments, 17-DMAG has also been observed to complement other therapies when they fall short. For example, p53 mutation causes resistance to apoptosis by single chemotherapeutic agents like doxorubicin. However, addition of 17-DMAG to the therapeutic regiment overcame this resistance [192]. Similarly, 17-DMAG was able to help overcome cisplatin resistance observed in treated bladder cancer cells [193]. However, 17-DMAG combination studies have been limited to *in vitro* studies mostly, and advanced *in vivo* and clinical studies need to be carried out to further evaluate the net therapeutic value of these combinational approaches.

The induction of the heat shock response appears to be an unfortunate drawback of all the N-domain inhibitors of HSP90, including the newer purine and 4,5-diaryisoxazole resorcinol compounds [194-196]. However it would appear that it can be avoided by switching to a recently-developed class of HSP90 inhibitor that binds with high-affinity to the C-terminal region of the chaperone. One of these C-terminal inhibitors, KU135, has been shown to promote client degradation, but not Heat shock factor (HSF)-1 induction, and to act as a potent inducer of mitochondria-mediated apoptosis [197].

Alternatively, it should be possible to overcome the detrimental effects of N-domain inhibitors inducing HSF-1 by using these inhibitors in combination with other drugs. In this respect inhibitors of the HSP70 family of molecular chaperones are potentially of tremendous potential. The status of HSP70 drug development has recently been reviewed [198,199], the targeting of HSP72 by one such compound enhancing HSP90 inhibitor-induced apoptosis in myeloma cells [200]. In cell culture the combinatorial use of 17-AAG and cisplatin has also shown promise, with cisplatin strongly suppressing the HSF-1 activation by 17-AAG [194]. These two agents act synergistically, leading to an increased tumor cell apoptosis as compared to the use of each agent alone [194]. Furthermore, since HDAC6 histone deacetylase is required for HSF-1 activation [201],

there is the prospect that HSP90 inhibitors might act synergistically with HDAC inhibitors in promoting the growth arrest and apoptosis of tumor cells. Small molecule inhibitors that act directly on HSF-1 have also been identified, including the natural flavonoid quercitin [202,203] and the benzylidene lactam compound KNK437 [204].

1.6.4.4. Other HSP90 inhibitors

The soluble stabilized hydroquinone form of 17-AAG, IPI-504 (retaspimycin hydrochloride) is currently in clinical trials. Evaluation of this drug was done in gastrointestinal stromal tumor, which is usually driven by the HSP90 client protein KIT, where the drug showed promising results [205]. In addition, encouraging activity has been seen in non–small cell lung cancer (NSCLC) patients and clinical evaluations in this setting are ongoing[206].

The tremendous success of work with geldanamycin analogs stimulated the race to discover synthetic small-molecule HSP90 inhibitors that could overcome some or all of the limitations of this class, such as by allowing the use of doses and schedules that provide sufficiently sustained client depletion while sparing the liver toxicity [207,208]. A large number of new HSP90 inhibitors that do not suffer from these constraints are now in clinical development.

Investigators seeking to discover new HSP90 drug candidates have benefited greatly from structure-based design using available X-ray crystal structures of HSP90 [209,210]. Success was achieved early on with the purine scaffold series, which was based initially on the prototype PU3 [211,212]and led to the clinical candidates BBIIB021 (CNF-2024) and BIIB028, as well as PU-H71 [213], now under evaluation in phase I clinical trials. In addition the resorcylic pyrazoles and isoxazoles led to development of NVP-

AUY922/VER52296, the resorcylic dihydroxybenzamide AT13387, and the structurally related KW-2478 [196,214]. A diverse range of chemical scaffolds have since emerged, as illustrated by the publication of 40 to 70 patents per year from 2005 to 2010, covering purines and resorcinols as well as pyrimidines, aminopyridines, azoles, and other chemotypes [215]. These include SNX-5422, which is a prodrug of the active benzamide SNX-2112, the orally active thienopyrimidine NVP-BEP800/VER-82576, the 8-arylthiopurine CUDC-305, which is orally bioavailable, blood-brain barrier–permeant, and active in an orthotopic brain tumor model, and the *N*-aryltropane XL888 [216-219]. Another promising agent currently in multiple clinical trials is STA-9090 [ganetespib] [220]. Although its full structure is undisclosed, it is thought to be a resorcinol-containing triazole.

The various new agents have the potential to be administered more frequently and to achieve a higher maximum dose (and hence better/more-prolonged target inhibition), in some cases with oral administration and blood-brain barrier penetration. New drugs also lack the significant hepatotoxicity that was limiting for members of the geldanamycin chemotype, consistent with this side effect being related to the quinone in those agents (see above) [206]. Encouraging early clinical data have been reported concerning these agents' pharmacodynamic and antitumor activities in diverse malignancies, again with the expected client protein and genetic profiles, including breast, NSCLC, and rectal cancer, as well as in melanoma and leukemia [163,185,221,222].

As we learn more about the role of HSP90 in modulating signaling networks and the sensitivity of various client proteins to HSP90 inhibition, a better understanding of HSP90 biology has already educated and will continue to inform the ongoing clinical development of HSP90 inhibitor-based therapy, in part by supporting the correct choice of tumor types and revealing additional molecular targets whose inhibition synergizes with HSP90 inhibition [165].

Although to date the vast majority of drug development efforts have focused on targeting the N-domain ATP binding site of HSP90, a second druggable site has been identified in the C-domain of the protein [223,224]. Coumarin antibiotics, such as novobiocin, are the prototypic inhibitors that interact at this site. Recently, investigators have made significant advances in improving the affinity of these compounds for HSP90 and have shown their ability to induce apoptosis in cancer cells, in some cases with superior efficacy compared with tanespimycin [225,226]. One potential benefit of these drugs is that some of the C-terminal inhibitors seem to be associated with significantly less-robust HSF1 activation than is characteristic of N-terminal inhibitors [227]. Existing data strongly support further medicinal chemistry optimization and preclinical evaluation of C-terminal HSP90 inhibitors.

When taken together, these data show that several tumor suppressor pathways may be deregulated following HSP90 inhibition. They further emphasize that the cumulative impact of an HSP90 inhibitor on both the individual and the cancer cell is multifactorial and will almost certainly be influenced by the duration of treatment, the disparate sensitivity to HSP90 inhibition of the various client proteins present in normal and cancer cells, the dependence of the particular cancer on the continued expression of one or more of these clients, and the local environmental context in which HSP90 inhibition occurs. Nonetheless, with these caveats in mind, the promising clinical responses that continue to be seen with several HSP90 inhibitors in a number of molecularly defined cancers certainly support the continued therapeutic development of these agents [165].

Although targeting HSP90 in cancer patients in order to achieve a significant therapeutic benefit is still a work in progress, a number of highly potent and pharmaceutically improved HSP90 inhibitors that avoid some of the drawbacks of the first-generation inhibitors, as discussed above, are now in clinical trial. In the long term, realizing the full therapeutic potential of HSP90 inhibitors may require concomitant inhibition of HSP70 isoforms or blockade of HSF1. In addition, differentiating tumors by client protein status and HSP90 expression level, more complete genetic profiling of tumors as a basis for patient selection, and careful HSP90 combinational therapies will help guide the field to a more efficacious use of HSP90 inhibiting drugs [165].

1.7. Cancer Immunity

1.7.1. Immunosurveillance: Immune response to cancer

The relationship between immune system and tumors is a complex one, as cells of the immune system can inhibit tumor progression, but under different circumstances, can aid tumor progression and angiogenesis as well. Immunodeficiency can predispose to tumor development, as observed in the case of Rag -/- mice [228]. In addition, established tumors often generate immunosuppressive microenvironments that can block productive antitumor immunity. Through a deeper understanding of the complicated relationship between tumors and the immune system, tumor immunology strives to harness the immune system to generate protective antitumor responses in patients [229,230].

Although the importance of antitumor immunity is most evident when considering tumors of viral origin, inherent immunodeficiencies in both mice and humans are associated with an increased incidence of tumors with no known infectious etiology, suggesting a role for spontaneous immunity in preventing tumor development . Extensive work in experimental systems has elucidated some of the mechanisms underlying spontaneous antitumor immunity, and has formed the basis for the cancer immunoediting hypothesis. This hypothesis divides the immune response to cancer into three phases: “elimination,” “equilibrium,” and “escape” [231]. The elimination phase occurs early during tumor growth, when productive antitumor immunity can efficiently eradicate malignant cells, preventing the growth of tumors. Based on work in animal models, tumor elimination by the immune system appears to involve the production of IFNγ, as well as the generation of tumor-reactive cytotoxic T cells. Several strains of immunodeficient mice, including mice lacking T cells and mice deficient in IFNγ, are also more susceptible to sporadic tumor development [232,233]. The second phase – equilibrium - occurs after a tumor has been established. During this phase, antitumor immunity effectively inhibits tumor progression, but does not fully eradicate the tumor,

leading to a stable disease stage. Recent studies in mice treated with low dose MCA have identified a subset of animals that develop tumors, which do not progress. Although these tumors remain stable for months in the absence of therapy, transient suppression of the adaptive immune system can induce rapid tumor growth, indicating that these stable tumors were controlled by adaptive immunity [232,233]. Following the equilibrium phase, tumors evolve to escape the immune response, enabling progressive tumor growth. Most tumors reach clinical attention after entering the escape phase, explaining the absence of effective, spontaneous antitumor immune responses in most patients [234].

Several lines of evidence suggest that, similar to animal models, spontaneous antitumor immunity can play a role in cancer patients. Immune infiltrates are common in many cancers, and infiltrates comprising activated $CD8^+$ and memory $CD45RO^+$ T cells have a strong correlation with a favorable prognosis. Several types of cancer are associated with distinct autoimmune syndromes, and cancer patients who have autoantibodies without frank autoimmune disease have improved prognosis. Equilibrium between tumor growth and immune rejection may also occur in some patients [235].

1.7.2. Tumor mediated immune suppression

The microenvironment of many tumors is highly immunosuppressive which presents a substantial barrier to tumor immunotherapy. The microenvironment of tumors is established through the coordinated activity of regulatory myeloid and lymphoid cells, as well as the secretion/expression of immune suppressive factors by tumors themselves. Tumor cells often secrete immunosuppressive cytokines, including IL-10, TGF-beta, PG-E2 and VEGF; these cytokines not only inhibit cytotoxic immune responses, but may also promote the formation or recruitment of additional regulatory cells [236]. In addition, unidentified tumor-derived factors modulate phosphorylation levels of signal transducers and activators of transcription (STAT)-3 and indoleamine 2,3-dioxygenase (IDO)

expression in DCs, resulting in so-called “tolerogenic” DC. IDO enzymatically depletes tryptophan, resulting in T cell anergy and in the apoptotic death of activated T cells due to the production of toxic metabolites, including kynurenines. Constitutive expression of IDO by tumors has also been reported and found to negatively impact T cell vitality and function within the tumor microenvironment [237,238].

A broad range of genetic alterations in tumor cells that have been linked to “immunoevasion”. Tumor cells may develop insensitivity against IFNγ produced by DCs, NK cell and T cells [232,239]. Loss of TRAIL (death receptor signaling) expression has also been observed to contribute to tumor cell survival in the face of active immunity [240].

It is also known that tumor cells down-regulate many molecules involved in the processing and presentation of antigenic peptides in MHC class I complexes [241,242]. For example, high variability in expression has been reported for human leukocyte antigen (HLA)-class I expression among ascitic cells from ovarian carcinoma, and for transporters associated with antigen processing (TAP) and β-2 microglobulin (β2m) on tumor cell lines [243]. Although, tumors exhibiting low levels of MHC class I expression may conversely be targeted by natural killer (NK) cells, tumor cells may also shed tumor associated stress-induced ligands (MICA and MICB), which can bind to activating NK cell receptors, such as NKG2D, and cause tumor lysis [244]. In addition, tumor cells may acquire expression of T cell death/inhibitory inducing molecules such as FasL and B7-H1. B7-H1 is a co-stimulatory molecule that can also inhibit activated PD-1+ effector T cells, is also frequently expressed on tumor cells in situ [245,246].

Tumor-derived (TD) exosomes can also exert immunoinhibitory effects and can be isolated from tumors and bodily fluids from patients with cancer. Tumors reported to release exosomes include cancers of the breast, oral cavity, colorectum, brain, ovary, bladder, prostate, and melanomas [247,248]. Exosomes contain molecules of the

neoplastic cells of origin, for example, urinary exosomes have molecular features of associated urologic malignancies, and exosomes from patients with melanomas contain Melan A/Mart 1 ([248]). TD exosomes variably contain epidermal growth factor receptor (EGFR), EGFRvIII, HSPs 27, 60, 70, 72, 73, 80, and 90 ([247,249,250], tumor necrosis factor alpha (TNFα), Fas ligand (FasL), and TGFβ [250,251].

Some tumors release exosomes that express FasL and/or tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; refs. [252-256]) and these can cause apoptosis in activated T cells [252,253,255-257]. In addition, TD exosomes containing $TGF\beta_1$ cause a downregulation of the NKG2D receptor, which is an activating receptor for NK and $CD8^+$T cells [258]. Thus, tumors-derived exosomes can partially suppress immune reactions using multiple mechanisms.

Because exosomes from DCs can potentiate crosspresentation of antigens to specific T effector cells that are capable of attacking tumor cells and limiting tumor growth and metastasis. [248,259-261], TD exosomes could mediate profound immunosuppression via their inhibition of APC function. In support of this, TD exosomes cause time-dependent inhibition of the maturation of immature DCs via a dose-dependent, increased expression of IL-6 and phosphorylation of Stat 3 [262,263].

In addition, HSP90A is released by invasive cancer cells in the form of exosomes, which can activate plasmin, a protease important in cancer cell invasion.

Suppression of tumor immunity by $CD4^+$ Tregs, which was originally described in the early 1980s, was largely ignored. However, the demonstration that Treg depletion in mouse models of cancer improves endogenous immune-mediated tumor rejection and tumor antigen–specific immunity quickly rekindled interest in the role of Tregs in tumor immunopathology [264,265]. Further work demonstrated that Treg depletion augments tumor immunotherapy, including vaccination and cytotoxic T lymphocyte–associated antigen 4 (CTLA4) blockade, and even augments immunologic rejection of brain tumors, which are difficult to treat using immunotherapeutic approaches because of the blood-brain barrier [266,267]. Tregs can inhibit tumor-specific $CD8^+$ and $CD4^+$ T cell effector functions through incompletely understood mechanisms including cell-cell contact and/or the production of soluble factors such as IL-10 or TGF-β [268-270]. Numbers of Tregs

are increased in the blood and populate the tumor mass and draining lymph nodes of patients with many different cancers. Interestingly, tumor cells may release chemokines, such as CCL22 and SDF-1, which attracts Tregs into tumor sites *in vivo* [271-278]. Exosomal suppression of immunity may also be caused by a transition of $CD4^+$, $CD25^-$ T cells to $CD4^+$, $CD25^+$, $Foxp3^+$ T-regulatory cells (Treg) via intrinsic phosphorylation of SMAD2/3 and Stat3. Such interactions in peripheral tissues mediated by TD exosomes may participate in the maintenance of a state of immune tolerance [279-281].

Tregs are of two origins — natural and adaptive. Natural $CD4^+CD25^+$ Tregs arise in the thymus under homeostatic conditions to safeguard against autoimmunity. Adaptive $CD4^+CD25^+$ Tregs arise during inflammatory processes such as infections and cancers and suppress immunity through heterogeneous mechanisms that include direct contact or the production of soluble factors such as IL-10 and TGF-β [282]. Tregs that infiltrate the tumor microenvironment are probably adaptive. The tumor itself and cells in the tumor microenvironment, such as DCs, induce the differentiation of Tregs through various mechanisms including the production of TGF-β and the expression of B7-H1 [283,284]. Different pathways of Treg differentiation in tumors probably lead to heterogeneous populations of infiltrating Tregs. At least some tumor-associated Tregs are specific for tumor antigens, although once activated, they can also suppress tumor antigen–independent immune responses though bystander mechanisms [285,286]. In addition to inhibiting the function of T cells, Tregs also inhibit the function of NK cells, B cells, and other immune cells, making Tregs an attractive inhibitory target for cancer therapy [287].

In addition to Tregs, certain myeloid lineage cells residing in the tumor stroma may down-modulate immune responses and/or directly support tumor growth. The myeloid derived suppressor cells (MDSCs), discovered by Bronte *et al.* as a population of cells expressing the markers Gr1 and CD11b. MDSCs represent an intrinsic part of the myeloid-cell lineage and are a heterogeneous population that is comprised of myeloid-cell progenitors and precursors of myeloid cells [288]. In healthy individuals, immature myeloid cells (IMCs) generated in bone marrow quickly differentiate into mature granulocytes, macrophages or dendritic cells (DCs). In pathological conditions such as

cancer, various infectious diseases, sepsis, trauma, bone marrow transplantation or some autoimmune disorders, a partial block in the differentiation of IMCs into mature myeloid cells results in an expansion of this population. Importantly, the activation of these cells results in the upregulated expression of immune suppressive factors such as arginase (encoded by *ARG1*) and inducible nitric oxide synthase (iNOS; also known as NOS2) and an increase in the production of NO (nitric oxide) and reactive oxygen species (ROS). Together, this results in the expansion of an IMC population that has immune suppressive activity; these cells are now collectively known as MDSCs [289]. Their accumulation has been documented in most patients and mice with cancer, where they are induced by various factors produced by tumor cells and/or by host cells in the tumor microenvironment. MDSC are considered a major contributor to the increased immune dysfunction of most patients with sizable tumor burdens. In tumor-bearing mice MDSC accumulate in the bone marrow, spleen, and peripheral blood, within primary and metastatic solid tumors, and to a lesser extent in lymph nodes. In cancer patients they are present in the blood, and it is not known whether they are present in other sites [290-293]. In both patients and experimental animals MDSC levels are driven by tumor burden and by the diversity of factors produced by the tumor and by host cells in the tumor microenvironment. Exosomes also increase MDSC development/maintenance via TGFβ and regulation of prostaglandin E2 (PGE2). In addition, HSP72 in exosomes may interact with toll-like receptor 2 and MyD88 expressed by MDSCs, leading to the activation of MDSCs in association with increased expression of IL-6 and phosphorylation of Stat 3 [249,294,295].

1.8. Immunotherapy

Cancer immunotherapy is the use of the immune system to reject cancer. The main idea is stimulating the patient's immune system to attack the malignant tumor cells that are responsible for the disease. This can be either through immunization of the patient (e.g.,

by administering a cancer vaccine), in which case the patient's own immune system is trained to recognize tumor cells as targets to be destroyed, or through the administration of therapeutic antibodies as drugs, in which case the patient's immune system is recruited to destroy tumor cells by the therapeutic antibodies. Cell based immunotherapy is another major entity of cancer immunotherapy. This involves immune cells such as the Natural killer Cells (NK cells), Lymphokine Activated killer cell (LAK), Cytotoxic T Lymphocytes(CTLs), Dendritic Cells (DC), etc., which are either activated *in vivo* by administering certain cytokines [296,297].

Many kinds of tumor cells that arise as a result of the onset of cancer are more or less tolerated by the patient's own immune system since the tumor cells are essentially the patient's own cells that are growing, dividing and spreading without proper regulatory control. In spite of this fact, however, many kinds of tumor cells display unusual antigens that are either inappropriate for the cell type and/or its environment, or are only normally present during the organisms' development (e.g. fetal antigens). Other kinds of tumor cells display cell surface receptors that are rare or absent on the surfaces of healthy cells, and which are responsible for activating cellular signal transduction pathways that cause the unregulated growth and division of the tumor cell. Examples include ErbB2, a constitutively active cell surface receptor that is produced at abnormally high levels on the surface of breast cancer tumor cells [298,299].

1.8.1. Monoclonal antibody therapy

Antibodies are a key component of the adaptive immune response, playing a central role in both in the recognition of foreign antigens and the stimulation of an immune response to them. It is not surprising therefore, that many immunotherapeutic approaches involve the use of antibodies. With monoclonal antibodies, it possible to raise antibodies against specific antigens such as the unusual antigens that are presented on the surfaces of

tumors. A number of therapeutic monoclonal antibodies have been approved for use in humans by the U.S. Food and Drug Administration (FDA) **[297]**.

Alemtuzumab is an anti-CD52 humanized IgG1 monoclonal antibody indicated for the treatment of Chronic lymphocytic leukemia (CLL), the most frequent form of leukemia in Western countries. The function of CD52 is unknown, but it is found on >95% of peripheral blood lymphocytes and monocytes. Upon binding to CD52, alemtuzumab initiates its cytotoxic effect by complement fixation and antibody-dependent cell-mediated cytotoxicity mechanisms **[300]**. Bevacizumab is a humanized IgG1 monoclonal antibody, which binds to and interferes with the vascular endothelial growth factor-A (VEGF-A), preventing receptor activation. Bevacizumab is indicated for colon cancer; but has been applied to numerous other cancers, especially renal cell carcinoma. Results obtained showed that bevacizumab increased the duration of survival, progression-free survival, the rate of response and the duration of response in a statistically relevant manner **[301]**. Rituximab is a chimeric monoclonal antibody specific for CD20. CD20 is widely expressed on B-cells. The exact mode of action of rituximab is also unclear, but it has been found to have a general regulatory effect on the cell cycle and on immune-receptor expression. Experiments involving primates showed that treatment with anti-CD20 reduced peripheral B-cells by 98%, and peripheral lymph node and bone marrow B-cells by up to 95% **[302]**. Trastuzumab is a monoclonal IgG1 humanized antibody specific for the epidermal growth factor receptor 2 protein (HER2). It received FDA-approval in 1998, and is clinically used for the treatment of breast cancer. The use of Trastuzumab is restricted to patients whose tumors over-express HER-2, as assessed by immunohistochemistry (IHC) and either chromogenic or Fluorescent in situ hybridization (FISH), as well as numerous PCR-based methodologies **[303,304]**.

1.8.1.1. Advances in monoclonal antibody immunotherapy

The development and testing of second generation immunotherapies are already under way. The targeted delivery of cytokines by anti-tumor antibodies is one example of using antibodies to delivery payloads rather than simply relying on the antibody to trigger an immune response against the target cell. Another strategy is to deliver a lethal radioactive dose directly to the target cell, which has been utilized in the case of the Zevalin therapeutic agent. Still another strategy is to deliver a lethal chemical dose to the target, as used in the Mylotarg therapeutic agent. Engineering the antibody-chemical pair in such a way that they separate after entry into a cell by endocytosis can potentially increase the efficacy of the payload. One strategy to accomplish this is the use of a disulfide linkage that could be severed by the reducing conditions in the cellular interior. However, recent evidence suggests that the actual intracellular trafficking of the antibody-payload after endocytosis is such to make this strategy not generally applicable. Other potentially useful linkage types include hydrazone and peptide linkages [305].

1.8.2. Adoptive T cell therapy

1.8.2.1. T-cell adoptive transfer

Adoptive cell transfer uses T cell-based cytotoxic responses to attack cancer cells. T cells that have been stimulated or genetically engineered reactivity to a patient's cancer are generated *in vitro* and then transferred back into the cancer patient. One study using autologous tumor-infiltrating lymphocytes was an effective treatment for patients with metastatic melanoma [306]. This was achieved by taking tumor-infiltrating lymphocytes (TILs) that are trained to multiply *in vitro* using high concentrations of IL-2, anti-CD3 and allo-reactive feeder cells. These T cells are then transferred back into the patient

along with exogenous administration of IL-2 to further boost their anti-cancer activity. So far, a 51% objective response rate has been observed; and in some patients, tumors shrank to undetectable size [307,308].

The initial studies of adoptive cell transfer using TIL, however, revealed that persistence of the transferred cells *in vivo* was too short [309]. Before reinfusion, lymphodepletion of the recipient is required to eliminate regulatory T cells as well as normal endogenous lymphocytes that compete with the transferred cells for homeostatic cytokines [310-312]. Transferred cells expanded *in vivo* and persisted in the peripheral blood in many patients, sometimes achieving levels of 75% of all $CD8^+$ T cells at 6–12 months after infusion. Clinical trials based on adoptive cell transfer of TILs for patients with metastatic melanoma are currently ongoing at a handful of institutes around the world [313].

1.8.2.2. Genetically engineered T cells

Genetically engineered T cells may be created by infecting patient's cells with a virus that contain a copy of a T cell receptor (TCR) gene that is specialized to recognize tumor antigens. The virus is not able to reproduce within the cell however integrates into the human genome. A patient's own T cells are exposed to these viruses and then expanded non-specifically or stimulated using the genetically engineered TCR. The cells are then transferred back into the patient and are ready to have an immune response against the tumor. Morgan *et al.* have demonstrated that the adoptive cell transfer of lymphocytes transduced with retrovirus encoding TCRs that recognize a cancer antigen are able to mediate anti-tumor responses in patients with metastatic melanomas. This therapy has been demonstrated to result in objective clinical responses in patients with refractory stage IV cancer [314]. The Surgery Branch of the National Cancer Institute (Bethesda, Maryland) is actively investigating this form of cancer treatment for patients suffering aggressive melanomas (Steven Rosenberg, personal communication). The use of adoptive

cell transfer with genetic engineered T cells is a promising new approach to the treatment of a variety of cancers.

1.8.2.3. TIL therapy

Adoptive transfer therapy with TILs requires the isolation of T cells from fresh patient biopsy specimens and the progressive selection of tumor-specific T cells ex vivo using high levels of IL-2 and various cell culture approaches. The adoptive transfer of these cells showed promise in preclinical models, but clinical experiences, with perhaps one exception, were almost uniformly disappointing [315-319]. However, recent studies at the National Cancer Institute suggest that prior host conditioning with chemotherapy increases the response to adoptive immunotherapy with TILs. When 13 patients with progressive metastatic melanoma were given cyclophosphamide and fludarabine, a drug regimen that is immunosuppressive but does not have anti-melanoma efficacy, 6 patients had partial responses as judged by Response Evaluation Criteria in Solid Tumors (RECIST), and 4 others had mixed responses, i.e., some of their tumors regressed but others remained [315,320]. This approximately 50% objective response was confirmed in a subsequent report from the same group [320]. Importantly, the TILs showed prolonged engraftment compared with TILs transfused to patients without prior treatment with these chemotherapeutics, and the levels of engraftment correlated with the clinical responses. Indeed, concomitant host immunosuppression seems to be important because only 34% of patients with melanoma who were treated with TIL administration and high-dose IL-2 and who received no prior chemotherapeutic conditioning therapy to induce lymphodepletion achieved objective clinical responses in trials previous to the incorporation of host lymphodepletion [318]. Most of the responses were transient, and the patients had limited persistence of the transferred cells in those trials. Adverse effects in the lymphodepletion trial included opportunistic infections and the frequent induction of vitiligo and uveitis, presumably due to autoimmunity. However, at this point, the results are difficult to interpret, as the ability to successfully generate TILs for therapy

could be a predictive biomarker of a more favorable clinical outcome [321,322]. However, in the absence of a randomized clinical trial it is not possible to determine how much lymphoablative chemotherapy, high-dose IL-2 administration, and TIL therapy contributed to the promising results in these recent trials. If it is confirmed that lymphodepletion augments TIL efficacy, the results from recent trials indicate that induction of immunosuppression in the host improves the antitumor efficacy of adoptive TIL therapy [320,323].

Technical issues with producing tumor-specific T cells currently present a formidable barrier to conducting randomized clinical trials using TILs. Only 30%–40% of biopsy specimens yield satisfactory T cell populations, and the process is labor and time intensive, requiring about 6 weeks to produce the T cells for infusion [313]. Furthermore, nearly all clinical experience with TILs has been with patients with melanoma because of the ready surgical availability of tumor biopsy tissue. However, should technical limitations of current tissue culture approaches be overcome, the recent studies indicating that the presence of TILs correlated positively with survival in ovarian and colorectal cancer [324] could extend the impact of this promising therapeutic approach to other commonly encountered epithelial cancers.

1.8.2.4. T cells engineered to express tumor antigen–specific receptors.

A principal limitation of adoptive T cell therapy for some tumors is that the tumors are poorly antigenic; therefore, neither T cells with high avidity for tumor-specific antigens, nor T cells with the desired specificity remain in the patient following chemotherapy. Two strategies to overcome this limitation are now being tested in the clinic. One approach has been to endow T cells with novel receptors by introduction of chimeric receptors that have antibody-based external receptor structures and cytosolic domains that encode signal transduction modules of the T cell receptor [325]. These constructs can function to retarget T cells *in vitro* in an MHC-unrestricted manner to attack the tumor

while retaining MHC-restricted specificity for the endogenous TCR. Three pilot clinical trials have recently been reported. A trial that tested T cells expressing a T body receptor specific for a folate-binding protein that is present on ovarian carcinoma cells indicated that the approach was safe, but poor expression and persistence of the transgene encoding the T body receptor were observed *in vivo* [268]. Similarly, a pilot study in children with neuroblastoma treated with autologous T cells retargeted for a tumor-associated adhesion molecule has indicated that the approach is safe but was limited by poor persistence of the T cells. In several of the patients in clinical studies, the engineered cells persisted for several days to weeks before elimination by host immune responses, indicating that a challenge for this approach is to prevent a host immune response from eliminating the adoptively transferred cells [268,326-328]. The other major issues with the approach currently involve improving receptor design by optimizing the ligand-binding domain and by trying to incorporate costimulatory signaling domains into the signaling molecule [328].

T cells are also being transduced to express natural αβTCR heterodimers of known specificity and avidity against MHC-presented tumor antigens [329]. In the first clinical trial using this approach, T cells were engineered to express a TCR specific for glycoprotein 100 (gp100), and lymphodepleted patients with melanoma were given a single infusion of these engineered T cells followed by an infusion of IL-2 [314]. A concern with this approach has been that it might generate additional, novel receptor specificities by pairing of the transgenes with the endogenous TCR chains. It is encouraging that no toxicity was observed in the pilot trial, and promising persistence of the engineered T cells was observed in some of the patients. However, one issue that arose was low cell-surface levels of expression of the gp100-specific TCR, which would be expected to lower the avidity of the TCR and therefore to limit effector functions. Another general limitation of this approach for humans is that each TCR is specific for a given peptide-MHC complex, such that each vector would only be useful for patients that shared both MHC alleles and tumor antigens [330].

1.8.2.5. The future of adoptive therapy with engineered T cells.

The field of adoptive therapy with engineered T cells is on the brink of substantial clinical advances that are now possible because of improved cell culture and gene transfer methods. The advent of lentiviral vectors has greatly increased the efficiency of human T cell engineering, and a recent pilot study with lentiviral engineered T cells that expressed an anti-sense HIV vector showed promise in patients infected with HIV. As mentioned above, insertional mutagenesis is a safety concern with any integrating viral vector. However, side-by-side tests in preclinical models indicate that lentiviral vectors are less prone to insertional mutagenesis [331-333]. Nevertheless, long-term observational studies with large patient safety data sets are required to determine the ultimate safety of this approach. Finally, a primary issue that could limit the ultimate efficacy of the approach is the immunogenicity of the proteins that the T cells are engineered to express; this is likely to be a larger problem in humans than in mice because activated human T cells, unlike mouse T cells, express MHC class II molecules and have been shown to function as effective APCs [334].

1.8.3. DC-based vaccines

Both immunity and tolerance are controlled by a network of professional APCs, the most important of which are known as DCs [335,336]. Tissue-resident DCs that capture pathogen-encoded antigens are activated by stimuli generated in the course of a pathogen-induced inflammatory response. Activation of DCs occurs in two phases, maturation and licensing, and is an essential step that enables the antigen-loaded DCs to migrate to the draining lymph nodes where they can activate T cells that recognize the antigens they are presenting (cognate T cells) [337]. Unlike infectious pathogens, tumors do not induce an effective inflammatory response conducive for optimal activation of DCs, and as a result the ensuing immune response is weak and ineffective. The primary

purpose of vaccinating individuals with cancer is to overcome this “defect” by channeling tumor antigens into DCs and providing the conditions for their optimal maturation into potent immunostimulatory APCs [338].

One approach that is gaining increasing popularity among tumor immunologists is to immunize cancer patients with autologous, patient-derived DCs loaded with tumor antigens ex vivo. The underlying premise of this approach is that the efficiency and control provided by ex vivo manipulation of the DCs generates an optimally activated APC and a superior method for stimulating immunity *in vivo* as compared with more traditional vaccination methods. Recent years have witnessed rapid and remarkable progress in developing DC-based vaccines, however the field is yet to make remarkable strides towards long-term elimination of cancer [338].

1.8.3.1. Ex vivo generation of immunocompetent DCs

The era of *ex vivo* DC vaccines was ushered in by the pioneering work of Inaba, Steinman, and colleagues, demonstrating that mouse DCs can be cultured ex vivo from bone marrow precursors [339]. In a similar fashion, human DCs can be generated in culture from $CD34^+$ hematopoietic progenitors and, more commonly, from peripheral blood–derived monocytes. For cancer vaccination, the goal is to generate ex vivo a population of antigen-loaded DCs that stimulates robust and long-lasting $CD4^+$ and $CD8^+$ T cell responses in the patient with cancer. What seems to be the rate-limiting step at present is the inability to fully recapitulate ex vivo the development of immunocompetent DCs, in particular the process of DC activation [340-342]. DC activation can be divided into two stages. In the periphery, immature DCs undergo a maturation process in response to inflammatory stimuli that gives the DCs the capacity to home to lymph nodes. DCs receiving the appropriate maturation stimuli upregulate expression of CC chemokine receptor 7 (CCR7) and become responsive to CC chemokine ligand 19 (CCL19) and CCL21, chemoattractants produced in the afferent lymphatics and the

lymph node. When reaching the lymph node, antigen-loaded mature DCs undergo an additional activation step, termed “licensing,” in response to various stimuli, notably CD40 ligand (CD40L) which is expressed on cognate $CD4^+$ T cells. For generating DC vaccines, therefore, the goal is to differentiate antigen-loaded DCs only to the point that they have acquired lymph node migratory capacity and become responsive to licensing stimuli when they reach the lymph node and encounter cognate T cells [343].

Immature DCs express low levels of CCR7 and cannot migrate to lymph nodes as effectively as mature DCs, which express high levels of CCR7. However, injecting immature DCs into skin that has been pre-exposed to adjuvants effectively induces the maturation of DCs *in situ*; the mature DCs then migrate to lymph nodes and, being superior to the ex vivo matured DCs, activate antitumor immunity. *In situ* maturation offers a simpler, effective method for generating DCs for cancer immunotherapy [344,345]. Other agents such as heat shock proteins (HSPs) also enhance the maturation and migration of DCs, thereby eliciting the antitumor immunity of DC vaccines. A recent report found that Hsp70-like protein 1 (Hsp70L1), a novel HSP derived from human DCs, promotes the maturation and activation of DCs, as well as increases CCR7 expression in DCs [346]. Furthermore, DCs pulsed with the recombinant fusion protein of Hsp70L1 and CEA (576-669) induce a more potent CEA (576-669)-specific CTL response than DCs pulsed with CEA (576-669) alone. Adoptive transfer of splenocytes from mice immunized with CEA (576-669)-Hsp70L1-pulsed DC inhibits tumor growth and prolongs the survival of colon carcinoma-bearing mice more markedly than splenocytes from mice immunized with CEA (576-669)-pulsed DCs. Thus, Hsp70L1 may be widely used as a Th1 adjuvant for enhancing the efficacy of DC vaccines for treatment of cancers [347].

1.8.3.2. Clinical trials with DC-based vaccines

Multiple clinical trials have been carried out to date targeting different cancers using different methods of generating DCs, different antigens, and different antigen-loading techniques [348]. At this early stage of clinical development, no indication or evidence has been obtained that DC vaccines represent a method of stimulating protective immunity in cancer patients that is superior to other vaccination strategies. In most studies, a fraction of patients, often half or less, exhibited immune responses against the vaccinating antigen [349]. Despite occasional correlations between immunological and clinical responses in such single-arm clinical trials, it is not known whether the modest clinical responses were caused by the vaccination or whether they reflect patients with better prognoses capable of mounting immune responses. The field of DC vaccines suffered a serious setback when a phase III clinical trial in patients with stage IV melanoma failed to demonstrate that DC vaccination provided increased benefit compared with standard DTIC chemotherapy [350]. The overall response was low in both patient groups (DTIC, 5.5%; DC vaccinated, 3.8). The study was therefore discontinued. It is conceivable that the suboptimal nature of the cytokine cocktail maturation protocol discussed above might have had an important role in the failure of this trial. In addition, it is important to appreciate the fact that a DC vaccination protocol is a complex, multi-step process and that a myriad of seemingly trivial steps such as how the cells are frozen and thawed, how long the cells are matured, the mechanics of their administration, and the time intervals between boosting can have a critical impact on the outcome of the treatment [338].

Another example offering a glimpse at the promise of DC vaccines is the clinical experience of Vieweg and colleagues, in which patients with prostate cancer vaccinated with DCs transfected with mRNA encoding tumor antigens such as PSA or TERT and patients with renal cancer vaccinated with DCs transfected with unfractionated tumor-derived mRNA developed tumor antigen–specific $CD8^+$ T cell responses. Virtually all vaccinated patients responded immunologically with the induction of measurable T cell

responses [351-354]. Furthermore, clinically related responses, such as reduction in PSA levels, were often seen in the vaccinated patients in the prostate cancer trials. As an indication of things to come, in a recent phase I/II clinical trial, Dannull *et al.* were able to show that partial removal of Tregs can further potentiate DC vaccine–induced immune responses in cancer patients [351].

Nevertheless, many immunologists maintain that DC vaccination remains a promising therapy for cancers; with a deeper understanding of DC biology, antitumor immunity, and the immune escape mechanisms, numerous novel and improved DC-based vaccines have been developed. These endeavors eventually led to the approval of the first DC vaccine, sipuleucel-T (Provenge) for the treatment of prostate cancer, by the US FDA in 2010 [355]. Sipuleucel-T is a perfect example of a DC vaccine used in translational medicine: several cycles of **“** bench to bedside and then back to bench**”** happen, during which the basic scientific understanding is applied to clinical treatments for cancers and then clinical feedback provides guidance for further scientific research.

1.9. SCOPE OF THE THESIS

The studies performed in this thesis were designed to assess the efficacy of integrating HSP90 inhibition with EphA2-specififc immunotherapies. Specifically, I looked at the ability of HSP90 inhibitor 17-DMAG to increase the presentation of EphA2-derived peptides in the context of MHC class I molecules on the tumor cell surface in order to break tumor-mediated immune tolerance. HSP90 is a critical protein for tumor growth and survival, giving HSP90 inhibition a distinct operational advantage – deregulation of multiple pro-tumorigenic pathways apart from increasing susceptibility of the tumor to the host immune system. My findings indicate that 17-DMAG enhances recognition of both tumor cells and tumor-associated stromal cells by HS90 client protein-specific CD8+ T cells. In addition, treatment of tumor-bearing mice with 17-DMAG for a short duration also increased the infiltration of inflammatory immune cells into the tumor site over a prolonged period of time. Integration of 17-DMAG in combination immunotherapeutic regimens targeting tumor EphA2 led to improved anti-tumor efficacy *in vivo*. I believe this work supports a general therapeutic paradigm allowing for the combined immune targeting of a broad range of tumor cell (over)expressed HSP90 client proteins such as survivin, p53, etc.,, thus rendering this approach extremely versatile for the coordinate targeting of tumor and its associated vasculature alike in most solid forms of cancer.

CHAPTER 2. HSP90 INHIBITOR COORDINATELY ENHANCES THE DEGRADATION OF MULTIPLE RECEPTOR TYROSINE KINASES IN MULTIPLE TUMOR CELL LINES

Aparna Rao, Mayumi Kawabe, Jennifer L. Taylor, and Walter J. Storkus*.

From the Departments of *Immunology and Dermatology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, and the University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213.

All the results reported in this study were obtained by Aparna Rao.

2.1. ABSTRACT

HSP90 is an important chaperone molecule that binds multiple oncoproteins that play important roles in tumorigenesis and vasculogenesis. In tumors, HSP90 stabilizes and mediated the proper (re)folding of these “client” proteins. Therefore, inhibiting the action of HSP90 using 17-DMAG would be expected to induce the concomitant degradation of multiple HSP90 clients including RTKs mediated by the proteasome. However, along with RTKs, various signaling molecules, especially ones that mediate immunological processes are also known to associate with HSP90, and these could also be downregulated upon inhibition of HSP90 function. Indeed, HSP90 inhibition mediated by geldanamycin and 17-AAG (predecessors of 17-DMAG) have been observed to perturb T cell function and antigen presentation mediated by DCs. In the current study, I investigated the effects of 17-DMAG on a range of RTKs (over)expressed by a number of unrelated tumor cell lines. In addition, I looked at the effect of 17-DMAG on immune cell phenotype and function *in vitro*. 17-DMAG induced a dose-dependent decrease in RTK expression in all tumor cell lines tested, indicating the general versatility of the drug. When I studied its effects on immune cells *in vitro*, 17-DMAG treatment at higher doses (or longer treatment duration) affected DC phenotype and function, as well as the ability of naïve T cells to become activated. These studies indicate that while using 17-DMAG to inhibit HSP90 could be a versatile approach for targeting multiple oncoproteins, the dose and duration of 17-DMAG treatment needs to be carefully regulated in order to avoid any untoward effects involving the host immune system.

2.2. INTRODUCTION

Receptor tyrosine kinases (RTK) are important for various cell processes like cell growth, differentiation and survival [9]. In tumors, RTKs are frequently overexpressed, leading to constitutive on-signaling and deregulated neoplastic growth and survival [1,356]. Mutated and overexpressed RTKs in tumor cells depend on the HSP90 co-chaperoning machinery for sustained protein stability and functionality [4]. Therefore, inhibiting HSP90 via the use of pharmacologic agents would lead to the simultaneous destabilization and degradation of multiple RTKs coordinately expressed in a given tumor cell, leading to broad spectrum inhibition of oncogenesis. HSP90 antagonism (and ultimately RTK degradation) leads to the uncoupling of pro-tumor pathways – including those linked to sustained growth and migration (i.e. metastasis) [5,6,182].

In addition to RTKs, HSP90 is known to interact with an extended list of client proteins (http://www.picard.ch/downloads/Hsp90interactors.pdf) that play important roles in cell signaling and in regulating the apoptotic cell death of normal as well as tumor cells [148]. Thus, inhibiting the function of HSP90 would lead to perturbed expression of anti-apoptotic proteins like Bcl-xl, resulting in the clinically-preferred demise of tumor cells [357]. However, even normal cell functions could be compromised by administration of HSP90 inhibitors; i.e. HSP90 has also been reported to aid in antigen presentation of peptides and the generation of specific immune responses (as a result of antigen crosspriming). A number of HSP client molecules have been reported to play roles in productive immune cell signaling (e.g. Zap70 is a signaling molecule that plays a role in TCR-mediated signaling, while NF-KB is a master transcription factor that mediated the transcription of a host of genes mediating an inflammatory immune response) [358-360]. Indeed, pharmacological HSP90 inhibitors have been observed to inhibit antigen presentation and to dampen immune responses [361]. In fact, HSP90 inhibitors have been touted as treatment options for patients with autoimmune diseases [362].

Hence, in some ways, clinical use of HSP90 inhibitors would be viewed as counterintuitive in the context of immunotherapeutic approaches. It should be mentioned

that the aforementioned studies were performed using Geldanamycin and 17-AAG, which have been generally observed to be markedly more toxic than 17-DMAG which I have used exclusively in my work. The increased toxicity of these predecessor drugs could be due to the fact that they had to be dissolved in organic solvent (DMSO) prior to administration, In contrast, 17-DMAG, is readily dissolved in water and exhibits greater bioavailability after ingestion [182]. The aims of the experiments performed in this chapter were two-fold: 1) to evaluate the effect of 17-DMAG on RTKs in addition to EphA2 in multiple tumor cell lines and 2) to determine any dose-dependent negative effects of 17-DMAG on immune cells like T cell and DC viability and function

2.3. MATERIALS AND METHODS

2.3.1. Tumor cell culture

MCA205 (sarcoma), B16 (melanoma), MC38 (colon carcinoma) and 4T1 (breast cancer) cell lines were purchased from the American Type Culture Collection. 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 (Life Technologies)] in a humidified incubator at 37°C and 5% CO_2. All cell lines were negative for known mouse pathogens and mycoplasma infections.

2.3.2. 17-DMAG treatment

HSP90 inhibitor 17-DMAG (NSC 707545) was obtained from National Cancer Institute (NCI). Lyophilized 17-DMAG was dissolved in sterile water as stock solution and diluted with RPMI 1640 before use. 17-DMAG at the required final concentration was added to cell culture in 6-well plates for 24 or 48 hours before termination of cell culture.

2.3.3. Western blot

Tumor cell lines were incubated with a range of 17-DMAG concentrations (10-500 nmol/L) in complete RPMI 1640 (CM) for 24 to 48 h. To assess the effect of proteasome function in protein degradation promoted by 17-DMAG, MG-132 (50 μmol/L; Peptides international) was added 3h prior to 17-DMAG addition. Harvested cells were then incubated with lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 0.2 mmol/L sodium orthovanadate, 0.5% NP-40 in PBS; all reagents from Sigma-Aldrich) containing a protease inhibitor cocktail (Complete mini; Roche Diagnostic) for 30 min at 4°C. Lysates were cleared by centrifugation at 13,500 × *g* for 10 min, and proteins in the lysate were separated by SDS-PAGE before electro-transfer onto polyvinylidene difluoride membranes (Millipore). Polyclonal anti-Her2 antibody, anti-VEGFR2 and anti-PDGFRB antibodies and horseradish peroxidase–conjugated goat anti-rabbit antibody (all from Santa Cruz Biotechnology) were used to detect the different RTKs. Probed proteins were visualized by Western Lighting chemiluminescence detection kit (Perkin-Elmer) and exposed to X-Omat film (Eastman Kodak) for 5-7 min.

2.3.4. T cell stimulation following 17-DMAG treatment

Spleens from naïve C59BL6/J mice were excised and dispersed into single cell suspensions. CD4+ or CD8+ cells were isolated by incubating the splenocytes with magnetically labeled anti-CD4 or anti-CD8 beads, respectively, and passing labeled cells through a magnetic column. The positively labeled cells were then flushed out of the column and washed three times with serum-free media before being cultured in Complete media (CM) with different concentrations of 17-DMAG (or left untreated) for 24 or 48 hours. After treatment, the cells were washed thoroughly with serum-free media and

stimulated with anti-CD3 at a concentration of 0.2 μg/well for 72 hours. Cell-free supernatants were harvested and assessed for levels of mIFNγ using a specific OptEIA ELISA set (BD Biosciences) as previously described. Data are reported as the mean ± SEM of quadruplicate determinations.

2.3.5. Effect of 17-DMAG on DC phenotype and function

BMDCs were obtained by differentiating cells from bone marrow of naïve C57BL6/J mice with GM-CSF and IL-4 for 7 days. CD11c+ DCs were isolated by MACS as described earlier. DCs were cultured in complete medium with different concentrations of 17-DMAG for 24 or 48 hours, after which, the phenotype of the DCs was assessed by flow cytometry. To determine their ability to stimulate T cells, 17-DMAG treated or untreated DCs were co-cultured with allogenic T cells (from naïve Balb/c mice) for 48 hours. The cell-free supernatant from this culture was assessed for levels of mIFNγ using a specific OptEIA ELISA set (BD Biosciences) as previously described. Data are reported as the mean ± SEM of quadruplicate determinations.

2.3.6. Flow cytometry

Before all stainings, cells were Fc blocked with anti-CD16/CD32 antibody (Becton Dickinson). Single-cell suspensions were stained using the following antibodies: APC - conjugated CD11c (eBioscience), FITC-conjugated H2-Kb and I-Ab (Becton Dickinson), PE-conjugated CD40, CD80 and CD86 (all BD bioscience), DAPI (Sigma) or matched, fluorochrome-labeled isotype control monoclonal antibody (mAb). Cells were analyzed using an LSR II flow cytometer (Beckman Coulter), with data analyzed using FlowJo software (version 7.6.1; Tree Star, Inc.).

2.3.7. Statistical analysis

All comparisons of intergroup means were performed using a two-tailed Student's *t* test, with $P < 0.05$ considered significant.

2.4. RESULTS

2.4.1. Degradation of RTKs upon 17-DMAG treatment

I first investigated the effect of 17-DMAG treatment on tumor cell expression of a number of RTKs known to be important for tumorigenesis and vasculogenesis (i.e. Her2, PDGFRβ and VEGFR2) in a variety of tumor cell lines (i.e. MC38 colon carcinoma, B16 melanoma, and MCA205 sarcoma cells). In all cases, 17-DMAG was observed to mediate a dose-dependent degradation of RTKs, as exemplified in the case of EphA2, compared to untreated tumor cells (Figure 5).

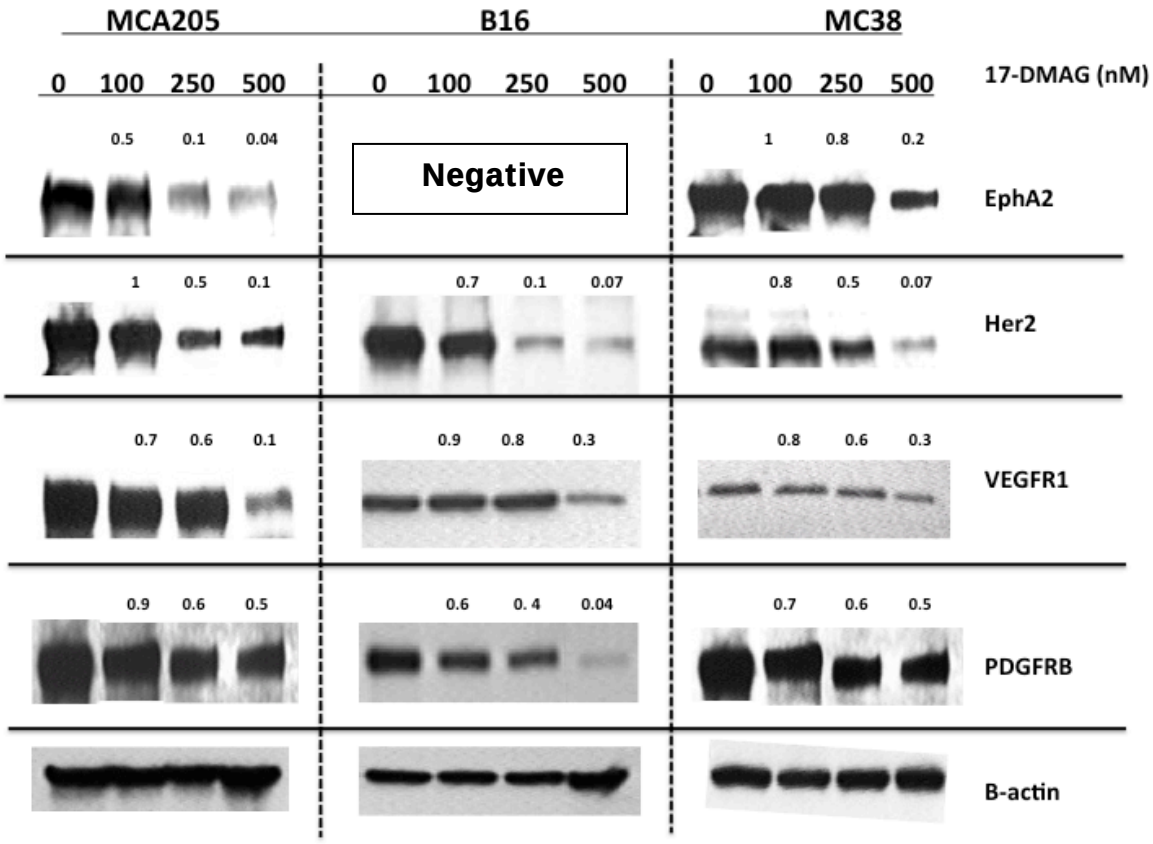


Figure 5: : 17-DMAG affects RTK expression in a dose-dependent manner

Cell lines MCA205 (sarcoma), B16 (melanoma) and MC38 (colon carcinoma) were treated with the indicated concentrations of 17-DMAG for 24 hours. After treatment, cells were lysed and assessed for RTK expression by Western Blot. Numbers indicate fold decrease vs untreated samples

2.4.2. 17-DMAG affects phenotype of DCs

Treatment of BMDCs with 17-DMAG appeared to result in changes in expression of MHC class I and class II molecules on DCs at higher doses (> 500 nm/L) compared to untreated DCs (Figure 7). The effect was observed to be more pronounced after 48 hours of treatment (versus 24 hour treatment). A similar effect of 17-DMAG was observed on other DC markers viz. CD40, CD80 and CD86 that play important costimulatory roles in T cell activation (Figure 6). Thus, in all, 17-DMAG was observed to affect DC surface markers that could potentially affect their function. To test if this effect was due to the increased toxicity of the drug (causing DC death) or due to its capacity to deregulate intrinsic signaling pathways in DCs, I repeated these experiments and stained the cells with DAPI in addition to the cell-type specific surface markers. When I evaluated the expression of DC surface markers after gating on DAPI-positive (live) cells, I did not observe any differences in expression of the assessed markers between treated versus untreated cells, suggesting that increased cell death in DCs was a possible reason for the noted decrease in expression of DC cell surface markers (Figure 8).

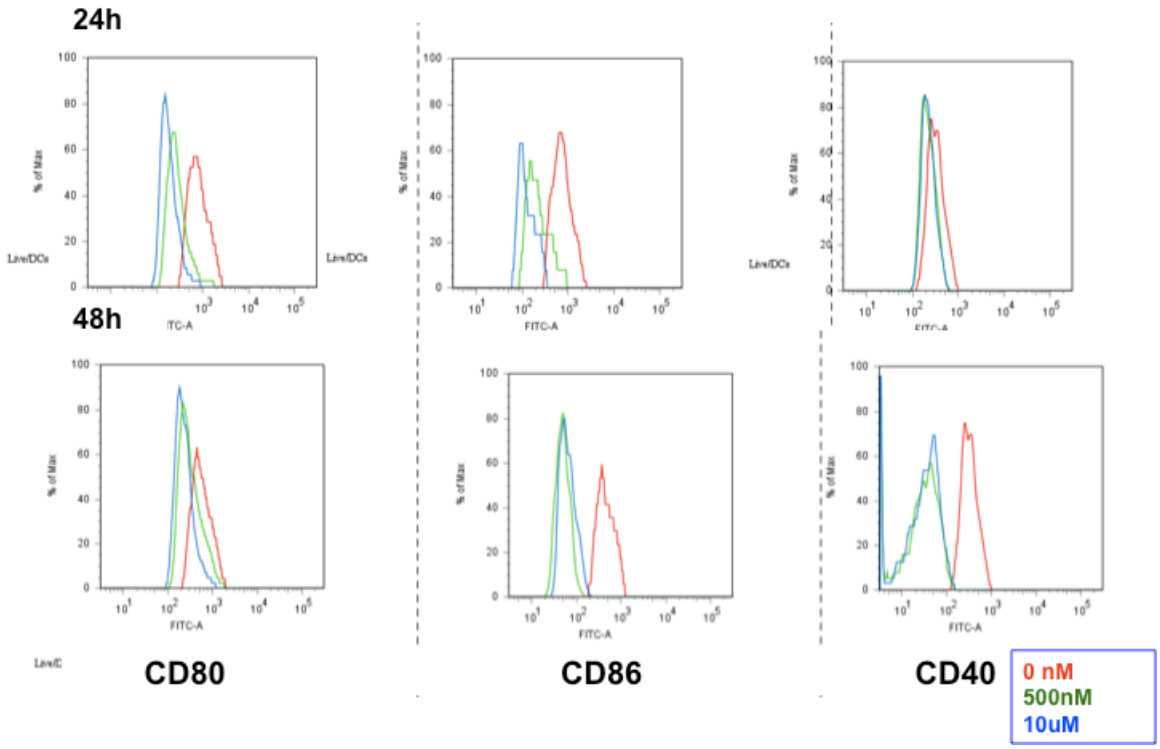


Figure 6: : 17-DMAG treatment decreases expression of co-stimulatory molecules on DCs

DCs were treated with 500nM (green histograms), 10 μM (blue histograms) or left untreated (red histograms) for 24h (top panel) or 48 hours (bottom panel). The markers evaluated were CD80 (left), CD86 (middle) and CD40 (right), with quantitation performed using flow cytometry.

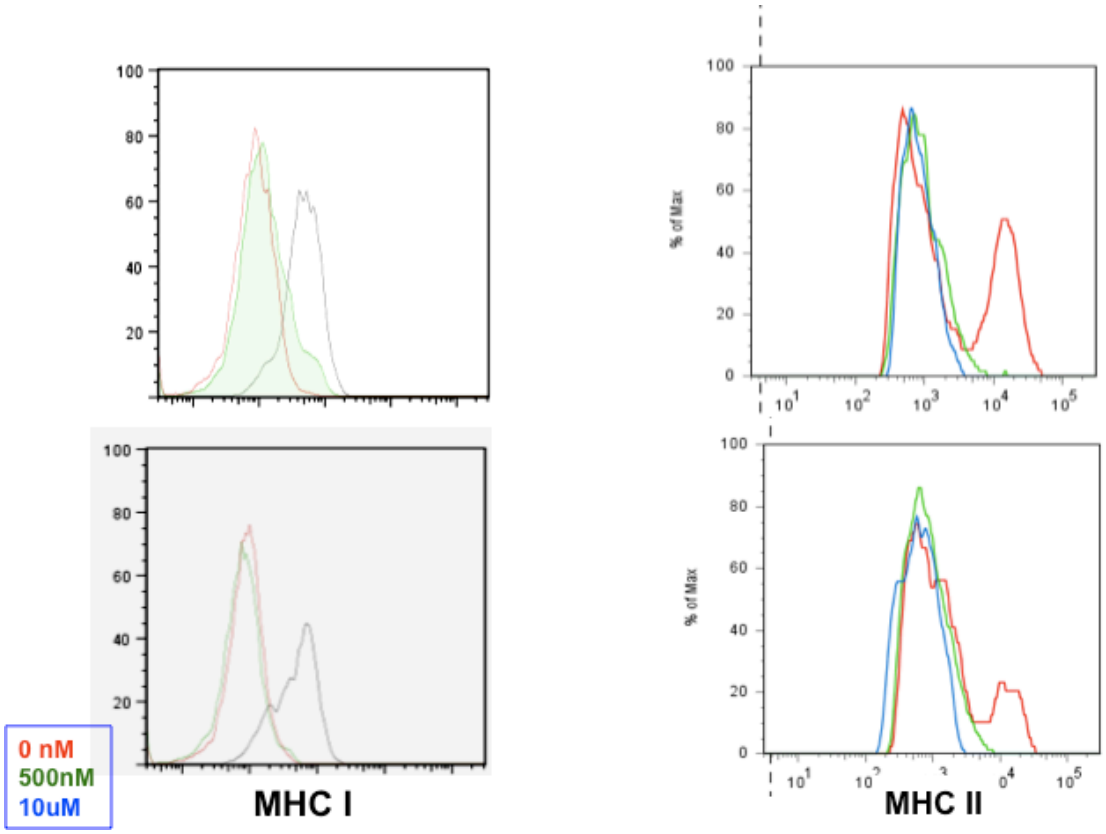


Figure 7: High doses of 17-DMAG affect MHC molecule expression on DCs

DCs were treated with 500nM (green histograms), 10 μM (blue histograms) or left untreated (red histograms) for 24h (top panel) or 48 hours (bottom panel) for MHC class I molecule expression (left) or MHC class II expression (right)

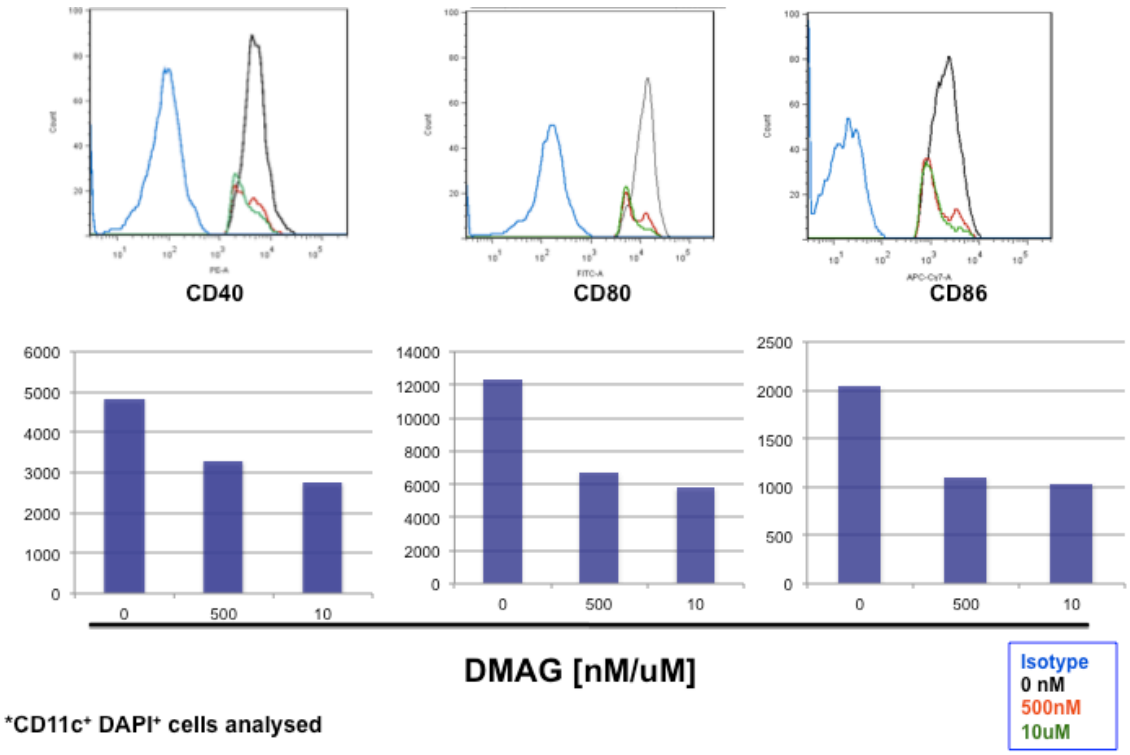


Figure 8: : Effect of 17-DMAG on DC phenotype is due to increased cell toxicity

DCs were treated with 500nM (red histograms), 10 μM (green histograms) or left untreated (black histograms) for 24h. The markers evaluated were CD80 (left), CD86 (middle) and CD40 (right), with live cell (DAPI+ cells) gated on and quantitation

2.4.3. 17-DMAG affects the ability of DCs to stimulate T cells

To look at the effect of 17-DMAG on DC function, we pre-treated BMDCs with different doses of 17-DMAG for 24 or 48 hours, after which, the DCs were thoroughly washed and co-cultured with naïve CD3+ T cells in an allogenic model (Balb/c mice) for 72 hours. Cell-free supernatant was harvested from the co-culture and analyzed for IFNγ levels by ELISA. It was observed that 17-DMAG- treated DCs had a significantly reduced capacity to stimulate allogenic T cells when compared to untreated DCs. As observed in other cases, the strongest ability to stimulate T cells was observed at higher doses of 17-DMAG and was more pronounced after 48 hours of drug treatment

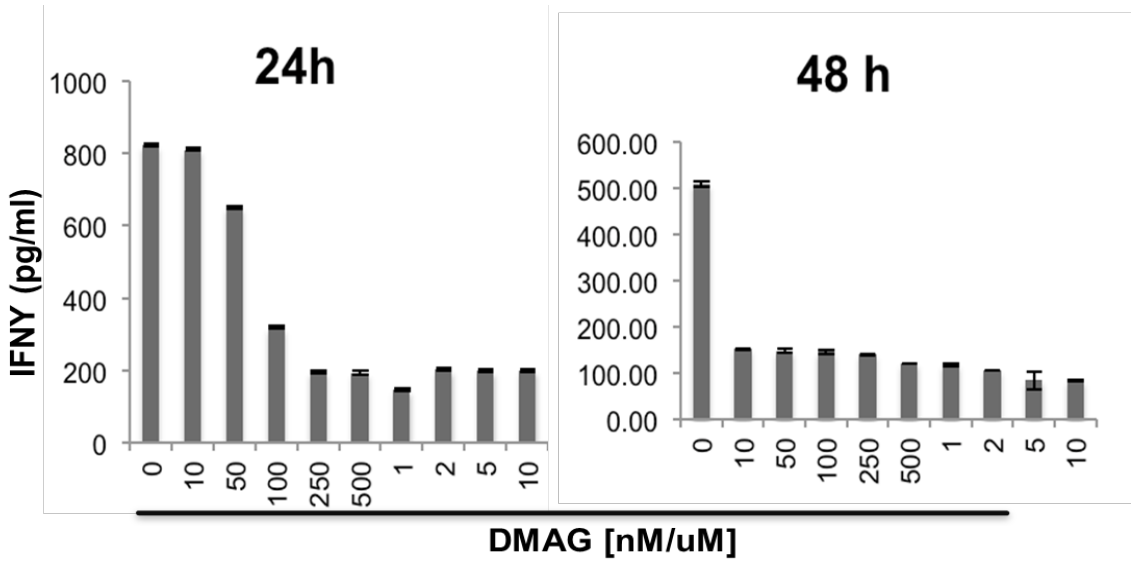


Figure 9: : 17-DMAG affects the ability to DCs to stimulate allogenic T cells

DCs from C57BL/6 mice were treated with different concentrations of 17-DMAG for 24h (left) or 48h (right) and later co-cultured with splenocytes from allogenic Balb/c mice for 72 hours. The supernatants from the co-culture were assessed for IFNγ levels.

2.4.4. 17-DMAG affects the ability of naive T cells to become activated in vitro

CD4+ and CD8+ T cells were isolated from naïve C57Bl6/J mice and treated with different doses of 17-DMAG for 24 or 48 hours before then being stimulated with mitogenic anti-CD3 antibody for an additional 48 hours. Cell-free supernatants were then harvested and assessed for IFNγ levels by ELISA. 17-DMAG treatment for 24 hours did not affect the ability of naïve T cells to become activated after CD3 ligation. However, $CD8^+$ T cells pre-treated for 48 hours with 17-DMAG, even at low concentrations (i.e. 100 nM), displayed a decreased ability to become mitogenically-activated, while $CD4^+$ T cells appeared largely unaffected in this regard.

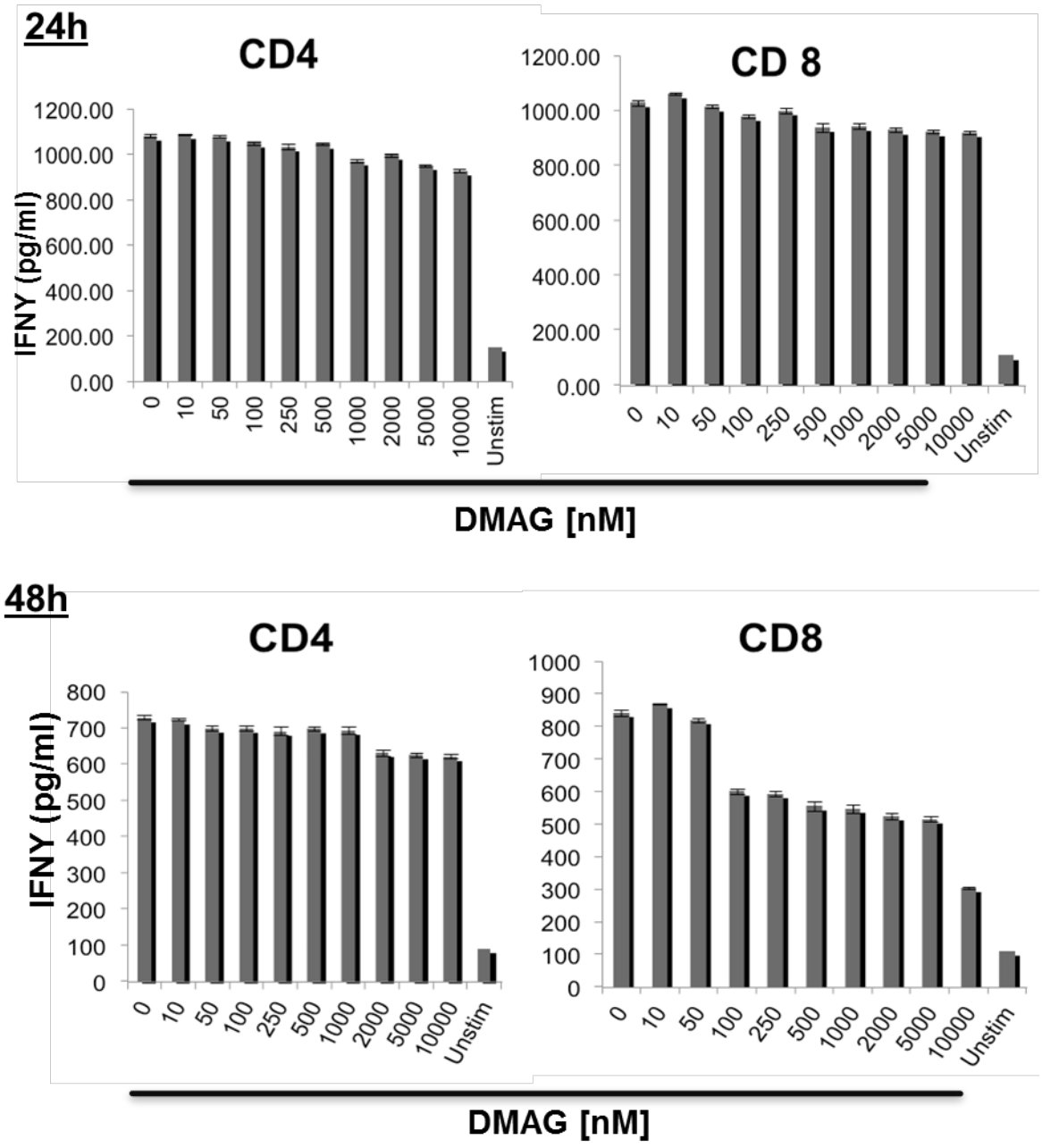


Figure 10: : 17-DMAG affects ability of naïve T cells to be stimulated

Naïve CD4+(left) or CD8+ T cells (right) were isolated from C57BL/6 mice and treated with different concentrations of 17-DMAG for 24h (top panel) or 48h (bottom panel) prior to being mitogenically activated using anti-CD3 mAb.

2.5. SUMMARY

To investigate the versatility of the RTK down-regulating effects of 17-DMAG, I assessed the effects of this drug on different RTKs expressed on a range of tumor cell lines. I consistently observed that 17-DMAG treatment led to a coordinate and dose-dependent degradation of multiple RTKs across the many cell lines examined. This suggests that my proposed combination therapy approach could be expanded to include a range of additional HSP90 client proteins as vaccine targets or inducers of specific T cells for use in adoptive co-therapy applications.

Since my intent is to combine 17-DMAG with immunotherapies, I was also held accountable to look at this drug potential inhibitory action on immune cell phenotypes and function (i.e. in DCs and responding T cell populations). My results suggest that any negative influence of 17-DMAG is due to the increased death of immune cells, and not due to the dysfunction of these cell types. Overall, my results indicate that careful inspection of the dichotomous effects of 17-DMAG on tumor versus immune cell types had to be taken into account in order to properly and equitably gauge the likely translational utility of such treatment options *in vivo*. In this regard, a pharmacologic dose that balanced these effects would need to be determined in order to be translatioanlly considered an effective and safe therapeutic option.

CHAPTER 3. COMBINATION IMMUNOTHERAPY INTEGRATING HSP90 INHIBITOR 17-DMAG RECONDITIONS THE TUMOR MICROENVIRONMENT FOR IMPROVED RECRUITMENT OF THERAPEUTIC T CELLS

Aparna Rao *, Jennifer L. Taylor**, Nina Chi-Sabins*, Mayumi Kawabe** and Walter J. Storkus*,**,†

From the Departments of *Immunology and **Dermatology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213 and the †University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213.

These data have been reported in *Cancer Res* Jun 6 2012. All data in this chapter was obtained by Aparna Rao. Nina Chi Sabins assisted with flow sorting for VECs for use in Figure 20 C.

3.1. ABSTRACT

Limitations in CD8+ T cell recognition of tumor cells due to defects in their antigen processing machinery or the selection of variants expressing low or absent levels of cognate tumor antigens have been previously identified as impediments to effective cancer immunotherapy. Hence, treatment regimens that coordinately promote enhanced activation of anti-tumor CD8+ T cells, improved delivery of such effector cells into tumor sites, and augmented recognition of tumor or tumor-associated stromal cells by therapeutic CD8+ T cells, would be expected to yield greater clinical benefit. Using an MCA205 sarcoma model, I 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: i.) slowed tumor growth; ii.) enhanced/prolonged recognition of tumor cells by anti-EphA2 CD8+ T cells; iii.) reduced levels of myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) in the tumor microenvironment (TME); and iv.) 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 cotreatment yielded a superior tumor therapeutic regimen that was capable of rendering animals free of disease.

3.2. INTRODUCTION

Receptor tyrosine kinases (RTKs) are an extended family of cell surface proteins [9] that bind growth factors and hormones and play important roles in cell survival, growth, migration and differentiation [356]. In neoplastic/cancerous tissues, RTK overexpression, mutation and/or constitutive activation may result in uncontrolled proliferation and increased malignant phenotype [363]. 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 [363]. 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 [23,39,79]. 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 [52,64,364]. As a consequence, EphA2 has become an attractive target for therapeutic intervention in patients with solid tumors [8].

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 [80,82,86,365,366]. 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 [4,7], 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 [2]. 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 [92,93], levels of circulating CD8+ T cells could also be amplified by vaccination for improved immune targeting of EphA2+ tumor cells *in vivo*. I 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.

3.3. MATERIALS AND METHODS

3.3.1. Mice

Six- to 10-week-old female C57BL/6 (H-2^b), and male and female B6;129S6-Epha2tm1Jrui (EphA2-/-; H-2^b) 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).

3.3.2. Tumor cell lines and tumor establishment.

The EphA2+ MCA205 sarcoma and EphA2neg B16 melanoma (H-2^b) 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_2. All cell lines were negative for known mouse pathogens, including mycoplasma. Tumors were established by injection of 5 x 10^5 MCA205 or 1 x 10^5 B16 tumor cells s.c. into the right flank of syngeneic mice, with tumor size (in mm^2) assessed every 3 to 4 days thereafter. Mice were sacrificed when

tumors became ulcerated or they reached a size of 400 mm^2, in accordance with IACUC guidelines.

3.3.3. 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 **μ**L, approximately 18 days after tumor inoculation, when tumors were ~100 mm^2 in area.

3.3.4. 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 [367]. 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 **μ**mol/L; Peptides International, Louisville, KY) and chloroquine (50 **μ**mol/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.

3.3.5. 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. [368]) alone or DC.IL12 pulsed with synthetic $mEphA2_{671–679}$ (FSHHNIIRL; H-$2D^b$ class I-presented; ref. [96]) and $mEphA2_{682–689}$ (VVSKYKPM; H-$2K^b$ 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 IFNγ
ELISA assays.

3.3.6. 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.

3.3.7. IFNγ analyses.

For tumor recognition assays, splenic CD8+ T cells were co-cultured with irradiated (100 Gy) tumor cells for 48 hours, after which, cell-free supernatants were harvested and assessed for mIFNγ 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 [369]. 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-K^b/-D^b 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 IFNγ 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 IFNγ in culture supernatants were quantified by specific ELISA.

3.3.8. Immunofluorescence staining and imaging.

Tumor tissue was processed and sectioned as previously reported [369], followed by immunofluorescence staining and microscopy. The following primary antibodies were used for staining sections: rat antimouse 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.

3.3.9. 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 Kb/Db, anti-Class I-A^b, 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 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.).

3.3.10. Statistical analysis.

All comparisons of intergroup means were performed using a two-tailed ANOVA test, with $P < 0.05$ considered significant.

3.4. RESULTS

3.4.1. 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 [5,184-187]. In preliminary *in vitro* studies, I determined that treatment of tumor cells with 17-DMAG resulted in their loss of EphA2 protein expression, with a clear drug dosedependency (Fig. 11A). Expression of alternate tumor RTKs and known HSP90 client proteins, such as erbB2/Her2 and VEGFR [1,4,7] 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 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 11B). Treatment of MCA205 cell cultures with 17-DMAG did not modulate the expression of MHC class I molecules on the tumor cell surface (Fig 11C) or tumor cell viability/apoptotic frequency (data not shown). Notably, EphA2-specific CD8+ T cells developed from EphA2 -/- mice (Fig. 12) demonstrated increased *in vitro* recognition of EphA2+ MCA205 (but not $EphA2^{neg}$ B16) tumor cells pretreated with 17-DMAG (Fig. 11D and Fig 12).

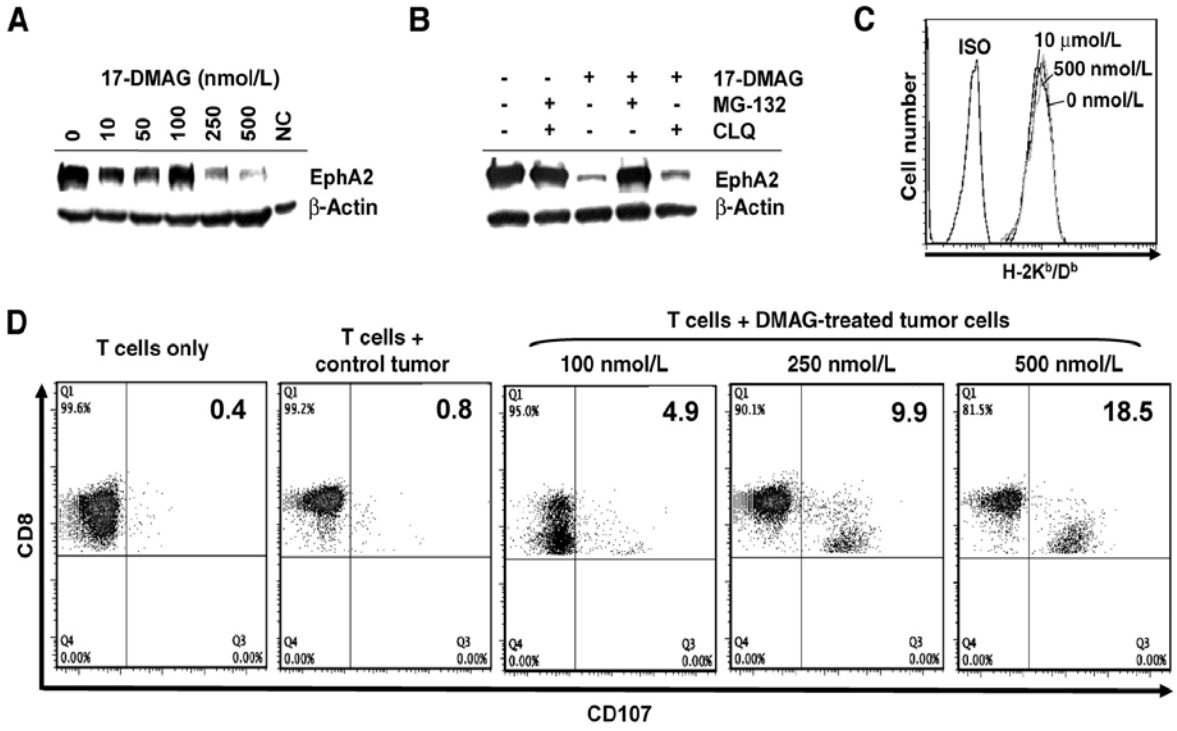


Figure 11: 17-DMAG promotes proteasome-dependent EphA2 protein degradation in MCA205 sarcoma cells and the enhanced recognition of tumor cells by anti-EphA2 CD8+ T cells *in vitro*

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. 2** 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.

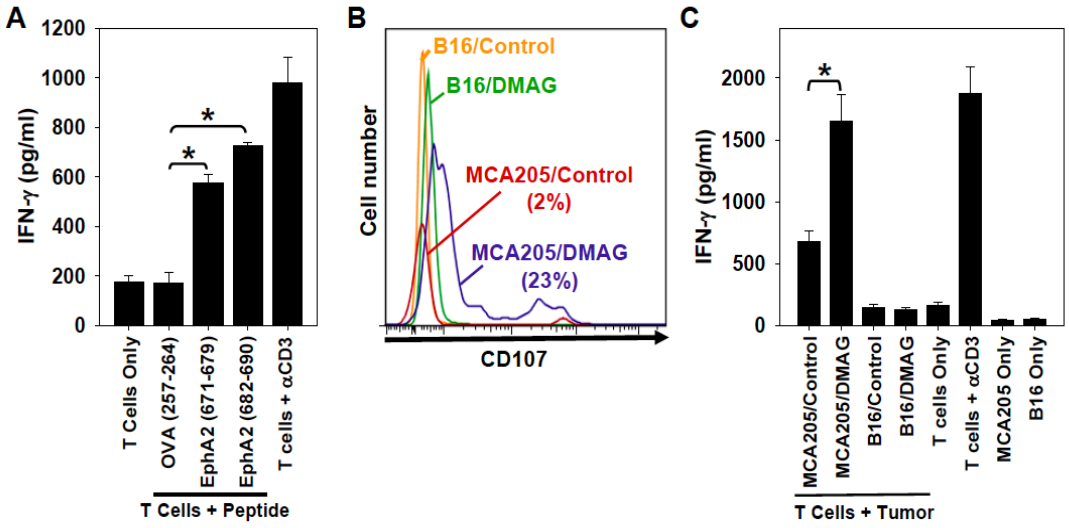


Figure 12: : EphA2-specific CD8+ T cells preferentially recognize DMAG-treated EphA2+ syngenic tumor target cells *in vitro*

A, EphA2 -/- (H-2^{b}) were vaccinated with syngenic DC.IL12 pulsed with the EphA2$_{671-679}$ and EphA2$_{682-689}$ peptides as outlined in Materials and Methods. One week after the last vaccination, CD8+ splenocytes were harvested and co-cultured with PBS, syngenic splenic monocytes pulsed with irrelevant OVA peptide, or either of the individual EphA2 peptides used in the vaccination, or they were stimulated with mitogenic anti-CD3 mAb. After 24h, cell-free supernatants were analyzed for IFNγ by specific ELISA. Data from triplicate determinations is reported as mean +/- SD. These same CD8+ T cells were also analyzed for reactivity against syngenic MCA205 sarcoma (EphA2+) or B16 (EphA2^{neg}) melanoma cells that had been left untreated or treated *in vitro* with 17-DMAG (500 ng/ml for 24h), with specific T cell responses detected in CD107 translocation (**B**) or IFNγ ELISA assays (**C**), as outlined in Materials and Methods

3.4.2. 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. 13A, 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 > 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 *in vitro*. I 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 (Fig. 13B).

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 IFNγ production assays (Fig. 13C and 13D). 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).

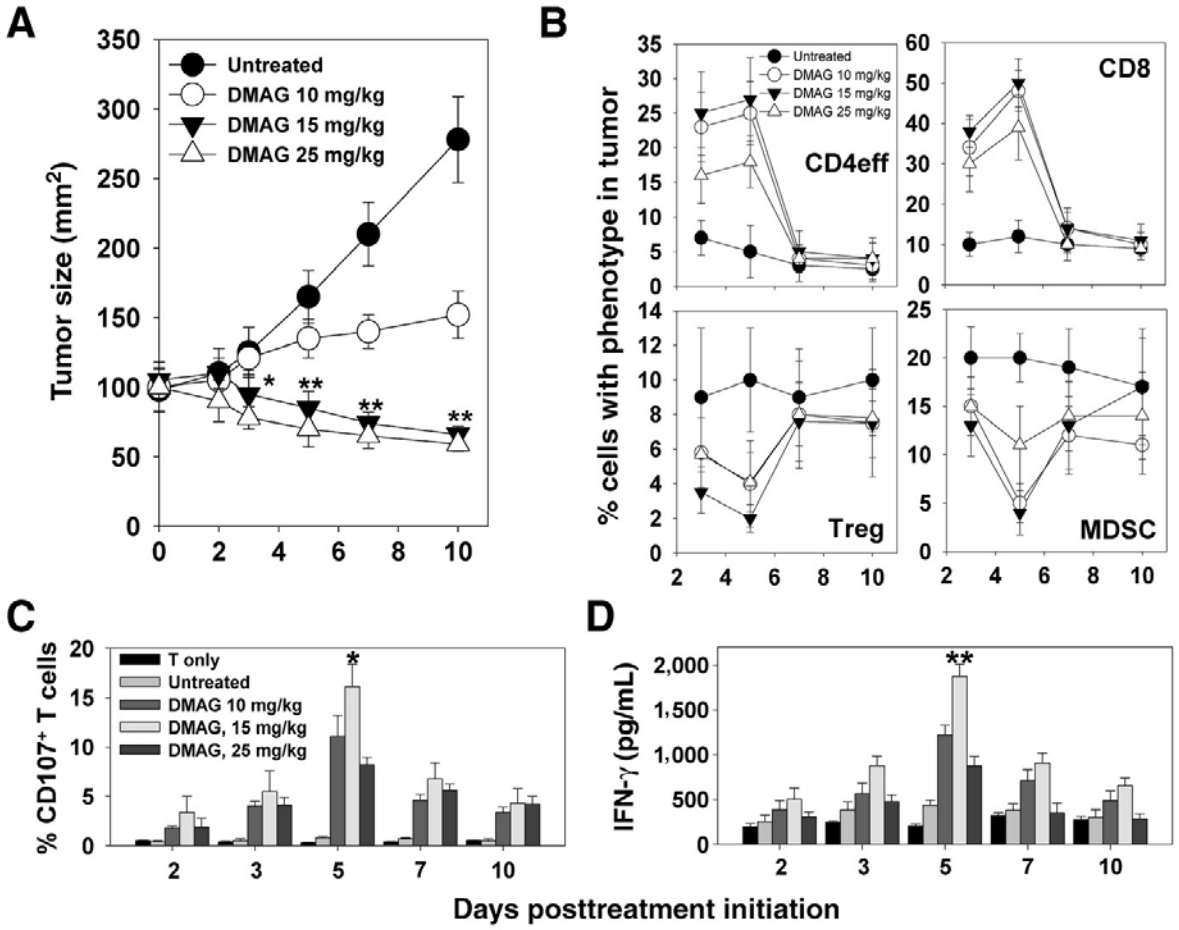


Figure 13: Treatment of mice bearing established MCA205 tumors with oral 17-DMAG transiently promotes a therapeutically preferred immunophenotype in the TME and is optimally effective in a 5-day regimen

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/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. **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 **B**) were also analyzed as target cells for anti-EphA2 CD8**.** T effector cells generated from EphA2 -/- mice (see **Fig. 2**) 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.

3.4.3. 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. 14A, 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 10-15 days after the discontinuation of drug (Fig. 14B). The ability of anti-EphA2 CD8+ T cells to recognize *in vivo* treated tumor cells remained significantly elevated through day 10-14 after treatment initiation (Fig. 14C) 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. 14D). I 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. 15). 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.15).

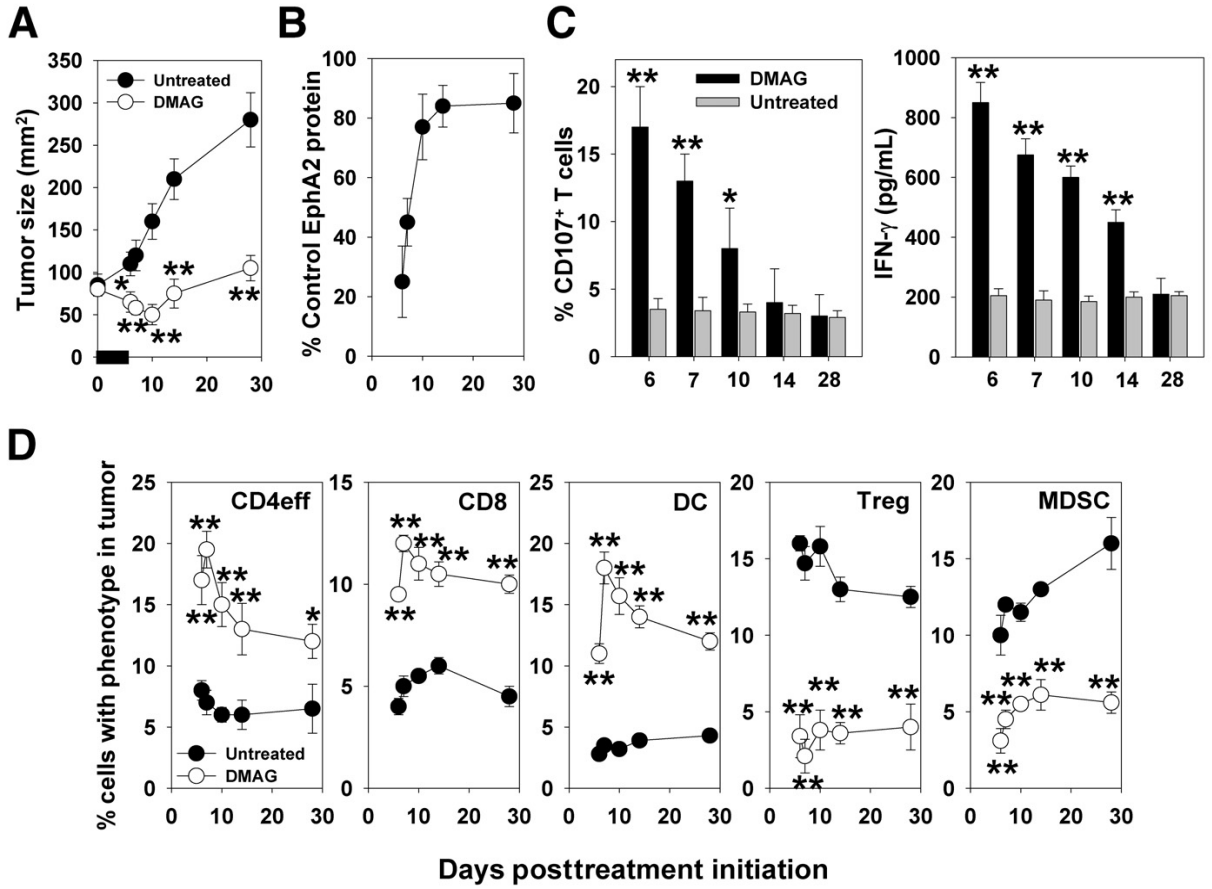


Figure 14: The impact of 17-DMAG-based therapy for 5 days persists after discontinuation of drug delivery

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.2**) 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 that obtained in 3 independent experiments. *$p < 0.05$ for treated versus untreated controls.

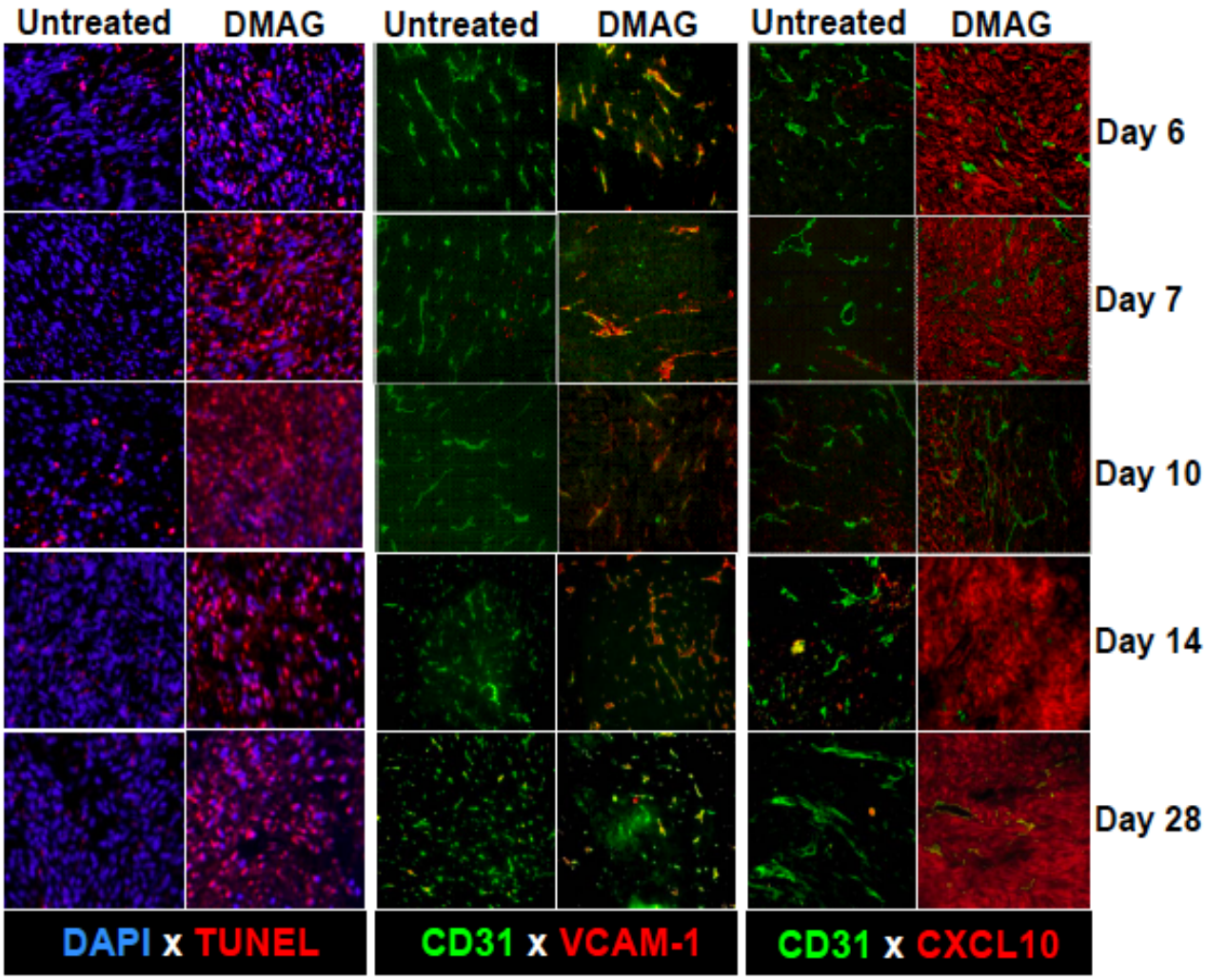


Figure 15: : DMAG promotes enhanced apoptosis and expression of VCAM-1 and CXCL10 in the MCA205 tumor microenvironment

MCA205-bearing mice were untreated or treated with a 5 day course of 17-DMAG (15 mg/kg/day provided via oral gavage per **Fig. 3A**). On the indicated days post-initiation of therapy, tumors were isolated from 2 mice/cohort and tissue sections analyzed for the indicated markers by fluorescence microscopy as described in Materials and Methods. Data derive from 1 of 3 experiments performed, with similar results obtained in each case.

3.4.4. 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, I 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. 16A, 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. 16A). An analysis of TIL harvested from the various treatment groups supports the superior induction/recruitment of Type-1 anti-EphA2 CD8+ T cells (Fig. 16B) and improved population of tumor lesions by CD4+ and CD8+ T effector cells and CD11c+ DC over regulatory immune cell subsets (Fig. 16C) after vaccine + 17-DMAG combination therapy.

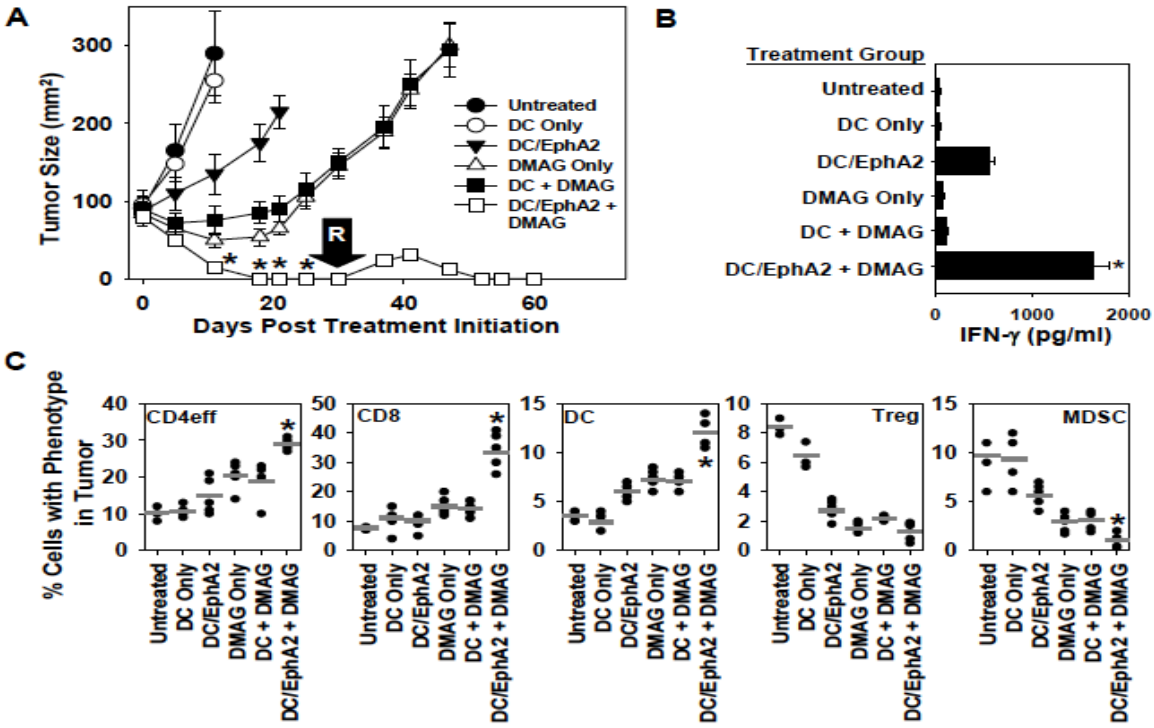


Figure 16: 17-DMAG administration improves the immunogenicity and anti-tumor efficacy of an EphA2 peptide-based vaccine in the MCA205 tumor model

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 -7 and 0 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 Rao *et al.* oral gavage). Tumor size (mean +/- SD) is reported in mm^2. 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 $EphA2_{671-679}$ + $EphA2_{682-689}$ 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. *$p < 0.05$ versus all other cohorts.

We have previously shown that EphA2 peptide-based vaccination was capable of slowing the growth of EphA2^{neg} 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, I 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. 17A, 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. 17B). To further investigate the therapeutic targeting of tumor-associated EphA2+ VEC in the B16 melanoma model, I 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. 17C) 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. 17D). I 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. 17C), but these T cells failed to recognize tumor-uninvolved kidney-associated VEC or cultured B16 tumor cells (Fig. 17C, 17D).

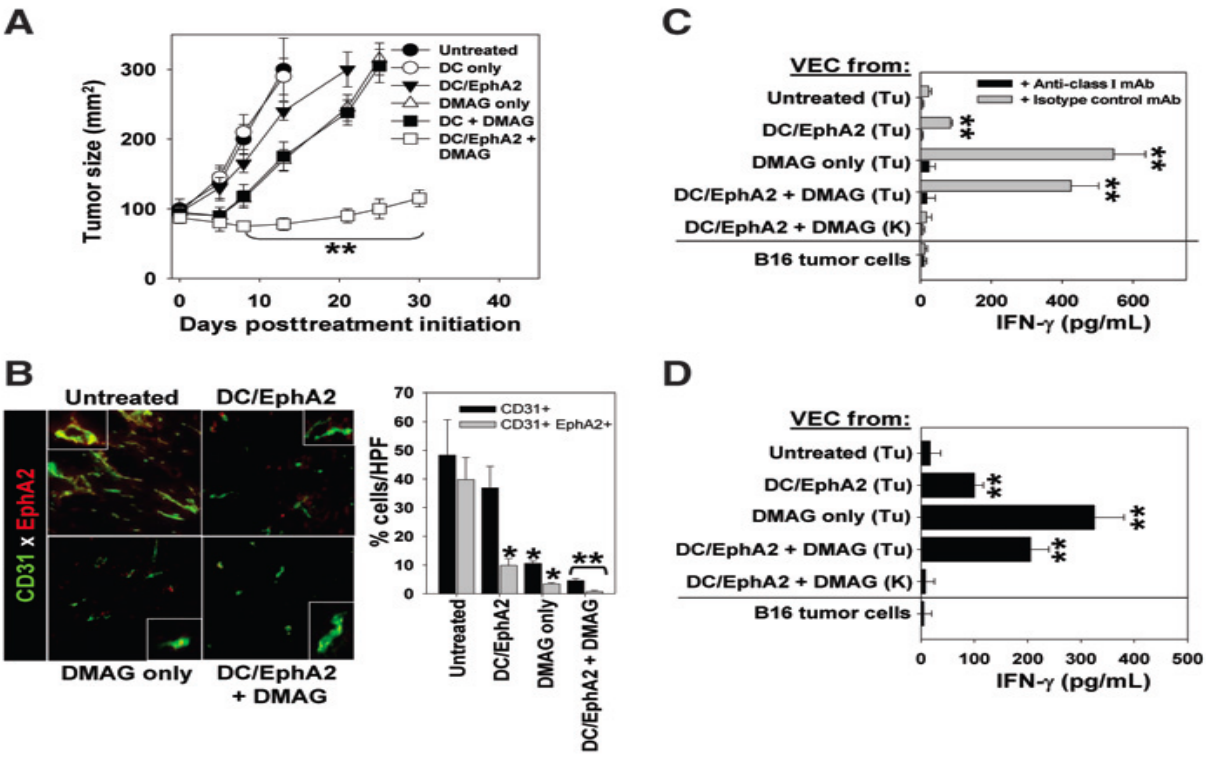


Figure 17: 17-DMAG improves the anti-tumor efficacy of an EphA2 peptide-based vaccine in the EphA2neg B16 melanoma model based on immune targeting of EphA2+ VEC

C57BL/6 mice bearing established s.c. B16 melanomas (right flank) were left untreated or treated as outlined in **Fig. 7A**, with tumor size (mean +/- SD) reported in mm2 followed for up to 30 days. *p < 0.05 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. Anti-EphA2 CD8+ T cells isolated from the spleens of immune EphA2 -/- mice (panel **C;** as outlined in **Fig. 2)**, 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. *p < 0.05 versus untreated controls (all panels) and versus replicate cultures containing blocking anti-MHC class I mAb (panel **C**). **p < all other cohorts (panel **B**). All data are representative of those obtained in 3 independent experiments.

3.4.5. 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 [306,314,370], I 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. 12). 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. 18). I 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. 19A), in concert with improved levels of CD4eff and CD8+ TIL and reduced levels of tumor-associated regulatory cell populations (Fig. 19B), and the accumulation of anti-EphA2+ Tc1 in the MCA205 TME (Fig. 19C). I 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).

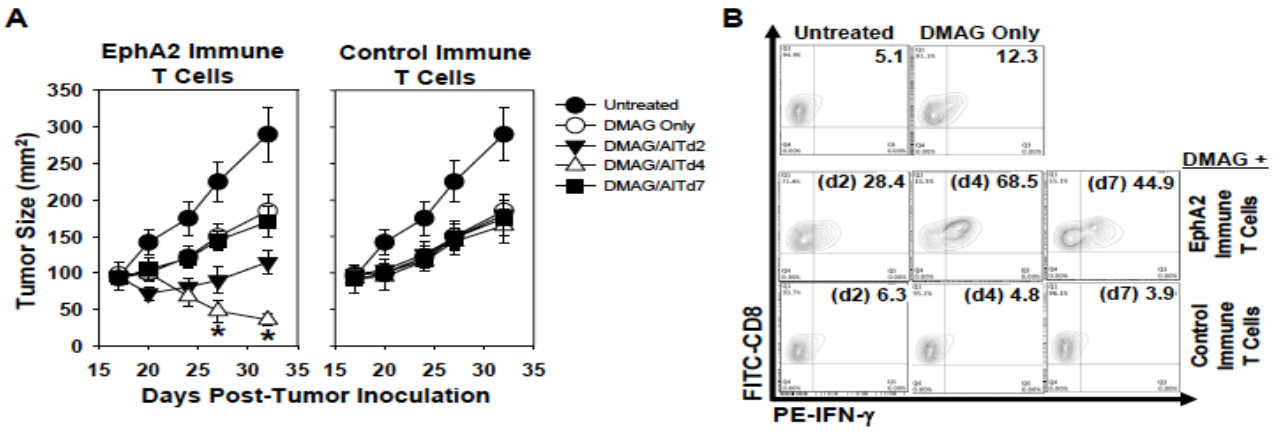


Figure 18: 17-DMAG improves the anti-tumor efficacy of adoptively-transferred anti-EphA2 CD8+ T cells with optimal anti-tumor benefit if T cell transfer occurs 4 days after initiating 17-DMAG administration.

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 days 2, 4 or 7 of treatment) of 5 x 10^6 CD8+ T cells isolated from EphA2 -/- mice previously vaccinated with DC.IL12 only (i.e. Control Immune T Cells) or DC.IL12 loaded with EphA2 peptides (i.e. EphA2 Immune T Cells) as described in Materials and Methods. Tumor size was monitored longitudinally and is reported (mean +/- SD) in mm^2 from 5 mice/group. In **B**, day 32 untreated or treated (as in panel **A**) tumors underwent enzymatic digestion, with CD8+ TIL analyzed for expression of intracellular IFN-γ by flow cytometry as described in Materials and Methods. *$p < 0.05$ versus all other cohorts. Data are from one representative experiment of 3 performed.

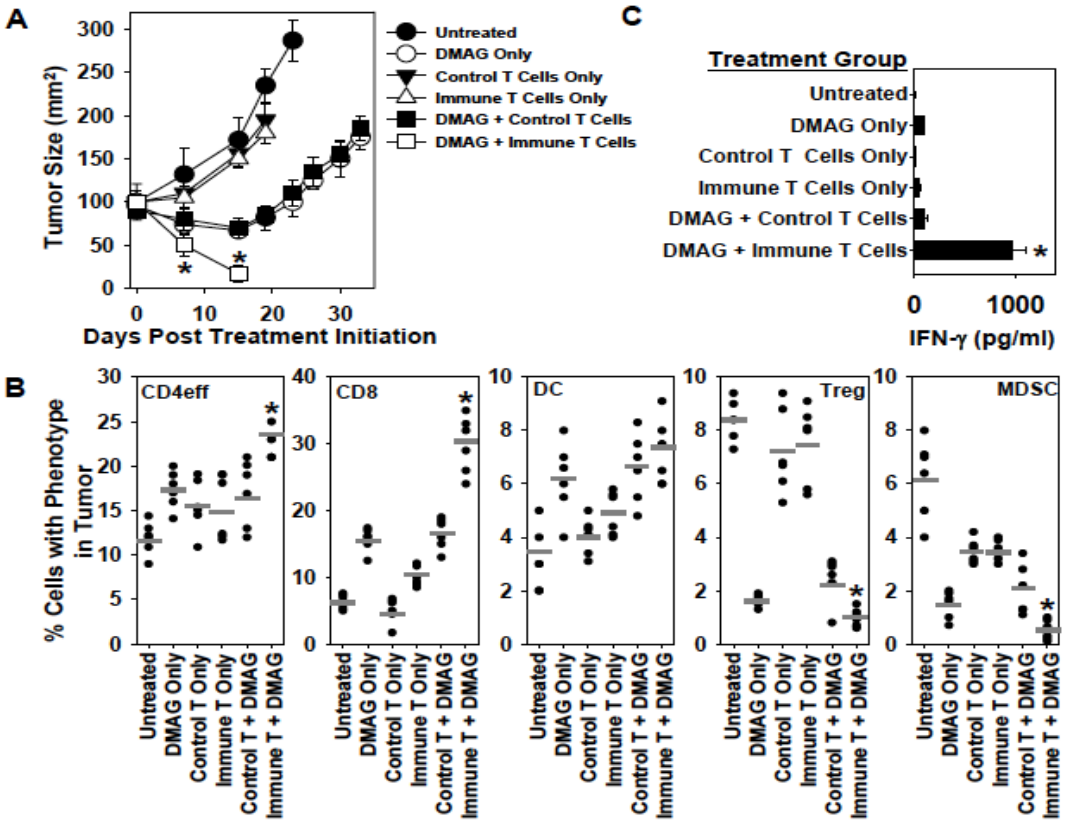


Figure 19: 17-DMAG improves the anti-tumor efficacy of adoptively-transferred anti-EphA2 CD8+ T cells in a combination therapy.

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. *$p < 0.05$ versus all other cohorts. All data are representative of those obtained in 3 independent experiments.

3.5. DISCUSSION

The major finding in this chapter 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 [64,358,371]. I 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. [38,51,361]) 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* (Fig. 16A, 17A) or the adoptive transfer of anti-EphA2 CD8+ T cells (Fig. 19A) 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 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+ 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 *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 antitumor efficacy and toxicity when administered as single agents [186,187,372-374]. 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 [186]. 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 [375-377], reinforcing the clinical need to evolve 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 [186,187,378-380], 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, cyclinB, EGFR, HER2/neu, IGF1-R, PDGFR, PIM-1, STAT3, survivin, TGF**β**R, VEGFR1, VEGFR2, among many others [1,4,381].

CHAPTER 4. 17-DMAG SIGNIFICANTLY IMPROVES ANTI-TUMOR EFFICACY IN COMBINATION WITH IMMUNOTHERAPY BY AFFECTING EXPRESSION OF INFLAMMATORY AND ANGIOGENIC GENES

Aparna Rao, Mayumi Kawabe, Jennifer L. Taylor, Walter J. Storkus

From the Departments of *Immunology and Dermatology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, and the University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213.

All the results reported in this study were obtained by Aparna Rao.

4.1. ABSTRACT

We have earlier shown that the HSP90 inhibitor 17-DMAG can mediate degradation of RTKs through the proteasome and cause a subsequent increase in the presentation of RTK-derived peptides in the tumor MHC class-I complexes expressed on the tumor cell surface. This allows for specific recognition of treated tumor cells by low-to-moderate avidity CD8+ T cells that are otherwise “tolerant” to normal self tissues. However, our preliminary results have also demonstrated that at higher doses and/or longer treatment times, 17-DMAG can adversely affect immune cell phenotype and function *in vitro*. When we assessed the effect of 17-DMAG on immune system *in vivo*, we replicated our *in vitro* findings at higher doses/treatment durations. However, we also observed (unexpectedly) that treatment with 17-DMAG at lower doses over short periods of time circumvented tumor-mediated immune suppression and orchestrated the increased infiltration of pro-inflammatory cells into the tumor microenvironment. The aim of this study was to elucidate the mechanisms of 17-DMAG-mediated immune cell infiltration into tumors and to assess the anti-tumor efficacy of 17-DMAG in a combinational immunotherapy setting targeting the RTK EphA2. We observed that 17-DMAG treatment upregulated expression of type-1 immune response genes in treated mice, and downregulation of immune suppressive/pro-angiogenic genes. In addition, in combination with two separate immunotherapeutic approaches, 17-DMAG treatment displayed significantly increased anti-tumor efficacy compared to mice receiving single modality treatments.

4.2. INTRODUCTION

17-DMAG is one of the more recently developed HSP90 pharmacological inhibitors, that is currently being evaluated in phase II clinical trials [186,187]. The biggest advantage 17-DMAG offers over its predecessors is its ability to be dissolved in water, thus giving the drug far greater bioavailability [5,6]. As mentioned earlier, along with playing a pro-tumorigenic role, HSP90 is known to foster inflammatory immune responses by directly impacting antigen presentation and immune cell activation [359,360]. Thus, even though my goal of using 17-DMAG was to inhibit HSP90 in order to block tumor growth and survival by affecting multiple tumorigenic and vasculogenic pathways, I was also concerned with potential inhibitory effects that this HSP90 inhibitor might have on immune responsiveness. If validated, such activity would be counterintuitive to my effort to achieve superior anti-tumor efficacy by combining 17-DMAG with EphA2-targeted immunotherapy.

Therefore, I initiated these studies by first looking at the effect(s) of 17-DMAG on DCs and T cell function *in vitro*. I observed that although 17-DMAG did not have any undesirable effects on immune cell phenotype and function at low concentrations, at doses higher than 500nM, it could negatively affect T cell and DC function (Chapter 2, figs 6,7,9,10). I also noted that the effects were associated with increased immune cell death (Chapter 2, fig 8). When I investigated the effects of 17-DMAG on immune cell infiltration in the tumors *in vivo*, I also observed that at high concentrations (>15 mg/Kg/day) and when given over a prolonged dosing schedule (> 5 consecutive days), 17-DMAG negatively affected immune cell infiltration into tumor lesions (Chapter 3, fig 13).

However, a more important (and unexpected) finding of my study was that treatment with a low dose of 17-DMAG for a short time increased infiltration of inflammatory T cells and DCs, and decreased the incidence of immunosuppressive cells like Tregs and MDSCs (Chapter 3, figs 13,14). Moreover, I observed that 17-DMAG treatment also led to increased expression of CXCL10 and VCAM-1 by tumor cells and the tumor-

associated vasculature, respectively. In addition, these pro-inflammatory effects of 17-DMAG were observed to persist even after 17-DMAG treatment was discontinued, for up to 28 days (Chapter 3. Fig 15).

4.3. MATERIALS AND METHODS

4.3.1. Mice

Six- to 10-week-old female C57BL/6 (H-2^b), and male and female B6;129S6-*Epha2*^{tm1Jrui} (EphA2-/-; H-2^b) 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).

4.3.2. Tumor establishment

The MCA205 (H-2^b) sarcoma cell line was purchased from the American Type Culture Collection. Cells 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 (Life Technologies)] in a humidified incubator at 37°C and 5% CO_2. Tumors were established by injection of 5×10^5 MCA205 tumor cells s.c. into the right flanks of syngeneic mice, with tumor size assessed every 3 to 4 days and recorded in mm^2. Mice were sacrificed when tumors became ulcerated or reached a maximum size of 400 mm^2 in accordance with IACUC guidelines.

4.3.3. 17-DMAG therapy

For *in vivo* uses, 17-DMAG was obtained from Cancer Therapy Evaluation Program (CTEP- NCI) and dissolved in sterile water. Tumor-bearing mice were orally administered 17-DMAG or distilled water in a total volume of 50 μL approximately 18 days after tumor inoculation when tumors were ~100 mm^2 in area. The optimal dose and duration of 17-DMAG treatment was 15mg/Kg and 5 days respectively.

4.3.4. Isolation of tumors

Tumors were resected from 17-DMAG treated or untreated mice and enzymatically digested with 0.1% (w/v) collagenase, 1% (w/v) hyaluronidase, and 0.1% (w/v) DNase (Sigma), and a single cell suspension was prepared. For isolation of tumor-infiltrating lymphocytes, tumor cells were loaded onto a lympholyte-M column (Cedarlane) and TILs were isolated as buoyant cells after discontinuous density gradient centrifugation as previously described [367].

4.3.5. Western blot

MCA205 tumor cells treated with 17-DMAG (or left untreated) were incubated with lysis buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 0.2 mmol/L sodium orthovanadate, 0.5% NP-40 in PBS; all reagents from Sigma-Aldrich) containing a protease inhibitor cocktail (Complete mini; Roche Diagnostic) for 30 min at 4°C. Lysates were cleared by centrifugation at 13,500 × *g* for 10 min, and proteins in the lysate were separated by SDS-PAGE before electro-transfer onto polyvinylidene difluoride membranes (Millipore). Polyclonal anti-STAT 3 and STAT 1 antibodies and horseradish peroxidase–conjugated goat anti-rabbit antibody (both from Santa Cruz Biotechnology) were used to detect STAT 3 and STAT 1 respectively. Probed

proteins were visualized by Western Lighting chemiluminescence detection kit (Perkin-Elmer) and exposed to X-Omat film (Eastman Kodak) for 5-7 min.

4.3.6. Reverse transcription-PCR

For semiquantitative reverse transcription-PCR (RT-PCR), tumors excised from 17-DMAG treated (or untreated) animals were digested to form a single-cell suspension (as described earlier) and used for RNA isolation and cDNA preparation using random hexamer primers (Applied Biosciences). PCR was performed using the primer pairs listed in Supplementary Table S1. Cycling times and temperature were as follows: initial denaturation at 94°C for 2 minutes (1 cycle); denaturation at 94°C for 30 seconds, annealing at 60°C to 65°C for 30 seconds, and elongation at 72°C for 1 minute (35–40 cycles); final extension at 72°C for 5 minutes (1 cycle). Following gel electrophoresis, PCR products were imaged and band density was quantified using LabWorks software (Perkin-Elmer).

4.3.7. DC.IL12 gene therapy

DCs were generated from mice bone marrow by differentiating with GM-CSF and IL-4. On day 5 of culture, the DCs were transduced with an adenoviral vector expressing murine IL12 for 48 hours. After transduction, 10^6 DCs were suspended in 100 μl and injected intra-tumorally in 17-DMAG treated/untreated mice on the last day of 17-DMAG treatment.

4.3.8. Immunization of EphA2 -/- mice to generate EphA2-specific T cells

EphA2 -/- mice were vaccinated with syngeneic DCs transduced with IL12 pulsed with EphA2 peptides H-2D^b-binding mEphA2$_{671–679}$ (FSHHNIIRL) and H-2K^b-binding

mEphA2$_{682–689}$ (VVSKYKPM) or non-pulsed DCs once a week or 15 days subcutaneously on the right flank. After 4 vaccinations, spleen was harvested and splenocytes in a single cell suspension were assessed for EphA2-specificity using a peptide recognition assay and for their function by CD107 cytotoxicity assay and tumor recognition IFNγ assay. For adoptive transfer, 5 x 10^6 $CD8^+$ T cells were suspended in 100ul of PBS and injected intra-tumorally (i.t.) into 17-DMAG treated/ untreated mice on the last day of 17-DMAG treatment.

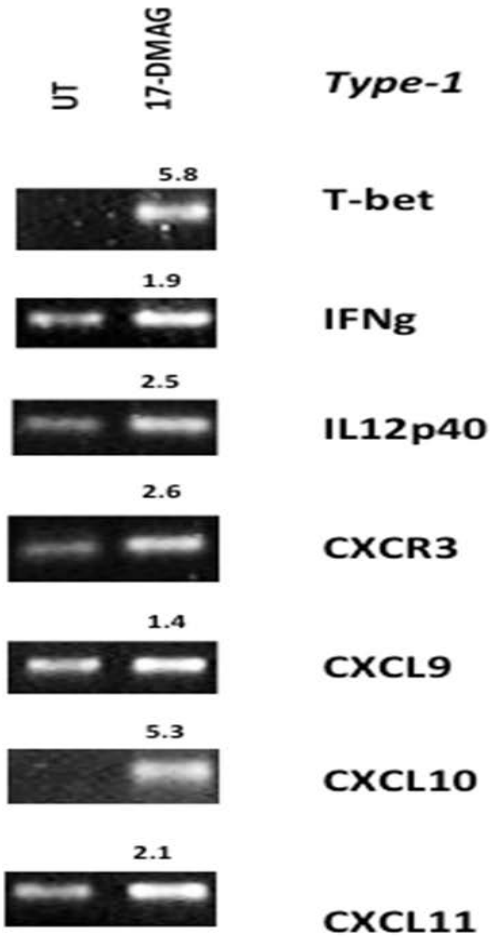
4.4. RESULTS

4.4.1. 17-DMAG treatment induces overexpression of type 1 immune response genes

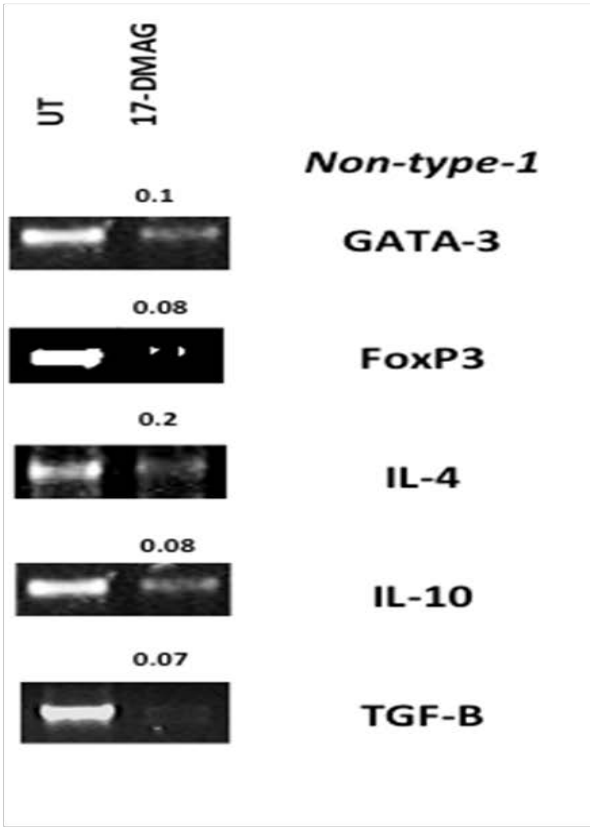
Tumors isolated from mice treated with 17-DMAG exhibited increased expression of genes indicative of type 1 immune responses like T-bet and IFNγ, and decreased expression of immune suppressive factors like FoxP3, IL-10 and TGFβ. In addition, 17-DMAG treated tumors also displayed increased levels of T cell recruiting chemokines and receptors such as CXCL9/10/11 and CXCR3, respectively. Importantly, 17-DMAG treatment decreased expression of pro-angiogenic factors like HIF1α/HIF2α, CXCL12 (aka stromal derived factor-1-alpha; SDF-1α) and its receptor, CXCR4.

17-DMAG treatment also slightly increased levels of p-STAT1 (associated with increased Type 1 immune function) and significantly decreased levels of p-STAT3 (typically associated with immunosuppression/Treg function and a pro-angiogenic microenvironment).

a)


UT
17-DMAG
Type-1
5.8
T-bet
1.9
IFNg
2.5
IL12p40
2.6
CXCR3
1.4
CXCL9
5.3
CXCL10
2.1
CXCL11

b)


UT
17-DMAG
Non-type-1
0.1
GATA-3
0.08
FoxP3
0.2
IL-4
0.08
IL-10
0.07
TGF-B

c)

d)

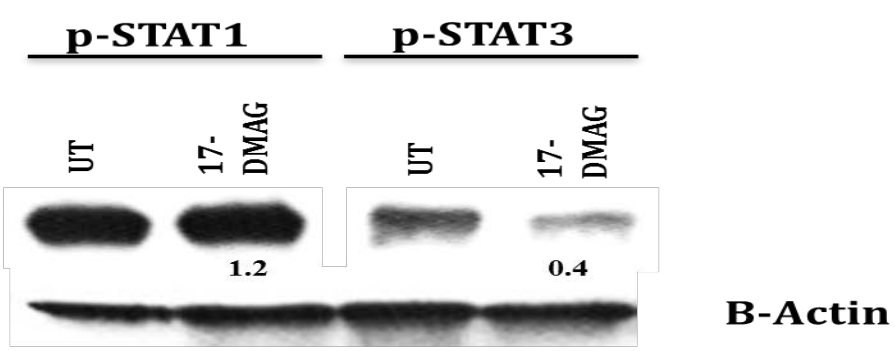


Figure 20: 17-DMAG treatment causes upregulation of type-1 immune response genes and decrease in non type-1/ angiogenic gene products

Tumors from 17-DMAG treated/untreated mice were excised and were either used for making tumor cell lysates or RNA isolation. mRNA gene expression of type-1 (a), non-type-1 (b) and angiogenic genes (c) were assessed by RT-PCR. p-STAT1 and p-STAT3 expression was assessed by western blot using specific antibody probes (d). Numbers indicate fold-increase/ decrease in expression

4.4.2. Combinational therapy of 17-DMAG with EphA2-specfic immunotherapy elicits superior anti-tumor efficacy

When 17-DMAG treatment was evaluated in combination with DC.IL12 gene therapy (DCs transduced with IL 12 and administered intratumorally on the last day of 17-DMAG treatment), I observed that mice receiving combinational therapy displayed significantly slower tumor growth rates than mice receiving a single mode of treatment or untreated mice. In addition, when I analyzed tumor infiltrating T cells for EphA2 specificity, I noted that mice treated with the combination therapy had significantly higher levels of EphA2-specific TILs.

Combining 17-DMAG with intra-tumoral adoptive transfer of EphA2-specific T cells resulted in significantly greater anti-tumor efficacy when compared to mice treated with monotherapies. In fact, in mice receiving the therapeutic combination of 17-DMAG with intra-tumorally transferred EphA2-specific T cells, the tumors showed significant hemorrhagic necrosis and complete liquefaction, thus making post-treatment analysis of the tumor impossible.

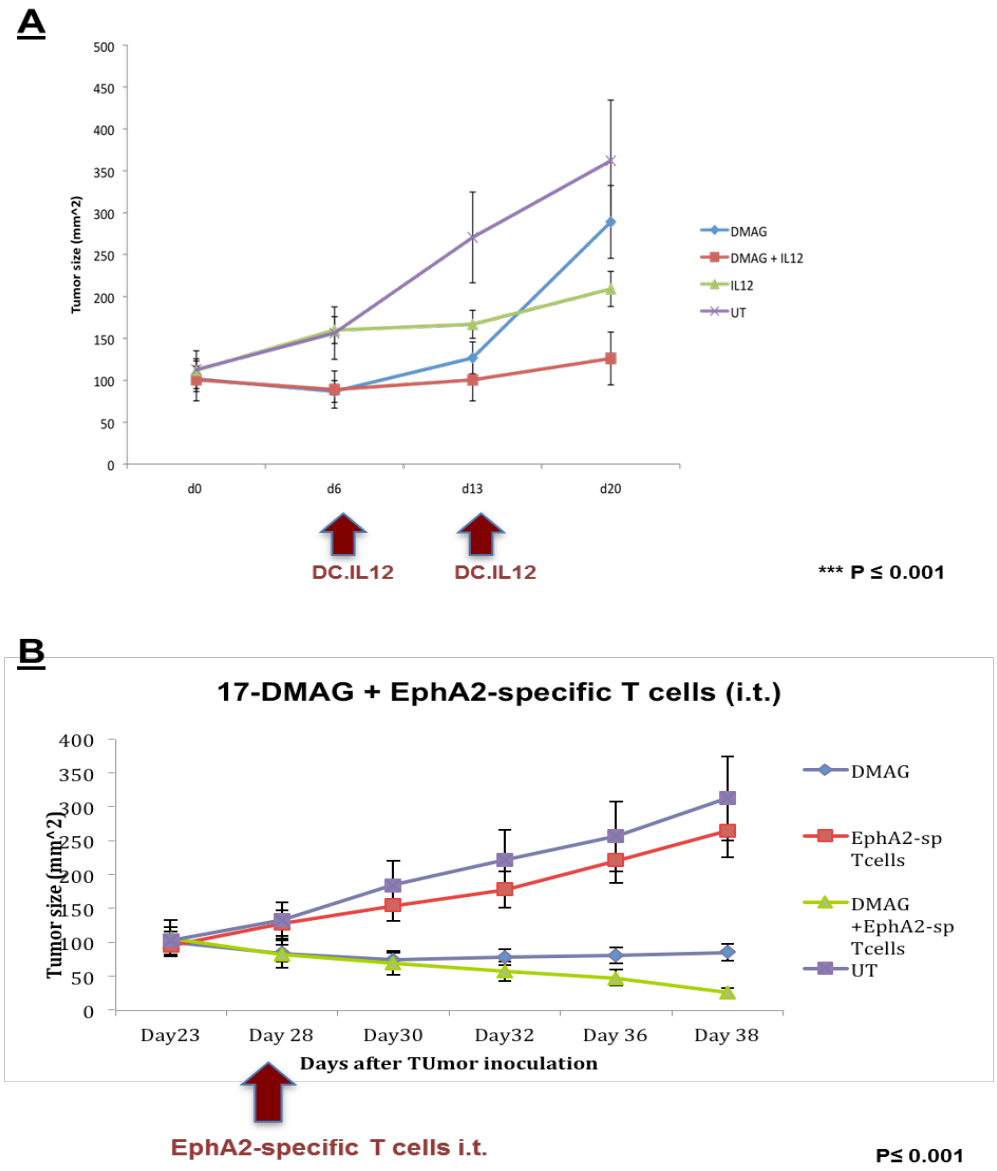


Figure 21: 17-DMAG combined with immunotherapy significantly improves anti-tumor efficacy

a) MCA205-tumor bearing mice were treated with 17-DMAG at 15 mg/Kg for 5 days and DCs transduced with IL12 gene were injected intra-tumorally in appropriate groups on the final day of treatment, with tumors monitored for the remainder of the study. b) MCA205-tumor bearing mice were treated with 17-DMAG at 15 mg/Kg for 5 days and $5x10^6$ cells were injected intra-tumorally on the final day (5) of DMAG treatment.

4.5. SUMMARY OF RESULTS

17-DMAG treatment of tumor bearing mice led to extensive infiltration of inflammatory CD4s, CD8s and DCs into the tumor microenvironment. I attempted to discern the underlying mechanisms of action of 17-DMAG and observed that the drug induced increased expression of type1 inflammatory genes and decrease expression of immunosuppressive factors within tumors. In addition, there was an increase in expression of anti-angiogenic factors and a concomitant decrease in pro-tumor angiogenic factors (HIFs, CXCL12, CXCR4 etc.). While I have not yet elucidated the exact temporal sequence of events that underlies the benefits of 17-DMAG treatment, my limited understanding of the changes occurring in the tumor microenvironment following 17-DMAG treatment has been enriched by the current study and is further expounded upon in General Discussion section of this thesis document.

In view of the immune adjuvant properties of 17-DMAG, I looked at the effects of combinational 17-DMAG-based immunotherapy in a sarcoma tumor model and observed that mice receiving combinational treatment had significantly slower/reduced tumor growth when compared to animals receiving single modes of treatment. In fact, when combined with the adoptive transfer of EphA2-specific T cells, this approach led to tumor liquefaction and hemorrhagic necrosis. Hence, combining 17-DMAG with EphA2-targeted immunotherapy could represent a particularly effective therapeutic approach for treating EphA2-positive solid tumors in the clinic.

CHAPTER 5. GENERAL DISCUSSION

Expression of receptor tyrosine kinases (RTKs) is upregulated in a number of cancers, where these molecules play important roles in tumorigenesis and vasculogenesis by mediating sustained tumor growth, angiogenesis and metastasis [1,9]. EphA2 is one such RTK that is overexpressed by both tumors as well as the tumor-associated vasculature, with its degree of overexpression linked to poor disease prognosis and an increased incidence of metastasis [8]. Given such clinical correlates, I have dedicated my Ph.D. project to defining means to effectively target and to modulate EphA2 expression in the tumor microenvironment via the induction of protein degradation, leading to enhanced immunity and reduced tumor growth and metastasis (as a consequences of negatively impacting tumor angiogenesis).

In tumors, HSP90 plays a central role in mediating the proper folding and stabilization of a range of oncoproteins (including RTK such as EphA2), with such target proteins considered to represent HSP90 “clients” [3,148,161]. Due to the post-translational action of HSP90 molecules, EphA2 protein can become overaccumulated in tumor cells. My work, and that of others in our laboratory indicate that agents like small molecule HSP90 inhibitors (i.e. 17-DMAG) and anti-EphA2 agonistic antibodies can effectively promote: 1) EphA2 degradation by re-route it for processing by the proteasome (instead of the lysosome), with the caveat that Ub-EphA2 would need to be onvestigated further in this regard, 2) possible loading/presentation of higher levels of EphA2-derived peptides (can only be truly validated using mass spectrometry applied to peptides extracted from affinity-purified tumor MHC class I molecules) and 3) the presentation of these MHC I/peptide complexes on the tumor cell surface. As a consequence, tumors treated with agonistic EphA2 antibody or 17-DMAG were observed to be significantly better

recognized by EphA2-specific (cloned or bulk) CD8+ T cell effector cells [2,7,382].

Tumors have evolved a number of mechanisms to evade the host immune response. Therefore, a focus has been placed on countering these mechanisms in order to generate more effective anti-tumor immune responses [230]. Immunotherapy for the treatment of cancer has gained popularity in recent years, as has been detailed in the Introduction section to this thesis, with several therapeutic approaches demonstrating some degree of success in the clinic, including the i) adoptive transfer of genetically-engineered patient T cells recognizing tumor antigens and ii.) injection of vaccines based on the patient's own dendritic cells loaded with tumor antigens in order to effectively stimulate specific anti-tumor T cells *in vivo* [306,338]. My studies as performed, did not allow me to discriminate whether improved T cell recognition also resulted (at least partially) due to HSP90 inhibitor-induced upregulation in tumor cell expressed costimulatory molecules or down-regulation of co-inhibitory molecules. These issues will need to be carefully evaluated in prospective studies. My research was also not designed to address the impact of HSP90 inhibitors on the level and composition of tumor-elaborated exosomes that may serve as regulators of immune responses by providing either negative signals or a source of antigen to fuel DC-based cross-presentation. Since HSP90 and RTKs are commonly found in tumor-derived exosomes, it is likely that HSP90 inhibitors will alter the nature of these vesicular mediators, thereby influencing both locoregional (to tumor) and peripheral immunity. These issues will need to be carefully assessed in future evaluations of single and combined modality HSP90 inhibitors *in vivo*.

However, immunotherapies as a whole, have displayed only modest efficacy in the clinic, despite their common ability to enhance circulating frequencies of tumor-specific T cells in the blood of many treated patients. This may be attributed to the low-to-moderate avidity of the negatively-selected T cell repertoire (reactive against non-mutated, "self" RTK peptides) that is activated by such therapies. Such T cell populations may become effectively activated by APCs presenting abundant (high) levels of tumor antigen-derived peptides, but they typically fail to recognize tumor cells that naturally present low stochastic levels of relevant MHC/tumor peptide complexes on their cell surface [296]. In

addition, many tumors downregulate components of their antigen presenting machinery (APM), particularly components required for cell surface MHC class I/peptide complexes, thus further hindering their ability to be regulated by the (CD8+) T cell arm of the adaptive immune response [241].

Cancer is a multi-factorial disease resulting from accumulation of multiple gene mutations and the deregulated expression of proteins aligned with pro-survival and –metastatic associated signaling pathways. Therefore, it is not surprising that in the clinic, the application of a single mode of treatment has resulted in limited effectiveness, as has been repeatedly observed in now thousands of clinical studies applied to patients with cancer [127,267]. The near-term future of tumor therapy lies in the development and clinical application of synergistic combination therapies that coordinately antagonist multiple survival, growth, metastatic pathways that are intrinsically important to heterogeneous populations of cancer cells within a given lesion in a given patient.

As outlined in the Introduction, the HSP90 inhibitor 17-DMAG has been used in combination with chemotherapy or radiotherapy in order to improve the anti-tumor impact of each single modality, in part, by overcoming the development of tumor chemo-/radio-resistance. Of note, 17-DMAG has been evaluated in combination with doxorubicin, cisplatin and HDAC inhibitors, with each of these combination therapies leading to improved treatment benefits when compared to either component single modality [191-193]. More recently, 17-DMAG has been used in combination with bortezomib (a proteasome inhibitor) as a improved treatment option in the setting of rhabdomyosarcoma [383]. Using this approach has demonstrated that inhibition of the proteasome re-routes the ubiquitinated proteins to the lysosomes for degradation. In addition, it has been reported that proteasome inhibition may activate autophagy which in turn may induce presentation of peptides on tumor MHC class I molecules [384]. This hypothesis, however, has not been confirmed in this study. HSP90 inhibition using 17-DMAG is also showing great promise in treatment of tumor cells with mutant forms of an oncoprotein, that are resistant to conventional chemotherapeutic agents that recognize/target the wild-type oncoproteins [192]. For example, 17-DMAG has been

used in combination with TKIs targeting EGFR in patients with EGFR-mutant non-small cell lung cancer that develop drug-resistance based on a compensatory shift to a c-met (HGFR)-mediated survival/growth signaling pathway [385]. In addition, the combination of 17-DMAG with radiotherapy, with 17-DMAG abrogating G2 → S phase transition and interfering with DNA damage repair in treated tumor cells. Ultimately, combination 17-DMAG/radiotherapy has demonstrated significantly higher anti-tumor efficacy *in vitro* and *in vivo* compared to single agent administration in a number of tumor models, including lung and breast cancers [188,378]. Overall, 17-DMAG has shown great promise in the combinational therapeutic setting, however, its efficacy as an "adjunct" to immunotherapy had not been determined *in vivo* prior to the performance of the current studies.

Based on our preliminary published data demonstrating that 17-DMAG treatment of EphA2+ human tumor cell lines leads to their increased recognition by specific, HLA-A2-restricted CD8+ T cells *in vitro* [7], I hypothesized that combining 17-DMAG with an anti-EphA2 immunotherapy approach (either adoptively-transferred EphA2-specific T cells or the active immunization against EphA2 using a DC-based vaccine) would provide superior anti-tumor efficacy when compared to either component treatment alone *in vivo*. Since our foundational studies were performed using human tumor cells *in vitro*, I began my studies in mouse models by evaluating the effects of 17-DMAG *in vitro.* My work showed that 17-DMAG caused a dose-dependent decrease in EphA2 "client protein" levels upon treatment with 17-DMAG, with no adverse effects on tumor cell expression of MHC class I levels. Although this suggested (indirectly) that there was no negative impact on the tumor antigen-processing machinery (APM), I could not directly assess the effect of 17-DMAG on other components of the APM like TAP1, TAP2, etc., due to unavailability of validated, specific probes reactive against these murine proteins. Furthermore merely the analysis of the protein content would not speak to any variance in their functional capacity pre- versus post-treatment with 17-DMAG. Hence, as was the case for our human *in vitro* studies, I observed that 17-DMAG promoted the proteasome (but not lysosome)-dependent degradation of tumor EphA2 molecules that resulted in subsequent increase in the ability of anti-EphA2 specific CD8+ T cells to recognize these

target cells *in vitro*.

As discussed in chapter 2, I also evaluated the effect of 17-DMAG on other RTKs – Her2/neu, VEGFR1 and PDGFRB across three different cell lines – MCA205 (sarcoma), B16 (melanoma) and MC38 (colon cancer). I found that, as was the case for EphA2, 17-DMAG coordinately mediated the degradation of multiple RTKs in all of these cell lines in a dose-dependent fashion. This suggests that my studies could be readily expanded in consideration of combinational therapies targeting other tumor RTKs, or even more broadly, to all HSP90 client proteins that are differentially overexpressed by tumor cells versus their normal cellular counterparts.

A number of previous reports have detailed the important role mediated by HSP90 in intrinsic antigen presentation and induction of inflammatory immune responses [358,371], as well as, the contribution that this HSP plays in the cytosolic translocation of extracellular antigens in the cross-presentation of T cells by dendritic cells (DC) [359,360]. Therapeutic inhibition of HSP90 (using 2nd and 3rd generation inhibitors) has been suggested as an effective treatment option for autoimmune diseases, in order to dampen the activity of self-reactive T cells [362,386]. However, these same attractive attributes in the setting of autoimmunity would likely prove disastrous in the cancer-bearing host, leading to rapid disease progression or recurrence.

To address this theoretical concern, I first evaluated the impact of 17-DMAG on immune cell phenotype and function *in vitro*. I noted that 17-DMAG treatment can negatively affected DC phenotype and function (the ability of DC to stimulate allogenic T cells) in a dose-, as well as, treatment duration-dependent fashion. When we looked at the effect of 17-DMAG on CD8+ T cell activation, a longer duration of treatment was required in order to discern any effect on these responder cells. In contrast, CD4+ T cells appeared resistant to the action of the HSP90 inhibitor. The apparent inhibition of immune cell function could be due to the differential toxicity of this drug, leading to biased cell death profiles, or to the deregulation of important intrinsic signaling pathways affecting DC/CD8+ T cells, but not CD4+ T cells. Indeed, NFKB, a master transcription factor

playing a role in transcription of inflammatory mediators and DC maturation, is an HSP90 client, as is Zap70 and several other T cell signaling molecules [358,359]. Overall, my murine *in vitro* data were consistent with other reports suggesting that 17-DMAG could have adverse (functional) effects on cells of the immune system, which was a source of major concern for me, as I planned the *in vivo* studies, leading me to stress an analysis of changes in the "immunophenotype" of the tumor and lymphoid organ microenvironments as a consequence of mice being treated with 17-DMAG.

In my preliminary *in vivo* experiments, I was careful to evaluate both the dose- and schedule-dependency of 17-DMAG administration on the tumor immunophenotype. I found that treatment of tumor-bearing animals with 17-DMAG for 5 days at a dose of 15 mg/Kg was optimal to enhance EphA2+ tumor cell recognition by EphA2-specific CD8+ T effector cells. Treatment at lower doses/times led to minimal impact on EphA2-specific T cell recognition of tumor cells, while treatment of animals for more than 5 days, or at doses higher than 15 mg/Kg, resulted in a reduction in the positive treatment effects observed using the 15 mg/Kg x 5 day protocol. This was clearly reflected in the tumor growth curves, since mice treated with 15 mg/Kg for 5 days show stabilization of tumor growth, while provision of higher doses of drug/longer treatment duration did not statistically improve suppression in tumor growth. When I looked at the effect of 17-DMAG on the immune cell composition in the tumors, I saw that 17-DMAG treatment for up to 5 days caused an increase infiltration of CD4+, CD8+ T cells and DCs, and decreased incidence of Tregs and MDSCs in the TME. However, treatment for more than 5 days reversed this phenomenon. Hence, it may be very possible that as observed *in vitro*, prolonged treatment fro more than 5 days with 17-DMAG may indeed be toxic to T cells and DCs, leading to their reduced prevalence in tumors and lymphoid organs. Tregs and MDSCs could be more refractory to the effects of 17-DMAG, and it possible that continued treatment of tumor-bearing animals with 17-DMAG for periods longer than 10 days might eventually have a negative effect on such regulatory cell populations as well. While such future studies are warranted, my current data suggest that a biologic/immunologic "prozone" exists for 17-DMAG-based therapy, with an optima in the mouse of 5 days at 15 mg/Kg/day.

In this regard, I have just begun to scratch the surface of the possible underlying mechanisms of action linked to the increase in inflammatory cells in the TME and decreased incidence of Tregs and MDSCs. The mRNA transcription profiles from 17-DMAG treated versus untreated mice suggests that 17-DMAG treatment causes an acute increase in type-1 immune response genes, and a decrease in non-type 1-associated gene transcripts. In addition, 17-DMAG caused a decrease in MDSC-function associated genes like arginase1, IDO and iNOS, which could be most readily explained by the decrease in MDSC recruiting molecules like CXCR4, CXCL12, S100A8 and S100A9 in the TME [288]. I also observed an increase in type-1 T cell chemokine/chemokine receptors like CXCR3, and its ligands, CXCL9 and CXCL10. At the protein level, I noted that 17-DMAG treatment caused an increase in expression of CXCL10 and VCAM-1 by the tumor and its associated stroma and blood vessels, respectively . In addition, high levels of pSTAT3 in the progressor TME were replaced by (type-1-associated) pSTAT1 after administration of 17-DMAG for up to 5 days *in vivo*.

A particularly interesting piece of this puzzle may relate to my observations pertaining to decreased levels of HIF1a and HIF2a transcripts expression in the TME after treatment with 17-DMAG. HIFs are HSP90 client proteins, and therefore when HSP90 is inhibited, HIF levels are reduced post-translationally [387]. Since STAT3 is a transcriptional regulator of HIFs, drug-induced reduction in pSTAT3 may also reduce HIF transcription *in vivo*. HIFs protect tumor cells by upregulating pro-survival pathways in tumor cells and cancer stem cells under low-oxygen conditions [388]. They also promote vascular endothelial growth factor (VEGF) in support of angiogenesis in the TME. In addition, HIFs may also promote a down-modulation in locoregional expression of adhesion molecule by tumor and tumor-associated stromal cells. Hence, perturbing HIF levels via the use of 17-DMAG may lead to increased expression of (type-1 T cell recruiting) VCAM-1 and CXCL9/10, and decreased angiogenesis and regulatory cell footprint in the TME [389].

Interestingly the anti-angiogenic tyrosine kinase inhibitors (TKIs) sunitinib, axitinib and

dasatinib appear to modify the TME *in vivo* in an operationally similar manner [390,391], Lowe *et al.*, unpublished data. Even though, I did not look at the effects of 17-DMAG on tumor-associated pericytes and cancer stem cells, given the known impact of hypoxia on these indices, it is predicted that 17-DMAG affects these cell populations, thereby sensitizing the TME and reconditioning it to more conducive to effector T cell recruitment and sustenance. Further studies will need to be performed to delineate causal underlying mechanisms of action associated with 17-DMAG anti-tumor efficacy and the development of protective and durable anti-tumor immunity. Figure 22 details our understanding of the biologic alterations in the TME following 17-DMAG treatment.

Since 17-DMAG treatment was for a short window of time, we expected the results to be short-lived and reversible once treatment was discontinued. While this was true in the case of tumor EphA2 degradation and the enhanced recognition of tumors by EphA2-specific T cells, we observed prolonged beneficial effects with regards to infiltration of inflammatory cells into the TME. This may relate to differential drug thresholds required to impact each of these biologic indices or the comparative ability of a given HSP90 client protein that is key in the biologic cascade supporting the endpoint index. Given my data, however, it is suggested that one may want to administer 17-DMAG as a “pulse therapy” in order to periodically and reiteratively activate the recruitment and function of protective immunity within the TME to optimized therapeutic benefit. Such effects may be further accentuated by increasing the number of circulating, “recruitable” anti-tumor T cells as a consequence of a co-therapeutic approach.

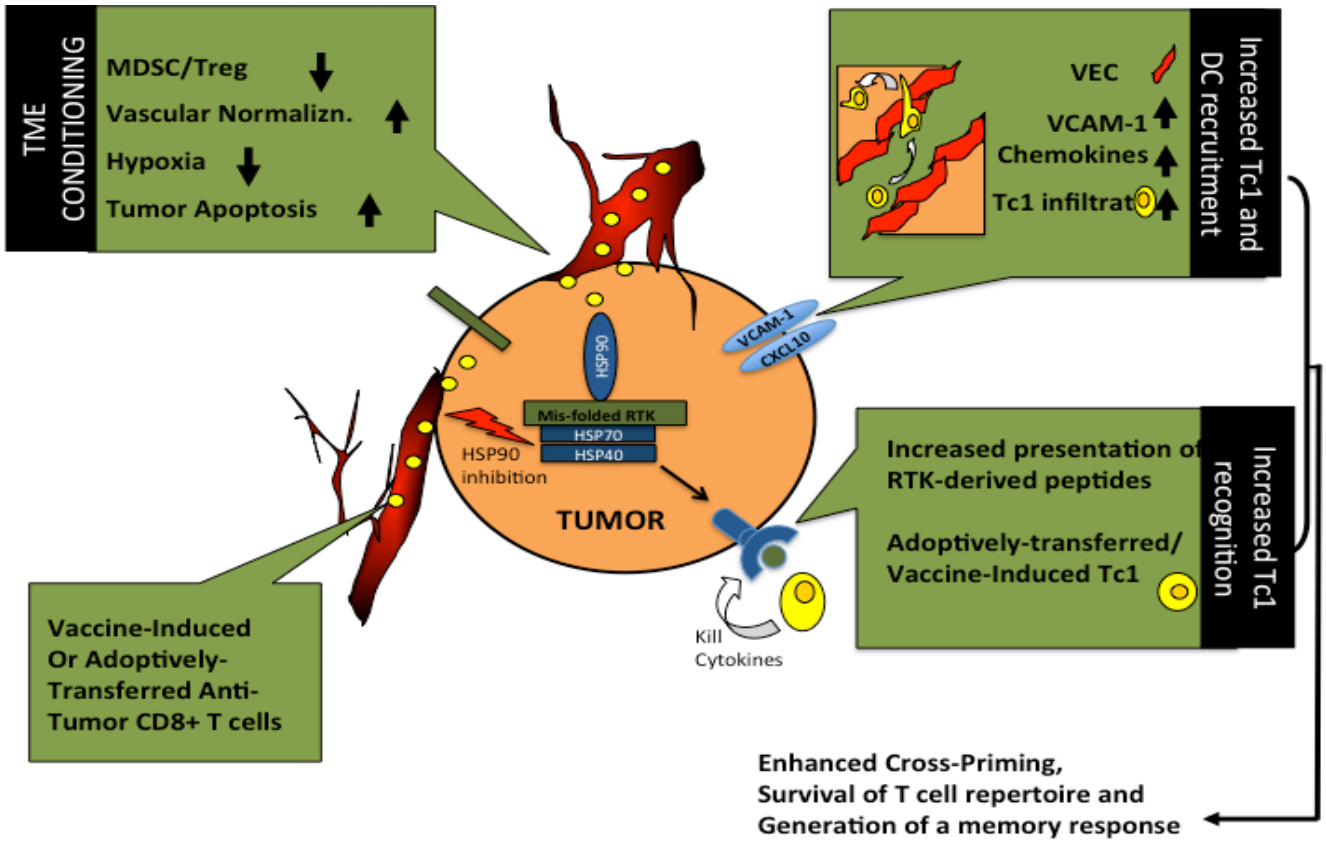


Figure 22: : A schematic of underlying immune effects of 17-DMAG

Inhibition of HSP90 by 17-DMAG causes a) increased presentation of RTK-derived peptides on tumor MHC class I molecules causing increased recognition of the tumor by specific Tc1 b) 17-DMAG mediated vascular normalization, decreased hypoxia and increased VCAM1/CXCR3 ligand expression leads to increased infiltration of protective Tc1 and DCs which in turn leads to increased cross-priming, prolonged sustenance of Tc1 and generation of a memory response.

In this context, I evaluated the efficacy of 17-DMAG in 4 different combinational therapy settings – i) with intratumoral IL12 gene therapy; ii) with DC-based EphA2 vaccines; iii) with adoptive transfer of EphA2 specific T cells provided wither intra-tumorally (i.t.) or iv) intra-venously (i.v.). In all cases, the combination therapy exhibited superior anti-tumor efficacy when compared to the component, single modalities, with the best therapies leading to the greatest infiltration of specific CD8+ T cells into the TME.

In my hands, 17-DMAG administration combined with DC/EphA2 peptide-based vaccination in the EphA2+ tumor setting (i.e. MCA205) was arguably the most effective combinational approach. Treatment resulted in the complete regression of tumors and generation of durable memory immune responses. Adoptive transfer of EphA2-specific T cells (i.t.) in combination with 17-DMAG also demonstrated potent anti-tumor effects, leading to extensive tumor necrosis and a complete liquefaction of the tumor in association with hemorrhaging in EphA2+ tumor blood vessels. 17-DMAG treatment did not hinder the trafficking of primed T cells to the tumor, as evidenced by the massive tumor necrosis observed in combinational therapy experiments with i.v. transfer of EphA2-specific T cells. This remarkable necrosis was specific for mice receiving the combinational therapy with EphA2-specific T cells (and not observed in DC-based vaccine approaches, which also involves elicitation of EphA2-specific T cell responses). This could be due to the higher functional avidity of (adoptively-transferred) EphA2-specific T cells generated by immunizing EphA2-/- mice, as opposed to comparatively low-to-moderate avidity anti-EphA2 T cells developed as a consequence of DC-based vaccines.

One of the biggest concerns with immunotherapy targeting tumor-associated antigens is the imminent threat of adverse autoimmune reactions against normal organs and tissues. In our case, EphA2 is expressed by a number of normal organs like kidney, intestine, lungs etc. In addition, HSP90 is a ubiquitous protein present in all cells. So combining HSP90 inhibition with EphA2-specific immunotherapy could potentially cause autoimmune reactions against other EphA2-positive organs. I did not observe any changes in the gross morphology of mouse organs like kidneys and lungs, nor did I note that treated animals underwent changes in their weight or behavior. Importantly, I was able to demonstrate that my combined therapy even worked in cases where $EphA2^{neg}$ tumors were being treated, based on anti-EphA2 recognition of tumor-associated EphA2+ vascular endothelial cells (VEC). These same protective T cells failed to recognize VEC isolated from the kidneys of these same mice receiving combinational therapy, which may directly relate to the higher EphA2 transcript/protein levels I observed for tumor-

versus-normal VEC, etc.. Overall, I did not observe tangible evidence of off-target, autoimmune sequelae in mice treated with 17-DMAG combination therapy approaches, suggesting that these may be seriously considered for translation into the clinic.

In all, my studies provide a proof-of-principle for the performance of analogous studies combining HSP90 with the immunotargeting of alternate HSP90 client proteins overexpressed by tumor cells or tumor-associated stromal cells. If a specific client protein can be demonstrated to undergo degradation via the proteasome, there is a good chance that HSP90 administration can promote its delivery into MHC class I complexes allowing for improved recognition by client protein-specific CD8+ T cells. My preliminary data suggests this will likely be the case for the combination therapeutic targeting of tumor-associated antigens such as EGFR, Her2/neu, VEGFR1 and PDGFRβ. Considering the wide range of HSP90 clients in the tumors mediating a diverse range of functions, combinational immunotherapy with HSP90 treatment could be applied to a wide range of tumors and their associated vasculature (primary or metastatic), thus making this therapeutic approach extremely versatile and effective. Furthermore targeted combinational therapeutics might be accentuated by the inclusion of antibodies specific for cell-surface tumor-associated HSP90 client proteins (i.e. RTKs, among others) given the ability of these agents to selectively promote their internalization/ degradation and potential MHC class I-presentation to CD8+ T cells.

CHAPTER 6. BIBLIOGRAPHY

1. Arslan MA, Kutuk O, Basaga H: **Protein kinases as drug targets in cancer**. *Curr Cancer Drug Targets* 2006, **6**:623-634.
2. Wesa AK, Herrem CJ, Mandic M, Taylor JL, Vasquez C, Kawabe M, Tatsumi T, Leibowitz MS, Finke JH, Bukowski RM, *et al.*: **Enhancement in specific CD8+ T cell recognition of EphA2+ tumors in vitro and in vivo after treatment with ligand agonists**. *J Immunol* 2008, **181**:7721-7727.
3. Neckers L: **Heat shock protein 90: the cancer chaperone**. *J Biosci* 2007, **32**:517-530.
4. Whitesell L, Lindquist SL: **HSP90 and the chaperoning of cancer**. *Nat Rev Cancer* 2005, **5**:761-772.
5. Egorin MJ, Lagattuta TF, Hamburger DR, Covey JM, White KD, Musser SM, Eiseman JL: **Pharmacokinetics, tissue distribution, and metabolism of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (NSC 707545) in CD2F1 mice and Fischer 344 rats**. *Cancer Chemother Pharmacol* 2002, **49**:7-19.
6. Eiseman JL, Lan J, Lagattuta TF, Hamburger DR, Joseph E, Covey JM, Egorin MJ: **Pharmacokinetics and pharmacodynamics of 17-demethoxy 17-[[(2-dimethylamino)ethyl]amino]geldanamycin (17DMAG, NSC 707545) in C.B-17 SCID mice bearing MDA-MB-231 human breast cancer xenografts**. *Cancer Chemother Pharmacol* 2005, **55**:21-32.
7. Kawabe M, Mandic M, Taylor JL, Vasquez CA, Wesa AK, Neckers LM, Storkus WJ: **Heat shock protein 90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin enhances EphA2+ tumor cell recognition by specific CD8+ T cells**. *Cancer Res* 2009, **69**:6995-7003.
8. Wykosky J, Debinski W: **The EphA2 receptor and ephrinA1 ligand in solid tumors: function and therapeutic targeting**. *Mol Cancer Res* 2008, **6**:1795-1806.
9. Robinson DR, Wu YM, Lin SF: **The protein tyrosine kinase family of the human genome**. *Oncogene* 2000, **19**:5548-5557.
10. Gschwind A, Fischer OM, Ullrich A: **The discovery of receptor tyrosine kinases: targets for cancer therapy**. *Nat Rev Cancer* 2004, **4**:361-370.
11. Huang H, Li T, Tian S, Gupta DK, Zhang X, Yang XE: **Role of EDTA in alleviating lead toxicity in accumulator species of Sedum alfredii H**. *Bioresour Technol* 2008, **99**:6088-6096.
12. Abella JV, Peschard P, Naujokas MA, Lin T, Saucier C, Urbe S, Park M: **Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation**. *Mol Cell Biol* 2005, **25**:9632-9645.

13. Peschard P, Fournier TM, Lamorte L, Naujokas MA, Band H, Langdon WY, Park M: **Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein**. *Mol Cell* 2001, **8**:995-1004.
14. Pawson T: **Protein modules and signalling networks**. *Nature* 1995, **373**:573-580.
15. Wells A, Welsh JB, Lazar CS, Wiley HS, Gill GN, Rosenfeld MG: **Ligand-induced transformation by a noninternalizing epidermal growth factor receptor**. *Science* 1990, **247**:962-964.
16. Dupre S, Volland C, Haguenauer-Tsapis R: **Membrane transport: ubiquitylation in endosomal sorting**. *Curr Biol* 2001, **11**:R932-934.
17. Muthuswamy SK, Gilman M, Brugge JS: **Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers**. *Mol Cell Biol* 1999, **19**:6845-6857.
18. Mancini A, Koch A, Wilms R, Tamura T: **c-Cbl associates directly with the C-terminal tail of the receptor for the macrophage colony-stimulating factor, c-Fms, and down-modulates this receptor but not the viral oncogene v-Fms**. *J Biol Chem* 2002, **277**:14635-14640.
19. Ridge SA, Worwood M, Oscier D, Jacobs A, Padua RA: **FMS mutations in myelodysplastic, leukemic, and normal subjects**. *Proc Natl Acad Sci U S A* 1990, **87**:1377-1380.
20. Lenferink AE, Pinkas-Kramarski R, van de Poll ML, van Vugt MJ, Klapper LN, Tzahar E, Waterman H, Sela M, van Zoelen EJ, Yarden Y: **Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers**. *EMBO J* 1998, **17**:3385-3397.
21. Pinkas-Kramarski R, Lenferink AE, Bacus SS, Lyass L, van de Poll ML, Klapper LN, Tzahar E, Sela M, van Zoelen EJ, Yarden Y: **The oncogenic ErbB-2/ErbB-3 heterodimer is a surrogate receptor of the epidermal growth factor and betacellulin**. *Oncogene* 1998, **16**:1249-1258.
22. Gale NW, Yancopoulos GD: **Ephrins and their receptors: a repulsive topic?** *Cell Tissue Res* 1997, **290**:227-241.
23. Cheng N, Brantley DM, Chen J: **The ephrins and Eph receptors in angiogenesis**. *Cytokine Growth Factor Rev* 2002, **13**:75-85.
24. Himanen JP, Chumley MJ, Lackmann M, Li C, Barton WA, Jeffrey PD, Vearing C, Geleick D, Feldheim DA, Boyd AW, *et al.*: **Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling**. *Nat Neurosci* 2004, **7**:501-509.
25. Smith FM, Vearing C, Lackmann M, Treutlein H, Himanen J, Chen K, Saul A, Nikolov D, Boyd AW: **Dissecting the EphA3/Ephrin-A5 interactions using a novel functional mutagenesis screen**. *J Biol Chem* 2004, **279**:9522-9531.
26. Gale NW, Holland SJ, Valenzuela DM, Flenniken A, Pan L, Ryan TE, Henkemeyer M, Strebhardt K, Hirai H, Wilkinson DG, *et al.*: **Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis**. *Neuron* 1996, **17**:9-19.
27. Coulthard MG, Duffy S, Down M, Evans B, Power M, Smith F, Stylianou C, Kleikamp S, Oates A, Lackmann M, *et al.*: **The role of the Eph-ephrin signalling system in the regulation of developmental patterning**. *Int J Dev Biol* 2002, **46**:375-384.

28. Wilkinson DG: **Multiple roles of EPH receptors and ephrins in neural development**. *Nat Rev Neurosci* 2001, **2**:155-164.
29. Mellitzer G, Xu Q, Wilkinson DG: **Control of cell behaviour by signalling through Eph receptors and ephrins**. *Curr Opin Neurobiol* 2000, **10**:400-408.
30. Wang HU, Chen ZF, Anderson DJ: **Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4**. *Cell* 1998, **93**:741-753.
31. Noren NK, Pasquale EB: **Eph receptor-ephrin bidirectional signals that target Ras and Rho proteins**. *Cell Signal* 2004, **16**:655-666.
32. Huynh-Do U, Vindis C, Liu H, Cerretti DP, McGrew JT, Enriquez M, Chen J, Daniel TO: **Ephrin-B1 transduces signals to activate integrin-mediated migration, attachment and angiogenesis**. *J Cell Sci* 2002, **115**:3073-3081.
33. Winning RS, Wyman TL, Walker GK: **EphA4 activity causes cell shape change and a loss of cell polarity in Xenopus laevis embryos**. *Differentiation* 2001, **68**:126-132.
34. Klein R: **Excitatory Eph receptors and adhesive ephrin ligands**. *Curr Opin Cell Biol* 2001, **13**:196-203.
35. Davy A, Robbins SM: **Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner**. *EMBO J* 2000, **19**:5396-5405.
36. Wahl S, Barth H, Ciossek T, Aktories K, Mueller BK: **Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase**. *J Cell Biol* 2000, **149**:263-270.
37. Miao H, Burnett E, Kinch M, Simon E, Wang B: **Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation**. *Nat Cell Biol* 2000, **2**:62-69.
38. Zantek ND, Azimi M, Fedor-Chaiken M, Wang B, Brackenbury R, Kinch MS: **E-cadherin regulates the function of the EphA2 receptor tyrosine kinase**. *Cell Growth Differ* 1999, **10**:629-638.
39. Surawska H SR: **The role of Ephrins and eph receptors in cancer**. *Cytokine Growth Factor Rev* 2004, **15**:419-433.
40. Heroult M, Schaffner F, Augustin HG: **Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression**. *Exp Cell Res* 2006, **312**:642-650.
41. Ganju P, Shigemoto K, Brennan J, Entwistle A, Reith AD: **The Eck receptor tyrosine kinase is implicated in pattern formation during gastrulation, hindbrain segmentation and limb development**. *Oncogene* 1994, **9**:1613-1624.
42. Bovenkamp DE, Greer PA: **Degenerate PCR-based cloning method for Eph receptors and analysis of their expression in the developing murine central nervous system and vasculature**. *DNA Cell Biol* 2001, **20**:203-213.
43. Lickliter JD, Smith FM, Olsson JE, Mackwell KL, Boyd AW: **Embryonic stem cells express multiple Eph-subfamily receptor tyrosine kinases**. *Proc Natl Acad Sci U S A* 1996, **93**:145-150.
44. Lindberg RA, Hunter T: **cDNA cloning and characterization of eck, an epithelial cell receptor protein-tyrosine kinase in the eph/elk family of protein kinases**. *Mol Cell Biol* 1990, **10**:6316-6324.

45. Potla L, Boghaert ER, Armellino D, Frost P, Damle NK: **Reduced expression of EphrinA1 (EFNA1) inhibits three-dimensional growth of HT29 colon carcinoma cells**. *Cancer Lett* 2002, **175**:187-195.
46. Munthe E, Finne EF, Aasheim HC: **Expression and functional effects of Eph receptor tyrosine kinase A family members on Langerhans like dendritic cells**. *BMC Immunol* 2004, **5**:9.
47. de Saint-Vis B, Bouchet C, Gautier G, Valladeau J, Caux C, Garrone P: **Human dendritic cells express neuronal Eph receptor tyrosine kinases: role of EphA2 in regulating adhesion to fibronectin**. *Blood* 2003, **102**:4431-4440.
48. Aasheim HC, Munthe E, Funderud S, Smeland EB, Beiske K, Logtenberg T: **A splice variant of human ephrin-A4 encodes a soluble molecule that is secreted by activated human B lymphocytes**. *Blood* 2000, **95**:221-230.
49. Bartley TD, Hunt RW, Welcher AA, Boyle WJ, Parker VP, Lindberg RA, Lu HS, Colombero AM, Elliott RL, Guthrie BA, *et al.*: **B61 is a ligand for the ECK receptor protein-tyrosine kinase**. *Nature* 1994, **368**:558-560.
50. Shao H, Pandey A, O'Shea KS, Seldin M, Dixit VM: **Characterization of B61, the ligand for the Eck receptor protein-tyrosine kinase**. *J Biol Chem* 1995, **270**:5636-5641.
51. Zelinski DP, Zantek ND, Stewart JC, Irizarry AR, Kinch MS: **EphA2 overexpression causes tumorigenesis of mammary epithelial cells**. *Cancer Res* 2001, **61**:2301-2306.
52. Kinch MS, Carles-Kinch K: **Overexpression and functional alterations of the EphA2 tyrosine kinase in cancer**. *Clin Exp Metastasis* 2003, **20**:59-68.
53. Walker-Daniels J, Riese DJ, 2nd, Kinch MS: **c-Cbl-dependent EphA2 protein degradation is induced by ligand binding**. *Mol Cancer Res* 2002, **1**:79-87.
54. Carles-Kinch K, Kilpatrick KE, Stewart JC, Kinch MS: **Antibody targeting of the EphA2 tyrosine kinase inhibits malignant cell behavior**. *Cancer Res* 2002, **62**:2840-2847.
55. Wang Y, Ota S, Kataoka H, Kanamori M, Li Z, Band H, Tanaka M, Sugimura H: **Negative regulation of EphA2 receptor by Cbl**. *Biochem Biophys Res Commun* 2002, **296**:214-220.
56. Dohn M, Jiang J, Chen X: **Receptor tyrosine kinase EphA2 is regulated by p53-family proteins and induces apoptosis**. *Oncogene* 2001, **20**:6503-6515.
57. Macrae M, Neve RM, Rodriguez-Viciana P, Haqq C, Yeh J, Chen C, Gray JW, McCormick F: **A conditional feedback loop regulates Ras activity through EphA2**. *Cancer Cell* 2005, **8**:111-118.
58. Rosenberg IM, Goke M, Kanai M, Reinecker HC, Podolsky DK: **Epithelial cell kinase-B61: an autocrine loop modulating intestinal epithelial migration and barrier function**. *Am J Physiol* 1997, **273**:G824-832.
59. Li Z, Tanaka M, Kataoka H, Nakamura R, Sanjar R, Shinmura K, Sugimura H: **EphA2 up-regulation induced by deoxycholic acid in human colon carcinoma cells, an involvement of extracellular signal-regulated kinase and p53-independence**. *J Cancer Res Clin Oncol* 2003, **129**:703-708.
60. Ivanov AI, Steiner AA, Scheck AC, Romanovsky AA: **Expression of Eph receptors and their ligands, ephrins, during lipopolysaccharide fever in rats**. *Physiol Genomics* 2005, **21**:152-160.

61. Walker-Daniels J, Coffman K, Azimi M, Rhim JS, Bostwick DG, Snyder P, Kerns BJ, Waters DJ, Kinch MS: **Overexpression of the EphA2 tyrosine kinase in prostate cancer**. *Prostate* 1999, **41**:275-280.
62. Zelinski DP, Zantek ND, Walker-Daniels J, Peters MA, Taparowsky EJ, Kinch MS: **Estrogen and Myc negatively regulate expression of the EphA2 tyrosine kinase**. *J Cell Biochem* 2002, **85**:714-720.
63. Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE: **EphA2: a determinant of malignant cellular behavior and a potential therapeutic target in pancreatic adenocarcinoma**. *Oncogene* 2004, **23**:1448-1456.
64. Herrem CJ, Tatsumi T, Olson KS, Shirai K, Finke JH, Bukowski RM, Zhou M, Richmond AL, Derweesh I, Kinch MS, *et al.*: **Expression of EphA2 is prognostic of disease-free interval and overall survival in surgically treated patients with renal cell carcinoma**. *Clin Cancer Res* 2005, **11**:226-231.
65. Wu N, Zhao X, Liu M, Liu H, Yao W, Zhang Y, Cao S, Lin X: **Role of microRNA-26b in glioma development and its mediated regulation on EphA2**. *PLoS One* 2011, **6**:e16264.
66. Chiarugi P, Taddei ML, Schiavone N, Papucci L, Giannoni E, Fiaschi T, Capaccioli S, Raugei G, Ramponi G: **LMW-PTP is a positive regulator of tumor onset and growth**. *Oncogene* 2004, **23**:3905-3914.
67. Kikawa KD, Vidale DR, Van Etten RL, Kinch MS: **Regulation of the EphA2 kinase by the low molecular weight tyrosine phosphatase induces transformation**. *J Biol Chem* 2002, **277**:39274-39279.
68. Malentacchi F, Marzocchini R, Gelmini S, Orlando C, Serio M, Ramponi G, Raugei G: **Up-regulated expression of low molecular weight protein tyrosine phosphatases in different human cancers**. *Biochem Biophys Res Commun* 2005, **334**:875-883.
69. Laprise P, Langlois MJ, Boucher MJ, Jobin C, Rivard N: **Down-regulation of MEK/ERK signaling by E-cadherin-dependent PI3K/Akt pathway in differentiating intestinal epithelial cells**. *J Cell Physiol* 2004, **199**:32-39.
70. Steinberg MS, McNutt PM: **Cadherins and their connections: adhesion junctions have broader functions**. *Curr Opin Cell Biol* 1999, **11**:554-560.
71. Hess AR, Seftor EA, Gruman LM, Kinch MS, Seftor RE, Hendrix MJ: **VE-cadherin regulates EphA2 in aggressive melanoma cells through a novel signaling pathway: implications for vasculogenic mimicry**. *Cancer Biol Ther* 2006, **5**:228-233.
72. Abraham S, Knapp DW, Cheng L, Snyder PW, Mittal SK, Bangari DS, Kinch M, Wu L, Dhariwal J, Mohammed SI: **Expression of EphA2 and Ephrin A-1 in carcinoma of the urinary bladder**. *Clin Cancer Res* 2006, **12**:353-360.
73. Merritt WM, Thaker PH, Landen CN, Jr., Deavers MT, Fletcher MS, Lin YG, Han LY, Kamat AA, Schmandt R, Gershenson DM, *et al.*: **Analysis of EphA2 expression and mutant p53 in ovarian carcinoma**. *Cancer Biol Ther* 2006, **5**:1357-1360.
74. Wykosky J, Gibo DM, Stanton C, Debinski W: **EphA2 as a novel molecular marker and target in glioblastoma multiforme**. *Mol Cancer Res* 2005, **3**:541-551.

75. Kirsch M, Schackert G, Black PM: **Metastasis and angiogenesis**. *Cancer Treat Res* 2004, **117**:285-304.
76. Ogawa K, Pasqualini R, Lindberg RA, Kain R, Freeman AL, Pasquale EB: **The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization**. *Oncogene* 2000, **19**:6043-6052.
77. Chen J, Hicks D, Brantley-Sieders D, Cheng N, McCollum GW, Qi-Werdich X, Penn J: **Inhibition of retinal neovascularization by soluble EphA2 receptor**. *Exp Eye Res* 2006, **82**:664-673.
78. Cheng N, Brantley D, Fang WB, Liu H, Fanslow W, Cerretti DP, Bussell KN, Reith A, Jackson D, Chen J: **Inhibition of VEGF-dependent multistage carcinogenesis by soluble EphA receptors**. *Neoplasia* 2003, **5**:445-456.
79. Cheng N, Brantley DM, Liu H, Lin Q, Enriquez M, Gale N, Yancopoulos G, Cerretti DP, Daniel TO, Chen J: **Blockade of EphA receptor tyrosine kinase activation inhibits vascular endothelial cell growth factor-induced angiogenesis**. *Mol Cancer Res* 2002, **1**:2-11.
80. Dobrzanski P, Hunter K, Jones-Bolin S, Chang H, Robinson C, Pritchard S, Zhao H, Ruggeri B: **Antiangiogenic and antitumor efficacy of EphA2 receptor antagonist**. *Cancer Res* 2004, **64**:910-919.
81. Brantley-Sieders DM, Zhuang G, Hicks D, Fang WB, Hwang Y, Cates JM, Coffman K, Jackson D, Bruckheimer E, Muraoka-Cook RS, *et al.*: **The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling**. *J Clin Invest* 2008, **118**:64-78.
82. Brantley DM, Cheng N, Thompson EJ, Lin Q, Brekken RA, Thorpe PE, Muraoka RS, Cerretti DP, Pozzi A, Jackson D, *et al.*: **Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo**. *Oncogene* 2002, **21**:7011-7026.
83. Lin YG, Han LY, Kamat AA, Merritt WM, Landen CN, Deavers MT, Fletcher MS, Urbauer DL, Kinch MS, Sood AK: **EphA2 overexpression is associated with angiogenesis in ovarian cancer**. *Cancer* 2007, **109**:332-340.
84. Coffman KT, Hu M, Carles-Kinch K, Tice D, Donacki N, Munyon K, Kifle G, Woods R, Langermann S, Kiener PA, *et al.*: **Differential EphA2 epitope display on normal versus malignant cells**. *Cancer Res* 2003, **63**:7907-7912.
85. Hu M, Carles-Kinch KL, Zelinski DP, Kinch MS: **EphA2 induction of fibronectin creates a permissive microenvironment for malignant cells**. *Mol Cancer Res* 2004, **2**:533-540.
86. Landen CN, Jr., Lu C, Han LY, Coffman KT, Bruckheimer E, Halder J, Mangala LS, Merritt WM, Lin YG, Gao C, *et al.*: **Efficacy and antivascular effects of EphA2 reduction with an agonistic antibody in ovarian cancer**. *J Natl Cancer Inst* 2006, **98**:1558-1570.
87. Lu M, Miller KD, Gokmen-Polar Y, Jeng MH, Kinch MS: **EphA2 overexpression decreases estrogen dependence and tamoxifen sensitivity**. *Cancer Res* 2003, **63**:3425-3429.
88. Koolpe M, Dail M, Pasquale EB: **An ephrin mimetic peptide that selectively targets the EphA2 receptor**. *J Biol Chem* 2002, **277**:46974-46979.
89. Kiewlich D, Zhang J, Gross C, Xia W, Larsen B, Cobb RR, Biroc S, Gu JM, Sato T, Light DR, *et al.*: **Anti-EphA2 antibodies decrease EphA2 protein levels in**

murine CT26 colorectal and human MDA-231 breast tumors but do not inhibit tumor growth. *Neoplasia* 2006, **8**:18-30.
90. Landen CN, Jr., Chavez-Reyes A, Bucana C, Schmandt R, Deavers MT, Lopez-Berestein G, Sood AK: **Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery**. *Cancer Res* 2005, **65**:6910-6918.
91. Nasreen N, Mohammed KA, Antony VB: **Silencing the receptor EphA2 suppresses the growth and haptotaxis of malignant mesothelioma cells**. *Cancer* 2006, **107**:2425-2435.
92. Alves PM, Faure O, Graff-Dubois S, Gross DA, Cornet S, Chouaib S, Miconnet I, Lemonnier FA, Kosmatopoulos K: **EphA2 as target of anticancer immunotherapy: identification of HLA-A*0201-restricted epitopes**. *Cancer Res* 2003, **63**:8476-8480.
93. Tatsumi T, Herrem CJ, Olson WC, Finke JH, Bukowski RM, Kinch MS, Ranieri E, Storkus WJ: **Disease stage variation in CD4+ and CD8+ T-cell reactivity to the receptor tyrosine kinase EphA2 in patients with renal cell carcinoma**. *Cancer Res* 2003, **63**:4481-4489.
94. Zhang JG, Eguchi J, Kruse CA, Gomez GG, Fakhrai H, Schroter S, Ma W, Hoa N, Minev B, Delgado C, *et al.*: **Antigenic profiling of glioma cells to generate allogeneic vaccines or dendritic cell-based therapeutics**. *Clin Cancer Res* 2007, **13**:566-575.
95. Hatano M, Eguchi J, Tatsumi T, Kuwashima N, Dusak JE, Kinch MS, Pollack IF, Hamilton RL, Storkus WJ, Okada H: **EphA2 as a glioma-associated antigen: a novel target for glioma vaccines**. *Neoplasia* 2005, **7**:717-722.
96. Hatano M, Kuwashima N, Tatsumi T, Dusak JE, Nishimura F, Reilly KM, Storkus WJ, Okada H: **Vaccination with EphA2-derived T cell-epitopes promotes immunity against both EphA2-expressing and EphA2-negative tumors**. *J Transl Med* 2004, **2**:40.
97. Nakamura R, Kataoka H, Sato N, Kanamori M, Ihara M, Igarashi H, Ravshanov S, Wang YJ, Li ZY, Shimamura T, *et al.*: **EPHA2/EFNA1 expression in human gastric cancer**. *Cancer Sci* 2005, **96**:42-47.
98. Noblitt LW, Bangari DS, Shukla S, Mohammed S, Mittal SK: **Immunocompetent mouse model of breast cancer for preclinical testing of EphA2-targeted therapy**. *Cancer Gene Ther* 2005, **12**:46-53.
99. Noblitt LW, Bangari DS, Shukla S, Knapp DW, Mohammed S, Kinch MS, Mittal SK: **Decreased tumorigenic potential of EphA2-overexpressing breast cancer cells following treatment with adenoviral vectors that express EphrinA1**. *Cancer Gene Ther* 2004, **11**:757-766.
100. Maffei A, Papadopoulos K, Harris PE: **MHC class I antigen processing pathways**. *Hum Immunol* 1997, **54**:91-103.
101. van Endert PM: **Genes regulating MHC class I processing of antigen**. *Curr Opin Immunol* 1999, **11**:82-88.
102. Imai J, Maruya M, Yashiroda H, Yahara I, Tanaka K: **The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome**. *EMBO J* 2003, **22**:3557-3567.

103. Correia MA, Sadeghi S, Mundo-Paredes E: **Cytochrome P450 ubiquitination: branding for the proteolytic slaughter?** *Annu Rev Pharmacol Toxicol* 2005, **45**:439-464.
104. Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I: **Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation**. *Nat Cell Biol* 2003, **5**:461-466.
105. Schrader EK, Harstad KG, Matouschek A: **Targeting proteins for degradation**. *Nat Chem Biol* 2009, **5**:815-822.
106. Babu JR, Geetha T, Wooten MW: **Sequestosome 1/p62 shuttles polyubiquitinated tau for proteasomal degradation**. *J Neurochem* 2005, **94**:192-203.
107. Li W, Ye Y: **Polyubiquitin chains: functions, structures, and mechanisms**. *Cell Mol Life Sci* 2008, **65**:2397-2406.
108. Paulsson KM: **Evolutionary and functional perspectives of the major histocompatibility complex class I antigen-processing machinery**. *Cell Mol Life Sci* 2004, **61**:2446-2460.
109. Cresswell P, Ackerman AL, Giodini A, Peaper DR, Wearsch PA: **Mechanisms of MHC class I-restricted antigen processing and cross-presentation**. *Immunol Rev* 2005, **207**:145-157.
110. Elliott T: **The 'chop-and-change' of MHC class I assembly**. *Nat Immunol* 2006, **7**:7-9.
111. Garbi N, Tanaka S, Momburg F, Hammerling GJ: **Impaired assembly of the major histocompatibility complex class I peptide-loading complex in mice deficient in the oxidoreductase ERp57**. *Nat Immunol* 2006, **7**:93-102.
112. Garbi N, Tanaka S, van den Broek M, Momburg F, Hammerling GJ: **Accessory molecules in the assembly of major histocompatibility complex class I/peptide complexes: how essential are they for CD8(+) T-cell immune responses?** *Immunol Rev* 2005, **207**:77-88.
113. Nakatsukasa K, Huyer G, Michaelis S, Brodsky JL: **Dissecting the ER-associated degradation of a misfolded polytopic membrane protein**. *Cell* 2008, **132**:101-112.
114. Heath WR, Belz GT, Behrens GM, Smith CM, Forehan SP, Parish IA, Davey GM, Wilson NS, Carbone FR, Villadangos JA: **Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens**. *Immunol Rev* 2004, **199**:9-26.
115. Ramirez MC, Sigal LJ: **The multiple routes of MHC-I cross-presentation**. *Trends Microbiol* 2004, **12**:204-207.
116. Shen L, Rock KL: **Cellular protein is the source of cross-priming antigen in vivo**. *Proc Natl Acad Sci U S A* 2004, **101**:3035-3040.
117. Reits EA, Vos JC, Gromme M, Neefjes J: **The major substrates for TAP in vivo are derived from newly synthesized proteins**. *Nature* 2000, **404**:774-778.
118. Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR: **Rapid degradation of a large fraction of newly synthesized proteins by proteasomes**. *Nature* 2000, **404**:770-774.
119. Kuckelkorn U, Ruppert T, Strehl B, Jungblut PR, Zimny-Arndt U, Lamer S, Prinz I, Drung I, Kloetzel PM, Kaufmann SH, *et al.*: **Link between organ-specific antigen**

processing by 20S proteasomes and CD8(+) T cell-mediated autoimmunity. *J Exp Med* 2002, **195**:983-990.
120. Goldberg AL, Cascio P, Saric T, Rock KL: **The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides**. *Mol Immunol* 2002, **39**:147-164.
121. Kloetzel PM: **Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII**. *Nat Immunol* 2004, **5**:661-669.
122. Toes RE, Nussbaum AK, Degermann S, Schirle M, Emmerich NP, Kraft M, Laplace C, Zwinderman A, Dick TP, Muller J, *et al.*: **Discrete cleavage motifs of constitutive and immunoproteasomes revealed by quantitative analysis of cleavage products**. *J Exp Med* 2001, **194**:1-12.
123. Van den Eynde BJ, Morel S: **Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome**. *Curr Opin Immunol* 2001, **13**:147-153.
124. Storkus WJ, Herrem C, Kawabe M, Cohen PA, Bukowski RM, Finke JH, Wesa AK: **Improving immunotherapy by conditionally enhancing MHC class I presentation of tumor antigen-derived Peptide epitopes**. *Crit Rev Immunol* 2007, **27**:485-493.
125. Wiley HS, Burke PM: **Regulation of receptor tyrosine kinase signaling by endocytic trafficking**. *Traffic* 2001, **2**:12-18.
126. Kono K, Sato E, Naganuma H, Takahashi A, Mimura K, Nukui H, Fujii H: **Trastuzumab (Herceptin) enhances class I-restricted antigen presentation recognized by HER-2/neu-specific T cytotoxic lymphocytes**. *Clin Cancer Res* 2004, **10**:2538-2544.
127. Wolpoe ME, Lutz ER, Ercolini AM, Murata S, Ivie SE, Garrett ES, Emens LA, Jaffee EM, Reilly RT: **HER-2/neu-specific monoclonal antibodies collaborate with HER-2/neu-targeted granulocyte macrophage colony-stimulating factor secreting whole cell vaccination to augment CD8+ T cell effector function and tumor-free survival in Her-2/neu-transgenic mice**. *J Immunol* 2003, **171**:2161-2169.
128. zum Buschenfelde CM, Hermann C, Schmidt B, Peschel C, Bernhard H: **Antihuman epidermal growth factor receptor 2 (HER2) monoclonal antibody trastuzumab enhances cytolytic activity of class I-restricted HER2-specific T lymphocytes against HER2-overexpressing tumor cells**. *Cancer Res* 2002, **62**:2244-2247.
129. Munster PN, Marchion DC, Basso AD, Rosen N: **Degradation of HER2 by ansamycins induces growth arrest and apoptosis in cells with HER2 overexpression via a HER3, phosphatidylinositol 3'-kinase-AKT-dependent pathway**. *Cancer Res* 2002, **62**:3132-3137.
130. Solit DB, Basso AD, Olshen AB, Scher HI, Rosen N: **Inhibition of heat shock protein 90 function down-regulates Akt kinase and sensitizes tumors to Taxol**. *Cancer Res* 2003, **63**:2139-2144.
131. Wegele H, Muller L, Buchner J: **Hsp70 and Hsp90--a relay team for protein folding**. *Rev Physiol Biochem Pharmacol* 2004, **151**:1-44.

132. Mosser DD, Morimoto RI: **Molecular chaperones and the stress of oncogenesis**. *Oncogene* 2004, **23**:2907-2918.
133. Yufu Y, Nishimura J, Nawata H: **High constitutive expression of heat shock protein 90 alpha in human acute leukemia cells**. *Leuk Res* 1992, **16**:597-605.
134. Jameel A, Skilton RA, Campbell TA, Chander SK, Coombes RC, Luqmani YA: **Clinical and biological significance of HSP89 alpha in human breast cancer**. *Int J Cancer* 1992, **50**:409-415.
135. Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G: **The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review**. *Pharmacol Ther* 1998, **79**:129-168.
136. Crevel G, Bates H, Huikeshoven H, Cotterill S: **The Drosophila Dpit47 protein is a nuclear Hsp90 co-chaperone that interacts with DNA polymerase alpha**. *J Cell Sci* 2001, **114**:2015-2025.
137. Chen B, Piel WH, Gui L, Bruford E, Monteiro A: **The HSP90 family of genes in the human genome: insights into their divergence and evolution**. *Genomics* 2005, **86**:627-637.
138. Pearl LH, Prodromou C: **Structure and in vivo function of Hsp90**. *Curr Opin Struct Biol* 2000, **10**:46-51.
139. Prodromou C, Pearl LH: **Structure and functional relationships of Hsp90**. *Curr Cancer Drug Targets* 2003, **3**:301-323.
140. Pearl LH, Prodromou C: **Structure, function, and mechanism of the Hsp90 molecular chaperone**. *Adv Protein Chem* 2001, **59**:157-186.
141. Wandinger SK, Richter K, Buchner J: **The Hsp90 chaperone machinery**. *J Biol Chem* 2008, **283**:18473-18477.
142. Buchner J: **Hsp90 & Co. - a holding for folding**. *Trends Biochem Sci* 1999, **24**:136-141.
143. Goetz MP, Toft DO, Ames MM, Erlichman C: **The Hsp90 chaperone complex as a novel target for cancer therapy**. *Ann Oncol* 2003, **14**:1169-1176.
144. Chiosis G, Caldas Lopes E, Solit D: **Heat shock protein-90 inhibitors: a chronicle from geldanamycin to today's agents**. *Curr Opin Investig Drugs* 2006, **7**:534-541.
145. Grenert JP, Sullivan WP, Fadden P, Haystead TA, Clark J, Mimnaugh E, Krutzsch H, Ochel HJ, Schulte TW, Sausville E, *et al.*: **The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation**. *J Biol Chem* 1997, **272**:23843-23850.
146. Xu Z, Horwich AL, Sigler PB: **The crystal structure of the asymmetric GroEL-GroES-(ADP)7 chaperonin complex**. *Nature* 1997, **388**:741-750.
147. Kampranis SC, Bates AD, Maxwell A: **A model for the mechanism of strand passage by DNA gyrase**. *Proc Natl Acad Sci U S A* 1999, **96**:8414-8419.
148. Picard D: **Heat-shock protein 90, a chaperone for folding and regulation**. *Cell Mol Life Sci* 2002, **59**:1640-1648.
149. Kimura Y, Matsumoto S, Yahara I: **Temperature-sensitive mutants of hsp82 of the budding yeast Saccharomyces cerevisiae**. *Mol Gen Genet* 1994, **242**:517-527.
150. Pratt WB, Morishima Y, Murphy M, Harrell M: **Chaperoning of glucocorticoid receptors**. *Handb Exp Pharmacol* 2006:111-138.
151. Grad I, Picard D: **The glucocorticoid responses are shaped by molecular chaperones**. *Mol Cell Endocrinol* 2007, **275**:2-12.

152. Pratt WB, Galigniana MD, Morishima Y, Murphy PJ: **Role of molecular chaperones in steroid receptor action**. *Essays Biochem* 2004, **40**:41-58.
153. Rafestin-Oblin ME, Couette B, Radanyi C, Lombes M, Baulieu EE: **Mineralocorticosteroid receptor of the chick intestine. Oligomeric structure and transformation**. *J Biol Chem* 1989, **264**:9304-9309.
154. Joab I, Radanyi C, Renoir M, Buchou T, Catelli MG, Binart N, Mester J, Baulieu EE: **Common non-hormone binding component in non-transformed chick oviduct receptors of four steroid hormones**. *Nature* 1984, **308**:850-853.
155. Redeuilh G, Moncharmont B, Secco C, Baulieu EE: **Subunit composition of the molybdate-stabilized "8-9 S" nontransformed estradiol receptor purified from calf uterus**. *J Biol Chem* 1987, **262**:6969-6975.
156. Catelli MG, Binart N, Jung-Testas I, Renoir JM, Baulieu EE, Feramisco JR, Welch WJ: **The common 90-kd protein component of non-transformed '8S' steroid receptors is a heat-shock protein**. *EMBO J* 1985, **4**:3131-3135.
157. Yamano T, Mizukami S, Murata S, Chiba T, Tanaka K, Udono H: **Hsp90-mediated assembly of the 26 S proteasome is involved in major histocompatibility complex class I antigen processing**. *J Biol Chem* 2008, **283**:28060-28065.
158. Lurje G, Lenz HJ: **EGFR signaling and drug discovery**. *Oncology* 2009, **77**:400-410.
159. Sawai A, Chandarlapaty S, Greulich H, Gonen M, Ye Q, Arteaga CL, Sellers W, Rosen N, Solit DB: **Inhibition of Hsp90 down-regulates mutant epidermal growth factor receptor (EGFR) expression and sensitizes EGFR mutant tumors to paclitaxel**. *Cancer Res* 2008, **68**:589-596.
160. Mohsin SK, Weiss HL, Gutierrez MC, Chamness GC, Schiff R, Digiovanna MP, Wang CX, Hilsenbeck SG, Osborne CK, Allred DC, *et al.*: **Neoadjuvant trastuzumab induces apoptosis in primary breast cancers**. *J Clin Oncol* 2005, **23**:2460-2468.
161. Calderwood SK, Khaleque MA, Sawyer DB, Ciocca DR: **Heat shock proteins in cancer: chaperones of tumorigenesis**. *Trends Biochem Sci* 2006, **31**:164-172.
162. Eustace BK, Sakurai T, Stewart JK, Yimlamai D, Unger C, Zehetmeier C, Lain B, Torella C, Henning SW, Beste G, *et al.*: **Functional proteomic screens reveal an essential extracellular role for hsp90 alpha in cancer cell invasiveness**. *Nat Cell Biol* 2004, **6**:507-514.
163. Follwell M, Burman D, Le LW, Wakimoto K, Seccareccia D, Bryson J, Rodin G, Zimmermann C: **Phase II study of an outpatient palliative care intervention in patients with metastatic cancer**. *J Clin Oncol* 2009, **27**:206-213.
164. Chiosis G, Vilenchik M, Kim J, Solit D: **Hsp90: the vulnerable chaperone**. *Drug Discov Today* 2004, **9**:881-888.
165. Neckers L, Workman P: **Hsp90 molecular chaperone inhibitors: are we there yet?** *Clin Cancer Res* 2012, **18**:64-76.
166. Kim YS, Alarcon SV, Lee S, Lee MJ, Giaccone G, Neckers L, Trepel JB: **Update on Hsp90 inhibitors in clinical trial**. *Curr Top Med Chem* 2009, **9**:1479-1492.
167. Uehara Y: **Natural product origins of Hsp90 inhibitors**. *Curr Cancer Drug Targets* 2003, **3**:325-330.

168. Mimnaugh EG, Chavany C, Neckers L: **Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin**. *J Biol Chem* 1996, **271**:22796-22801.
169. Schneider C, Sepp-Lorenzino L, Nimmesgern E, Ouerfelli O, Danishefsky S, Rosen N, Hartl FU: **Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90**. *Proc Natl Acad Sci U S A* 1996, **93**:14536-14541.
170. Roe SM, Prodromou C, O'Brien R, Ladbury JE, Piper PW, Pearl LH: **Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin**. *J Med Chem* 1999, **42**:260-266.
171. Workman P, Collins I: **Probing the probes: fitness factors for small molecule tools**. *Chem Biol* 2010, **17**:561-577.
172. Banerji U, O'Donnell A, Scurr M, Pacey S, Stapleton S, Asad Y, Simmons L, Maloney A, Raynaud F, Campbell M, *et al.*: **Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies**. *J Clin Oncol* 2005, **23**:4152-4161.
173. Banerji U, Walton M, Raynaud F, Grimshaw R, Kelland L, Valenti M, Judson I, Workman P: **Pharmacokinetic-pharmacodynamic relationships for the heat shock protein 90 molecular chaperone inhibitor 17-allylamino, 17-demethoxygeldanamycin in human ovarian cancer xenograft models**. *Clin Cancer Res* 2005, **11**:7023-7032.
174. Modi S, Stopeck AT, Gordon MS, Mendelson D, Solit DB, Bagatell R, Ma W, Wheler J, Rosen N, Norton L, *et al.*: **Combination of trastuzumab and tanespimycin (17-AAG, KOS-953) is safe and active in trastuzumab-refractory HER-2 overexpressing breast cancer: a phase I dose-escalation study**. *J Clin Oncol* 2007, **25**:5410-5417.
175. Modi S, Stopeck A, Linden H, Solit D, Chandarlapaty S, Rosen N, D'Andrea G, Dickler M, Moynahan ME, Sugarman S, *et al.*: **HSP90 inhibition is effective in breast cancer: a phase II trial of tanespimycin (17-AAG) plus trastuzumab in patients with HER2-positive metastatic breast cancer progressing on trastuzumab**. *Clin Cancer Res* 2011, **17**:5132-5139.
176. Chavany C, Mimnaugh E, Miller P, Bitton R, Nguyen P, Trepel J, Whitesell L, Schnur R, Moyer J, Neckers L: **p185erbB2 binds to GRP94 in vivo. Dissociation of the p185erbB2/GRP94 heterocomplex by benzoquinone ansamycins precedes depletion of p185erbB2**. *J Biol Chem* 1996, **271**:4974-4977.
177. Solit DB, Zheng FF, Drobnjak M, Munster PN, Higgins B, Verbel D, Heller G, Tong W, Cordon-Cardo C, Agus DB, *et al.*: **17-Allylamino-17-demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts**. *Clin Cancer Res* 2002, **8**:986-993.
178. Kelland LR, Sharp SY, Rogers PM, Myers TG, Workman P: **DT-Diaphorase expression and tumor cell sensitivity to 17-allylamino, 17-demethoxygeldanamycin, an inhibitor of heat shock protein 90**. *J Natl Cancer Inst* 1999, **91**:1940-1949.

179. Basso AD, Solit DB, Munster PN, Rosen N: **Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2**. *Oncogene* 2002, **21**:1159-1166.
180. Burger AM, Fiebig HH, Stinson SF, Sausville EA: **17-(Allylamino)-17-demethoxygeldanamycin activity in human melanoma models**. *Anticancer Drugs* 2004, **15**:377-387.
181. Guo W, Reigan P, Siegel D, Zirrolli J, Gustafson D, Ross D: **Formation of 17-allylamino-demethoxygeldanamycin (17-AAG) hydroquinone by NAD(P)H:quinone oxidoreductase 1: role of 17-AAG hydroquinone in heat shock protein 90 inhibition**. *Cancer Res* 2005, **65**:10006-10015.
182. Smith V, Sausville EA, Camalier RF, Fiebig HH, Burger AM: **Comparison of 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17DMAG) and 17-allylamino-17-demethoxygeldanamycin (17AAG) in vitro: effects on Hsp90 and client proteins in melanoma models**. *Cancer Chemother Pharmacol* 2005, **56**:126-137.
183. Hollingshead M, Alley M, Burger AM, Borgel S, Pacula-Cox C, Fiebig HH, Sausville EA: **In vivo antitumor efficacy of 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin hydrochloride), a water-soluble geldanamycin derivative**. *Cancer Chemother Pharmacol* 2005, **56**:115-125.
184. Kummar S, Gutierrez ME, Gardner ER, Chen X, Figg WD, Zajac-Kaye M, Chen M, Steinberg SM, Muir CA, Yancey MA, *et al.*: **Phase I trial of 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), a heat shock protein inhibitor, administered twice weekly in patients with advanced malignancies**. *Eur J Cancer* 2010, **46**:340-347.
185. Lancet JE, Gojo I, Burton M, Quinn M, Tighe SM, Kersey K, Zhong Z, Albitar MX, Bhalla K, Hannah AL, *et al.*: **Phase I study of the heat shock protein 90 inhibitor alvespimycin (KOS-1022, 17-DMAG) administered intravenously twice weekly to patients with acute myeloid leukemia**. *Leukemia* 2010, **24**:699-705.
186. Pacey S, Wilson RH, Walton M, Eatock MM, Hardcastle A, Zetterlund A, Arkenau HT, Moreno-Farre J, Banerji U, Roels B, *et al.*: **A phase I study of the heat shock protein 90 inhibitor alvespimycin (17-DMAG) given intravenously to patients with advanced solid tumors**. *Clin Cancer Res* 2011, **17**:1561-1570.
187. Ramanathan RK, Egorin MJ, Erlichman C, Remick SC, Ramalingam SS, Naret C, Holleran JL, TenEyck CJ, Ivy SP, Belani CP: **Phase I pharmacokinetic and pharmacodynamic study of 17-dimethylaminoethylamino-17-demethoxygeldanamycin, an inhibitor of heat-shock protein 90, in patients with advanced solid tumors**. *J Clin Oncol* 2010, **28**:1520-1526.
188. Koll TT, Feis SS, Wright MH, Teniola MM, Richardson MM, Robles AI, Bradsher J, Capala J, Varticovski L: **HSP90 inhibitor, DMAG, synergizes with radiation of lung cancer cells by interfering with base excision and ATM-mediated DNA repair**. *Mol Cancer Ther* 2008, **7**:1985-1992.
189. Dote H, Cerna D, Burgan WE, Camphausen K, Tofilon PJ: **ErbB3 expression predicts tumor cell radiosensitization induced by Hsp90 inhibition**. *Cancer Res* 2005, **65**:6967-6975.

190. Huston A, Leleu X, Jia X, Moreau AS, Ngo HT, Runnels J, Anderson J, Alsayed Y, Roccaro A, Vallet S, *et al.*: **Targeting Akt and heat shock protein 90 produces synergistic multiple myeloma cell cytotoxicity in the bone marrow microenvironment**. *Clin Cancer Res* 2008, **14**:865-874.
191. Rao R, Lee P, Fiskus W, Yang Y, Joshi R, Wang Y, Buckley K, Balusu R, Chen J, Koul S, *et al.*: **Co-treatment with heat shock protein 90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin (DMAG) and vorinostat: a highly active combination against human mantle cell lymphoma (MCL) cells**. *Cancer Biol Ther* 2009, **8**:1273-1280.
192. Robles AI, Wright MH, Gandhi B, Feis SS, Hanigan CL, Wiestner A, Varticovski L: **Schedule-dependent synergy between the heat shock protein 90 inhibitor 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin and doxorubicin restores apoptosis to p53-mutant lymphoma cell lines**. *Clin Cancer Res* 2006, **12**:6547-6556.
193. Tatokoro M, Koga F, Yoshida S, Kawakami S, Fujii Y, Neckers L, Kihara K: **Potential role of Hsp90 inhibitors in overcoming cisplatin resistance of bladder cancer-initiating cells**. *Int J Cancer* 2012, **131**:987-996.
194. McCollum AK, Lukasiewicz KB, Teneyck CJ, Lingle WL, Toft DO, Erlichman C: **Cisplatin abrogates the geldanamycin-induced heat shock response**. *Mol Cancer Ther* 2008, **7**:3256-3264.
195. McCollum AK, TenEyck CJ, Stensgard B, Morlan BW, Ballman KV, Jenkins RB, Toft DO, Erlichman C: **P-Glycoprotein-mediated resistance to Hsp90-directed therapy is eclipsed by the heat shock response**. *Cancer Res* 2008, **68**:7419-7427.
196. Eccles SA, Massey A, Raynaud FI, Sharp SY, Box G, Valenti M, Patterson L, de Haven Brandon A, Gowan S, Boxall F, *et al.*: **NVP-AUY922: a novel heat shock protein 90 inhibitor active against xenograft tumor growth, angiogenesis, and metastasis**. *Cancer Res* 2008, **68**:2850-2860.
197. Shelton SN, Shawgo ME, Matthews SB, Lu Y, Donnelly AC, Szabla K, Tanol M, Vielhauer GA, Rajewski RA, Matts RL, *et al.*: **KU135, a novel novobiocin-derived C-terminal inhibitor of the 90-kDa heat shock protein, exerts potent antiproliferative effects in human leukemic cells**. *Mol Pharmacol* 2009, **76**:1314-1322.
198. Powers MV, Jones K, Barillari C, Westwood I, van Montfort RL, Workman P: **Targeting HSP70: the second potentially druggable heat shock protein and molecular chaperone?** *Cell Cycle* 2010, **9**:1542-1550.
199. Powers MV, Clarke PA, Workman P: **Death by chaperone: HSP90, HSP70 or both?** *Cell Cycle* 2009, **8**:518-526.
200. Davenport EL, Zeisig A, Aronson LI, Moore HE, Hockley S, Gonzalez D, Smith EM, Powers MV, Sharp SY, Workman P, *et al.*: **Targeting heat shock protein 72 enhances Hsp90 inhibitor-induced apoptosis in myeloma**. *Leukemia* 2010, **24**:1804-1807.
201. Ryhanen T, Viiri J, Hyttinen JM, Uusitalo H, Salminen A, Kaarniranta K: **Influence of Hsp90 and HDAC inhibition and tubulin acetylation on perinuclear protein aggregation in human retinal pigment epithelial cells**. *J Biomed Biotechnol* 2011, **2011**:798052.

202. Nagai N, Nakai A, Nagata K: **Quercetin suppresses heat shock response by down regulation of HSF1**. *Biochem Biophys Res Commun* 1995, **208**:1099-1105.
203. Lee YJ, Erdos G, Hou ZZ, Kim SH, Kim JH, Cho JM, Corry PM: **Mechanism of quercetin-induced suppression and delay of heat shock gene expression and thermotolerance development in HT-29 cells**. *Mol Cell Biochem* 1994, **137**:141-154.
204. Yokota S, Kitahara M, Nagata K: **Benzylidene lactam compound, KNK437, a novel inhibitor of acquisition of thermotolerance and heat shock protein induction in human colon carcinoma cells**. *Cancer Res* 2000, **60**:2942-2948.
205. Floris G, Debiec-Rychter M, Wozniak A, Stefan C, Normant E, Faa G, Machiels K, Vanleeuw U, Sciot R, Schoffski P: **The heat shock protein 90 inhibitor IPI-504 induces KIT degradation, tumor shrinkage, and cell proliferation arrest in xenograft models of gastrointestinal stromal tumors**. *Mol Cancer Ther* 2011, **10**:1897-1908.
206. Sequist LV, Gettinger S, Senzer NN, Martins RG, Janne PA, Lilenbaum R, Gray JE, Iafrate AJ, Katayama R, Hafeez N, *et al.*: **Activity of IPI-504, a novel heat-shock protein 90 inhibitor, in patients with molecularly defined non-small-cell lung cancer**. *J Clin Oncol* 2010, **28**:4953-4960.
207. Samuni Y, Ishii H, Hyodo F, Samuni U, Krishna MC, Goldstein S, Mitchell JB: **Reactive oxygen species mediate hepatotoxicity induced by the Hsp90 inhibitor geldanamycin and its analogs**. *Free Radic Biol Med* 2010, **48**:1559-1563.
208. Guo W, Reigan P, Siegel D, Ross D: **Enzymatic reduction and glutathione conjugation of benzoquinone ansamycin heat shock protein 90 inhibitors: relevance for toxicity and mechanism of action**. *Drug Metab Dispos* 2008, **36**:2050-2057.
209. Janin YL: **ATPase inhibitors of heat-shock protein 90, second season**. *Drug Discov Today* 2010, **15**:342-353.
210. Biamonte MA, Van de Water R, Arndt JW, Scannevin RH, Perret D, Lee WC: **Heat shock protein 90: inhibitors in clinical trials**. *J Med Chem* 2010, **53**:3-17.
211. Wright L, Barril X, Dymock B, Sheridan L, Surgenor A, Beswick M, Drysdale M, Collier A, Massey A, Davies N, *et al.*: **Structure-activity relationships in purine-based inhibitor binding to HSP90 isoforms**. *Chem Biol* 2004, **11**:775-785.
212. Chiosis G, Timaul MN, Lucas B, Munster PN, Zheng FF, Sepp-Lorenzino L, Rosen N: **A small molecule designed to bind to the adenine nucleotide pocket of Hsp90 causes Her2 degradation and the growth arrest and differentiation of breast cancer cells**. *Chem Biol* 2001, **8**:289-299.
213. Caldas-Lopes E, Cerchietti L, Ahn JH, Clement CC, Robles AI, Rodina A, Moulick K, Taldone T, Gozman A, Guo Y, *et al.*: **Hsp90 inhibitor PU-H71, a multimodal inhibitor of malignancy, induces complete responses in triple-negative breast cancer models**. *Proc Natl Acad Sci U S A* 2009, **106**:8368-8373.
214. Woodhead AJ, Angove H, Carr MG, Chessari G, Congreve M, Coyle JE, Cosme J, Graham B, Day PJ, Downham R, *et al.*: **Discovery of (2,4-dihydroxy-5-isopropylphenyl)-[5-(4-methylpiperazin-1-ylmethyl)-1,3-dihydrois oindol-2-yl]methanone (AT13387), a novel inhibitor of the molecular chaperone Hsp90 by fragment based drug design**. *J Med Chem* 2010, **53**:5956-5969.

215. Messaoudi S, Peyrat JF, Brion JD, Alami M: **Heat-shock protein 90 inhibitors as antitumor agents: a survey of the literature from 2005 to 2010**. *Expert Opin Ther Pat* 2011, **21**:1501-1542.
216. Lyman SK, Crawley SC, Gong R, Adamkewicz JI, McGrath G, Chew JY, Choi J, Holst CR, Goon LH, Detmer SA, *et al.*: **High-content, high-throughput analysis of cell cycle perturbations induced by the HSP90 inhibitor XL888**. *PLoS One* 2011, **6**:e17692.
217. Fadden P, Huang KH, Veal JM, Steed PM, Barabasz AF, Foley B, Hu M, Partridge JM, Rice J, Scott A, *et al.*: **Application of chemoproteomics to drug discovery: identification of a clinical candidate targeting hsp90**. *Chem Biol* 2010, **17**:686-694.
218. Brough PA, Barril X, Borgognoni J, Chene P, Davies NG, Davis B, Drysdale MJ, Dymock B, Eccles SA, Garcia-Echeverria C, *et al.*: **Combining hit identification strategies: fragment-based and in silico approaches to orally active 2-aminothieno[2,3-d]pyrimidine inhibitors of the Hsp90 molecular chaperone**. *J Med Chem* 2009, **52**:4794-4809.
219. Bao R, Lai CJ, Qu H, Wang D, Yin L, Zifcak B, Atoyan R, Wang J, Samson M, Forrester J, *et al.*: **CUDC-305, a novel synthetic HSP90 inhibitor with unique pharmacologic properties for cancer therapy**. *Clin Cancer Res* 2009, **15**:4046-4057.
220. McCleese JK, Bear MD, Fossey SL, Mihalek RM, Foley KP, Ying W, Barsoum J, London CA: **The novel HSP90 inhibitor STA-1474 exhibits biologic activity against osteosarcoma cell lines**. *Int J Cancer* 2009, **125**:2792-2801.
221. Cleary JM, Sadow PM, Randolph GW, Palmer EL, Lynch TP, Nikiforov YE, Wirth LJ: **Neoadjuvant treatment of unresectable medullary thyroid cancer with sunitinib**. *J Clin Oncol* 2010, **28**:e390-392.
222. Haferlach T, Kohlmann A, Wieczorek L, Basso G, Kronnie GT, Bene MC, De Vos J, Hernandez JM, Hofmann WK, Mills KI, *et al.*: **Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group**. *J Clin Oncol* 2010, **28**:2529-2537.
223. Marcu MG, Chadli A, Bouhouche I, Catelli M, Neckers LM: **The heat shock protein 90 antagonist novobiocin interacts with a previously unrecognized ATP-binding domain in the carboxyl terminus of the chaperone**. *J Biol Chem* 2000, **275**:37181-37186.
224. Marcu MG, Schulte TW, Neckers L: **Novobiocin and related coumarins and depletion of heat shock protein 90-dependent signaling proteins**. *J Natl Cancer Inst* 2000, **92**:242-248.
225. Zhao H, Donnelly AC, Kusuma BR, Brandt GE, Brown D, Rajewski RA, Vielhauer G, Holzbeierlein J, Cohen MS, Blagg BS: **Engineering an antibiotic to fight cancer: optimization of the novobiocin scaffold to produce anti-proliferative agents**. *J Med Chem* 2011, **54**:3839-3853.
226. Donnelly A, Blagg BS: **Novobiocin and additional inhibitors of the Hsp90 C-terminal nucleotide-binding pocket**. *Curr Med Chem* 2008, **15**:2702-2717.
227. Conde R, Belak ZR, Nair M, O'Carroll RF, Ovsenek N: **Modulation of Hsf1 activity by novobiocin and geldanamycin**. *Biochem Cell Biol* 2009, **87**:845-851.

228. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD: **IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity**. *Nature* 2001, **410**:1107-1111.
229. Dunn GP, Koebel CM, Schreiber RD: **Interferons, immunity and cancer immunoediting**. *Nat Rev Immunol* 2006, **6**:836-848.
230. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD: **Cancer immunoediting: from immunosurveillance to tumor escape**. *Nat Immunol* 2002, **3**:991-998.
231. Dunn GP, Old LJ, Schreiber RD: **The three Es of cancer immunoediting**. *Annu Rev Immunol* 2004, **22**:329-360.
232. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD: **Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice**. *Proc Natl Acad Sci U S A* 1998, **95**:7556-7561.
233. Dighe AS, Richards E, Old LJ, Schreiber RD: **Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors**. *Immunity* 1994, **1**:447-456.
234. Odunsi K, Old LJ: **Tumor infiltrating lymphocytes: indicators of tumor-related immune responses**. *Cancer Immun* 2007, **7**:3.
235. Mlecnik B, Bindea G, Pages F, Galon J: **Tumor immunosurveillance in human cancers**. *Cancer Metastasis Rev* 2011, **30**:5-12.
236. Zou W: **Immunosuppressive networks in the tumour environment and their therapeutic relevance**. *Nat Rev Cancer* 2005, **5**:263-274.
237. Munn DH, Mellor AL: **Indoleamine 2,3-dioxygenase and tumor-induced tolerance**. *J Clin Invest* 2007, **117**:1147-1154.
238. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, Niu G, Kay H, Mule J, Kerr WG, *et al.*: **Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity**. *Nat Med* 2005, **11**:1314-1321.
239. Smyth MJ, Cretney E, Kershaw MH, Hayakawa Y: **Cytokines in cancer immunity and immunotherapy**. *Immunol Rev* 2004, **202**:275-293.
240. Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MR, Yagita H: **Nature's TRAIL--on a path to cancer immunotherapy**. *Immunity* 2003, **18**:1-6.
241. Seliger B, Maeurer MJ, Ferrone S: **Antigen-processing machinery breakdown and tumor growth**. *Immunol Today* 2000, **21**:455-464.
242. Slingluff CL, Jr., Speiser DE: **Progress and controversies in developing cancer vaccines**. *J Transl Med* 2005, **3**:18.
243. Norell H, Carlsten M, Ohlum T, Malmberg KJ, Masucci G, Schedvins K, Altermann W, Handke D, Atkins D, Seliger B, *et al.*: **Frequent loss of HLA-A2 expression in metastasizing ovarian carcinomas associated with genomic haplotype loss and HLA-A2-restricted HER-2/neu-specific immunity**. *Cancer Res* 2006, **66**:6387-6394.
244. Kaiser BK, Yim D, Chow IT, Gonzalez S, Dai Z, Mann HH, Strong RK, Groh V, Spies T: **Disulphide-isomerase-enabled shedding of tumour-associated NKG2D ligands**. *Nature* 2007, **447**:482-486.
245. O'Connell J, Bennett MW, Nally K, Houston A, O'Sullivan GC, Shanahan F: **Altered mechanisms of apoptosis in colon cancer: Fas resistance and**

counterattack in the tumor-immune conflict. *Ann N Y Acad Sci* 2000, **910**:178-192; discussion 193-175.
246. Thompson RH, Dong H, Kwon ED: **Implications of B7-H1 expression in clear cell carcinoma of the kidney for prognostication and therapy**. *Clin Cancer Res* 2007, **13**:709s-715s.
247. Graner MW, Cumming RI, Bigner DD: **The heat shock response and chaperones/heat shock proteins in brain tumors: surface expression, release, and possible immune consequences**. *J Neurosci* 2007, **27**:11214-11227.
248. Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, Flament C, Pouzieux S, Faure F, Tursz T, *et al.*: **Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming**. *Nat Med* 2001, **7**:297-303.
249. Chalmin F, Ladoire S, Mignot G, Vincent J, Bruchard M, Remy-Martin JP, Boireau W, Rouleau A, Simon B, Lanneau D, *et al.*: **Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells**. *J Clin Invest* 2010, **120**:457-471.
250. Graner MW, Alzate O, Dechkovskaia AM, Keene JD, Sampson JH, Mitchell DA, Bigner DD: **Proteomic and immunologic analyses of brain tumor exosomes**. *FASEB J* 2009, **23**:1541-1557.
251. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO: **Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells**. *Nat Cell Biol* 2007, **9**:654-659.
252. Kim SH, Bianco N, Menon R, Lechman ER, Shufesky WJ, Morelli AE, Robbins PD: **Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive**. *Mol Ther* 2006, **13**:289-300.
253. Abusamra AJ, Zhong Z, Zheng X, Li M, Ichim TE, Chin JL, Min WP: **Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis**. *Blood Cells Mol Dis* 2005, **35**:169-173.
254. Kapsogeorgou EK, Abu-Helu RF, Moutsopoulos HM, Manoussakis MN: **Salivary gland epithelial cell exosomes: A source of autoantigenic ribonucleoproteins**. *Arthritis Rheum* 2005, **52**:1517-1521.
255. Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S, Whiteside TL: **Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes**. *Clin Cancer Res* 2005, **11**:1010-1020.
256. Taylor DD, Gercel-Taylor C: **Tumour-derived exosomes and their role in cancer-associated T-cell signalling defects**. *Br J Cancer* 2005, **92**:305-311.
257. Sabapatha A, Gercel-Taylor C, Taylor DD: **Specific isolation of placenta-derived exosomes from the circulation of pregnant women and their immunoregulatory consequences**. *Am J Reprod Immunol* 2006, **56**:345-355.
258. Clayton A, Mitchell JP, Court J, Linnane S, Mason MD, Tabi Z: **Human tumor-derived exosomes down-modulate NKG2D expression**. *J Immunol* 2008, **180**:7249-7258.
259. Valenti R, Huber V, Filipazzi P, Pilla L, Sovena G, Villa A, Corbelli A, Fais S, Parmiani G, Rivoltini L: **Human tumor-released microvesicles promote the**

differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res* 2006, **66**:9290-9298.
260. Andre F, Schartz NE, Movassagh M, Flament C, Pautier P, Morice P, Pomel C, Lhomme C, Escudier B, Le Chevalier T, *et al.*: **Malignant effusions and immunogenic tumour-derived exosomes**. *Lancet* 2002, **360**:295-305.
261. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, Ricciardi-Castagnoli P, Raposo G, Amigorena S: **Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes**. *Nat Med* 1998, **4**:594-600.
262. Yu S, Liu C, Su K, Wang J, Liu Y, Zhang L, Li C, Cong Y, Kimberly R, Grizzle WE, *et al.*: **Tumor exosomes inhibit differentiation of bone marrow dendritic cells**. *J Immunol* 2007, **178**:6867-6875.
263. Liu C, Yu S, Kappes J, Wang J, Grizzle WE, Zinn KR, Zhang HG: **Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host**. *Blood* 2007, **109**:4336-4342.
264. Shimizu J, Yamazaki S, Sakaguchi S: **Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity**. *J Immunol* 1999, **163**:5211-5218.
265. Bursuker I, North RJ: **Generation and decay of the immune response to a progressive fibrosarcoma. II. Failure to demonstrate postexcision immunity after the onset of T cell-mediated suppression of immunity**. *J Exp Med* 1984, **159**:1312-1321.
266. Steitz J, Bruck J, Lenz J, Knop J, Tuting T: **Depletion of CD25(+) CD4(+) T cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon alpha-induced, CD8(+) T-cell-dependent immune defense of B16 melanoma**. *Cancer Res* 2001, **61**:8643-8646.
267. Sutmuller RP, van Duivenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, Allison JP, Toes RE, Offringa R, Melief CJ: **Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses**. *J Exp Med* 2001, **194**:823-832.
268. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, White DE, Wunderlich JR, Canevari S, Rogers-Freezer L, *et al.*: **A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer**. *Clin Cancer Res* 2006, **12**:6106-6115.
269. Chen ML, Pittet MJ, Gorelik L, Flavell RA, Weissleder R, von Boehmer H, Khazaie K: **Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo**. *Proc Natl Acad Sci U S A* 2005, **102**:419-424.
270. Levings MK, Sangregorio R, Roncarolo MG: **Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function**. *J Exp Med* 2001, **193**:1295-1302.
271. Wang RF, Peng G, Wang HY: **Regulatory T cells and Toll-like receptors in tumor immunity**. *Semin Immunol* 2006, **18**:136-142.
272. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, *et al.*: **Specific recruitment of regulatory**

T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004, **10**:942-949.
273. Sasada T, Kimura M, Yoshida Y, Kanai M, Takabayashi A: **CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression**. *Cancer* 2003, **98**:1089-1099.
274. Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B: **Increase of regulatory T cells in the peripheral blood of cancer patients**. *Clin Cancer Res* 2003, **9**:606-612.
275. Javia LR, Rosenberg SA: **CD4+CD25+ suppressor lymphocytes in the circulation of patients immunized against melanoma antigens**. *J Immunother* 2003, **26**:85-93.
276. Somasundaram R, Jacob L, Swoboda R, Caputo L, Song H, Basak S, Monos D, Peritt D, Marincola F, Cai D, *et al.*: **Inhibition of cytolytic T lymphocyte proliferation by autologous CD4+/CD25+ regulatory T cells in a colorectal carcinoma patient is mediated by transforming growth factor-beta**. *Cancer Res* 2002, **62**:5267-5272.
277. Woo EY, Yeh H, Chu CS, Schlienger K, Carroll RG, Riley JL, Kaiser LR, June CH: **Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation**. *J Immunol* 2002, **168**:4272-4276.
278. Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR, June CH: **Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer**. *Cancer Res* 2001, **61**:4766-4772.
279. Szajnik M, Czystowska M, Szczepanski MJ, Mandapathil M, Whiteside TL: **Tumor-derived microvesicles induce, expand and up-regulate biological activities of human regulatory T cells (Treg)**. *PLoS One* 2010, **5**:e11469.
280. Wang GJ, Liu Y, Qin A, Shah SV, Deng ZB, Xiang X, Cheng Z, Liu C, Wang J, Zhang L, *et al.*: **Thymus exosomes-like particles induce regulatory T cells**. *J Immunol* 2008, **181**:5242-5248.
281. Ichim TE, Zhong Z, Kaushal S, Zheng X, Ren X, Hao X, Joyce JA, Hanley HH, Riordan NH, Koropatnick J, *et al.*: **Exosomes as a tumor immune escape mechanism: possible therapeutic implications**. *J Transl Med* 2008, **6**:37.
282. Bluestone JA, Abbas AK: **Natural versus adaptive regulatory T cells**. *Nat Rev Immunol* 2003, **3**:253-257.
283. Larmonier N, Marron M, Zeng Y, Cantrell J, Romanoski A, Sepassi M, Thompson S, Chen X, Andreansky S, Katsanis E: **Tumor-derived CD4(+)CD25(+) regulatory T cell suppression of dendritic cell function involves TGF-beta and IL-10**. *Cancer Immunol Immunother* 2007, **56**:48-59.
284. Curiel TJ, Wei S, Dong H, Alvarez X, Cheng P, Mottram P, Krzysiek R, Knutson KL, Daniel B, Zimmermann MC, *et al.*: **Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity**. *Nat Med* 2003, **9**:562-567.
285. Zou W: **Regulatory T cells, tumour immunity and immunotherapy**. *Nat Rev Immunol* 2006, **6**:295-307.

286. Wang HY, Lee DA, Peng G, Guo Z, Li Y, Kiniwa Y, Shevach EM, Wang RF: **Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy**. *Immunity* 2004, **20**:107-118.
287. Lim HW, Hillsamer P, Banham AH, Kim CH: **Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells**. *J Immunol* 2005, **175**:4180-4183.
288. Gabrilovich DI, Nagaraj S: **Myeloid-derived suppressor cells as regulators of the immune system**. *Nat Rev Immunol* 2009, **9**:162-174.
289. Bronte V, Apolloni E, Cabrelle A, Ronca R, Serafini P, Zamboni P, Restifo NP, Zanovello P: **Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells**. *Blood* 2000, **96**:3838-3846.
290. Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ: **Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy**. *Cancer Immunol Immunother* 2009, **58**:49-59.
291. Ochoa AC, Zea AH, Hernandez C, Rodriguez PC: **Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma**. *Clin Cancer Res* 2007, **13**:721s-726s.
292. Mirza N, Fishman M, Fricke I, Dunn M, Neuger AM, Frost TJ, Lush RM, Antonia S, Gabrilovich DI: **All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients**. *Cancer Res* 2006, **66**:9299-9307.
293. Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP, Gabrilovich DI: **Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer**. *J Immunol* 2001, **166**:678-689.
294. Liu Y, Xiang X, Zhuang X, Zhang S, Liu C, Cheng Z, Michalek S, Grizzle W, Zhang HG: **Contribution of MyD88 to the tumor exosome-mediated induction of myeloid derived suppressor cells**. *Am J Pathol* 2010, **176**:2490-2499.
295. Xiang X, Poliakov A, Liu C, Liu Y, Deng ZB, Wang J, Cheng Z, Shah SV, Wang GJ, Zhang L, *et al.*: **Induction of myeloid-derived suppressor cells by tumor exosomes**. *Int J Cancer* 2009, **124**:2621-2633.
296. Rosenberg SA, Yang JC, Restifo NP: **Cancer immunotherapy: moving beyond current vaccines**. *Nat Med* 2004, **10**:909-915.
297. Waldmann TA: **Immunotherapy: past, present and future**. *Nat Med* 2003, **9**:269-277.
298. Florescu A, Amir E, Bouganim N, Clemons M: **Immune therapy for breast cancer in 2010-hype or hope?** *Curr Oncol* 2011, **18**:e9-e18.
299. Gonzalez-Angulo AM, Litton JK, Broglio KR, Meric-Bernstam F, Rakkhit R, Cardoso F, Peintinger F, Hanrahan EO, Sahin A, Guray M, *et al.*: **High risk of recurrence for patients with breast cancer who have human epidermal growth factor receptor 2-positive, node-negative tumors 1 cm or smaller**. *J Clin Oncol* 2009, **27**:5700-5706.
300. Keating MJ, Cazin B, Coutre S, Birhiray R, Kovacsovics T, Langer W, Leber B, Maughan T, Rai K, Tjonnfjord G, *et al.*: **Campath-1H treatment of T-cell prolymphocytic leukemia in patients for whom at least one prior chemotherapy regimen has failed**. *J Clin Oncol* 2002, **20**:205-213.

301. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, *et al.*: **Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer**. *N Engl J Med* 2004, **350**:2335-2342.
302. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, Anderson DR: **Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20**. *Blood* 1994, **83**:435-445.
303. Jones FE, Stern DF: **Expression of dominant-negative ErbB2 in the mammary gland of transgenic mice reveals a role in lobuloalveolar development and lactation**. *Oncogene* 1999, **18**:3481-3490.
304. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, *et al.*: **Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer**. *Science* 1989, **244**:707-712.
305. Austin CD, Wen X, Gazzard L, Nelson C, Scheller RH, Scales SJ: **Oxidizing potential of endosomes and lysosomes limits intracellular cleavage of disulfide-based antibody-drug conjugates**. *Proc Natl Acad Sci U S A* 2005, **102**:17987-17992.
306. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME: **Adoptive cell transfer: a clinical path to effective cancer immunotherapy**. *Nat Rev Cancer* 2008, **8**:299-308.
307. Motohashi S, Nakayama T: **Natural killer T cell-mediated immunotherapy for malignant diseases**. *Front Biosci (Schol Ed)* 2009, **1**:108-116.
308. Khattar M, Chen W, Stepkowski SM: **Expanding and converting regulatory T cells: a horizon for immunotherapy**. *Arch Immunol Ther Exp (Warsz)* 2009, **57**:199-204.
309. Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson EM, Lotze MT, Yang JC, Topalian SL, *et al.*: **Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction**. *N Engl J Med* 1990, **323**:570-578.
310. Gattinoni L, Finkelstein SE, Klebanoff CA, Antony PA, Palmer DC, Spiess PJ, Hwang LN, Yu Z, Wrzesinski C, Heimann DM, *et al.*: **Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells**. *J Exp Med* 2005, **202**:907-912.
311. Antony PA, Piccirillo CA, Akpinarli A, Finkelstein SE, Speiss PJ, Surman DR, Palmer DC, Chan CC, Klebanoff CA, Overwijk WW, *et al.*: **CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells**. *J Immunol* 2005, **174**:2591-2601.
312. Dummer W, Niethammer AG, Baccala R, Lawson BR, Wagner N, Reisfeld RA, Theofilopoulos AN: **T cell homeostatic proliferation elicits effective antitumor autoimmunity**. *J Clin Invest* 2002, **110**:185-192.
313. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, *et al.*: **Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes**. *Science* 2002, **298**:850-854.

314. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, *et al.*: **Cancer regression in patients after transfer of genetically engineered lymphocytes**. *Science* 2006, **314**:126-129.
315. Dreno B, Nguyen JM, Khammari A, Pandolfino MC, Tessier MH, Bercegeay S, Cassidanius A, Lemarre P, Billaudel S, Labarriere N, *et al.*: **Randomized trial of adoptive transfer of melanoma tumor-infiltrating lymphocytes as adjuvant therapy for stage III melanoma**. *Cancer Immunol Immunother* 2002, **51**:539-546.
316. Kono K, Takahashi A, Ichihara F, Amemiya H, Iizuka H, Fujii H, Sekikawa T, Matsumoto Y: **Prognostic significance of adoptive immunotherapy with tumor-associated lymphocytes in patients with advanced gastric cancer: a randomized trial**. *Clin Cancer Res* 2002, **8**:1767-1771.
317. Figlin RA, Thompson JA, Bukowski RM, Vogelzang NJ, Novick AC, Lange P, Steinberg GD, Belldegrun AS: **Multicenter, randomized, phase III trial of CD8(+) tumor-infiltrating lymphocytes in combination with recombinant interleukin-2 in metastatic renal cell carcinoma**. *J Clin Oncol* 1999, **17**:2521-2529.
318. Rosenberg SA, Yannelli JR, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, Parkinson DR, Seipp CA, Einhorn JH, White DE: **Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2**. *J Natl Cancer Inst* 1994, **86**:1159-1166.
319. Alexander RB, Rosenberg SA: **Long-term survival of adoptively transferred tumor-infiltrating lymphocytes in mice**. *J Immunol* 1990, **145**:1615-1620.
320. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, Royal RE, Kammula U, White DE, Mavroukakis SA, *et al.*: **Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma**. *J Clin Oncol* 2005, **23**:2346-2357.
321. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, *et al.*: **Effector memory T cells, early metastasis, and survival in colorectal cancer**. *N Engl J Med* 2005, **353**:2654-2666.
322. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C, *et al.*: **Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer**. *Proc Natl Acad Sci U S A* 2005, **102**:18538-18543.
323. Wrzesinski C, Paulos CM, Gattinoni L, Palmer DC, Kaiser A, Yu Z, Rosenberg SA, Restifo NP: **Hematopoietic stem cells promote the expansion and function of adoptively transferred antitumor CD8 T cells**. *J Clin Invest* 2007, **117**:492-501.
324. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, *et al.*: **Type, density, and location of immune cells within human colorectal tumors predict clinical outcome**. *Science* 2006, **313**:1960-1964.
325. Eshhar Z, Waks T, Bendavid A, Schindler DG: **Functional expression of chimeric receptor genes in human T cells**. *J Immunol Methods* 2001, **248**:67-76.

326. Park JR, Digiusto DL, Slovak M, Wright C, Naranjo A, Wagner J, Meechoovet HB, Bautista C, Chang WC, Ostberg JR, *et al.*: **Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma**. *Mol Ther* 2007, **15**:825-833.
327. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, Gratama JW, Stoter G, Oosterwijk E: **Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience**. *J Clin Oncol* 2006, **24**:e20-22.
328. Sadelain M, Riviere I, Brentjens R: **Targeting tumours with genetically enhanced T lymphocytes**. *Nat Rev Cancer* 2003, **3**:35-45.
329. Schumacher TN: **T-cell-receptor gene therapy**. *Nat Rev Immunol* 2002, **2**:512-519.
330. June CH: **Adoptive T cell therapy for cancer in the clinic**. *J Clin Invest* 2007, **117**:1466-1476.
331. Ellis J: **Silencing and variegation of gammaretrovirus and lentivirus vectors**. *Hum Gene Ther* 2005, **16**:1241-1246.
332. Levine BL, Humeau LM, Boyer J, MacGregor RR, Rebello T, Lu X, Binder GK, Slepushkin V, Lemiale F, Mascola JR, *et al.*: **Gene transfer in humans using a conditionally replicating lentiviral vector**. *Proc Natl Acad Sci U S A* 2006, **103**:17372-17377.
333. Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, Sergi Sergi L, Benedicenti F, Ambrosi A, Di Serio C, *et al.*: **Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration**. *Nat Biotechnol* 2006, **24**:687-696.
334. Mauri D, Wyss-Coray T, Gallati H, Pichler WJ: **Antigen-presenting T cells induce the development of cytotoxic CD4+ T cells. I. Involvement of the CD80-CD28 adhesion molecules**. *J Immunol* 1995, **155**:118-127.
335. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K: **Immunobiology of dendritic cells**. *Annu Rev Immunol* 2000, **18**:767-811.
336. Banchereau J, Steinman RM: **Dendritic cells and the control of immunity**. *Nature* 1998, **392**:245-252.
337. Reis e Sousa C: **Dendritic cells as sensors of infection**. *Immunity* 2001, **14**:495-498.
338. Gilboa E: **DC-based cancer vaccines**. *J Clin Invest* 2007, **117**:1195-1203.
339. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM: **Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor**. *J Exp Med* 1992, **176**:1693-1702.
340. Banchereau J, Palucka AK: **Dendritic cells as therapeutic vaccines against cancer**. *Nat Rev Immunol* 2005, **5**:296-306.
341. Nestle FO, Farkas A, Conrad C: **Dendritic-cell-based therapeutic vaccination against cancer**. *Curr Opin Immunol* 2005, **17**:163-169.
342. Schuler G, Schuler-Thurner B, Steinman RM: **The use of dendritic cells in cancer immunotherapy**. *Curr Opin Immunol* 2003, **15**:138-147.
343. Akira S, Uematsu S, Takeuchi O: **Pathogen recognition and innate immunity**. *Cell* 2006, **124**:783-801.

344. Randolph GJ, Angeli V, Swartz MA: **Dendritic-cell trafficking to lymph nodes through lymphatic vessels**. *Nat Rev Immunol* 2005, **5**:617-628.
345. Adema GJ, de Vries IJ, Punt CJ, Figdor CG: **Migration of dendritic cell based cancer vaccines: in vivo veritas?** *Curr Opin Immunol* 2005, **17**:170-174.
346. Wan T, Zhou X, Chen G, An H, Chen T, Zhang W, Liu S, Jiang Y, Yang F, Wu Y, *et al.*: **Novel heat shock protein Hsp70L1 activates dendritic cells and acts as a Th1 polarizing adjuvant**. *Blood* 2004, **103**:1747-1754.
347. Fang H, Wu Y, Huang X, Wang W, Ang B, Cao X, Wan T: **Toll-like receptor 4 (TLR4) is essential for Hsp70-like protein 1 (HSP70L1) to activate dendritic cells and induce Th1 response**. *J Biol Chem* 2011, **286**:30393-30400.
348. Cranmer LD, Trevor KT, Hersh EM: **Clinical applications of dendritic cell vaccination in the treatment of cancer**. *Cancer Immunol Immunother* 2004, **53**:275-306.
349. Lesterhuis WJ, de Vries IJ, Adema GJ, Punt CJ: **Dendritic cell-based vaccines in cancer immunotherapy: an update on clinical and immunological results**. *Ann Oncol* 2004, **15 Suppl 4**:iv145-151.
350. Schadendorf D, Ugurel S, Schuler-Thurner B, Nestle FO, Enk A, Brocker EB, Grabbe S, Rittgen W, Edler L, Sucker A, *et al.*: **Dacarbazine (DTIC) versus vaccination with autologous peptide-pulsed dendritic cells (DC) in first-line treatment of patients with metastatic melanoma: a randomized phase III trial of the DC study group of the DeCOG**. *Ann Oncol* 2006, **17**:563-570.
351. Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, Zhang A, Dahm P, Chao N, Gilboa E, *et al.*: **Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells**. *J Clin Invest* 2005, **115**:3623-3633.
352. Su Z, Dannull J, Yang BK, Dahm P, Coleman D, Yancey D, Sichi S, Niedzwiecki D, Boczkowski D, Gilboa E, *et al.*: **Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8+ and CD4+ T cell responses in patients with metastatic prostate cancer**. *J Immunol* 2005, **174**:3798-3807.
353. Su Z, Dannull J, Heiser A, Yancey D, Pruitt S, Madden J, Coleman D, Niedzwiecki D, Gilboa E, Vieweg J: **Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells**. *Cancer Res* 2003, **63**:2127-2133.
354. Heiser A, Coleman D, Dannull J, Yancey D, Maurice MA, Lallas CD, Dahm P, Niedzwiecki D, Gilboa E, Vieweg J: **Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors**. *J Clin Invest* 2002, **109**:409-417.
355. Gaines KK: **Sipuleucel-T (Provenge): immunotherapy for metastatic prostate cancer**. *Urol Nurs* 2012, **32**:95-98.
356. Zwick E, Bange J, Ullrich A: **Receptor tyrosine kinase signalling as a target for cancer intervention strategies**. *Endocr Relat Cancer* 2001, **8**:161-173.
357. Duus J, Bahar HI, Venkataraman G, Ozpuyan F, Izban KF, Al-Masri H, Maududi T, Toor A, Alkan S: **Analysis of expression of heat shock protein-90 (HSP90) and the effects of HSP90 inhibitor (17-AAG) in multiple myeloma**. *Leuk Lymphoma* 2006, **47**:1369-1378.

358. Castro JE, Prada CE, Loria O, Kamal A, Chen L, Burrows FJ, Kipps TJ: **ZAP-70 is a novel conditional heat shock protein 90 (Hsp90) client: inhibition of Hsp90 leads to ZAP-70 degradation, apoptosis, and impaired signaling in chronic lymphocytic leukemia**. *Blood* 2005, **106**:2506-2512.
359. Imai T, Kato Y, Kajiwara C, Mizukami S, Ishige I, Ichiyanagi T, Hikida M, Wang JY, Udono H: **Heat shock protein 90 (HSP90) contributes to cytosolic translocation of extracellular antigen for cross-presentation by dendritic cells**. *Proc Natl Acad Sci U S A* 2011, **108**:16363-16368.
360. Oura J, Tamura Y, Kamiguchi K, Kutomi G, Sahara H, Torigoe T, Himi T, Sato N: **Extracellular heat shock protein 90 plays a role in translocating chaperoned antigen from endosome to proteasome for generating antigenic peptide to be cross-presented by dendritic cells**. *Int Immunol* 2011, **23**:223-237.
361. Bae J, Mitsiades C, Tai YT, Bertheau R, Shammas M, Batchu RB, Li C, Catley L, Prabhala R, Anderson KC, *et al.*: **Phenotypic and functional effects of heat shock protein 90 inhibition on dendritic cell**. *J Immunol* 2007, **178**:7730-7737.
362. Shimp SK, 3rd, Chafin CB, Regna NL, Hammond SE, Read MA, Caudell DL, Rylander M, Reilly CM: **Heat shock protein 90 inhibition by 17-DMAG lessens disease in the MRL/lpr mouse model of systemic lupus erythematosus**. *Cell Mol Immunol* 2012, **9**:255-266.
363. Bache KG, Slagsvold T, Stenmark H: **Defective downregulation of receptor tyrosine kinases in cancer**. *EMBO J* 2004, **23**:2707-2712.
364. Kinch MS, Moore MB, Harpole DH, Jr.: **Predictive value of the EphA2 receptor tyrosine kinase in lung cancer recurrence and survival**. *Clin Cancer Res* 2003, **9**:613-618.
365. Ansuini H, Meola A, Gunes Z, Paradisi V, Pezzanera M, Acali S, Santini C, Luzzago A, Mori F, Lazzaro D, *et al.*: **Anti-EphA2 Antibodies with Distinct In vitro Properties Have Equal In vivo Efficacy in Pancreatic Cancer**. *J Oncol* 2009, **2009**:951917.
366. Yamaguchi S, Tatsumi T, Takehara T, Sakamori R, Uemura A, Mizushima T, Ohkawa K, Storkus WJ, Hayashi N: **Immunotherapy of murine colon cancer using receptor tyrosine kinase EphA2-derived peptide-pulsed dendritic cell vaccines**. *Cancer* 2007, **110**:1469-1477.
367. Pardee AD, McCurry D, Alber S, Hu P, Epstein AL, Storkus WJ: **A therapeutic OX40 agonist dynamically alters dendritic, endothelial, and T cell subsets within the established tumor microenvironment**. *Cancer Res* 2010, **70**:9041-9052.
368. Komita H, Zhao X, Katakam AK, Kumar P, Kawabe M, Okada H, Braughler JM, Storkus WJ: **Conditional interleukin-12 gene therapy promotes safe and effective antitumor immunity**. *Cancer Gene Ther* 2009, **16**:883-891.
369. Zhao X, Bose A, Komita H, Taylor JL, Kawabe M, Chi N, Spokas L, Lowe DB, Goldbach C, Alber S, *et al.*: **Intratumoral IL-12 gene therapy results in the crosspriming of Tc1 cells reactive against tumor-associated stromal antigens**. *Mol Ther* 2011, **19**:805-814.
370. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, *et al.*: **Sipuleucel-T immunotherapy for castration-resistant prostate cancer**. *N Engl J Med* 2010, **363**:411-422.

371. Kiang JG, Smith JT, Agravante NG: **Geldanamycin analog 17-DMAG inhibits iNOS and caspases in gamma-irradiated human T cells**. *Radiat Res* 2009, **172**:321-330.
372. Gartner EM, Silverman P, Simon M, Flaherty L, Abrams J, Ivy P, Lorusso PM: **A phase II study of 17-allylamino-17-demethoxygeldanamycin in metastatic or locally advanced, unresectable breast cancer**. *Breast Cancer Res Treat* 2012, **131**:933-937.
373. Lu X, Xiao L, Wang L, Ruden DM: **Hsp90 inhibitors and drug resistance in cancer: The potential benefits of combination therapies of Hsp90 inhibitors and other anti-cancer drugs**. *Biochem Pharmacol* 2011.
374. Weigel BJ, Blaney SM, Reid JM, Safgren SL, Bagatell R, Kersey J, Neglia JP, Ivy SP, Ingle AM, Whitesell L, *et al.*: **A phase I study of 17-allylaminogeldanamycin in relapsed/refractory pediatric patients with solid tumors: a Children's Oncology Group study**. *Clin Cancer Res* 2007, **13**:1789-1793.
375. Gaspar N, Sharp SY, Pacey S, Jones C, Walton M, Vassal G, Eccles S, Pearson A, Workman P: **Acquired resistance to 17-allylamino-17-demethoxygeldanamycin (17-AAG, tanespimycin) in glioblastoma cells**. *Cancer Res* 2009, **69**:1966-1975.
376. McCollum AK, Teneyck CJ, Sauer BM, Toft DO, Erlichman C: **Up-regulation of heat shock protein 27 induces resistance to 17-allylamino-demethoxygeldanamycin through a glutathione-mediated mechanism**. *Cancer Res* 2006, **66**:10967-10975.
377. Zajac M, Gomez G, Benitez J, Martinez-Delgado B: **Molecular signature of response and potential pathways related to resistance to the HSP90 inhibitor, 17AAG, in breast cancer**. *BMC Med Genomics* 2010, **3**:44.
378. Bear AS, Cruz CR, Foster AE: **T cells as vehicles for cancer vaccination**. *J Biomed Biotechnol* 2011, **2011**:417403.
379. Chang CN, Huang YC, Yang DM, Kikuta K, Wei KJ, Kubota T, Yang WK: **A phase I/II clinical trial investigating the adverse and therapeutic effects of a postoperative autologous dendritic cell tumor vaccine in patients with malignant glioma**. *J Clin Neurosci* 2011, **18**:1048-1054.
380. Joniau S, Abrahamsson PA, Bellmunt J, Figdor C, Hamdy F, Verhagen P, Vogelzang NJ, Wirth M, Van Poppel H, Osanto S: **Current vaccination strategies for prostate cancer**. *Eur Urol* 2012, **61**:290-306.
381. Richardson PG, Mitsiades CS, Laubach JP, Lonial S, Chanan-Khan AA, Anderson KC: **Inhibition of heat shock protein 90 (HSP90) as a therapeutic strategy for the treatment of myeloma and other cancers**. *Br J Haematol* 2011, **152**:367-379.
382. Rao A, Taylor JL, Chi-Sabins N, Kawabe M, Gooding WE, Storkus WJ: **Combination therapy with HSP90 inhibitor 17-DMAG reconditions the tumor microenvironment to improve recruitment of therapeutic T cells**. *Cancer Res* 2012.
383. Peron M, Bonvini P, Rosolen A: **Effect of inhibition of the Ubiquitin-Proteasome System and Hsp90 on growth and survival of Rhabdomyosarcoma cells in vitro**. *BMC Cancer* 2012, **12**:233.
384. Chemali M, Radtke K, Desjardins M, English L: **Alternative pathways for MHC class I presentation: a new function for autophagy**. *Cell Mol Life Sci* 2011, **68**:1533-1541.

385. Koizumi H, Yamada T, Takeuchi S, Nakagawa T, Kita K, Nakamura T, Matsumoto K, Suda K, Mitsudomi T, Yano S: **Hsp90 Inhibition Overcomes HGF-Triggering Resistance to EGFR-TKIs in EGFR-Mutant Lung Cancer by Decreasing Client Protein Expression and Angiogenesis**. *J Thorac Oncol* 2012, **7**:1078-1085.
386. Madrigal-Matute J, Lopez-Franco O, Blanco-Colio LM, Munoz-Garcia B, Ramos-Mozo P, Ortega L, Egido J, Martin-Ventura JL: **Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis**. *Cardiovasc Res* 2010, **86**:330-337.
387. Bohonowych JE, Gopal U, Isaacs JS: **Hsp90 as a gatekeeper of tumor angiogenesis: clinical promise and potential pitfalls**. *J Oncol* 2010, **2010**:412985.
388. Noman MZ, Janji B, Kaminska B, Van Moer K, Pierson S, Przanowski P, Buart S, Berchem G, Romero P, Mami-Chouaib F, *et al.*: **Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression**. *Cancer Res* 2011, **71**:5976-5986.
389. Ahluwalia A, Tarnawski AS: **Critical role of hypoxia sensor--HIF-1alpha in VEGF gene activation. Implications for angiogenesis and tissue injury healing**. *Curr Med Chem* 2012, **19**:90-97.
390. Bose A, Lowe DB, Rao A, Storkus WJ: **Combined vaccine+axitinib therapy yields superior antitumor efficacy in a murine melanoma model**. *Melanoma Res* 2012, **22**:236-243.
391. Bose A, Taylor JL, Alber S, Watkins SC, Garcia JA, Rini BI, Ko JS, Cohen PA, Finke JH, Storkus WJ: **Sunitinib facilitates the activation and recruitment of therapeutic anti-tumor immunity in concert with specific vaccination**. *Int J Cancer* 2011, **129**:2158-2170.