DEVELOPING COMBINATIONAL IMMUNOTHERAPIES TARGETING TUMOR RECEPTOR TYROSINE KINASES

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Current immunotherapies designed to stimulate specific T cell-mediated immunity have thus far yielded modest objective clinical response rates, despite the increase of tumor-specific T cells have been observed in treated patient blood. Since the majority of tumor antigens being targeted in immunotherapies are non-mutated, "self" antigens, current clinical results may relate, in part, to the low-to-moderate avidity, negatively-selected T cell repertoire in patients that is being asked to regulate tumor progression. In the current thesis, I hypothesized that by conditionally enhancing the proteasomal degradation of tumor antigens, I could generate a "synchronized" pool of derivative peptides that could then be presented in a "wave-like" temporal fashion in MHC class I complexes on the tumor cell surface. For at least a transient period thereafter, I theorized that specific $CD8^+$ T cell recognition and anti-tumor activities would be improved. I selected a family of tumor-associated antigens, receptor tyrosine kinases (RTK) for study, as their overexpression has been linked with poor clinical prognosis in many forms of cancer. In this thesis, I show that EphA2 agonists, as well as, HSP90 inhibitors effectively promote EphA2 degradation via a proteasome- dependent manner, providing the delivery of EphA2 peptides into the classical MHC class I presentation pathway. I also show that specific $CD8^+$ T cell recognition of EphA2 peptides derived from both the extracellular and intracellular domains of this transmembrane protein was improved as a consequence of tumor cell treatment with these agents being in consistent with the use of TAP- and ER-associated degradation. Notably, the combination of both drugs further enhanced anti-EphA2 T cell recognition of tumor cells, suggesting these modalities work via complementary, but not identical mechanisms. Importantly, complete tumor eradication was achieved *in vivo* (in a Hu-SCID tumor model) using a combinational therapy consisting of agonist administration just prior to the adoptive transfer of human anti-EphA2 $CD8^+$ T cells, where either single modality was minimally beneficial.

Preliminary data from additional studies targeting the tumor cell-overexpressed RTKs, Her2/neu and EGFR, suggest that this core treatment paradigm may be generalizeable to many (if not all) RTKs.

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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's, but also understanding with and support when I went through various family issues one after another. If my mentor were not Dr. Storkus, my graduation would never have been achieved. 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 and have provided me with the help, support and friendship I needed.

Finally, I would like to extend a special thank you to my family in Japan and here. My late mother Masako and father Fusakazu have given me lots of love and education. Without this foundation, I would have never made it this far. I am also grateful to my husband Touichi who has always given me support and love, and to my little daughter Megumi who somehow understands the "work" and makes me the happiest mother in the world. I dedicate this thesis to all of you with love.

1. Introduction

Many observations, including our own, suggest that most forms of cancer coordinately overexpress multiple receptor tyrosine kinases (RTKs), leading to constitutive kinase activation and enforcement of the proliferative or metastatic potential of neoplastic cells [1]. One goal for cancer therapy is, therefore, to decrease RTK levels of expression or their associated kinase activity. Numerous pharmacological agents that interfere with RTK-mediated signal transduction have been developed, however, in clinical trials these agents have proven only modestly effective and have exhibited toxicities when applied as single modalities [2]. A second major goal for cancer (immuno)therapy, we believe, may be to increase the frequency of RTK-derived antigenic peptides presented in MHC class I complexes on the tumor cell surface [3]. Such complexes are critical for RTK-specific $CD8^+$ T cell mediated eradication of cancer cells. How one may conditionally and selectively manipulate tumor cell presentation of T cell epitopes derived from endogenous antigens remains an understudied area of translational/clinical research. Of the many RTKs that have clinical implications in the cancer setting, I have chosen to focus predominantly on EphA2, which is overexpressed/dysregulated in virtually all solid cancers [4-6], where its degree of overexpression is associated with a poor clinical prognosis [4-7]. An additional rationale for this choice of target antigen was that we have previously defined EphA2 peptide epitopes that are recognized by HLA-A2-restricted $CD8^+$ T cells [8], making my experiments feasible. My data suggest that $CD8^+$ T cell recognition of $EphA2^+$ tumor cells may be dramatically improved by promoting the conditional proteasomal processing of the EphA2 protein as a consequence of tumor cell treatment with RTK agonists or HSP90 inhibitors. While my EphA2 modeling provides "proof-of-principle" data, I have also investigated the generality of these findings in parallel models based on the RTKs EGFR (ErbB1) and Her2/neu (ErbB2), which are also commonly overexpressed/dysregulated in a broad range of cancer histologies.

1.1. Receptor Tyrosine Kinesis

Receptor tyrosine kinases (RTKs) are a subclass of cell-surface growth-factor receptors that exhibit an intrinsic, ligand-controlled tyrosine-kinase activity. They are comprised of 58 known members, that are distributed among 20 sub-families (Table 1), which regulate diverse cellular functions including proliferation, differentiation, migration, and survival [1].

Table 1. Human receptor tyrosine kinases (RTKs).

Subfamily	Member
EGFR (epidermal growth factor receptor)	**ErbB1(EGFR)**, **ErbB2(Her2/neu)**, **ErbB3***, **ErbB4**
InsR (insulin receptor)	INSR, **IGF-1R**, IRR
PDGFR (platelet-derived growth factor receptor)	**PDGFR-α**, **PDGFR-β**, **CSF-1R**, **KIT(SCFR)**, **FLK2(FLT3)**
VEGFR (vascular endothelial growth factor receptor)	**VEGFR-1**, **VEGFR-2**, **VEGFR-3**
FGFR (fibroblast growth factor receptor)	**FGFR-1**, **FGFR-2**, **FGFR-3**, **FGFR-4**
KLG/CCK (colon carcinoma kinase)	CCK4*
NGFR (nerve growth factor receptor)	**TRKA**, TRKB, **TRKC**
HGFR (hepatocyte growth factor receptor)	**MET, RON**
EphR (ephrin receptor)	EphA1, **EphA2**, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphB1, **EphB2**, EphB3, **EphB4**, EphB5, EphB6
Axl	**AXL**, MER, TYRO3
TIE (tyrosine kinase receptor in endothelial cells)	**TIE**, **TEK**
RYK (receptor related to tyrosine kinases)	RYK*
DDR (discoidin domain receptor)	DDR1, DDR2
Ret (rearranged during transfection)	**RET**
ROS (RPTK expressed in some epithelial cell types)	**ROS**
LTK (leukocyte tyrosine kinase)	LTK, **ALK**
ROR (receptor orphan)	ROR1, ROR 2
MuSK (muscle-specific kinase)	MUSK
LMR (Lemur)	AATYK, AATYK-2, AATYK-3
?	RTK106

RTK members in bold type are implicated in human malignancies. An asterisk indicates that the member is devoid of intrinsic kinase activity.

In normal cells, the binding of ligand to the RTK promotes RTK dimerization/oligomerization and phosphorylation, creating binding sites for adaptor proteins that allow for the transmission of biological signals into the cell. During this process, the RTK protein is ubiquitinated by Cbl family ubiquitin-protein ligases. C-Cbl contains an internal SH2 domain that binds to phosphorylated RTKs (pRTKs), and a ubiquitin E3-ligase responsible for the addition of ubiquitin molecules to pRTKs [9, 10]. The ubiquitinated RTK is internalized via clathrin-coated pits, forming a sorting endosome [11, 12], which is then targeted towards a lysosomal compartment for proteolytic degradation (Figure 1). Prior to degradation this process is reversible, so that de-phosphorylated and/or non-ubiquitinated receptors may be recycled to the cell surface, where their functional lifespan may be temporally extended [11, 13, 14]. RTK-mediated signaling is normally modulated reversibly by the action of specific protein tyrosine phosphatases (PTPs), as well as, irreversibly by lysosomal degradation [11]. In addition, an alternative degradation route has been revealed by recent studies, in which polyubiquitinated RTK may be delivered to the proteasome for degradation [15].

Signaling processes associated with RTKs are often altered in tumor cells, in support of more aggressive cellular growth and/or the increased invasiveness of tumor cells, resulting in an overall poor prognosis for patients [7, 16-22]. Slightly more than half of the known RTKs have been reported to be either mutated or overexpressed in cancer cells; e.g. EGFR, ErbB2-4, PDGFR-α/β, VEGFR1-3, FGFR1-4, MET, RON, EphA2, EphB2/4, AXL, TIE, TEK [1, 7, 16-18, 20-23]. Cellular overexpression of RTK increases dimer concentrations, and causes constitutive kinase activation, sometimes even in the absence of specific ligand binding. Many of the mutated RTKs acquire increased kinase activity [24, 25] or avoid homeostatic down-regulation by loss of interaction with the E3-ligase c-Cbl [11].

As a result, RTKs and their down-stream signaling molecules have become rational targets for therapeutic intervention, prompting the development of novel pharmacological inhibitors. Such agents include neutralizing antibodies against RTK ligands, anti-RTK antibodies targeting either overexpressed receptors or receptor heterodimers, and small-molecule inhibitors of RTK-associated kinase activity [2]. In addition to targeting RTK themselves, one may also consider the therapeutic targeting of regulatory PTPs, such as low molecular weight protein tyrosine

phosphatase (LMW-PTP) and PTP-1B, that have been recently reported to be overexpressed in numerous cancer types in concert with overexpressed and hypo-phosphorylated RTKs [26, 27].

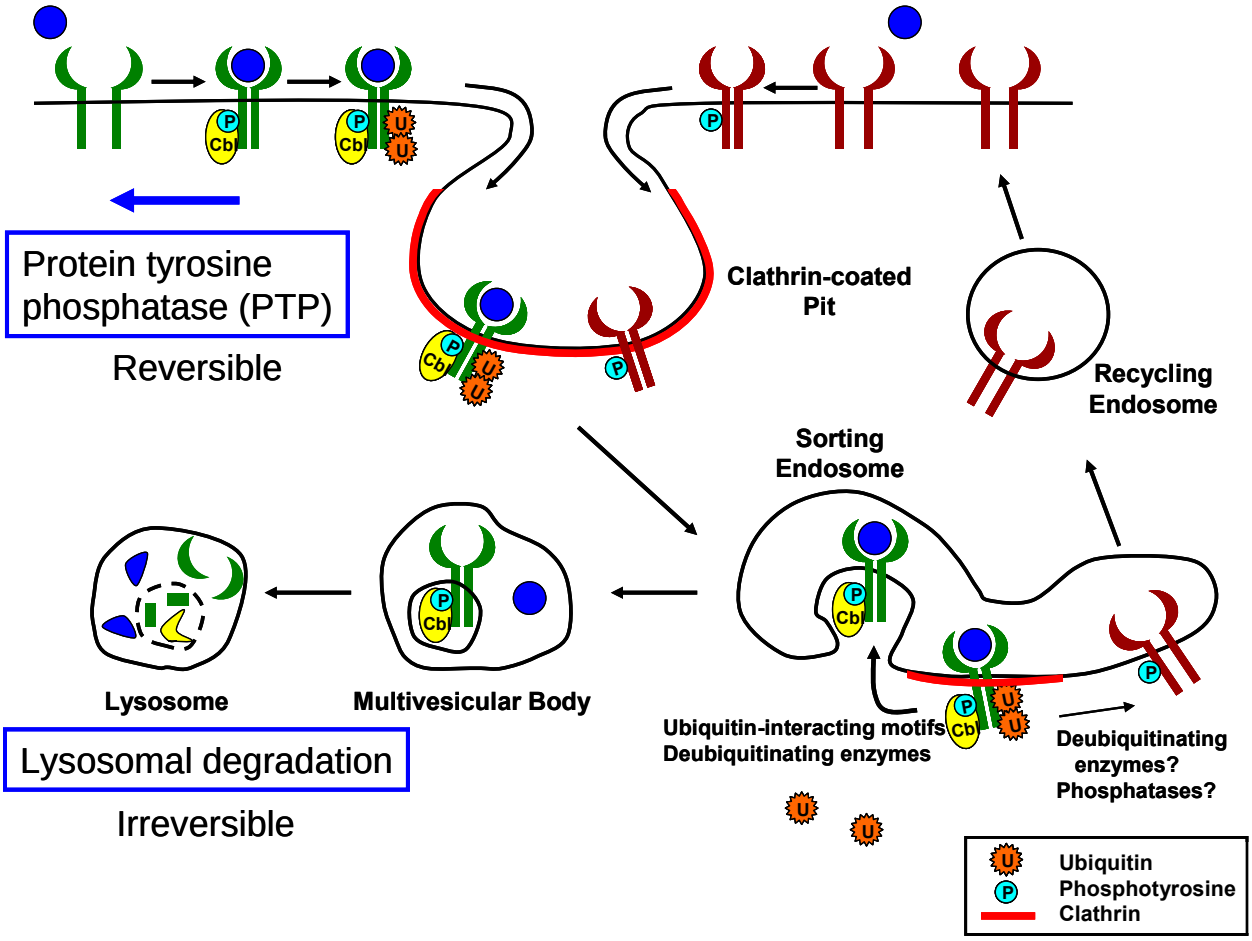

Figure 1. RTK ubiquitination and downregulation of cell surface RTK.
Binding of ligand to the RTK promotes RTK dimerization/oligomerization and its phosphorylation, creating binding sites for adaptor proteins in order to relay biological signals. During this process, the RTK protein is ubiquitinated by Cbl family ubiquitin-protein ligases, and internalized via clathrin-coated pits, forming a sorting endosome which is then targeted towards a lysosomal compartment for proteolytic degradation. Prior to lysosomal delivery this process is reversible, so that de-phosphorylated and/or non-ubiquitinated receptors may be recycled to the cell surface where their functional lifespan may be temporally extended. RTK signaling is normally modulated reversibly by the action of specific protein tyrosine phosphatases (PTPs), as well as, irreversibly by lysosomal degradation.

1.2.EphA2 Overview

1.2.1. Eph Receptor and Ephrin Ligands

The Eph family of molecules contains 16 members, comprising the largest known cohort of receptor tyrosine kinases (RTK) [28] (for nomenclature, refer to http://eph-nomenclature.med.harvard.edu/cell_letter.html). Based on sequence similarity and binding affinities, two classes of receptors, EphA and EphB, and two classes of corresponding ligands, ephrin-A and ephrin-B, have been defined. The EphA receptor family consists of 10 members (EphA1 – EphA10), and primarily bind 6 ephrin-A ligands (ephrinA-1 – ephrinA-6). Ephrin-A ligands are attached to the cell surface via glycosylphosphotidylinositol (GPI) anchors and tend to bind only EphA receptors. EphB receptor family consists of 6 members (EphB1 – EphB6), that primarily bind 3 ephrin-B transmembrane ligands (ephrinB1 – ephrinB3). However, Eph-ephrin binding is promiscuous in some cases, as there is some crossover in the interactions between Eph receptors and ephrin ligands. For instance, Ephrin-B molecules bind with low affinity to EphA4, whereas ephrin-A5 is known to interact with EphB2 [29, 30].

Like other RTK, Eph receptors are type 1 transmembrane proteins [31], having multiple domains as shown in Figure 2. The extracellular N-terminus of these molecules consists of a highly conserved ligand binding region, followed by a cysteine-rich region, and two membrane-proximal fibronectin Type-III repeats. The intracellular region of Eph receptors is characterized by a juxtamembrane segment containing two major auto-phosphorylation sites, followed by a conserved kinase domain, which phosphorylates secondary adapter proteins and can also phosphorylate Eph receptors themselves [32]. The C-terminal region contains a sterile a motif (SAM), and a PDZ binding domain. The PDZ domain may be involved in receptor oligomerization [33], facilitating the binding of adapter proteins [34]. The binding of Eph receptors to their corresponding ephrin ligands occurs at a 1:1 stoichiometric ratio, with the complex forming a circularized heterotetramer [35].

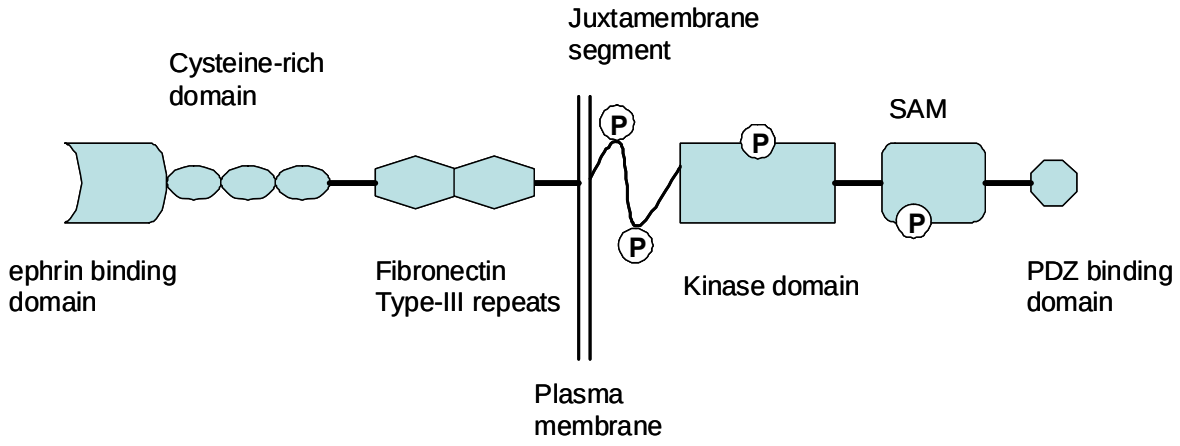

Figure 2. Schematic view of structural and signaling components of an Eph receptor.
The extracellular N-terminus of Eph receptor consists of ephrin binding domain, cysteine-rich domains and fibronectin Type-III repeats. The intracellular region of Eph receptors has a juxtamembrane segment, a conserved kinase domain, a sterile a motif (SAM) and a PDZ binding domain. P represents a tyrosine phosphorylation site.

Eph receptors were originally defined based on their involvement in various embryonic developmental processes, such as the formation of tissue boundaries, neural crest cell migration, axon guidance, and vascular system organization [16, 36-40]. However the developing embryo is not the only area where the Eph/ephrin system exerts its biologic influence. Eph/ephrin binding and subsequent signaling regulates the attachment, shape and motility of adult cells due to modulation of integrins/adhesion molecules on the cell surface and/or re-organization of the cell cytoskeleton [41-48]. Furthermore, some Eph receptors (in particular EphA2), have also been shown to play an important role(s) in neo-angiogenesis in normal adult tissues, as well as, tumors [4, 5, 49-51].

1.2.2. EphA2

One Eph receptor, EphA2, is of particular interest due to recent evidence suggesting it may play a role in the development and progression of cancer. The EphA2 gene is located on human chromosome 1, and encodes a 130 kDa Type-1 glycoprotein. EphA2 binds to ephrin-A1, -A3, -A4, -A5, but does not require ligand binding for its enzymatic activity [51]. Along with other

Eph/ephrin family members, EphA2 is involved in the organization of the developing nervous system [52] and vasculature [53], and is even expressed in embryonic stem cells [54]. In normal adult tissues, EphA2 is expressed at low levels on a broad range of epithelial tissues [55], 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 [48, 56]. EphA2 is also expressed by endothelial cells where it contributes to normal tissue (and tumor) neovascularization in the adult [49]. More recently 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 [57-59].

1.3. Regulation of EphA2 Expression

1.3.1. 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 [48] which is anchored to the surface of neighboring cells [60, 61]. In contrast, malignant cells express high levels of EphA2 protein, but these only poorly bind ligand [48, 62]. The EphA2 gene is expressed most highly in tissues that contain a high proportion of epithelial cells (e.g., skin, small intestine, lung, and ovary), with somewhat lower expression levels typically observed in kidney, brain, spleen, and submaxillary gland, and very low expression levels noted for heart, skeletal muscle, liver, testes, and thymus. These results diverge from studies of EphA2 protein expression, where strong expression has been reported for epithelial cells of kidney and lung, with weaker expression suggested for liver, small intestine and skin in the rat [55]. Our own studies using Western blot analysis of mouse organ lysates showed that moderate levels of EphA2 protein were expressed in the spleen, liver and lung, whereas no detectable expression was noted for brain, heart and skeletal muscle tissues

[63]. The observed disconnect between mRNA and protein level assessments in the various tissues likely reflects differences in post-translational stability (i.e. the "lifecycle" of EphA2 protein) in a given organ system.

Upon ligand binding, EphA2 seems to follow a similar destiny with other RTKs as described in section 1.1. Using ephrinA1-Fc fusion protein or EphA2-specific agonistic antibody, it has been shown that; (i.) cell surface EphA2 is internalized and degraded in response to ligand- or antibody-mediated stimulation, (ii.) EphA2 interacts with the c-Cbl adaptor protein in a stimulation-dependent manner, and (iii.) the proteasome serves as the major degrader of internalized EphA2 protein [64-67]. Overall, these results suggest that cellular levels of EphA2 protein are sensitive to turnover based on ligand-induced activation, as well as, relative levels of "regulatory" protein interactions (i.e. Cbl, PTPs, etc.) that dictate the fate of internalized EphA2 molecules.

Of note, one striking difference between EphA2 and most other RTKs is that the enzymatic activity associated with EphA2 is not dependent on ligand binding or receptor autophosphorylation, likely due to the lack of an 'activation loop' tyrosine in the EphA2 cytoplasmic domain [48, 66, 68]. Therefore, in the absence of ligand binding signals, EphA2 exhibits constitutively active enzyme activity, yet this does not appear to accelerate the turnover of EphA2 protein. Such dysregulation may result in the "accumulation" of EphA2 protein (overexpression) and a progressive accumulation of EphA2 signal strength (supporting tumorigenesis, etc.) [62].

1.3.2. EphA2 and PTPs

Tyrosine phosphorylation of RTKs in both resting and ligand-activated cells is modulated by the dynamic equilibria of tyrosine kinase and protein tyrosine phosphatase (PTP) activities. With regard to the latter class of enzymes, the PTP superfamily has approximately 70 members. These can be classified into four major categories based on enzyme function, structure, and sequence: (i.) tyrosine-specific phosphatases, (ii.) VH1-like dual specificity PTPs, (iii.) cdc25, and (iv.) low molecular weight phosphatases [69].

Of these, low molecular weight-protein tyrosine phosphatases (LMW-PTP) have been reported to play a major role in acting on pEphA2 as a substrate [26, 70, 71]. LMW-PTPs are a group of 18 kDa cytosolic enzymes that are constitutively expressed by most cell types [72]. Under normal conditions, LMW-PTP removes the phosphate groups on cytoplasmic tyrosine residues that are initially added to EphA2 as a consequence of agonist ligand binding. This dephosphorylation event is necessary for the homeostatic regulation of secondary signals that are generated upon ligand binding. Indeed, LMW-PTP has been reported to negatively regulate the ephrinA1-mediated repulsive response, cell proliferation, cell adhesion and spreading, and the formation of retraction fibers, as a result of its dephosphorylation of EphA2, suggesting LMW-PTP acts as a terminator of ephrinA1 signaling through EphA2 kinase activity [70].

Interestingly, LMW-PTP has been reported to be overexpressed in several cancer types [26, 71, 73]. This is consistent with additional reports that tumor EphA2 is largely non-phosphorylated, while it is prominently tyrosine phosphorylated in nontransformed cells [48, 62, 68]. Hence, high levels of EphA2 protein are coordinately observed in cells that overexpress LMW-PTP. This is a logical outcome, since elevated levels of LMW-PTP would be expected to be associated with decreased levels of pEphA2, leading to enhanced recycling versus degradation of EphA2 protein in cancer cells.

Another PTP reported to be involved in pEphA2 dephosphorylation is the SH2-domain-containing PTP2 (SHP2), which may regulate changes in cell-morphology that occur as a consequence of EphA2 activation [5, 47]. And more recently, SHIP2 (Src homology 2 domain-containing phosphoinositide 5-phosphatase 2) has also been reported to be recruited to activated EphA2 via a heterotypic sterile α motif (SAM)-SAM domain interaction, leading to the inhibition of EphA2 internalization [74]. The relative hierarchy of LMW-PTP versus SHP2 versus SHIP2 in regulating EphA2 phosphorylation status and EphA2 accumulation has not been comprehensively investigated in normal cells or tumor cells to date.

1.3.3. Genetic Regulation of EphA2

Several stimuli appear competent to efficiently up-regulate *EphA2* gene transcription. Most interestingly, p53, known as guardian of the genome or gatekeeper for growth and division, has been shown to upregulate EphA2 transcription [75-78]. Upregulated expression of p53 family members (p53, p73, and p63) increased both EphA2 transcript and protein levels, and a p53 binding site located within the *EphA2* promoter was defined and shown to be responsive to wild-type p53, p73, and p63, but not mutant p53 or p73 [78]. This turned out not to be a consensus p53 binding site, but rather a novel 10-bp perfect palindromic decanucleotide (GTGACGTCAC) binding site in the EphA2 promoter region [75]. While at face value, it might appear strange that wild-type p53 upregulates EphA2 transcription, it is important to note that levels of p53 are upregulated as a consequence of stabilization by mutant p53 protein in many forms of cancer [79, 80], hence p53 facilitated EphA2 expression becomes a rather logical conclusion.

EphA2 transcription is also enhanced as a result of activating the Ras-Raf-MAPK signaling pathway. Increased EphA2 protein level was observed previously in Ras-transformed mammary epithelial cells [68], with more direct evidence demonstrated by Macrae *et al.* [81], with Raf activation shown to stimulate both EphA2 mRNA and protein expression. They also demonstrated that EphA2 is a direct transcriptional target of the Ras-Raf-MAPK pathway and that ligand-stimulated EphA2 attenuates the growth factor-induced activation of Ras, suggesting that a negative feedback loop is created that impacts Ras activity [81]. It is noteworthy that ligand binding itself has been shown to upregulate EphA2 mRNA via the MAPK pathway [82].

Various stimuli/stressors that invoke the Ras-Raf-MAPK signaling cascade are potent inducers of EphA2 expression. Interleukin (IL)-1ß, IL-2, EGF, which are known to activate Ras, stimulate expression of EphA2 [83]. Deoxycholic acid, a well-known constituent of bile acid and cancer promoter, upregulates EphA2, at least partially through activation of the MAPK pathway [84]. Hypertonic stress and urea-associated stress increase Ras activity in renal epithelial cells [85], in concert with enhancing EphA2 mRNA and protein expression [86]. Thus, EphA2 expression in the renal medulla may represent an adaptive response to hypertonicity or exposure to urea. In renal ischemia-reperfusion injury, EphA2 mRNA may be increased as a consequence

of src kinase activation [87]. In addition, systemic injection of lipopolysaccharide (LPS; a MAPK activator via TLR2/4 stimulation) increased EphA2 mRNA expression in the liver [88].

Negative regulators of EphA2 expression have also been identified, with the most notable examples being signaling through the estrogen receptor and c-*myc* [89]. Exposure of non-transformed breast epithelial cells to physiological levels of estradiol was sufficient to silence EphA2 expression. Levels of EphA2 expression in breast cancer cells have also been shown to be inversely related to estrogen receptor expression, suggesting that loss of hormone sensitivity may override this repressive mechanism and contribute to widespread overexpression of EphA2.

1.4. Signaling Through EphA2

1.4.1. MAPK

The mitogen-activated protein kinase (MAPK) pathway is a signal transduction pathway that couples intracellular responses to the binding of growth factors with their corresponding cell surface receptors. Given the complexity of this signaling pathway, it is perhaps not surprising that investigations of how EphA2 receptor ligation impacts MAPK pathway activation have proven equivocal. Pratt *et al.*. demonstrated that ligand stimulation of EphA2 activates MAPK/ERK in breast cancer cells and that pEphA2 interacts with the PTB and SH2 domains of SHC [82, 90]. Another study also reported that ligand-mediated activation of EphA2 activated MAPK/ERK pathway and increased cell proliferation of glioblastoma cells [91]. In contrast, Miao *et al.* reported that EphA2 activation via ligand-binding potently inhibited the Ras/ERK signaling cascade in fibroblasts, endothelial cells, as well as normal and transformed epithelial cells. Moreover, EphA2 activation was shown to antagonize Ras/ERK activation in response to EGF, VEGF and PDGF in concert with reduced cellular proliferation [92]. More recently, this same group showed that ephrin-A1 stimulation of wild-type, but not EphA2-null, keratinocytes attenuates ERK1/2 activation [93]. Similarly, the attenuation of VEGF-induced MAPK pathway

activation by ligand-stimulated EphA2 has been confirmed by other groups in analyses of breast cancer or retinal endothelial cells [81, 94]. While these discrepancies may be simply due to differences in the cell type analyzed [93], more investigations are clearly needed to further clarify the impact of EphA2 stimulation on MAPK activity.

1.4.2. FAK

Focal-adhesion kinase (FAK), a cytoplasmic kinase localized to sites of cellular adhesion (i.e. where cells make contact with the ECM via integrins, etc.), is a major phosphoprotein species associated with changes in both the actin and microtubule cytoskeleton in adhering cells [95-97]. Phosphorylated FAK exhibits stronger kinase activity versus unphosphorylated FAK and appears important in recruiting signaling and adaptor proteins important to the strength of cellular adhesion to matrix. Miao *et al.* reported that activation of EphA2 results in the dephosphorylation of pFAK, which in turn, suppresses integrin function [47]. They showed that EphA2 is constitutively (physically) associated with focal-adhesion kinase (FAK) in resting cells, and that upon stimulation with the ligand ephrin-A1, the protein tyrosine phosphatase SHP2 is recruited to EphA2, followed by the rapid dephosphorylation of FAK (within two minutes) and dissociation of the FAK-EphA2 complex. As a result, integrins assume an inactive conformation, with cell spreading, migration and integrin-mediated adhesion co-coordinately inhibited. In contrast, Carter *et al.* showed that EphA2 activation by ephrinA1 induces FAK phosphorylation and cell adhesion and actin cytoskeletal assembly in fibroblasts [98]. These apparent discrepancies may be simply the result of when FAK phosphorylation status was evaluated after EphA2 activation (i.e. FAK may be quickly dephosphorylated, then re-phosphorylated at later time points). When studies of steady-state cells were performed using EphA2 siRNA or EphA2 transduction procedures, EphA2 silencing induced FAK dephosphorylation and reduced cellular invasiveness [99]. On the other hand, ectopic EphA2 overexpression in cells results in increased FAK phosphorylation, however, after one hour of ephrin-A1 ligation, FAK phosphorylation is reduced and the invasive phenotype attenuated [100].

1.5. Role of EphA2 in Cancer

1.5.1. Overexpression of EphA2 in Cancer

Overexpression of EphA2 has been observed in an ever-expanding range of cancer types [4-6]. For example, EphA2 has been reported to be overexpressed at levels 10-100 times greater in metastatic prostate carcinoma cells when compared to non-invasive prostate epithelial cells [101]. EphA2 protein is also expressed in greater amounts in malignant mammary tissue when compared to benign mammary epithelia [62, 89]. Similarly, EphA2 is vastly overexpressed in aggressive MUM-2B melanoma cells when compared to poorly invasive MUM-2C melanoma cells [102], and EphA2 mRNA levels were significantly higher in cell lines derived from distant metastases versus primary melanomas [103]. PANC1, a poorly-differentiated pancreatic adenocarcinoma that is highly invasive, overexpresses EphA2 when compared to the non-invasive Capan-2 pancreatic cancer cell line [99]. In renal cell carcinoma (RCC), we have reported that EphA2 is expressed in metastatic RCC cell lines to a degree exceeding that for primary RCC cell lines, a trend recapitulated when analyzing freshly-resected RCC clinical specimens [8]. More recently, we compared RCC and normal adjacent kidney (NAK) tissues and found that EphA2 expression varied between different tumors and that RCC lesions expressing higher levels of EphA2 were significantly higher in grade and tended to be larger, more-vascularized tumors [7].

Given such strong evidence supporting an association between EphA2 overexpression and tumor aggressiveness and metastatic potential, one would predict that the degree and distribution of EphA2 protein expression by primary tumors may prove predictive of clinical outcome in patients with $EphA2^{+}$ tumors. Indeed, it has been recently suggested that the quantitative levels of EphA2 expressed by lung cancer cells or esophageal squamous cell carcinoma might provide useful prognostic information with regard to the metastatic potential of human carcinomas in situ and the clinical course of cancer patients [17, 18, 64]. Consistent with this hypothesis, our group has also shown that the degree of EphA2 overexpression by biopsied RCC tissues (versus normal matched autologous kidney tissue) is predictive of short-term (<1 year) versus longer-term (≥1 year) disease-free interval, as well as, overall survival [7]. Among the RCC patients evaluated, individuals with tumors exhibiting the lowest levels of EphA2 expression were more likely to

remain disease-free for a longer period of time after surgery, whereas those patients with tumors expressing high levels of EphA2 relapsed quickly and tended to survive for a shorter period of time [7] .

1.5.2. Mechanisms of EphA2 Overexpression in Cancer

The dominant mechanism by which EphA2 is over-expressed and consequently promotes metastasis has not yet been conclusively elucidated. Several different possibilities impacting cellular expression of EphA2 include: gene amplification, decreased rates of protein degradation, and increased or stabilized mRNA transcription/translation. An attractive current hypothesis associated with EphA2 over-expression by tumor cells involves the disruption of EphA2 homeostatic protein degradation.

In order for EphA2 to be properly degraded, it needs to become phosphorylated and any disruption of this process may result in the accumulation of cell surface EphA2 molecules. As discussed above, LMW-PTP, which preferentially dephosphorylates pEphA2, is overexpressed in several cancers [26, 71, 73], and tumor cell expressed EphA2 protein is typically not phosphorylated [48, 62, 68]. Overexpression of LMW-PTP by tumor cells is associated with decreased levels of pEphA2, and enhanced levels of EphA2 due to enhanced protein stability.

Additional/alternative mechanism(s) linked to elevated EphA2 expression may involve changes observed for E-cadherin expression in cancer cells. E-cadherin is a major protein involved in cellular adhesion, and is localized in adherent junctions formed between adjoining cells in tissues [104, 105]. In order to mediate proper signaling, EphA2 needs to bind its corresponding ligand (i.e. ephrin-A1) on adjacent cells, and in the cancer setting, this ability appears to be perturbed due to unstable cell-cell contact. Notably, $EphA2^{+}$ tumors that lose expression of E-cadherin, tend to express the highest levels of EphA2 protein and EphA2 is no longer restricted to sites of cell-to-cell contact. Instead, EphA2 adopts a diffuse expression pattern throughout the cell [48]. Consistent with this notion are observations made by Hess *et al.* investigating the transient knockout of E-cadherin [106]. In this study, the authors found that E-cadherin and EphA2 were typically co-localized in cell-cell adhesion junctions, and that E-cadherin regulated EphA2

expression by modulating its ability to interact with, and become activated by, ligand ephrin-A1 [106]. Thus, E-cadherin serves as a "rheostat" in controlling EphA2 expression at the cell surface, with an inverse correlation reported between E-cadherin and EphA2 expression in bladder carcinoma [107].

Furthermore, as discussed in Genetic Regulation of EphA2 (section 1.3.3.), mutated/decreased levels of p53 and decreased levels of estrogen receptor may contribute to tumor cell overexpression of EphA2. Indeed, an analysis of ovarian cell lines and human ovarian cancers showed that EphA2 overexpression occurred in 91% of tumors with p53 null mutations versus only 68% in tumors with wild-type or missense mutations ($p = 0.027$) [108], suggesting the possible role of p53 in EphA2 accumulation in cancer cells.

1.5.3. Role(s) of EphA2 in Tumorigenesis

The role(s) of RTKs in carcinogenesis have been increasingly characterized over the last decade, in particular, with recent research demonstrating that tumor cell EphA2 expression levels may correlate with disease progression to metastasis [7, 17, 18, 64]. Metastasis is the process whereby cancerous cells leave their tissue of origin; invade the extracellular matrix and traffic via either the lymphatics or the bloodstream to distal tissue sites. Metastatic cancer is most deadly and a difficult form of disease to effectively treat.

A foundational study linking EphA2 and tumorigenesis was performed by Zelinski *et al* [62], in which enforced overexpression of EphA2 (via transfection of specific cDNA) was shown to be sufficient to promote transformation of mammary epithelial cells, allowing them to form invasive tumors/metastases in immunecompromised (athymic) mice. EphA2 cDNA transduced cells exhibited defects in adhesion, EphA2 subcellular distribution, and decreased P-Tyr content of EphA2, all of which were reminiscent of the phenotype observed for metastatic breast cancer cells [48]. The noted transforming capacity of overexpressed EphA2 appeared related to the inability of transgenic EphA2 to interact appropriately with its natural ligand, ephrin-A1. However, artificial stimulation of EphA2 was able to reverse the growth and invasiveness of

EphA2-transformed cells in this model. This suggests that similar manipulations may be effective in the clinical management of $EphA2^+$ cancers.

In addition, enforced overexpression of the EphA2-associated PTP, LMW-PTP, was found to be sufficient to transform normal epithelial cells *in vitro* [71], with LMW-PTP acting as a positive regulator of tumor onset and progression in *in vivo* animal models. This supports the oncogenic potential of LMW-PTP, likely via its ability to efficiently dephosphorylate EphA2, leading to EphA2 accumulation [26]. Since tyrosine phosphorylation of EphA2 is not necessary for its intrinsic enzymatic activity, non-phosphorylated, overexpressed EphA2 may serve as a potent oncoprotein [26, 48, 62, 66].

While the detailed mechanism(s) by which overexpressed, hypophosphorylated EphA2 promotes malignant transformation remain poorly-defined, one may speculate that this phenotype is associated with altered cell adhesion properties (i.e. disruption of E-cadherin and integrin binding, [47, 100, 106]). Additionally, since EphA2 activation via ligand-binding may inhibit the MAPK pathway (important for cellular responses to growth factors), the state of EphA2 hypophosphorylation in cancer cells may be linked to enhanced growth potential [81, 92-94], although this conclusion remains somewhat controversial. When taken together, it is likely that cellular overexpression/hypophosphorylation of EphA2 results in an abnormal distribution of this RTK, disruption of cell-to-cell contacts, and an enhancement in cell-to-extracellular matrix attachment, yielding cells with increased motility and invasive properties. The hypothetical role(s) of the EphA2/ephrinA1 system in tumor cells is summarized in Figure 3.

1.5.4. Angiogenesis

As tumors increase in size, they require more nutrients and oxygen for their continued and sustained growth. The inability of progressive tumors to gain access to a new blood supply puts the tumor at risk of reaching a growth maxima, above which anoxia and necrosis are likely to occur. Hence the successful tumor microenvironment is conducive to neoangiogenesis [109]. Establishment of neo-vascular vessels also provides primary solid tumors portals through which they may metastasize and eventually colonize distant sites. Indeed, neoangiogenesis has been

correlated with metastasis and poor-prognosis in several cancers, including breast and pancreatic carcinomas [110, 111].

Many factors, including EphA2 and its ligands, have been implicated in the process of angiogenesis. EphA2, along with its ligand ephrin-A1, are expressed in growing neovessels found in breast tumors and sarcomas [112], and EphA2-deficient endothelial cells fail to become incorporated into nascent tumor microvessels *in vivo* [113]. While ephrin-A1 (i.e. EphA2-ligand) is expressed in both tumor and normal vascular endothelial cells, EphA2 appears to be differentially expressed by tumor-associated vascular endothelial cells [114]. Notably, EphA2 overexpression in both tumor cells and tumor-associated endothelial cells has been linked to higher lesional vascularity/angiogenesis and poor clinical outcome in the setting of renal and ovarian carcinoma [7, 115]. Soluble EphA2 receptor, which antagonizes EphA2-mediated signaling, inhibits VEGF-mediated and ephrin-A1-mediated angiogenesis, and can antagonize tumor neoangiogenesis [116, 117] and growth in vivo [114, 118, 119]. 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.

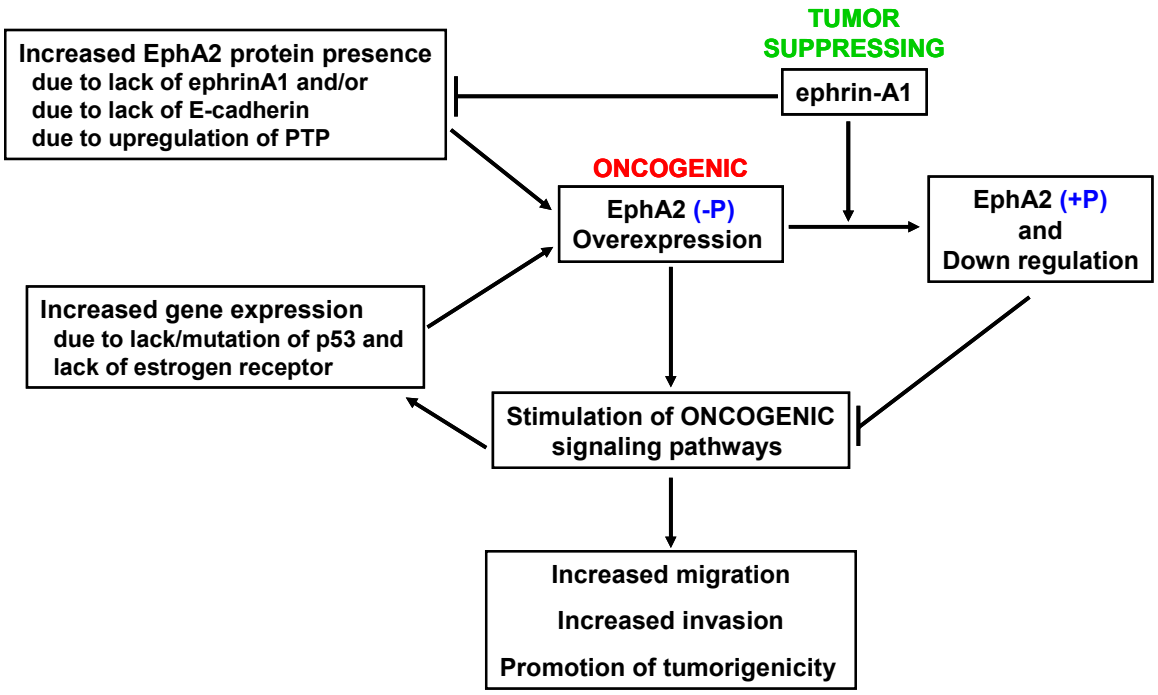

Figure 3. Hypothetical role of the EphA2/ephrinA1 system in solid tumor cells.
EphA2 becomes overexpressed possibly due to increased gene expression or a lack of ephrinA1-induced receptor down-regulation. Tumor cell overexpressed EphA2 protein is frequently non-phosphorylated and the phenotype is associated with cells exhibiting increased migration, invasiveness and malignancy. The binding of ephrinA1 causes receptor phosphorylation and subsequent down-regulation of receptor, both of which likely contribute to the tumor-suppressing effects of the ligand in tumor cells. (−P), nonphosphorylated; (+P), phosphorylated; PTP, protein tyrosine phosphatase.

1.6. Therapeutic Approaches Targeting EphA2

1.6.1. Interventions Targeting EphA2 Activation/Degradation

1.6.1.1. Anti-EphA2 Antibodies

Carles-Kinch *et al.* were the first to report that an agonist anti-EphA2 antibody could reverse the aggressive phenotype of EphA2^{+} tumors [66]. They generated monoclonal antibodies to extracellular domain of EphA2, and found that a subset of EphA2 monoclonal antibodies (mAb)

induced EphA2 phosphorylation, followed by the internalization and destruction of EphA2 protein. Notably, monoclonal antibodies identified a conformationally distinct determinant that was differentially expressed by tumor cell-associated EphA2 versus normal cell-associated EphA2. These mAbs were effective in inhibiting human tumor growth in xenograft models, in concert with decreased EphA2 protein expression levels and an increased apoptotic index in treated tumor lesions [66, 120, 121]. Given their specificity for EphA2 expressed on malignant cells, such antibodies have significant potential for clinical benefit, and lessen concern regarding untoward toxicity directed against normal $EphA2^{+}$ tissues. In addition, combinational therapies implementing EphA2 agonistic antibodies and chemotherapeutic drugs (such as paclitaxel or tamoxifen) may be readily envisioned, as this regimen has been reported to yield superior tumor growth inhibition versus either single modality, when assessed in pre-clinical models [122, 123]. Such results may be dependent on the relative dominance of EphA2-dependent oncogenic processes in tumor cells, however, since a contradictory report suggests that at least in some models, agonist anti-EphA2 Abs have little impact on tumor growth [124].

1.6.1.2. Ephrin-A1 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 [23, 100]. 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 [125]. 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 [125, 126]. With regard to their potential clinical utility, one concern in using ephrin-A1 Fc (protein or gene constructs) as a therapeutic agent reflects 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 [125], further safety studies prior to clinical translation of this agent would appear warranted.

1.6.1.3. Peptide Mimetics

Koolpe *et al.* employed a phage display approach and identified two peptides that bind selectively to the extracellular domains of EphA2 and antagonize ephrin ligand binding [127]. One of these peptides stimulates EphA2 tyrosine phosphorylation and signaling, suggesting the potential therapeutic utility of this peptide, based on analogy to similar effects mediated by either agonist anti-EphA2 mAb or ephrin-A1 Fc. In addition, they found that this peptide targets the delivery of phage particles to $EphA2^+$ cells, suggesting potential therapeutic value in selectively delivering therapeutic agents to $EphA2^+$ tumor sites [127].

1.6.2. Interventions Targeting EphA2 Ligands

Soluble EphA2-Fc, a chimeric receptor of EphA2 fused with an IgG Fc fragment, antagonizes EphA2 signaling, and has been found to inhibit VEGF-mediated and ephrin-A1-mediated angiogenesis. Local or systemic administration of soluble EphA2 inhibits tumor angiogenesis, growth [114, 118], and even metastasis [119] in *in vivo* tumor models. Interestingly, VEGF induces ephrin-A1 expression, which in turn activates EphA2-dependent angiogenesis [117]. Hence soluble EphA2 would be anticipated to suppress tumor-associated VEGF-, but not basic fibroblast growth factor (FGF)-, induced angiogenesis [117]. With regard to safety concerns, soluble EphA2 significantly inhibits pathologic retinal angiogenesis without affecting normal intraretinal vessels [116], suggesting that this agent may not inflict untoward toxicity on normal $EphA2^+$ tissues.

1.6.3. Gene Silencing by siRNA

Since most human cancers, including RCC, overexpress EphA2, EphA2 gene silencing is a plausible therapeutic strategy to reverse malignancy. A limited cohort of recent studies suggest that application of EphA2 siRNA suppresses EphA2 protein expression and cellular invasiveness, and that systemic administration of EphA2 siRNA retards tumor growth and inhibits metastasis *in vivo* [99, 124, 128, 129] via the induction of tumor cell apoptosis mediated via caspase 9 activation [129].

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 preliminary data demonstrated that LMW-PTP silencing with siRNA reduced EphA2 protein expression of metastatic RCC cells (Wesa *et al.*, Manuscript in preparation, 2009), suggesting the possibility for an alternative therapeutic method.

1.6.4. EphA2-based Vaccines

Seven human leukocyte antigen (HLA)-A2 binding and three HLA-DR4 binding peptides derived from EphA2 have been identified by our group and by Alves *et al*. [8, 130]. These peptides are immunogenic, being recognized by $CD8^+$ or $CD4^+$ T cells generated from normal donors or cancer patients bearing the appropriate HLA types, and by $CD8^+$ T cells developed in HLA-A*0201-transgenic HHD mice [8, 130]. 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 plasma membrane of tumor cells, in a manner that allows for effector T cell reactivity (i.e. interferon-γ production, cytolysis). Such EphA2-specific T cells have been identified in the peripheral blood of patients with RCC [8], prostate cancer [130] or glioma [131, 132], suggesting that these responses may be naturally-primed during cancer progression. In RCC patients, we observed that anti-EphA2 $CD4^+$ and $CD8^+$ T cells were most pronounced in patients that had no evidence of disease after therapy or that were long-term survivors in the face of limited tumor load [8]. In the anti-EphA2 reactive $CD4^+$ T cell compartment, we noted that T cells isolated from patients with active disease were more prone to produce Th2- or Treg-associated cytokines, such as IL-4, IL-5 and TGF-β, while patients without disease, or those with better clinical outcomes, were more biased towards Th1-type cytokine production (i.e. IFN-γ). The lack of Th1-type T cells reacting against EphA2 in RCC patients with active disease may reflect the high frequency of pro-apoptotic T cell in these populations (Wesa *et al.*, Manuscript in preparation, 2009). This suggests that therapeutic/protective $EphA2^+$ cancer vaccines may need to expand,

appropriately polarize (i.e. Type-1) and protect anti-EphA2 T cells against tumor-induced immune-deviation and death.

In this regard, active vaccination against EphA2, in order to elicit and sustain specific T cells is a logical endpoint that would be anticipated to provide clinical benefit in $EphA2^+$ cancer patients. Regardless of whether an EphA2 peptide, recombinant protein or gene vector (plasmid, viral or bacterial) is contemplated as a therapeutic agent in the setting of $EphA2^+$ cancers, their application in the context of a DC-based vaccination would be attractive, given the ability of the this antigen presenting cell type to prime, polarize and extend the survival of antigen-specific T cells [133, 134]. Indeed, Hatano *et al.* recently demonstrated that DC pulsed with murine EphA2 peptide epitopes effectively elicit specific CTL responses *in vivo* that are capable of inhibiting syngenic tumor progression in C57BL/6 mice [63]. 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 [63] (Komita *et al.*, Manuscript in preparation, 2009). This may reflect greater densities of EphA2 epitopes presented on the surface of tumor cells versus normal tissues, with T cells exhibiting moderate avidity only 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.

1.7. Cancer Immunity

1.7.1. Immunosurveillance: Immune response to cancer

Cancer immunosurveillance is a hypothesis proposed by Burnet and Thomas [135], some fifty years ago, which posits that the immune system protects the host against the development of cancers of non-viral origin. This theory was largely discarded until the 1990s due to the lack of

appropriate mouse models that allowed for its detailed inspection, however, many recent studies support the working paradigm of immunosurveillance [136-138]. One of the first definitive studies, performed by Shankaran *et al.*, showed that RAG2−/− mice (lack T cell, B cell, and NKT cell) had higher risks of developing spontaneous carcinoma, as well as, carcinogen-induced sarcomas [139]. Subsequent studies employing various strains of immunodeficient mice (with defects in innate and/or adaptive immunity) revealed that these animals indeed develop spontaneous and carcinogen-induced tumors at a higher rate than their immunocompetent counterparts [138]. Furthermore, in humans, a correlation between the number of tumor infiltrating lymphocytes (TILs) and better prognosis has been reported for a broad range of tumors [140]. The observation that patients with severe deficits of immunity are more likely to develop tumors of non-viral origin provides strong support to the generalized immunosurveillance theory [141, 142].

The current consensus regarding immunosurveillance is that there is continuous cross-talk between cancer cells and the immune system throughout tumor growth/development which occurs within the tumor site, as well as, in the tumor draining lymph node(s) [136, 138, 143]. Such operational reciprocity is reflected in: i.) temporal changes in the antigenic repertoire expressed by tumor cells; ii.) the disruption of local tissue homeostasis by infiltrating macrophages, NKT cells and NK cells; iii.) local inflammation, then recruitment of DCs that may acquire tumor antigens and traffic to draining lymph nodes where they may "crossprime" antigen-specific T (effector) cells and B (antibody-producing) cells; and iv.) recruitment of primed immune cells into the tumor microenvironment where they may mediate direct killing of tumor cells and elaborate chemokines/cytokines that facilitate killing of tumor cells and further infiltration of tumor lesions by host innate and adaptive immune cells [136, 138, 143]. Under such re-iterated rounds of immune attack, cancer cells may be eradicated, or alternatively they may accumulate genomic/proteomic alterations that permit escape from immune detection/clearance.

1.7.2. Immunoevasion

The evolution of a more malignant cancer cell phenotype under selective immune pressure supports the theory that superior immunotherapeutic results will be gained with early disease detection and the treatment of early-stage disease, at a time when low numbers of (less aggressive) cancer cells may be most effectively managed by host immunity. This is also consistent with the overall immune responsiveness of patients, which has been perceived as "normal" in early-stage disease, but which frequently exhibits immunosuppressed behavior/exhaustion at later stages of disease [144, 145].

There is a broad range of genetic alterations in tumor cells that have been linked to "immunoevasion". For instance, tumor cells may develop insensitivity against interferon γ (IFNγ) produced by DCs, NK cell and T cells [146]. IFNγ is known to exert multiple functions on tumor cells, including direct antiproliferatic/apoptic effect on tumor cells, sensitization of tumor cells for death receptor-mediated apoptosis and enhancement of recognition by T cells through upregulation of MHC class I and II and induction of immunoproteasome as well as other members of the MHC antigen-processing machinery (APM) [147]. Loss of TRAIL (death receptor signaling) expression has also been observed to contribute to tumor cell survival in the face of active immunity [148].

It has now been well-established that tumor cells down-regulate many molecules involved in the processing and presentation of antigenic peptides in MHC class I complexes [149-151]. 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 [152]. Tumors exhibiting low levels of MHC class I expression may conversely be targeted by natural killer (NK) cells, however, 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 serve as antagonists for tumor lysis [153].

In addition, tumor cells may acquire expression of T cell death/inhibitory inducing molecules such as FasL and B7-H1, among others. Colon tumors have been reported to constitutively

express FasL which may interact with Fas expressed on T cells, thus depleting tumor-infiltrating lymphocytes via the induction of their apoptotic death [154]. B7-H1, a co-stimulatory molecule that can also inhibit activated PD-1^+ effector T cells, has also frequently been found to be expressed on tumor cells *in situ* [155].

Besides intrinsic tumor changes that allow for immunoevasion, tumor cells may also develop mechanisms to prevent the initial activation/priming of anti-tumor T cells by interfering with antigen-presenting cell function(s). $CD8^+$ T cell stimulation by (tumor-conditioned) immature DC causes the $CD8^+$ T cells to hyporesponsive/anergic T cells in the tumor-draining lymph-node. This result has been suggested to depend on tumor cell elaboration of suppressive (to DC) mediators including vascular endothelial growth factor (VEGF), prostaglandin (PG)-E2, IL-10 and TGF-β [156]. 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 [157, 158], 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.

Accumulation of T regulatory cells (Tregs: $CD4^+CD25^+$) within tumor sites and draining lymph nodes also supports immnoevasion. Tregs control immunological tolerance to self-antigens by impeding the generation and activation of effector T cells, and which results in the suppression of anti-tumor T effector cells [159, 160]. Tumor cell-produced TGF-β induces the differentiation of naïve $CD4^+$ T cells into Tregs, and interestingly, tumor cells may release chemokines, such as CCL22 and SDF-1, which attracts Tregs into tumor sites *in vivo* [161].

In addition to tumor cells themselves, 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 [162], produce immunosuppressive factors such as nitric oxide and reactive oxygen species which suppress the proliferation of $CD4^+$ and $CD8^+$ T cells and inhibit the function of $CD56^{dim}$ NK cells [163]. Moreover, tumor-associated macrophages

(TAMs) are known to release pro-tumoral cytokines, such as IL-4, IL-13 and IL-10 that contribute to depress Type-1 T cell responsiveness that is required for effective regulation of tumor growth [164].

1.7.3. Immunotherapy

Over the past decade, immune therapies, such as monoclonal antibodies, immune adjuvants, and vaccines against oncogenic viruses, have become a standard treatment for a variety of cancers [165]. Fueled by these successes, various modes of immunotherapy have been intensively investigated for cancer patients.

Monoclonal antibody has proven a successful mode of immunotherapy against cancer. Nine monoclonal antibodies, targeting six tumor-associated proteins, have been licensed for the treatment of cancer [165, 166]. Five of these antibodies bind surface proteins (CD52, CD33 and CD20) that are highly-expressed on hematologic tumors. The remaining 4 antibodies target either RTK or its ligand; EGFR (Cetuximab, Pantitumumab), Her2/neu (Trastuzumab), or VEGF. Infused monoclonal antibodies mediate direct effects against the cancer microenvironment and are minimally impacted by active suppression mechanisms in place within tumor lesions. Abs recognize "tumor-associated" antigens expressed by tumor cells or cells within the tumor-associated stroma without being "restriction" by MHC molecules (as in the case of T cells), and therefore, Abs may be applied to all patients regardless of their HLA type. By binding to their respective target proteins, antibodies exercise their functions through several mechanisms, including steric inhibition and neutralization, complement activation leading to direct cytotoxicity of tumor cells, and activation of antibody-dependent cellular cytotoxicity (ADCC) mediated by FcR^+ effector cells. Due to their high-degree of specificity and general safety profiles, a variety of antibodies are now being investigated as second generation agents designed to productively modulate anti-tumor immune cells that would otherwise be impaired in cancer patients. Targets for therapeutic Abs include negative regulatory receptors on immune cells (CTLA-4 and PD-1; [167, 168]), immunosuppressive cytokines (IL-10, TGF-β; [169, 170]), TNF family costimulatory receptors (GITR, OX40, CD137, CD40; [171, 172]), and regulatory T cells (CD25, CCL22, CCR4, IL-35; [173]).

Another class of immunotherapies involves the local (paracrine) administration of immune-activating agents (adjuvants) which induce tumor-associated inflammation and protective immunity. BCG is currently used for superficial bladder cancer as standard of care, and TLR7 agonist imiquimod is approved for the treatment of some type of tumor [165]. A next generation of immune adjuvants, including TLR9 agonists has also been extensively evaluated and shown to mediate considerable efficacy in preclinical models [174].

Vaccines continue to represent a primary modality for cancer prevention and treatment. While prophylactic vaccination against hepatitis B virus (HBV) and human papilloma virus (HPV) are approved for use in patients with hepatocellular carcinoma or cervical cancer, respectively, the development of effective therapeutic cancer vaccines targeting tumor histologies that exhibit no known viral etiology remains a substantial challenge [165]. Many strategies for generating therapeutic immune responses to cancer have been extensively investigated. Of these, DC-based vaccines are now considered as one of the most promising methods by which to stimulate T cell mediated immunity. The vaccines typically consist of autologous monocyte-derived DCs loaded *ex vivo* with antigenic peptides, proteins, or gene vectors ([175, 176]) that are then administrated i.v., s.c., i.l., or i.t. to induce antigen specific T cell responses. DC-based vaccines have been shown to increase circulating levels of tumor specific T cells in the majority of patients analyzed in clinical trials, however, the objective clinical responses noted in these phase I/II evaluations have been modest (i.e. less than 10%; [177]).

A concept that has now gained wide-acceptance in the field of cancer vaccine research is that vaccine monotherapy is unlikely to deliver the necessary stimulus to generate robust and long-lasting (memory) immune responses that are competent to overcome tolerance, immune escape and the immunosuppressive microenvironment of the tumor. Thus, the synergy of vaccines and existing (chemo)immunotherapies are envisioned to lead to superior clinical benefits [178, 179].

While technically laborious and costly, adoptive T cell transfer represents another currently *en vogue* modality of immunotherapy that may (at least temporarily) circumvent immunosuppressive mechanisms prevalent in cancer patients. Tumor-specific cytotoxic T cells (CTLs) are commonly generated/expanded *in vitro* by repetitive stimulation of peripheral blood mononuclear cells with antigen-presenting cells (APCs) “presenting” tumor antigens of interest.

CTL lines have traditionally been cultured in the medium containing IL-2 to support T cell survival and proliferation. The T cells recovered late in these cultures, however, develop a terminal effector phenotype characterized by poor proliferation, low IL-2 production, reduced survival *in vivo*, and an inferior ability to traffic into tumor sites *in vivo* [180, 181]. Accumulating evidence now favors the transfer of memory T cells which exhibit enhanced survival, proliferative potential and ability to home into lymphoid tissues, where they may then respond to tumor antigens cross-presented by endogenous APC. Cytokines which support memory T cell generation (IL-7, IL-12, IL-15, and IL-21) are also now being considered as supportive factors to be co-applied along with T cell transfer in cancer patients [182, 183]. The relative importance of sub-classes of memory T cells (T_{CM} and T_{EM}) to anti-tumor efficacy has not yet been conclusively demonstrated, and both subsets of T cells have been reported to confer some degree of protection in different tumor models [182-185].

Another important aspect of the conventional adoptive T cell transfer approach is that it relies on the endogenous anti-tumor reactivity of the transferred CTLs. Isolated and *ex vivo* expanded tumor associated antigen (TAA)-specific T cells, however, frequently possess low-affinity/avidity T cell receptors (Tars) and are less effective at killing tumor cells expressing modest levels of TAA-derived antigens and/or MHC complexes. To circumvent these limitations, genetical modifications of T cells with receptors capable of recognizing low levels of TAA/MHC complexes on the tumor cell surface have been studied. Such engineered T cells express TCR from high-affinity TAA-specific T cell clones, or chimeric antigen receptors that recognize tumor through single-chain variable fragments (scFv) of TAA-specific antibodies [186, 187].

1.8. $CD8^+$ Cytotoxic T cell-Mediated Immunity

Many studies have been performed to identify the importance of $CD8^+$ T cell mediated immunity to cancer prevention/regression. Antibody-mediated depletion of $CD8^+$ T cells in both

fibrosarcoma and lymphoma models showed that these lymphocytes are critical for controlling tumor growth and progression [139, 188]. Activated, effector T cells have been shown to be capable of infiltrating tumors in both mice and humans. For example, $CD8^+$ T-cell infiltration has been shown to occur in primary cutaneous melanoma lesions [189] and in lymph node (LN) metastases [190] in association with a better clinical prognosis. In the vast majority of cases evaluated, $CD8^+$ cytotoxic T cell (CTLs) are considered as the key effector cells required to eliminate tumor cells *in vivo* [191, 192]. These T cells are commonly referred to as cytotoxic T lymphocytes (CTL) due to their potent killing activities.

1.8.1. Generation and Functions Mediated by $CD8^+$ Cytotoxic T cells

The generation of effector CTL, development of effector function and clonal expansion, requires multiple signals contributed by APC and the immediate tissue microenvironment [193, 194]. Signal 1 is delivered through TCR/MHC interactions and provides specificity to the response, since only T cells that can recognize antigen in the context of MHC class I can be activated. Signal 2 is delivered by interaction of a variety of costimulatory molecules expressed on activated DCs (e.g. CD80 and CD86) and $CD8^+$ T cells (CD28), and dictates the fate of the response (activation/tolerance) providing a regulatory mechanism. Delivery of signal 1 without sufficient costimulation is thought to lead to functional tolerance, while inhibitory costimulatory molecules, such as CTLA-4 and PD-1, play a role in the active suppression of CTL responses. Signal 3 is required for the development of optimal CTL responses and is delivered by cytokines (such as IL-12, Type-1 IFN) or by inflammatory signals supplied by TLR ligands or alternate "danger signals".

Following stimulation with Signals 1-3, naïve $CD8^+$ T cells may quickly differentiate into activated effector $CD8^+$ T cells as defined by their production of IFNγ, cytolytic function, or both. Highly-activated effector T cells exhibit the capacity to traffic into tumor sites, while memory T cells display enhanced survival, proliferative potential and the ability to home to lymphoid tissues where they may respond to tumor antigens cross-presented by APC. Memory T cells may be further categorized into two populations, central memory T cells (T_{CM}) and effector memory T cells (T_{EM}), based on their differential expression of the lymphoid homing markers CCR7 and

CD62L [195]. $CD8^+$ T_{EM} cells secrete IFNγ and TNFα, while $CD8^+$ T_{CM} cells secrete IL-2 in addition to IFNγ and TNFα. It still remains controversial whether the transition of activated effector T cells to T_{EM} cells and T_{CM} cells is linear, as appears to be the case for $CD4^+$ T cells, or whether both populations arise and are sustained independently [196, 197]. Continued (chronic) antigenic stimulation of $CD8^+$ T cells can lead to the terminal differentiation of effector cells, which only produce IFNγ, and are destined for senescence. Terminally-differentiated effector T cells display impaired proliferation/survival and poor migration to lymphoid tissues, and given these deficiencies, these cells may not represent an optimal population for use in adoptive transfer therapies [182, 194, 198].

Effector $CD8^+$ T cells recognize target cells in an antigen- and MHC class I-restricted manner and then mediate target cell death via apoptotic/necrotic pathways. Apoptosis is considered a major mode of target cell death and can be induced by three pathways [199, 200]; 1) granule-dependent exocytosis pathway, 2) Fas-FasL intercellular linkage-mediated pathway, and 3) cross-linking of TNF and TNFR type I. In the first pathway, lytic molecules such as perforin, granzymes, and granulysin are exocytosed into the extracellular space between a CTL and a target cell. Three models are currently proposed for internalization of those molecules, in which perforin and granzyme receptor play critical roles [199, 200]. Once released into the cytoplasm of the target cell, granzyme B can initiate apoptotic cell death through the direct cleavage of pro-caspase-3 or, indirectly, through caspase-8 activation. In addition, cleavage of BID results in mitochondrial dysfunction which can lead to necrotic death and the release of factors mediating caspase-independent cell death. In the second pathway, the interaction of the FasL-trimer on the CTL membrane with target cell-expressed Fas induces the trimerization of Fas-associated death domain (FADD) molecules of target cell that recruit procaspase 8 or 10. This results in the activation of caspase-8 and eventually leads to the activation of caspase-3, which degrades chromosomal DNA resulting in target cell death. The third pathway follows a similar mechanism using the Fas-FasL pathway after trimerization of TNF receptor (TNFR) on the target cell membrane.

In addition to the direct killing activity mediated by CTLs, IFNγ produced by CTLs may also exert beneficial anti-tumor effects by promoting DC differentiation/maturation, enhancement of

NK cell activity, and induction of Th1-type immune responses [147]. Furthermore, IFNγ may exert anti-proliferative/pro-apoptotic effects in treated tumor cells, and may sensitize tumor cells for death receptor-mediated apoptosis and enhanced recognition by T cells based on its ability to upregulate MHC class I and II expression and/or the MHC class I/II APM [147, 201].

1.8.2. MHC class I Antigen Presentation Pathway

Major histocompatibility complex (MHC) class I molecules are constitutively expressed by virtually all nucleated 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 at least proteasome, ERP1/ERAAP, transporter associated with antigen presentation complex (TAP, heterodimer of TAP1 and TAP2), general ER chaperones and tapasin [202]. Two distinct pathways, direct and cross-presentation pathways, are well known for MHC class I restricted peptide presentation (Figure 4) [202, 203]. 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.

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 (composed of the class I heavy chain and non-covalently bound β2-microglobulin). Peptides may be trimmed by an ER-associated aminopeptidase (ERAAP or ERAP1) to a preferred (MHC class I) loading length of 8–10 amino acids. Essential molecules for optimal peptide loading into MHC class I complexes include TAP,

tapasin, calreticulin and ERp57 [204-206]. Fully-assembled class I molecules are then transported through the Golgi to the cell surface.

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 correct (native) conformations may be ubiquitinylated and retrotranslocated from the ER back into to the cytoplasm, where they face degradation by the proteasome [203, 207]. 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 [208, 209]. Exogenous antigens are first phagocytosed, endocytosed (via specific or scavenger-type receptors), pinocytosed, or macropinocytosed by APC. During formation of the phagocytic cup (a structure formed at the base of the phagocytic substrate during the initial stages of phagocytosis), ER fuses with the nascent phagosome to form early phagosomes that contain ER proteins, including all the components required for MHC-I antigen presentation (e.g. TAP, tapasin, MHC-I), ubiquitin-conjugating enzymes and translocon (proteins associated with translocation such as Sec61). From here three alternative routes have been discovered [209]. In the phagosome–cytosol–ER route, 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 onto MHC-I. In the vacuolar route, the antigen does not leave the phagosome, but is instead processed by enzymes within the phagosomal lumen and then loaded into new or recycled MHC class I molecules. In the phagosome–cytosol–phagosome route, antigens are exported to the cytosol, ubiquitinated, processed by the associated proteasomes, and then transported back into the same phagosome by TAP, where they may be loaded into MHC class I complexes.

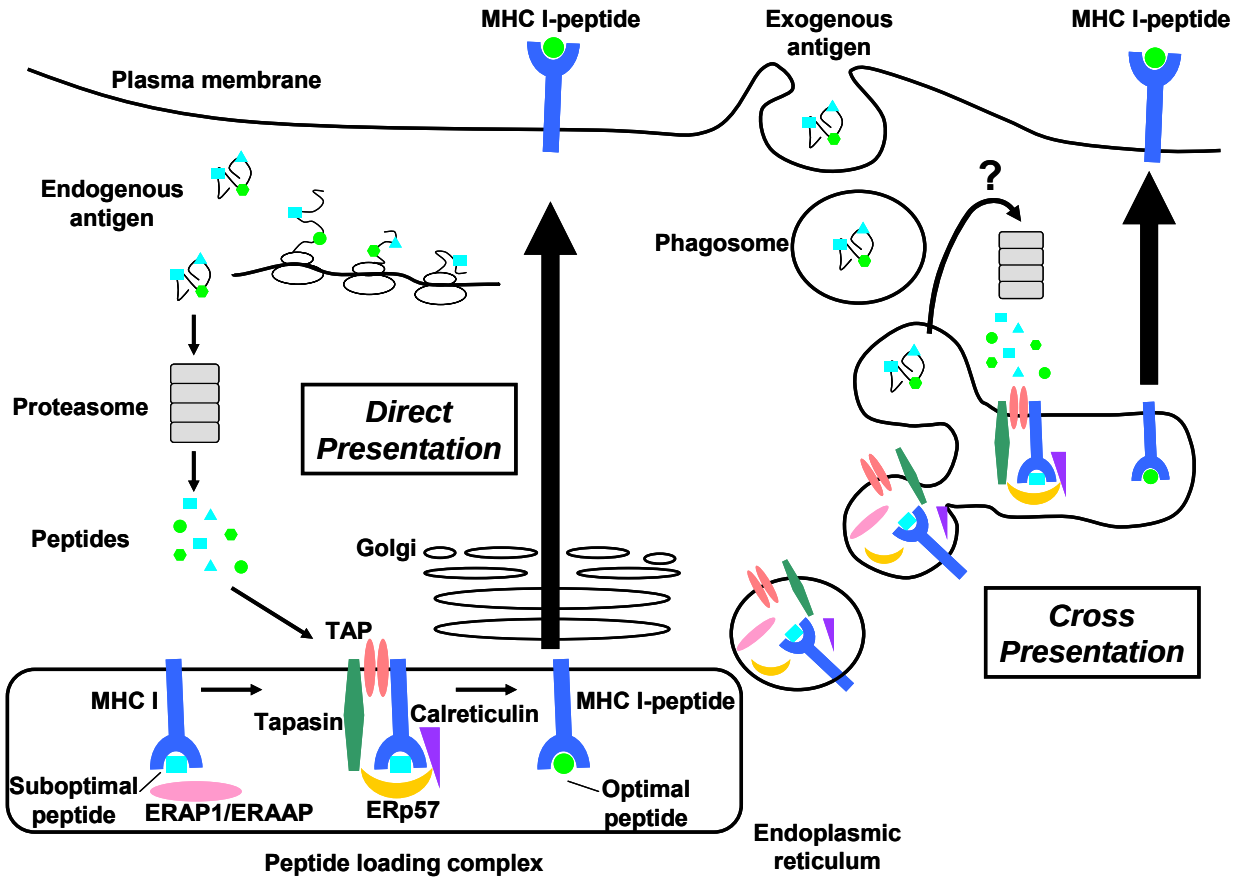

Figure 4. Major histocompatibility complex (MHC) class I presentation pathways.
In the direct presentation pathway, endogenous proteins are degraded by the proteasome into peptides within the cell cytosol, which are then transported by the TAP complex into the endoplasmic reticulum (ER) for loading into 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 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. PLC: peptide loading complex.

Both the direct- and cross-presentation pathways rely on the cleavage of polypeptides by the proteasome [210-212]. The subunit composition of the constitutive proteasome varies in different tissues [213]. 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 (high activity of cleavage on the C-terminal side of hydrophobic or basic residues) as well as a different cleavage rate. The immunoproteasome generally favors the production of MHC-binding peptides and its activity serves to increase the efficiency of antigen presentation in cells, since the C-terminal anchor residues of antigenic peptides are, in most cases, either hydrophobic or basic amino acids [214-216]. Depending on the specific protein substrate, however, the activity of the immunoproteasome can either negatively or positively influence antigenic peptide production. Therefore, the repertoire of peptides presented at the cell surface in MHC class I complexes depends on the type of proteasome active within that cell's cytoplasm. Thus, the preferential presentation of certain peptides in APC versus cancer cells in the absence of inflammation is only partially overlapping and CTL crossprimed by DC (that have acquired tumor proteins) may fail to recognize tumor cells in certain instances [217] .

1.8.3. Tumor Antigens

Tumor specific antigens (TSA) are antigens expressed endogenously only in cancer cells, which typically result from the inherent genetic instability of cancer cells (i.e. they derive from mutated proteins or proteins that result from chromosomal translocations, etc.). TSAs are ideal targets for cancer immunotherapy because they are exclusively expressed by the cancer cell but not by non-malignant tissues, thereby minimizing the risk of autoimmune destruction of normal cells as a result of antigen-specific immunotherapy [218]. Due to their unique mechanisms of expression, tumor-specific antigens are, however, uniquely expressed in a given patients' cancer cells or in rare cohorts of patients with a given type of cancer. Treatments based on the targeting of TSA represent customized therapies that have been traditionally viewed as too costly and time-consuming to be adopted as a standard of care [218, 219].

Tumor-associated antigens (TAA) are generally normal cellular proteins, which due to changes in epigenetic control, are expressed in cancer cells and by only a limited range of normal tissues. TAAs can be subdivided into four major categories according to patterns of expression [218]. Oncospermatogonal antigens (cancer-testis antigens) are expressed by cancer cells but are normally found on spermatocytes/spermatogonia (i.e. MAGE, GAGE, BAGE and NY-ESO-1). Cancer Differentiation antigens are molecules expressed on non-malignant cells of the same cell lineage as the tumor (i.e. TRP-1, gp100, MART-1, tyrosinase, CD20 and EpCAM). Oncofetal antigens are antigens found on embryonic and fetal tissues as well as certain cancers (i.e. alpha fetoprotein, carcinoembryonic antigen CEA and 5T4). Over-expressed antigens are normal proteins whose expression is up-regulated in cancer cells (i.e. PSA, wild-type p53, EphA2, Her2/neu and EGFR). Many TAAs have been identified and investigated for their use in vaccines, however, such vaccines many times have proven imperfect in breaking operational tolerance that exists against "self (tumor) antigens".

In addition to those antigens expressed on tumor cells themselves (i.e. TSA and TAA), it has also become feasible to consider the immune targeting of antigens that may be quantitatively or qualitatively expressed in a differential manner by cells that make up the tumor stroma. The vascular endothelia, supporting fibroblasts, and infiltrating leukocytes comprising the stroma are uniquely conditioned in the hypoxic tumor microenvironment, and contribute to tumor growth and invasion. Recent studies suggest that vaccines targeting antigens expressed by such non-cancerous supportive tissue (i.e. VEGF, VEGF receptor 2, fibroblast activating protein, HBB) can effectively eliminate established tumors [220-222], even in cases where tumor cells cannot be directly recognized by host T cells.

1.8.4. $CD8^+$ Cytotoxic T cells Specific for Tumor Antigens

Despite aberrant expression of TAAs in tumor cells, many of these proteins are also expressed at some level in non-malignant peripheral adult tissues. Because of this, the immune system may recognize TAAs as self-antigens and limit the T cell immune response through physiological tolerance mechanisms, both recessively and dominantly (via regulatory T cells).

Recessive tolerance, operating at several levels, deletes or functionally inactivates self antigen-reactive T cells. In the thymus, the ectopic expression of many, otherwise tissue-restricted, self antigens leads to clonal deletion, limiting the escape of the high-avidity, naïve T cell repertoire to the periphery [223]. Naïve T cell escapees may subsequently be deleted in peripheral lymph nodes following encounter with their cognate antigen [224, 225]. If the self antigen is not adequately presented by cells in the periphery, T cells escaping all these processes exist in an "ignorant" status. Thus, the naïve T cell repertoire may persist if cognate antigens are poorly processed and presented by professional APC, and if cognate antigens (i.e. antigenic peptides) have a low-intermediate affinity for MHC presenting molecules.

Due to imperfections in tolerance mechanisms, even though most TAA-specific T cells have been reported to have moderate-to-low functional avidity, they can be commonly found in normal donors and cancer patients. For example, MART-$1_{26–35}$-HLA-A2.1 multimer^{+} CD8^{+} T cells are detectable in most healthy HLA-A2 donors [226]. HLA-A2^{+} breast cancer patient and healthy donors were shown to possess CD8^{+} T cells specific for at least one of MUC-1, Her2/neu, carcinoembryonic antigen, NY-ESO-1, or SSX-2 derived peptides [227, 228]. In addition to Her2/neu, CD8^{+} T cells specific for other RTKs such as EphA2, EGFR and c-Met, have also been identified in the peripheral blood of normal donors and patients with cancer [8, 130, 229, 230]. Such circulating, anti-TAA CD8^{+} T cells appear to represent recent thymic emigrants [231].

1.9 Approaches to Increase RTK-derived Epitope Presentation by Tumor Cell MHC Class I Complexes

Interferons have been used extensively in the clinic, in hopes of improving tumor cell antigen presentation via the up-regulation of its antigen processing machinery (APM). However, CD8^{+} T cell recognition of tumor cells *in vivo* is reported to be only modestly increased as a consequence of treatment with interferons, and moreover, interferons do not increase specific

antigen presentation in a selective manner. 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 (Figure 5). 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 shunting 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.

1.9.1. RTK Agonists and PTP Inhibition

As described in sections 1.1 and 1.3.1, 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 de-phosphorylated 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 [15]. 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* [232-234]. In addition to anti-Her2/neu

antibodies, many antibodies targeting various RTKs have been investigated in tumor models, however, their abilities to increase RTK-derived peptide presentation in MHC class I complexes have not been studied.

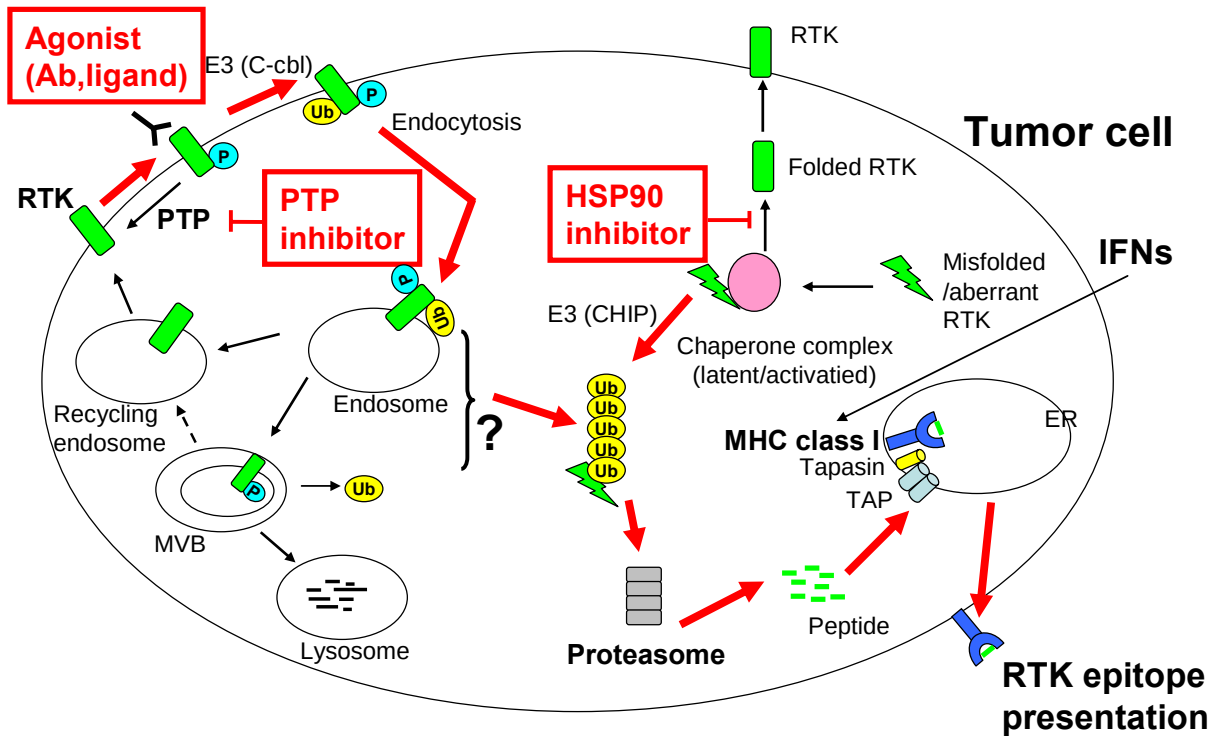

Figure 5. Strategies to increase RTK-derived epitope presentation in tumor cell MHC class I complexes.
Three possible reagent groups, that allow for biased improvement in tumor cell (MHC class I) presentation of RTK-derived peptide epitopes, are depicted; RTKs agonists, PTP inhibitors and HSP90 inhibitors. RTKs agonists and PTP inhibitors promote RTK internalization and subsequent proteasomal degradation, while HSP90 inhibitors prevent the salvage of improperly folded RTK proteins by inhibiting chaperone function, leading to the shunting 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.

1.9.2. HSP90 Inhibition

Another 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 selection process of whether a misfolded "client" protein is recycled or degraded in cells. HSP90 is a constitutively expressed molecule that guides the normal folding, intracellular disposition and proteolytic turnover of its client proteins (including RTKs, such as Her2/neu and EGFR) [235-238]. Furthermore the increased expression of HSP90 by tumor cells has been observed in many tumor types and is presumed to allow for mutant/mal-folded client proteins to be retained, while permitting tumor cells to better survive the pathologic consequences of imbalanced signaling pathways [238-241]. Therefore when HSP90 function is inhibited, we hypothesized that overexpressed and misfolded proteins are delivered to the proteasome, degraded, and presented by MHC class I through the direct presentation pathway.

1.9.2.1. HSP90

HSP90 is one of the most abundant molecular chaperones that regulate folding, maturation, stabilization, and renaturation of proteins [237, 238]. Unlike other heat shock protein family members, which are involved in the general maintenance of protein folding, Hsp90 only interacts with a set of proteins, called client proteins (see http://www.picard.ch/downloads/Hsp90interactors.pdf).

Multiple HSP90 client proteins are overexpressed in cancer cells, and involved in six hallmarks of cancer [242] mediating 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. Indeed it has become apparent that chaperones can serve as biochemical buffers at the phenotypic level for the genetic instability that is characteristic of many human cancers. HSP90 thus allows tumor cells to tolerate the mutation of multiple critical signaling molecules that would otherwise be lethal.

The HSP90 chaperoning cycle is a dynamic process in which client proteins bind to HSP90 in an intermediate complex containing the co-chaperones Hsp70, Hsp40, Hip and Hop [243, 244]. Upon ATP binding and hydrolysis, HSP90 forms a mature complex, containing p23, p50/cdc37 and immunophilins (IP), which catalyzes the conformational maturation of HSP90 client proteins. Association of HSP90 with client proteins is regulated by the activity of the N-terminal ATPase domain, which binds and hydrolyses ATP to mediate a series of association-dissociation cycles between HSP90 and client substrates. Interestingly HSP90 exists in an equilibrium between an 'activated state' prevalent in transformed cells and a 'latent state' predominant in normal cells. In activated state HSP90 is exclusively complexed with co-chaperones in a state of high affinity for ATP/ADP, whereas HSP90 exist primarily in uncomplexed, low affinity form in the latent state.

1.9.2.2 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 perturbing the C-terminal domain of HSP90 [238, 245].

The N-terminal region of HSP90 contains a regulatory pocket that binds and hydrolyzes ATP. N-terminal domain targeted inhibitors, therefore, impact ATP binding and hydrolysis, thereby locking HSP90 in an intermediate complex. Bound client proteins may then be more readily (poly)ubiquitinated and shunted to the proteasome for degradation. Drugs in this category include ansamycins (e.g. Geldanamycin; GA) and its derivatives (17-AAG, 17-DMAG), Radicicol, purine-scaffold derivatives, Pyrazole (CCT018159). GA, a first generation drug, was found to promote severe (liver) toxicity, while second generation inhibitors, such as 17-AAG, was far better tolerated in phase I/II clinical trials. 17-DMAG, one of the newest generations of HSP90 inhibitors based on geldanamycin, is water-soluble, far less toxic and capable of being provided via oral administration. 17-DMAG, however, retains dose-limiting toxicity in gastrointestine, kidney, gallbladder and bone marrow as reported in preclinical experiments

using rats and dogs [246], and has exhibited limited efficacy against tumor progression in mice at well-tolerated doses [247]. Therefore enhancing the therapeutic efficacy of 17-DMAG through combinational applications with alternate treatment modalities will be required to optimize its "clinical" potential. This is consistent with a widely-accepted concept in the field of cancer therapy which supports the necessity of combinational therapies to affect cures [3, 178, 179]. Both 17-AAG and 17-DMAG are currently being evaluated in Phase I/II clinical trials as cancer therapeutic agents at the University of Pittsburgh and elsewhere ([248, 249]; Dr. Merrill Egorin, personal communication).

N-terminal binders have potential to also interact with additional HSP90 family member proteins in tumor cells, since the N-terminal ATP-binding site is highly-conserved among HSP90 family proteins (HSP90, gp96 and Trap1). While Ge *et al.* reported that 17-AAG and 17-DMAG have comparable affinities for human HSP90 and canine gp96 [250], Chandarlapaty *et al.* found that GA, 17-AAG and 17-DMAG exhibit (5-7X) higher affinity to human HSP90 versus human gp96 [251]. The binding of GA to gp96 requires a rearrangement of the Gly hinge and leads to a conformational change in gp96, while the binding of GA to HSP90 does not require any substantive structural changes, suggesting a possible explanation for the superior binding capacity of GA and its derivatives to HSP90 versus gp96 [252].

The contribution of the C-terminal domain region to HSP90 function remains unknown, however, it has been hypothesized that this domain might regulate the ATPase activity of the N-terminal region, and therefore, the cycling of the HSP90 machinery. C-terminal domain targeted inhibitors include chemotherapeutic agents such as Novobiocin and Cisplatin.

Interestingly HSP90 in normal cells (uncomplexed form) has a low-affinity for HSP90 inhibitors, resulting in the poor accumulation of HSP90 inhibitors and poor drug sensitivity in normal tissues [245]. On the contrary, complexed HSP90, as observed in cancer cells, exhibits high-affinity binding to HSP90 inhibitors. Thus, tumor tissues tend to selectively accumulate HSP90 inhibitors and exhibit significant sensitivity to drug action in vivo. Indeed, it has been reported that HSP90 derived from tumor cells binds to 17-AAG up to 100 times more tightly than does HSP90 isolated from normal cells [253].

Scope of This Thesis

The studies performed in my thesis were designed to identify strategies that would allow an existing immune system to better recognize tumor cells in order to define a novel immunotherapy approach. In particular, I have focused on means to improve tumor cell MHC presentation of peptides derived from a class of tumor-associated antigens, receptor tyrosine kinases (RTK), allowing for the improved reactivity of specific cytotoxic T cells. Such T cells have been intimately linked to the ability of the host to resist tumor development and progression, and when activated as a consequence of therapy, they may effectively mediate complete tumor regression *in vivo*. My findings suggest that treatment of tumor cells with RTK agonists and/or HSP90 inhibitors leads to enhanced immune recognition *in vitro* and *in vivo*, via a mechanism that enforces the generation of, and MHC class I loading with, RTK-derived peptides. Such conditional manipulation of tumor cells allows for the design of "pulse" therapies in which anti-RTK $CD8^+$ T cells may be activated by specific vaccination or be adoptively transferred along with the administration of RTK agonists and/or HSP90 inhibitors to optimize T cell recognition of cancer cells in situ, and thereby the clinical benefit associated with such cytotoxic cells.

Preface to Chapter 2

My goal was to determine whether EphA2 agonists (ephrin-A1-Fc and anti-EphA2 mAb), which promote the internalization and degradation of this RTK on/in tumor cells, result in the improved recognition of treated tumor cells by anti-EphA2 $CD8^+$ T cells. Western blot analyses were performed to monitor EphA2 phosphorylation and degradation status, while ELISPOT/ELISA analyses, as well as, ^{51}Cr-release assays were used to assess differential T cell responses against agonist-treated versus control tumor cells *in vitro*. The efficacy of combinational therapy *in vivo* was tested in a Hu-SCID tumor model. We found that both agonists promote EphA2 autophosphorylation, rapid internalization and proteasomal degradation/processing, with a corollary improvement observed in tumor recognition by moderate-to-low functional avidity anti-EphA2 $CD8^+$ T cells *in vitro* and *in vivo*. Notably, $CD8^+$ T cells recognizing peptide epitopes derived from the extracellular (i.e. $EphA2_{58\text{-}66}$) or the intracellular (i.e. $EphA2_{883\text{-}891}$) domains of the EphA2 protein exhibited higher IFN-γ production in response to agonist-treated versus control tumor cells. This suggests that both domains of this transmembrane protein are driven into the proteasomal processing and MHC class I presentation pathways. Of perhaps greatest importance, the adoptive anti-EphA2 $CD8^+$ T cell therapy was only able to promote complete tumor regression in concert with the co-provision of EphA2 agonists *in vivo*.

2. Enhancement in Specific CD8$^+$ T Cell Recognition of EphA2$^+$ Tumors after Treatment with Ligand Agonists

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These data have been reported in *Journal of Immunology 181:* 7721-7727, 2008. Western blot analyses and Flow cytometry analyses in Figures 6, 7A and 9A were performed by C.J. Herrem, C. Vasquez and M. Kawabe. Generation of SLR20.A2 cell line used in Figures 7B, 7C, 8C, 8D, 9B was performed by M. Kawabe. Generation of T cell bulk and clone used in Figures 7B, 7C, 8, 9B was performed by A.K. Wesa and M Mandic. ELISPOT analyses in Figures 7B, 7C, 8, 9B were performed by A.K. Wesa, M. Mandic and M. Kawabe. Cr^{51} release assay (Figure 10) and Hu-SCID mice experiments (Figure 11 and Table 2) were performed by E. Bruckheimer and M.S. Kinch at MedImmune.

2.1. ABSTRACT

EphA2, a member of the receptor tyrosine kinase (RTK) family, is commonly expressed by a broad range of cancer types, where its level of (over)expression correlates with poor clinical outcome. We and others have shown that EphA2 contains multiple peptide epitopes that can be recognized by effector $CD4^+$ and $CD8^+$ T cells isolated from tumor-bearing patients. Specific $CD8^+$ T cells, however, typically exhibit only moderate-to-low functional avidity (given "tolerance" against the "self" antigen EphA2), rendering them marginally competent to recognize $EphA2^+$ tumor cells *in vitro* or *in vivo.* We hypothesized that conditional promotion of tumor cell EphA2 degradation would lead to the selective enhancement in EphA2 peptide presentation by MHC class I, rendering tumor cells better targets for recognition by previously inept anti-EphA2 T cells. In this chapter, agonistic mAbs and ligand (ephrinA1)-Fc fusion protein were selected as agonists to treat tumor cells. We show that these agonists trigger the proteasome-dependent degradation of EphA2 and improve EphA2-derived peptide presentation by MHC class I on the tumor cell surface *in vitro* (as manifested by increased recognition by EphA2-specific $CD8^+$ T cells). Notably, peptides derived from both the extracellular and intracellular domains of EphA2 appear to be better presented after agonist treatment, supporting the effective enzymatic processing of the full-length transmembrane protein. These results suggest that strategies targeting the conditional proteasome-mediated destruction of tumor cell EphA2 may be effective in improving the anti-tumor efficacy of anti-EphA2 $CD8^+$ T effector cells. We also observed that single administration of ephrinA1-Fc fusion protein or agonistic mAb into $EphA2^+$ tumor lesions failed to halt tumor progression in a humanized SCID model, however, when combined with the adoptive transfer of modestly therapeutic (human) anti-EphA2 $CD8^+$ CTL, complete tumor eradication was attained. These results support a novel combinational therapy strategy to improve the therapeutic benefits of T cell-based immunotherapies.

2.2. INTRODUCTION

EphA2, a member of the RTK family of molecules, is a 130kDa (Type I) glycoprotein that mediates intercellular interactions via binding to its natural ligands Ephrin-A1, -A3, -A4 and -A5 expressed on an opposing cell surface [51]. This RTK is expressed at low levels on a broad range of epithelial tissues in normal adults, including lung, spleen, kidney and liver [63], where it is primarily localized to sites of cell-to-cell contact and plays a role in contact inhibition of cell growth/migration that is critical for the organization and formation of epithelial layers in EphA2$^+$ tissues [48]. In addition to epithelial cells, activated endothelial cells also express EphA2 in association with tissue neovascularization in adults [49].

In contrast to non-transformed cells, EphA2 is commonly overexpressed in a range of cancer types, including melanoma and many carcinomas [4, 7, 8, 18, 49, 99, 107, 254], where it serves as an oncoprotein and a facilitator of metastasis [48, 62]. Clinical observations suggest that the level of EphA2 overexpression by tumor cells is an indicator of poor prognosis, since it has been linked to reduced time to disease recurrence, and enhanced disease progression and metastatic spread [7, 17, 18, 48, 62, 64].

As a consequence, EphA2 represents an attractive target for therapeutic intervention in the majority of patients with solid tumors, with several treatment strategies considered for translation into the clinic. In particular, a class of agonistic EphA2 Abs and Ephrin-A1 Fc have been developed that can induce EphA2 internalization and degradation, thereby reducing expression of this powerful oncoprotein. Ephrin-A1 Fc is a dimerized Ephrin-A1 fused to human immunoglobulin G (IgG) Fc having increased agonistic function and stability. Repeated administration of these reagents has proven successful in inhibiting tumor cell growth in both *in vitro* and *in vivo* models, as well as, enhancing the survival of tumor-bearing mice [66, 119-121]. Another potent strategy to target EphA2$^+$ tumor is dendritic cell based vaccine. Immunogenic human and murine EphA2 derived peptides have recently identified by us and others [8, 130, 132], and dendritic cell-based vaccines incorporating mEphA2 peptides have been reported to promote protective T cell responses in murine melanoma and colon cancer models [63, 255]. Since EphA2-specific CD8$^+$ T cells have been detected in the peripheral blood of patients with

renal cell carcinoma (RCC, [8]), prostate carcinoma [130] or glioma [132], the frequencies of these protective T cells would be anticipated to be augmented as a consequence of active vaccination [63, 255].

Those CTLs are, however, expected to be moderate-to-low avidity, given the fact that EphA2 is a non-mutated, ”self” antigen that would be predicted to induce central and peripheral tolerance mechanisms [256, 257]. Moderate-to-low avidity T cells typically fail to recognize target cells, including tumor cells, because tumor cells often exhibit defects in their antigen presentation machinery (APM), which can lead to their escape from immune surveillance [150, 151]. Despite EphA2 protein overexpression in many cancers, such defects may preclude the effective processing and loading of a sufficient number of tumor MHC complexes to allow for the activation of specific T cells. In this light, interferons have been used clinically hoping to improve the general ability of antigen presentation via its well-known up-regulating effects on many components of the APM. However, MHC class I-restricted T cell responses are typically only modestly induced by interferons [258], and moreover, interferons fail to selectively increase the processing and MHC class I presentation of peptides derived from a particular antigen. Hence, the clinical expectation for single modality vaccines based on EphA2 is that this approach would fail to be optimally efficacious, however, combinational therapies that sensitize $EphA2^+$ tumors for specific $CD8^+$ T cell eradication might yield enhanced clinical benefits [3].

Therefore, I was interested in evaluating agents that had the potential to alter the life cycle of tumor cell EphA2 protein, particularly with regard to its ultimate degradation via the proteasome. Classically, endocytosed membrane receptors (such as EphA2) expressed by non-professional APC have been believed to be exclusively degraded in mature lysosomes, however, recent studies have revealed that such receptors may also gain access to the proteasome [15]. I, therefore, selected the ephrin-A1 Fc and agonistic anti-EphA2 mAb208 reagents to trigger the internalization of tumor cell EphA2 protein. Herein, I investigated whether the treatment of $EphA2^+$ human tumor cells with these specific agonists would induce the proteasome-dependent degradation of EphA2 protein and the consequent presentation of its derivative peptides on tumor cell MHC class I complexes, thus allowing for improved recognition by anti-EphA2 $CD8^+$ T cells *in vitro* and *in vivo*.

2.3. MATERIALS AND METHODS

2.3.1. Cell lines and Media

SLR20 ($EphA2^{+}$, $HLA\text{-}A2^{neg}$; [8]), SLR24 ($EphA2^{+}$, $HLA\text{-}A2^{+}$; [8]), SLR20.A2 ($EphA2^{+}$, $HLA\text{-}A2^{+}$) and T2 ($EphA2^{neg}$, $HLA\text{-}A2^{+}$) cell lines were used as peptide presenting cell in ELISPOT and ELISA assays. SLR20.A2 cell line was established via transduction of SLR20 with a recombinant retrovirus encoding HLA-A2.1 (provided by Dr. Peter Cresswell, Yale University), and selection by puromycin. SLR20.A2 and SLR24 were tested for their EphA2 protein expression under various conditions, while PC-3 prostate carcinoma cell line served EphA2 positive control. All cell lines were free of mycoplasma contamination and maintained in RPMI-1640 media supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 10 mM L-glutamine (all reagents from Invitrogen, Carlsbad, CA, unless otherwise indicated).

2.3.2. Peptides

HLA-A2 presented EphA2 peptides, $EphA2_{58\text{-}66}$ (IMNDMPIYM; [130]) and $EphA2_{883\text{-}891}$ (TLADFDPRV; [8]), as well as HIV-$nef_{180\text{-}189}$ (VLEWRFDSRL) were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry by University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Facility, as previously described [8]. The purity of peptides was >96% based on high performance liquid chromatography, with peptide identity validated by mass spectrometric (MS/MS) analyses performed by the UPCI Protein Sequencing Facility (a Shared Resource).

2.3.3. Mice

Six- to 8-wk-old female C.B-17 *scid*/*scid* mice were obtained from Taconic and maintained in microisolator cages. Animals were handled under aseptic conditions as per an Institutional Animal Care and Use Committee-approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

2.3.4. EphA2 agonists

EphrinA1-Fc (R&D Systems) is a chimeric protein consisting of the ligand binding domain of the EphA2 ligand ephrinA1 fused with the Fc portion of a human IgG Ab. mAb208 (kindly provided by MedImmune) is a mouse IgG mAb specific for hEphA2 [100]. EphrinB1-Fc (Sigma-Aldrich) and MOPC21 mAb (mouse IgG; Sigma-Aldrich) were used as specificity controls for ephrinA1-Fc and mAb208, respectively.

2.3.5. Western Blot Analyses

SLR20.A2 and SLR24 cell lines at 80-90% confluency were incubated with 10μg/ml of EphA2 agonists (ephrinA1-Fc or mAb208) or their controls in RPMI-1640 media for 24-48h, as indicated in text. Harvested cells were then incubated with lysis buffer (1% TritonX-100, 150 mM NaCl, 10 mM Tris-HCl pH7.4, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% NP-40 in PBS; all reagents from Sigma-Aldrich) containing a protease inhibitor cocktail (Complete mini, Roche Diagnostic, Mannheim, Germany) for 30 minutes at 4^{o}C. Lysates were cleared by centrifugation at 13,500 x g for 10 min, and proteins in the lysate resolved by SDS-PAGE before electro-blotting onto PVDF membranes (Millipore, Bedford, MA). Polyclonal rabbit anti-EphA2 Ab (C-20) or polyclonal rabbit anti-EGFR (sc-03) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody as secondary antibody were used to detect EphA2 or EGFR (all from Santa Cruz Biotechnology, Santa Cruz, CA). Mouse anti-β-actin Ab (clone AC-15, Abcam) and goat anti-mouse mAb (BioRad, CA) were used to detect β-actin as a loading control. Probed proteins were visualized by Western Lighting^{TM} chemiluminescence detection kit (Perkin Elmer, Boston, MA) and exposed to X-Omat film (Eastman Kodak, Rochester, NY) for 1-5 min. Immunoprecipitations for EphA2 were performed using the anti-EphA2 Ab D7 (Millipore). Anti-phosphotyrosine Ab (clone py99, Santa Cruz Biotechnology) was then used to assess pEphA2 content.

2.3.6. Flow Cytometry

For phenotypic analyses of control or ligand agonist-treated tumor cells, PE- or FITC-conjugated mAbs against total HLA class I complexes (W6/32; pan-class I specific; Serotec), HLA-A2 complexes (BB7.2 and MA2.1; American Type Culture Collection), or empty HLA-A2 molecules (HC-A2, [259], the kind gift of Dr. H. Ploegh, Massachusetts Institute of Technology) and appropriate isotype controls (purchased from BD Biosciences) were used, and flow cytometric analyses were performed using a FACScan (BD Biosciences) flow cytometer. Cell surface expression of EphA2 protein was analyzed using direct immunofluorescence staining monitored by flow cytometry. After treatment for 0–24 h at 37°C with 10 μg/ml ephrinA1-Fc, ephrinB1-Fc, mAb208, or the MOPC21 mAb, tumor cells were stained for 30 min at 4°C with FITC-conjugated anti-EphA2 mAb B2D6 (Millipore; note that this mAb is not sterically inhibited by the binding of ephrinA1-Fc or mAb208 to EphA2, data not shown) before washing with PBS and analysis by flow cytometry. The results of these assays are reported as percentage control (untreated) tumor cell expression based on a comparison of arbitrary mean fluorescence intensity (MFI) units obtained for experimental versus control specimens.

2.3.7. Proteasome Dependency Assessment

The impact of proteasome inhibition in Western blot (evaluating agonist-induced EphA2 degradation in tumor cells) and T cell ELISPOT (evaluating enhanced anti-EphA2 T cell recognition of agonist-treated tumor cells) assays was assessed by treatment of tumor cells with either MG-132 (Sigma-Aldrich) or clasto-lactacystin β-lactone (hereafter designated as lactacystin; BIOMOL International), as outlined in relevant text and figure legends.

2.3.8. T cell lines and Clones

Bulk $CD8^+$ T cell lines and clones specific for $EphA2_{58\text{-}66}$ or $EphA2_{883\text{-}891}$ were generated as previously described [8]. Briefly, mature dendritic cells (DC) were developed from peripheral blood mononuclear cells (PBMC; obtained with written consent under an IRB-approved protocol) isolated from normal HLA-$A2^+$ donors in 7 day cultures containing rhGM-CSF

(Sargramostim; Amgen, Thousand Oaks, CA) and rhIL-4 (Peprotech, Rocky Hill, NJ), then pulsed with either the $EphA2_{58-66}$ or $EphA2_{883-891}$ peptides (10 μM) for 4h at 37°C at 5% CO_2 tension. Autologous $CD8^+$ T cells were stimulated on a weekly basis with peptide-pulsed autologous DC for 3-4 cycles (at a T:DC ratio of 10:1) to generate a bulk population of peptide-specific $CD8^+$ T cells. These T cells were used in tumor recognition assays and for cloning via limiting dilution assays; [260]). T cells were maintained in IMDM media supplemented with 10% human AB serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM L-glutamine and MEM Non-essential amino acids (all reagents from Invitrogen, except human AB serum that was purchased from Sigma-Aldrich). After their initial isolation, T cell clones were expanded *in vitro* in IMDM media supplemented with 1μg/ml phytohemagglutinin (PHA; Sigma-Aldrich) and 200 U/ml rhIL-2 (Peprotech).

2.3.9. Tumor Recognition Assays

Tumor recognition by anti-EphA2 T cells was evaluated by IFN-γ ELISPOT assays as described before [8] or using a commercial hIFN-γ ELISA (BD-Biosciences). For both the ELISPOT and ELISA protocols, tumor cells were treated with 10 μg/ml anti-EphA2 mAb208 or control IgG (MOPC21 mAb) for 24-48h, prior to their harvest using Trypsin-EDTA (Invitrogen). After washing with PBS (Invitrogen), tumor cells were co-cultured with anti-EphA2 T cell lines/clones at an effector:target cell ratio of 1:1 for 24h at 37°C and 5% CO_2 tension. To assess the impact of proteasome function on EphA2 agonists-treated tumor cells, SLR20.A2 cells were pretreated with MG-132 (10 μM) or lactacystin (20 μM) for 3h prior to 24h treatment with EphA2 agonists (mAb208 or EphrinA1-Fc). After harvest, tumor cells were washed twice with PBS, prior to using these cells as targets for T cell recognition.

2.3.10. Cytotoxicity Assays

$CD8^+$ T cell clones E883 (anti-$EphA2_{883–891}$) and 2E4 (anti-HLA-A2) were evaluated for their capacity to lyse $EphA2^+$, HLA-$A2^+$ SLR24 tumor cells using standard 4-h ^{51}Cr-release assays, as previously described [261]. Briefly target cells (SLR24) were untreated or treated with either ephrinA1-Fc, ephrinB1-Fc or mAb208 for 24h, before radiolabeling with 100 μCi of Na_2-$^{51}CrO_4$

(New England Nuclear-DuPont, Bedford, MA) for 1 h at 37°C. Labeled target cells were washed with medium and co-cultured with either E883 or 2E4 T cell clone for 4h in 96 well round bottom plate, then ^{51}Cr released into culture supernatants was monitored by gamma counter. BB7.2 mAb was added to target cell 30min before cocluture for the HLA-A2 blocking cohort. Target cells incubated in medium alone or in medium containing 5% Triton X-100 (Sigma Chemical Co.) were used to determine spontaneous and maximum ^{51}Cr release, respectively. The percentage of specific ^{51}Cr release was calculated as 100 x (experimental release - spontaneous release) / (maximum release - spontaneous release).

2.3.11. Hu-SCID Tumor Model

1 x 10^6 SLR24 ($EphA2^+$, $HLA\text{-}A2^+$) RCC cells were s.c. injected to right flank of C.B-17 *scid/scid* mice and tumors were allowed to establish to a size of ~30 mm^2 (i.e., day 18 postinjection). The tumor-bearing mice were then randomized into groups (five animals per group) that received either no treatment, a single intratumoral injection of ephrinA1-Fc, ephrinB1-Fc, or mAb208 (all 50 µg in 50 µl saline) on day 18, a single tail vein injection with $CD8^+$ T cells (anti-$EphA2_{883–891}$ clone E883 or allospecific anti-HLA-A2 clone 2E4; 5 x 10^6 cells in 100 µl saline) on day 19, or the combined d18 (EphA2 agonist) plus d19 ($CD8^+$ T cell adoptive transfer) regimen. Animals were evaluated every 3–4 days for tumor size, with tumor-free status noted on day 44 post-tumor inoculation. For the Western blot analyses of EphA2 content in SLR24 tumor lesions, tumors pre- and postadministration of agonists or their controls were surgically resected from euthanized mice. Tumors were then digested into single-cell suspensions using a DNase, hyaluronidase, collagenase mixture (all reagents purchased from Sigma-Aldrich) as previously described [262], and filtered through Nitex mesh (Tetko) before generating lysates for Western blotting analyses, as outlined above.

2.3.12. Statistical Analyses

Two-tailed Student's t tests were used to evaluate the difference between groups, with p values < 0.05 considered significant.

2.4. RESULTS

2.4.1. EphA2 agonists induce the phosphorylation and proteasome-dependent degradation of EphA2 in tumor cells *in vitro*.

EphA2 is phosphorylated upon interaction with its ligands, then internalized and degraded following polyubiqutination. Previous studies have demonstrated that tumor cells exhibit unstable cell-cell contacts and that this impairs the ability of EphA2 to interact with its ephrinA1 ligand on neighboring cells [64, 120, 263], and that EphA2 protein in malignant cells is generally observed to be in a hypophosphorylated state [101]. Therefore EphA2 levels of RCC tumor cell lines, SLR20.A2 and SLR24, were analyzed along with their phosphorylation status after exposure to EphA2 ligands (ephrinA1-Fc and mAb208). High expression levels of EphA2 protein were observed in both renal cell carcinoma cell lines, with undetectable levels of p-EphA2. Treatment of tumor cells with agonists, however, induced rapid phosphorylation within 10 min, while total EphA2 levels were preserved (Figure 6, A and B). Treatment of the EphB1^{+} SLR20.A2 or SLR24 tumor cells with ephrinB1-Fc (a ligand for EphB1, but not EphA2) or control mIgG MOPC21 failed to induce EphA2 phosphorylation (Figure 6, A and B). Substantial EphA2 protein degradation was, however, observed after 24h treatment with agonists but not with their control ephrinB1-Fc or MOPC21 mAb (Figure 6C). Such degradation was EphA2-specific, as neither agonists altered expression levels of the control proteins EGFR (another cancer-related RTK) or β-actin (Figure 6C). A repeat of these experiments in the absence or presence of chloroquine or MG-132 suggested that EphA2 degradation in SLR20.A2 cells was predominantly 26S proteasome-dependent (Figure 6D). Flow cytometric analysis of agonist-treated SLR20.A2 tumor cells indicated that cell surface EphA2 is rapidly lost (i.e., internalized) within hours, preceding loss of total tumor cell EphA2 protein as analyzed by Western blotting, after treatment with ephrinA1-Fc and mAb208, but not with ephrinB1-Fc or MOPC21 mAb (Figure 6E).

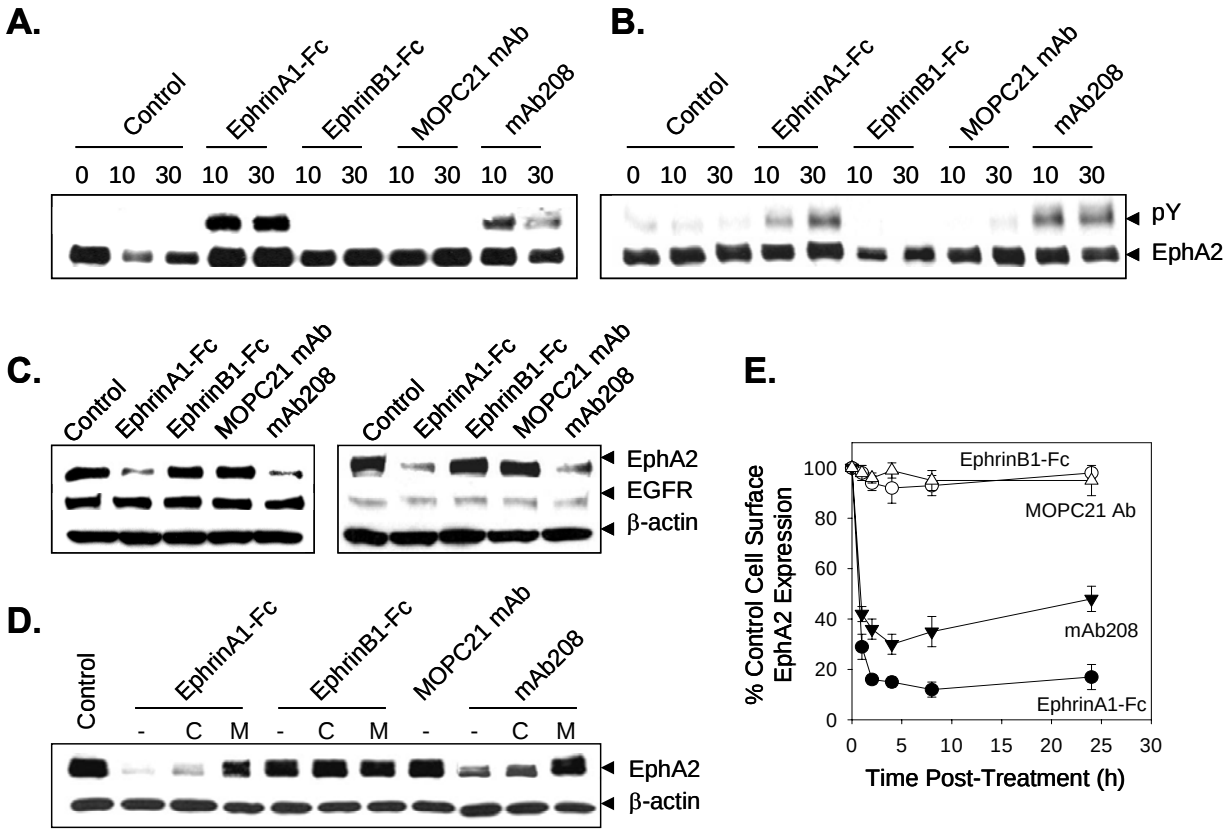

Figure 6. EphA2 agonists induce the phosphorylation and proteasome-dependent degradation of tumor cell EphA2 *in vitro*.

To determine whether agonist treatment promotes EphA2 phosphorylation, SLR20.A2 (**A**) and SLR24 (**B**) renal carcinoma cells (2–4 x 10^6) were left untreated or were treated for 10 or 30 min with ephrinA1-Fc, ephrinB1-Fc, MOPC21 mAb, or mAb208 (each at 10 μg/ml) at 37°C. Cellular lysates were resolved by SDS-PAGE, and EphA2 protein was immunoprecipitated using the anti-EphA2 Abs D7 in pull-down assays. Western blot analyses were then performed using anti-EphA2 and anti-phosphotyrosine Abs, respectively. To determine whether agonist treatment induces EphA2-specific degradation, SLR20.A2 (left panel of **C**) and SLR24 (right panel of **C**) tumor cells were treated as in **A** above for 24h with consequent cell lysates resolved by SDS-PAGE and Western blot analyses performed using polyclonal anti-EphA2 and control anti-β-actin Abs. Anti-EGFR Ab was used to image identically prepared lysates as a specificity (negative) control in these experiments. To assess the proteasome dependence of agonist-induced EphA2 degradation, MG-132 (50 μM) or chloroquine (100 μM) were also added to SLR20.A2 cell cultures, where indicated, 30 min before the addition of ephrin-Fc proteins or mAb208 (**D**). After 24h, cell lysates were generated and Western blot analyses were then performed using anti-EphA2 Abs and negative control anti-β-actin Abs. In **E**, the kinetics of EphA2 down-modulation on the surface of treated (with the indicated agents) SLR20.A2 cells was investigated by flow cytometry. Data are reported as percentage control EphA2 cell surface expression (versus untreated cells) based on MFI values obtained. All data are representative of three independent experiments performed. Data in Figure 6 were generated by C.J. Herrem, C. Vasquez and M. Kawabe.

2.4.2. Anti-EphA2 mAb208 sensitizes the EphA2$^+$, HLA-A2$^+$ tumor cell line SLR20.A2 to recognition by anti-EphA2 CD8$^+$ T cells *in vitro*.

Since agonistic Abs triggered the proteasomal destruction of EphA2, we hypothesized that this could preferentially increase presentation of EphA2 peptides in tumor cell surface MHC class I complexes. Such a change would be expected to be tied directly to the ability of EphA2-specific CD8$^+$ T cells to recognize agonist-treated tumor cells. To address this question, EphA2$^+$ tumor cell lines were incubated with mAb208 for 24 or 48 h before evaluating the ability of these target cells to be recognized by HLA-A2-restricted CD8$^+$ T cell lines and clones specific for the EphA2$_{58–66}$ (located in EphA2 extracellular domain) or EphA2$_{883–891}$ (located in the EphA2 intracellular domain) peptide epitopes. Rather than assessing differential tumor sensitivity to T cell killing (which could involve changes in both T cell and tumor cell functions induced by agonists), we instead chose to more directly interrogate changes in T cell functional recognition of treated tumor cells using the IFNγ ELISPOT assay as readout for effector T cell reactivity.

Pretreatment of SLR20.A2 (EphA2$^+$, HLA-A2$^+$, Figure 7A) tumor cells with mAb208 significantly enhanced their recognition by both anti-EphA2 CD8$^+$ T cell lines (Figure 7, B and C) and moderate avidity (Figure 8, A and B) CD8$^+$ T cell clones 15/9 and 3C1 (Figure 8, C and D, respectively). Notably, improved T cell recognition of treated tumor cells was sustained for a period of at least 48 h (Figure 7, B and C, Figure 8, C and D). SLR20 (EphA2$^+$, HLA-A2^{neg}) tumor cells failed to be recognized by any of these T cell populations, even after treatment with mAb208 (that promotes EphA2 degradation of SLR20, data not shown). Furthermore, treatment of SLR20.A2 cells with control IgG (MOPC21 mAb) failed to enhance tumor cell recognition by anti-EphA2 CD8$^+$ T cells (Figure 7, B and C, Figure 8, C and D).

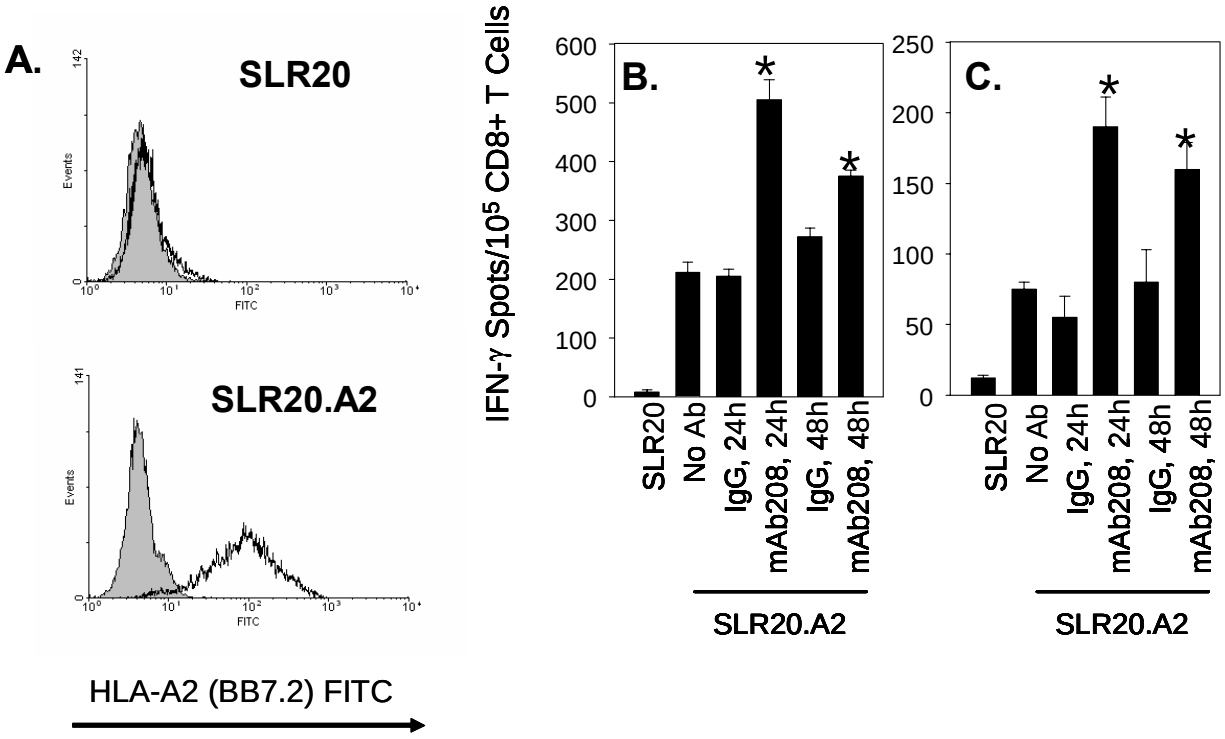

Figure 7. Anti-EphA2 mAb208 sensitizes the EphA2^+, HLA-A2^+ tumor cell line SLR20.A2 to recognition by anti-EphA2 CD8^+ bulk T cells *in vitro*.

A. SLR20.A2 tumor cells were analyzed for expression of cell surface HLA-A2 and by flow cytometry using specific mAbs (open profiles) versus isotype control mAbs (filled profiles). Bulk $CD8^+$ T cell lines were developed from HLA-$A2^+$ normal donors against the $EphA2_{58–66}$ (**B**) and $EphA2_{883–891}$ (**C**) peptides, as described in *Materials and Methods*, and evaluated for their differential recognition of SLR20 (EphA2^+, HLA-$A2^{neg}$) and SLR20.A2 (EphA2^+, HLA-$A2^+$) tumor cell targets using IFNγ ELISPOT assays. SLR20.A2 cells were pretreated with no Ab, control IgG (MOPC21 mAb), or mAb208 (10 µg/ml each) for 24 or 48h, as indicated, before ELISPOT analyses. Data are reported as mean IFNγ-specific spots/10^5 $CD8^+$ T cells ± SD from triplicate determinations. SLR20.A2 cell line was generated by M. Kawabe, T cell bulks and clones were generated by A.K. Wesa and M Mandic, ELISPOT analyses were performed by A.K. Wesa, M. Mandic and M. Kawabe.

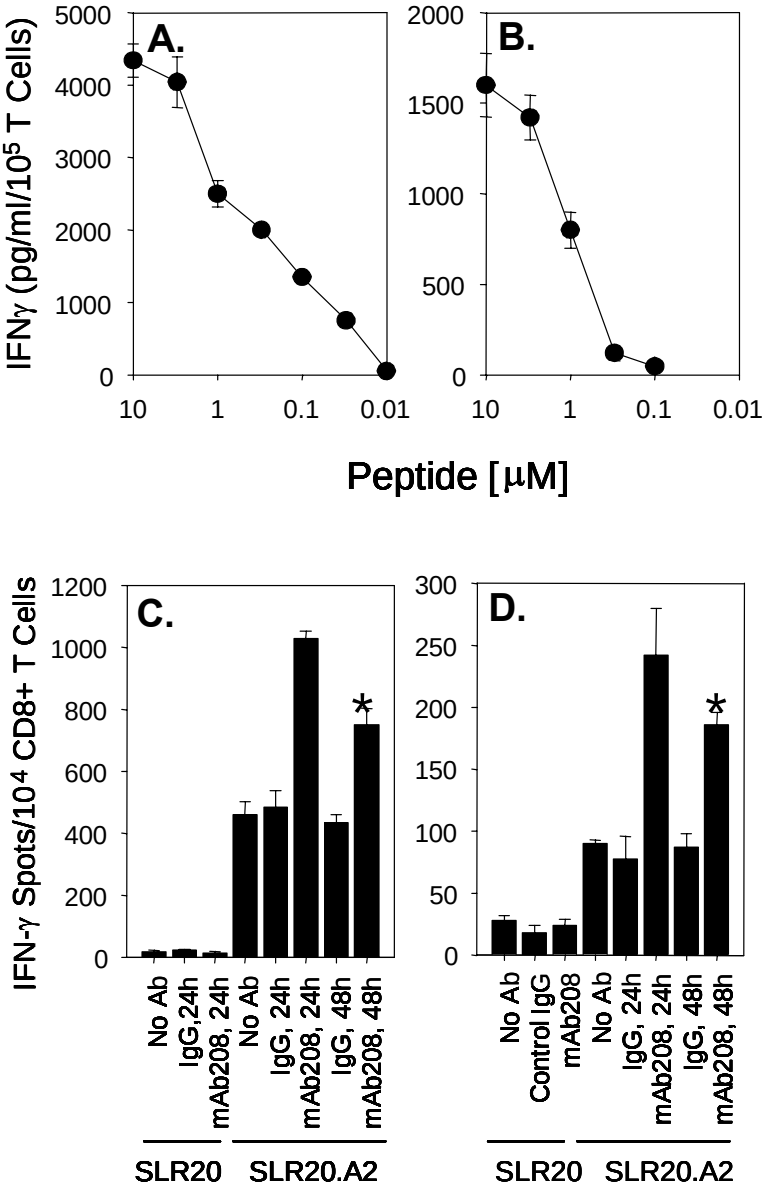

Figure 8. Anti-EphA2 mAb208 sensitizes the EphA2$^+$, HLA-A2$^+$ tumor cell line SLR20.A2 to recognition by anti-EphA2 CD8$^+$ clone T cells *in vitro*.
The HLA-A2-restricted CD8$^+$ T cell clones 15/9 (specific for EphA2$_{58–66}$) (**A**) and 3C1 (specific for EphA2$_{883–891}$) (**B**) were developed, as described in *Materials and Methods*, and exhibited moderate to low functional avidity against specific peptide-pulsed T2 cells (EC_{50} ~1 μM peptide for both clones) as assessed by IFNγ ELISA (mean ± SD from triplicate determinations). These clones recognize SLR20.A2 tumor cells selectively in IFNγ ELISPOT assays, with recognition increased at 24 and 48h by pretreatment of tumor cells with mAb208, but not with control IgG. Data are reported as mean IFNγ-specific spots/10^4 CD8$^+$ 15/9 (**C**) and 3C1 (**D**) T cells ± SD from triplicate determinations. All data are representative of three independent experiments performed. *, $p < 0.05$ versus control IgG-treated tumor cells. SLR20.A2 cell line was generated by M. Kawabe, T cell bulks and clones were generated by A.K. Wesa and M Mandic, ELISPOT analyses were performed by A.K. Wesa, M. Mandic and M. Kawabe.

2.4.3. EphA2 agonist-enhanced recognition of SLR20.A2 tumor cells by anti-EphA2 T cell clone is proteasome-dependent.

To demonstrate whether enhanced $CD8^+$ T cell recognition of tumor cells was due to processing through the proteasome, SLR20.A2 tumor cells were pretreated with MG-132 or lactacystin (reversible or irreversible 20S/26S proteasome inhibitors, respectively). Due to concerns for the toxicity of proteasome inhibitors during a prolonged exposure period, we chose a short 3h period for tumor pretreatment, which showed no negative impact on tumor cell morphology or viability (data not shown). Cells were then cultured with EphA2 agonists for 24-48h, before use as targets for $CD8^+$ T cell recognition. Consistent with the data depicted in Figure 6D, co-application of MG-132 or lactacystin prevented agonist-induced EphA2 degradation (Figure 9A). These proteasome inhibitors also completely abrogated any enhancement in recognition of SLR20.A2 cells by anti-EphA2 T cells resulting from treatment with either of the EphA2 agonists (Figure 9B).

2.4.4. EphrinA1-Fc and mAb208 sensitize $EphA2^+$, $HLA\text{-}A2^+$ SLR24 tumor cells to lysis mediated by anti-EphA2, but not by anti-HLA2 allospecific $CD8^+$ T cell clones in vitro.

INFγ production assay indicates that $CD8^+$ T cell recognizes tumor cells and is activated. To confirm the increased IFNγ production observed above indeed links to the actual tumor killing function of $CD8^+$ T cells, we performed ^{51}Cr-release assay that is a more direct measure of killing activity of $CD8^+$ T cell. SLR24 tumor cells were treated with either EphA2 agonist (ephrinA1-Fc or mAb208) or control protein (ephrinB1-Fc) for 24h before targeted by either $CD8^+$ T cell clone reactive to $EphA2_{883–891}$ (E883) or HLA-A2 (2E4). Anti-HLA-A2 mAb treatment was included to demonstrate the observed lysis is a specific lysis in HLA-A2 restricted manner. As depicted in Figure 10A, clone E883 mediates the lysis of SLR24 tumor cells *in vitro* in an HLA-A2-restricted manner, with cytolysis dramatically increased when the tumor cell line is pretreated with either ephrinA1-Fc or mAb208, but not with ephrinB1-Fc. Those sensitization of SLR24 cells to T cell-mediated cytolysis is specific to anti-EphA2 $CD8^+$ T cells, since alloreactive (anti-HLA-A2) $CD8^+$ T cell clone lysed SLR24 tumor cells to a comparable degree regardless of pretreatment conditions applied to the tumor cells (Figure 10B).

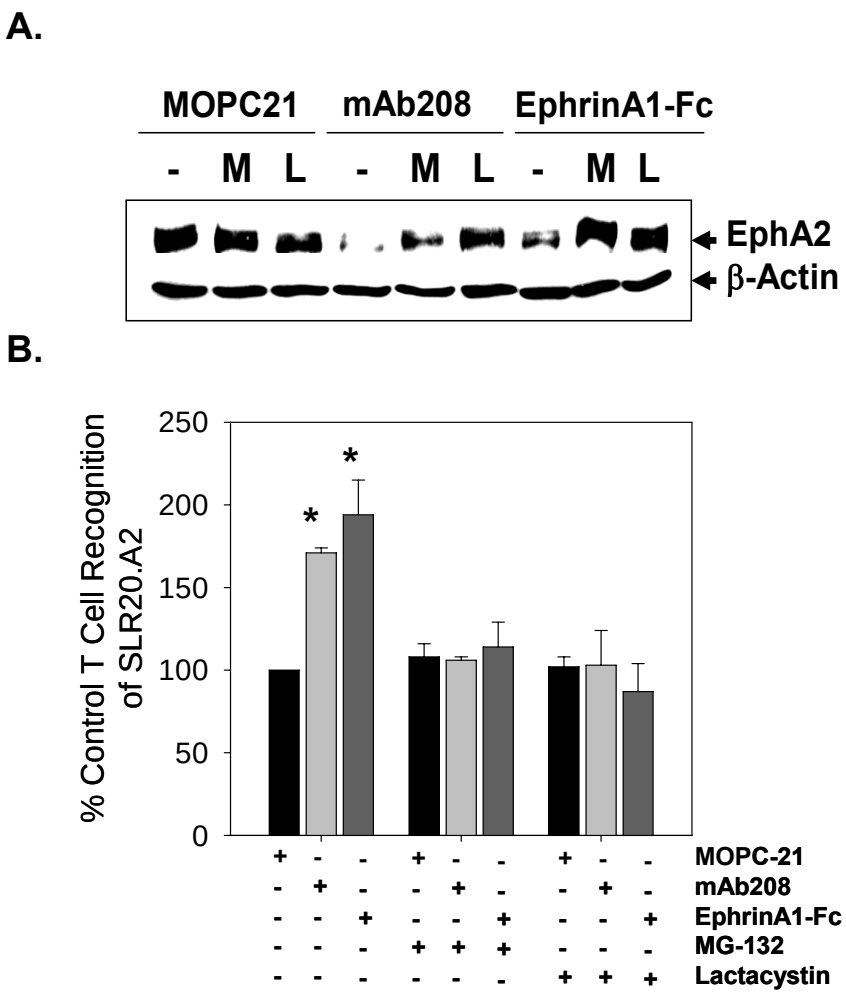

Figure 9. EphA2 agonist-enhanced *in vitro* recognition of SLR20.A2 tumor cells by anti-EphA2$_{58–66}$ T cell clone 15/9 is proteasome-dependent.
SLR20.A2 cells were pretreated with MG-132 (10 μM) or lactacystin (20 μM) or media alone for 3h, before being treated with 1 μg/ml MOPC-21 (control IgG), 1 μg/ml mAb208, or 0.1 μg/ml ephrinA1-Fc for an additional 3h. No toxicity was observed under any conditions. The effect of proteasomal inhibitors on agonist-induced degradation of EphA2 expression was confirmed by Western blot (**A**). After harvesting and washing the treated tumor cells extensively, these cells were used as targets for clone 15/9 (anti-EphA2$_{58–66}$) in IFN-γ ELISPOT assays (**B**) as outlined in *Materials and Methods*. Data from a representative experiment are reported as percentage control (mean ± SD) T cell response to SLR20.A2 versus tumor cells treated with control IgG (i.e., MOPC21). *, $p < 0.05$ versus control IgG-treated tumor cells. Western blot analyses were performed by C.J. Herrem, C. Vasquez and M. Kawabe, SLR20.A2 cell line was generated by M. Kawabe, T cell bulks and clones were generated by A.K. Wesa and M Mandic, ELISPOT analyses were performed by A.K. Wesa, M. Mandic and M. Kawabe.

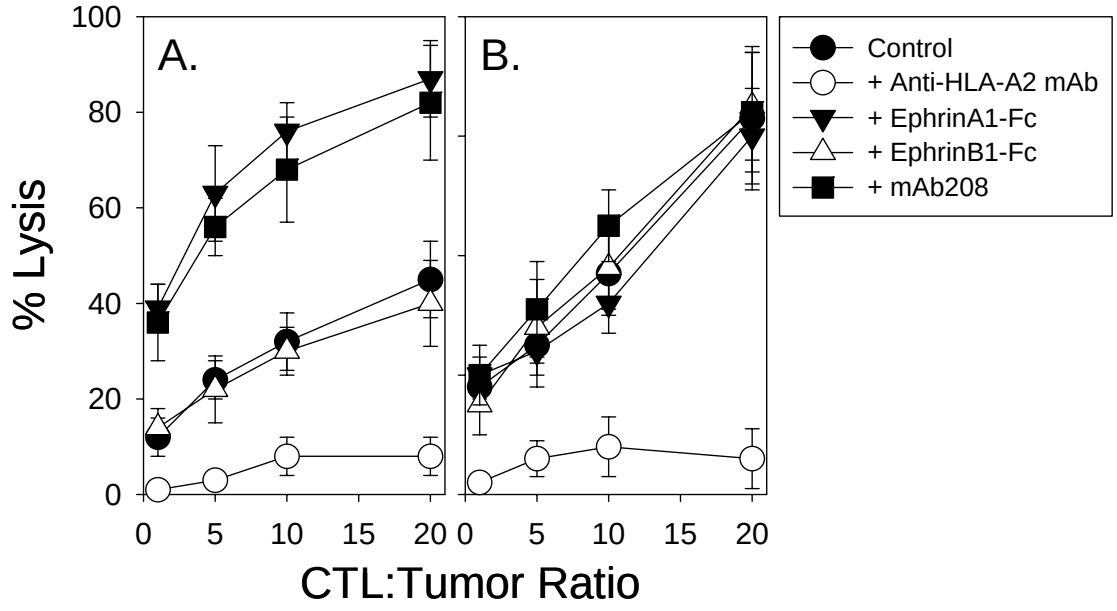

Figure 10. EphrinA1-Fc and mAb208 sensitize EphA2$^+$, HLA-A2$^+$ SLR24 tumor cells to enhanced lysis mediated by anti-EphA2, but not by anti-HLA2 allospecific CD8$^+$ T cell clones *in vitro*.

SLR24 tumor cells were untreated or pretreated for 24h with ephrinA1-Fc, ephrinB1-Fc, or mAb208 before their use as target cells in 4h ^{51}Cr-release assays. Anti-EphA2$_{883–891}$ CD8$^+$ T cell clone E883 and anti-HLA-A2 allo-specific CD8$^+$ T cell clone 2E4 (developed as described in *Materials and Methods*) were used as effector cells at the indicated E:T ratios in **A** and **B**, respectively. Anti-HLA-A2 mAb BB7.2 was added to wells to demonstrate the HLA-A2-restricted nature of T cell recognition of SLR24 tumor cells. Data are reported as the mean ± SD of triplicate determinations and are representative of three independent experiments performed. These data were generated by E. Bruckheimer and M.S. Kinch at MedImmune.

2.4.5. EphrinA1-Fc and mAb208 treatment of tumor induces substantial therapeutic efficacy of adoptively transferred anti-EphA2 CD8$^+$ T cells in a Hu-SCID tumor model.

To address whether the conditional (agonist-induced) enhancement of EphA2$^+$ tumor cell recognition by EphA2 specific CD8$^+$ T cells could be of therapeutically significant, we established a Hu-SCID tumor model system for the analysis of combinational adoptive immunotherapy. Human SLR24 (EphA2$^+$, HLA-A2$^+$) renal carcinoma cells were s.c. injected

into the right flanks of C.B-17 *scid*/*scid* mice and allowed to progress to a size of ~30 mm^2, at which time animals were either left untreated or were treated with intratumoral injection of ephrinA1-Fc, ephrinB1-Fc, or mAb208 and/or i.v. administration of $CD8^+$ T cells specific for either $EphA2_{883–891}$ or HLA-A2. Clone E883 (anti-EphA2) was used due to its ability to be expanded to the high numbers of T cells required for these experiments.

Intratumoral injection of 50 μg ephrinA1-Fc, ephrinB1-Fc, or mAb208 (on day 18 posttumor inoculation) had minimal effect on the continued progressive growth of SLR24 lesions (Figure 11A), despite a profound reduction in EphA2 protein expression *in situ* at 24 h after injection with ephrinA1-Fc or mAb 208, but not with ephrinB1-Fc (Figure 11B). These data are contradictory to some previous reports that demonstrated tumor regression after a single administration of such agonists in xenograft tumor models [66, 120, 121] [125, 126]. However, additional studies failed to show any impact of anti-EphA2 mAb on tumor growth [124], consistent with my findings, which may suggest that discrepancies are attributable to the differential tumor models employed in each case.

The adoptive transfer of 5 x 10^6 $CD8^+$ T cells alone (either the anti-EphA2 clone E883 or the anti-HLA-A2 clone 2E4 on day 19 post-tumor inoculation) also failed to significantly alter consequent tumor growth *in vivo* (Figure 11, C and D). However, the combined application of ephrinA1-Fc or mAb208 (on day 18) along with the adoptive transfer of E883 T cells (on day 19) resulted in complete tumor eradication in all treated animals (Figure 11C, Table 2). In contrast, combined use of ephrinB1-Fc and E883 T cells yielded a tumor growth curve that was indistinguishable from single agent administration, with no animals rejecting their tumors (Figure 11C, Table 2). Although the (anti-HLA-A2) allospecific 2E4 $CD8^+$ T cell clone efficiently kills SLR24 tumor cells *in vitro* (Figure 10B), administration of 2E4 T cells alone failed to halt the progression of lesional tumor growth, and when applied in combinational approaches with agonists, these T cells did not improved therapeutic efficacy (Figure 11D). This result is consistent with the failure of SLR24 pretreatment with ephrinA1-Fc or mAb208 to augment tumor sensitivity to 2E4 T cell-mediated lysis *in vitro* (Figure 10B).

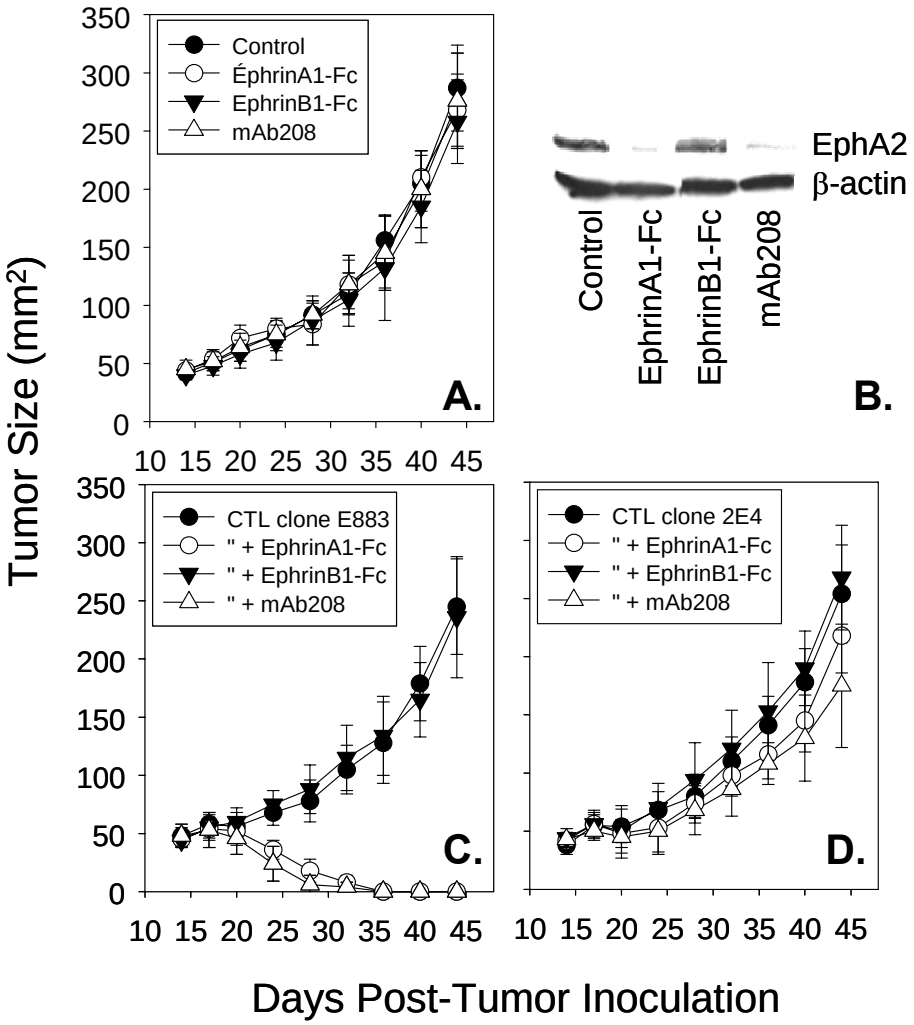

Figure 11. EphrinA1-Fc and mAb208 promote EphA2 down-regulation *in situ* and sensitize EphA2$^+$, HLA-A2$^+$ SLR24 tumors to enhanced eradication mediated by adoptively transferred anti-EphA2, but not by anti-HLA2 allospecific CD8$^+$ T cell clones *in vivo*.
A. Female C.B-17 *scid*/*scid* mice were injected with 1 x 10^6 human SLR24 (HLA-A2$^+$/EphA2$^+$) RCC cells s.c. in the right flank and allowed to establish to a size of ~30 mm^2 (i.e., day 18). Animals were then randomized into four cohorts (six animals each) receiving no treatment (control) or intratumoral injections of ephrinA1-Fc (50 μg), ephrinB1-Fc (50 μg), or mAb208 (50 μg) on day 18. Tumor size was evaluated every 3–4 days, with results reported in mean mm^2 ± SD. **B.** Tumors were resected from one mouse per cohort on day 19 (i.e., 24h after treatment and Western blots performed to validate EphA2 degradation in situ). In **C** and **D**, 5 x 10^6 CD8$^+$ T cells (either anti-EphA2$_{883–891}$ clone E883 or anti-HLA-A2 allospecific clone 2E4, respectively) were adoptively transferred by tail vein injection on day 19 posttumor inoculation alone or in combination with prior day 18 intratumoral injections of ephrinA1-Fc, ephrinB1-Fc, or mAb208 (50 μg each). Tumor size was evaluated every 3–4 days, with results reported in mean mm^2 ± SD. Data are representative of three independent experiments performed. These data were generated by E. Bruckheimer and M.S. Kinch at MedImmune.

Table 2. Combined application of EphA2 agonists and the adoptive transfer of Anti-EphA2 $CD8^+$ T cells results in the therapeutic regression of human SLR24 renal cell carcinomas in C.B-17 SCID.

Treatment Group	Fraction of Tumor-Free Mice (d44)*
Control	0/15
EphrinA1-Fc only	0/15
EphrinB1-Fc only	0/10
mAb208 only	0/10
Clone E883 only	0/15
Clone 2E4 only	0/15
EphrinA1-Fc + Clone E883	14/15
EphrinB1-Fc + Clone E883	0/10
mAb208 + Clone E883	10/10
EphrinA1-Fc + Clone 2E4	0/10
EphrinB1-Fc + Clone 2E4	0/10
mAb208 + Clone 2E4	0/10

*Aggregate results obtained from 3 independent experiments performed as described in Figure 11.

Data in Table 2 were generated by E. Bruckheimer and M.S. Kinch at MedImmune.

2.5. DISCUSSION

Immunotherapies designed to stimulate specific T cell-mediated immunity have thus far yielded only modest objective clinical response rates [177], despite they are typically capable of increasing tumor-specific T cells in the majority of treated patients. Since most tumor-associated antigens are non-mutated ("self") proteins, treatment-enhanced T cells are believed to be derived from a low-to-moderate avidity repertoire that has survived negative selection [256, 257]. These T cells may become effectively activated by peptide-based vaccines, but they frequently fail to recognize tumor cells that naturally present only low levels of relevant MHC/tumor peptide complexes [264], often times due to defects in the tumor cell APM [150, 151].

If tolerance selection restricts the anti-tumor $CD8^+$ T cell repertoire to a degree that limits their clinical utility, I hypothesized that tumor cells might instead be manipulated in order to exceed the cognate antigen threshold requirements for effective immune surveillance. In particular, I believe that by conditionally enhancing the proteasome-mediated processing of tumor antigens, such as EphA2, the level of class I/EphA2 peptide complexes might be (at least transiently) increased on the tumor cell surface, allowing for improved recognition by modest avidity anti-EphA2 $CD8^+$ T cells [3].

Recombinant ligand (ephrinA1-Fc) and agonistic anti-EphA2 mAb208 were selected as candidate reagents for this purpose, since they have been demonstrated to be capable of selected promotion of EphA2 degradation and reduction of EphA2 expression/signaling. The major finding of the present study is; 1) the treatments of tumor cells with agonists promote EphA2 autophosphorylation, rapid internalization and proteasomal degradation/processing, 2) those treatment result in improved recognition by EphA2-specific and moderate-to-low functional avidity $CD8^+$ T cells both *in vitro* and *in vivo*, 3) as a consequence, moderate-to-low functional avidity (i.e., about 1 μM ED_{50} for peptide recognition on T2 cells) EphA2-reactive $CD8^+$ T cells are rendered to be capable of mediating the $EphA2^+$ tumor regression *in vivo*.

Several mechanisms of EphA2 overexpression have been suggested including gene amplification, decreased rates of protein degradation, and increased or stabilized mRNA transcription/translation. Despite our findings that constitutive tumor cell-expressed EphA2

exists in a hypophosphorylated state, the chosen agonists readily promoted the rapid phosphorylation and internalization of EphA2 protein, prior to its subsequent destruction via a proteasome-dependent pathway. The impairment of endogenous EphA2 protein degradation in tumor cells under standard conditions may stem from the disrupted interaction of EphA2 with its ligands, or from overexpression of protein tyrosine phosphatase (PTP) that enforces the recycling of internalized EphA2. Western blot analyses of our RCC cell lines for PTPs (LMW-PTP, PTP-1b, SHS-PTP), however, were not conclusive in demonstrating gross protein overexpression (Appendix Figure 1). Hence, I believe the mechanism underlying EphA2 overexpression by my tumor cell lines may be due to a destabilization in intercellular cell-cell contacts involving EphA2 and its ligands (i.e. both the SLR20.A2 and SLR24 cell lines express the EphA2 ligands ephrinA1 and ephrin A5; Appendix Figure 2) expressed by proximal tumor cells.

Notably, EphA2 was capable of yielding epitopes ($EphA2_{58–66}$ and $EphA2_{883–891}$, located in the extracellular domain and intracellular domain of EphA2, respectively), which could consequently be presented by MHC class I molecules for extended periods of time. This suggests the superior processing/presentation of peptides derived from the full-length, transmembrane protein may be anticipated as a consequence of agonist application. However, how both domains of the EphA2 transmembrane protein become accessible to proteasome-dependent degradation in the cytosol remains unclear. The classical direct presentation pathway describes a mechanism whereby peptides derived from cytosolic proteins may become presented in the context of MHC class I complexes. In this model, proteasomally-cleaved peptides may be transported by TAP into the ER and then be loaded into nascent MHC class I complexes that are consequently exported to the cell surface. However transmembrane proteins, such as EphA2, are generally thought to undergo degradation within the acidic microenvironment of mature lysosomes after ligand-induced internalization [15]. This would suggest a non-classical mechanism of antigen processing is involved in the case of EphA2. Such processing could occur via retrotransportation/retrotranslocation, in which an internalized protein may be shunted from an early endosome through the trans-Golgi/ER and then back into the cytosol [207, 265-267]. This is an intracellular trafficking mechanism employed by several bacterial toxins and viral proteins and is associated with their pathology. Alternatively, $EphA2^{+}$ endosomes may fuse with the ER which possesses the machinery required to mediate the retrotranslocation of transmembrane

proteins into the cytosol. Another plausible explanation for EphA2 entry into the cytoplasm is the loss of endocytic vesicle integrity, resulting in the release of internalized EphA2 into the cytosol. We are currently undertaking pharmacologic studies to delineate the relevance of these various pathways by which agonist agents impact cell membrane EphA2 proteins give rise to enhanced, specific tumor recognition by anti-EphA2 $CD8^+$ T cells. A better understanding of the mechanism(s) involved in agonist-induced EphA2 processing may allow for the therapeutic accentuation of relevant pathways, allowing for enhanced treatment benefits to be obtained in the setting of patients harboring $EphA2^+$ tumors.

While unlikely, I also considered the trivial explanation that increased tumor cell recognition by anti-EphA2 $CD8^+$ T cells could be the result of a general up-regulation in tumor cell expression of HLA-A2 class I molecules (and hence a compensatory increase in the cohort of HLA-A2 complexes containing EphA2-derived peptides). To address this possibility, the SLR20.A2 and SLR24 cell lines were treated with ephrinA1-Fc or mAb208 and analyzed by flow cytometry for cell surface expression levels of total HLA class I complexes, empty HLA-class I complexes, peptide-loaded HLA-A2 complexes, and empty HLA-A2 complexes. The empty molecules were defined based on their immunoreactivity with (positive for) mAbs HC-10 and/or HC-A2 [259]. We noted no significant changes in the MFIs of any of these parameters as a consequence of tumor treatment with these EphA2 agonists (data not shown), suggesting that increased IFNγ production by specific $CD8^+$ T cells is the direct result of increased MHC class I presentation of EphA2-derived peptides on the tumor cell surface.

Both bulk T cell lines and cloned T cells exhibiting low-to-moderate functional avidity recognized agonist-treated tumor cells to a greater extent than control, untreated tumor cells. This is an important finding since tolerance circuitry limits the avidity of self-reactive (i.e. anti-EphA2) $CD8^+$ T cells, which may be functionally incompetent to mediate anti-tumor effects in the clinical setting (i.e. the tumor does not provide signals sufficient to exceed the activation threshold of the T cells). Therefore, it may prove clinically important that agonist treatment of tumor cells conditionally enables such “weak” T cells to now effectively recognize and respond to cancer cells (based on increased IFNγ production and the frequency of responder T cells as analyzed by ELISPOT in Figure 7 and Figure 8). While bulk T cell populations reactive against

tumor antigens are a clinically-relevant (adoptive therapeutic) modality, analyses of cloned T cells with various functional avidities might provide a more detailed picture as to the comparative benefits imparted to T cell recognition of tumor cells as a consequence of agonist treatment.

It is rather surprising that the combinational therapy works or fails *in vivo*, presumably based on an ~2–4-fold increase in tumor cell recognition by anti-EphA2 $CD8^+$ T cells after agonist treatment *in vitro* (measured by IFNγ production or cytotoxic analyses; Figure 7-10). This may suggest that additional mechanisms of action are in play *in vivo*. Clearly one might envision that EphA2 processing/presentation might be more efficient *in vivo* than *in vitro* for a given tumor cell line. Although overall efficiency of APM might be estimated by the expression level of cell surface HLA molecule, EphA2-specific processing/presentation can only be addressed by mass spectrometry analyses for the $EphA2_{58–66}$ and $EphA2_{833–891}$ peptides extracted from HLA-A2 complexes expressed on SLR24 tumor cells. We are currently preparing SLR24 tumor cell extracts from cultured tumor cells versus resected tumors (*in vivo*), with and without agonist treatment for 24 h (via addition to culture or intratumoral injection). Another possible mechanism might be distinct from any peptide presentation mechanism, but act via improved T cell activation, recruitment, and/or survival in the tumor microenvironment. Since our T cell lines/clones do not express discernable levels of EphA2 (data not shown), it is not likely that EphA2 agonists impact T cells directly. Rather EphA2 agonists might act on tumor cells to induce a change in the expression pattern of molecules that contribute to preferred (in the cancer setting) Type-1 $CD8^+$ T cell-mediated immune responses. Since agonists are capable of inducing the phosphorylation of EphA2, and down-stream signaling (i.e. MAPK), hence corollary protein synthesis would be anticipated as a result of such activation. We have not yet identified such byproduct proteins, however, I would hypothesize that such changes might include alterations in inflammatory chemokines expression (e.g. IP-10, Mig, I-TAC), in integrin expression (e.g. VLA-4) and in activatory/inhibitory co-stimulatory molecules expression (e.g. B7 family molecules) by tumor cells, each of which would improve T cell trafficking into tumor lesions *in situ* and/or effector T cell reactivity (for an extended period of time) against tumor cells. To address such possibilities, we will repeat the adoptive transfer studies and harvest tumors 24h after injection of CFSE-labeled $CD8^+$ T cells in order to determine whether the recruitment of transferred $CD8^+$ T

cells into the tumor site has been appreciably altered as a consequence of agonist "conditioning". Another possibility is that host macrophages and NK cells may play important anti-tumor roles (in addition to the transferred $CD8^+$ T cells), since those immune cell functions remain intact in SCID mice. Although both the ephrinA1-Fc and mAb208 agents would be anticipated to be rapidly internalized along with EphA2, it remains formally possible that FcR^+ mouse macrophage and NK cells could recognize the Fc portion of these agonists on the tumor cell surface, allowing for antibody-dependent cellular cytotoxicity to occur. The destruction of tumor cells initiated by transferred $CD8^+$ T cells might also activate and/or may recruit innate FcR^+ effector cells into the tumor site, leading to a more effective elimination of tumor cells in the context of our combinational treatment group. Alternative, and perhaps of greatest intrigue, agonists may allow for the immune targeting of the $EphA2^+$ tumor-associated vasculature [112] by specific $CD8^+$ T cells, thereby providing multiple targets (i.e. tumor cells and the associated tumor stroma) for immune intervention. Indeed, we have recently shown that the vaccination of mice with peptides derived from the murine EphA2 protein inhibits the growth of EphA2-negative tumor cells *in vivo,* at least in part, by limiting neoangiogenesis in the tumor microenvironment [63]. This data suggests that combinational treatments using EphA2 agonists and T cell-based immunotherapies will likely have multiple strategic $EphA2^+$ cellular targets within the tumor microenvironment, potentially opening patient accrual to individuals harboring any form of vascularized (solid) cancer.

Overall our present results strongly suggest the potential therapeutic benefits of using "off-the-shelf"' agonists to sensitize $EphA2^+$ tumor cells to anti-EphA2 $CD8^+$ T cells that could be preactivated via specific immunization [8, 130] or provided by the adoptive transfer of Ag-specific ex vivo-expanded, autologous $CD8^+$ T cell populations.

Preface Chapter 3

My goal was to determine whether HSP90 inhibitors, which disrupt refolding/stabilization of misfolded HSP90 client proteins, induce proteasomal degradation of EphA2 and the subsequent improvement in MHC class I presentation of EphA2 derived peptides on the tumor cell membrane. Enhanced presentation was monitored as improved reactivity mediated by anti-EphA2 $CD8^+$ T cells *in vitro*. I also aimed to discern the mechanisms by which such increased "antigenicity" of tumor cells occurs and whether treatments consisting of EphA2 agonists and HSP90 inhibitors would yield to even further improvement in specific T cell recognition of tumor cells. Western blot and ELISPOT/ELISA analyses were performed to monitor EphA2 degradation and the MHC-presentation of EphA2-derived peptides on tumor cells, respectively. I determined that EphA2 is a novel client protein of HSP90 and that the HSP90 inhibitor, 17-DMAG, promotes EphA2 degradation in tumor cells via a mechanism that is dependent on Sec61-mediated retrotranslocation, proteasome-mediated cleavage and TAP-mediated delivery of EphA2 peptides into the MHC class I biosynthetic pathway. I noted that 17-DMAG treatment of $EphA2^+$ tumor cells improves their recognition by low-to-moderate avidity anti-EphA2 $CD8^+$ T cells for at least a period of 24h, and that such recognition could be further enhanced using a combinational treatment including both 17-DMAG and EphA2 agonists. These data were reported in an in press *Cancer Research* report (2009), on which I was the first author.

The studies in **Chapter 3** suggest that clinical-grade 17-DMAG may help in defining novel combinational immunotherapies (in concert with vaccines to elicit anti-EphA2 $CD8^+$ T cells or adoptively transferred anti-EphA2 $CD8^+$ T cells) for the treatment of $EphA2^+$ cancers. These studies using drugs that act in a mechanistically distinct manner from those used in **Chapter 2**, again support my hypothesis that the conditioning of tumor cells to promote acute EphA2 protein degradation leads to improve immune recognition by $CD8^+$ T cells of modest functional avidity.

3. HSP90 Inhibitor 17-DMAG Enhances EphA2$^+$ Tumor Cell Recognition by Specific CD8$^+$ T cell

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All the results reported in this study were obtained by Mayumi Kawabe, except for ELISPOT analyses which were performed in collaboration with Drs. Maja Mandic and Amy Wesa.

3.1 ABSTRACT

EphA2, a member of the receptor tyrosine kinase (RTK) family, is commonly expressed by a broad range of cancer types, where its level of (over)expression correlates with poor clinical outcome. Since tumor cell expressed EphA2 is a non-mutated "self" protein, specific $CD8^+$ T cells are subject to self-tolerance mechanisms and typically exhibit only moderate-to-low functional avidity, rendering them marginally competence to recognize $EphA2^+$ tumor cells *in vitro* or *in vivo*. We have demonstrated in Chapter 2 that the ability of specific $CD8^+$ T cells to recognize $EphA2^+$ tumor cells can be augmented after the cancer cells are pretreated with EphA2 agonists that promote proteasomal degradation and upregulate expression of EphA2/class I complexes on the tumor cell membrane. In the current study we show that treatment of $EphA2^+$ tumor cells with the irreversible HSP90 inhibitor, 17-DMAG, similarly enhances their recognition by EphA2-specific $CD8^+$ T cell lines and clones *in vitro* via a mechanism that is dependent on proteasome and TAP function, as well as, the retrotranslocation of EphA2 into the tumor cytoplasm. When 17-DMAG and agonist anti-EphA2 mAb are co-applied, T cell recognition of tumor cells is further increased over that observed for either agent alone. These studies suggest that EphA2 represents a novel HSP90 client protein and that the treatment of cancer patients with 17-DMAG-based "pulse" therapy may improve the anti-tumor efficacy of $CD8^+$ T effector cells reactive against EphA2-derived epitopes.

3.2. INTRODUCTION

EphA2 represents an attractive target for therapeutic intervention in the majority of patients with solid tumors, due to the observations that EphA2 is commonly overexpressed in a broad range of cancer types, and that the level of EphA2 overexpression by tumor cells link to poor prognosis. Among several treatment strategies considered for translation into the clinic, one strategy involves the implementation of EphA2 agonists (agonist mAb or recombinant ligands) that promote the proteasome-mediated degradation of tumor EphA2 protein, thereby limiting its oncogenic function [120, 125]. As reported in Chapter 2, we have determined that such agonists also promote a corollary enhancement in tumor cell presentation of EphA2 peptides in tumor cell MHC class I complexes, thereby facilitating tumor cell recognition by low-to-modest avidity $CD8^+$ T cells [260]. Since EphA2-specific $CD8^+$ T cells have been detected in the peripheral blood of patients with renal cell carcinoma (RCC [8]), prostate carcinoma [130] or glioma [132], the frequencies of these protective T cells would be anticipated to be augmented as a consequence of active vaccination [63, 255]. Novel combinational therapies that sensitize $EphA2^+$ tumors for specific $CD8^+$ T cell eradication, therefore, may yield enhanced clinical benefits in the cancer setting [3].

Interestingly, some RTK, such as EGFR and Her2/neu, serve as client proteins for the molecular chaperone HSP90 (see http://www.picard.ch/downloads/Hsp90interactors.pdf), a protein designed to stabilize or refold denatured proteins into their native conformations to preserve their function and utility in normal and stressed cells [238]. HSP90 is commonly overexpressed in tumor cells, where it is believed to protect client oncogenic/survival proteins important to tumor progression and metastasis, in part, by preventing their proteasome-dependent destruction [242]. As a consequence, many small molecules have been developed to inhibit HSP90 function as potential clinical agents. Among these, 17-DMAG represents one of the newest generation of HSP90 inhibitors, exhibiting an increased solubility, the ability to be administered orally, and a lower toxicity index versus older generation drugs [246, 249].

Although EphA2 had not been previously reported to represent a client protein for HSP90, given that several alternate RTKs had been identified as HSP90 clients, I felt this was a logical

possibility. Therefore, I hypothesized that inhibition of HSP90 function might lead to the degradation of EphA2 proteins most probably via the proteasome and that derivative peptides might gain access to nascent MHC class I molecules through the direct presentation pathway. Data detailed in the current chapter suggests that EphA2 indeed represents a previously unknown HSP90 client protein. Furthermore, treatment of tumor cells with 17-DMAG for 24-48h, at clinically well-tolerated doses of this agent [242, 246], results in the proteasome-dependent degradation of tumor cell-expressed EphA2 protein and in the augmentation of tumor cell recognition by anti-EphA2 $CD8^+$ T cells *in vitro*.

3.3. MATERIALS AND METHODS

3.3.1. Cell lines and Media

SLR20 ($EphA2^{+}$, $HLA\text{-}A2^{neg}$), SLR22 ($EphA2^{+}$, $HLA\text{-}A2^{+}$) and SKOV3 ($EphA2^{+}$, $HLA\text{-}A2^{neg}$), as well as, the SLR20.A2 ($EphA2^{+}$, $HLA\text{-}A2^{+}$) and SKOV3.A2 ($EphA2^{+}$, $HLA\text{-}A2^{+}$) cell lines (established via transduction of the corresponding parental cell lines with a recombinant retrovirus encoding HLA-A2.1 provided by Dr. Peter Cresswell, Yale University) were used both in Western blot analyses and ELISPOT/ELISA analyses. SLR24, Mel397, Mel526, Mel624, SKBR3 were used in Western blot analyses. SLR [8] and Mel [268], cell lines were previously established from renal cell carcinoma and melanoma respectively, and SKOV3 and SKBR3 cell lines (both kindly provided by Dr. Nora Disis, University of Washington) were previously established from ovarian carcinoma and breast carcinoma respectively. All cell lines were maintained in RPMI-1640 media supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 10 mM L-glutamine (all reagents from Invitrogen, Carlsbad, CA) and free of mycoplasma contamination.

3.3.2. HSP90 inhibitor and Peptides

HSP90 inhibitors, 17-(allylamino)-17-demethoxygeldanamycin (17AAG, NSC 330507) and 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG, NSC 707545) were obtained by National Cancer Institute (Bestheda, Maryland). Lyophilized 17-AAG and 17-DMAG were dissolved in DMSO or sterile water respectively as stock solution and diluted with RPMI-1640 before use. HLA-A2 presented EphA2 peptides, $EphA2_{58\text{-}66}$ (IMNDMPIYM; [130]) and $EphA2_{883\text{-}891}$ (TLADFDPRV; [8]) were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry by University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Facility, as previously described [8]. The purity of peptides was >96% based on high performance liquid chromatography, with peptide identity validated by mass spectrometric (MS/MS) analyses performed by the UPCI Protein Sequencing Facility (a Shared Resource). The $ICP47_{1\text{-}35}$ and $ICP47_{35\text{-}1}$ synthetic peptides [265] were kindly provided by Dr. Peter Cresswell.

3.3.3. Western Blot Analyses

Tumor cell lines at 80-90% confluency were incubated with 17-DMAG (10-1000 nM) in 2% human serum supplemented RPMI-1640 media for 24-48h, as indicated in text. To assess the impact of proteasome function, endosomal acidification and retrotranslocation on EphA2 protein degradation promoted by the HSP90 inhibitor, MG-132 (5-10 μM; Sigma-Aldrich, St. Louis, MO), chloroquine (30-100 μM; Sigma-Aldrich) and *Pseudomonas aeruginosa* exotoxin A (10-50 μg/ml; Sigma-Aldrich), respectively, were added at the initiation of 24h tumor cell cultures, as indicated in individual experiments. Harvested cells were then incubated with lysis buffer (1% TritonX-100, 150 mM NaCl, 10 mM Tris-HCl pH7.4, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% NP-40 in PBS; all reagents from Sigma-Aldrich) containing a protease inhibitor cocktail (Complete mini, Roche Diagnostic, Mannheim, Germany) for 30 minutes at 4^{o}C. Lysates were cleared by centrifugation at 13,500 x g for 10 min, and proteins in the lysate were resolved by SDS-PAGE before electro-blotting onto PVDF membranes (Millipore, Bedford, MA). Polyclonal anti-EphA2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), CAIX mAb (R&D, MD) and MART-1 mAb (NeoMarkers, Fremont, CA) were used to detect each protein. Monoclonal antibodies against TAP-1 and TAP-2 (NOB-1 and NOB-2, respectively) were kindly provided by Dr. Soldano Ferrone, University of Pittsburgh. Monoclonal antibodies against β-actin (clone AC-15, Abcam), GAPDH and HLA-A2 heavy chain (HC-A2; the kind gift of Dr. H. Ploegh, Massachusetts Institute of Technology, [259]) were used for the detection of loading control. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or HRP-conjugated goat anti-mouse IgG (BioRad) were used as secondary antibodies. Probed proteins were then visualized by Western Lighting^{TM} chemiluminescence detection kit (Perkin Elmer, Boston, MA) and exposed to X-Omat film (Eastman Kodak, Rochester, NY) for 1-5 min.

3.3.4 Flow Cytometry

Control or treated tumor cells were harvested with Trypsin-EDTA (Invitrogen), washed and then incubated with anti-EphA2 mAb (B2D6, Upstate Biologicals, Inc., Lake Placid, NY) for 30 minutes at 4^{o}C. After washing with PBS/0.2% BSA/0.02% NaN3, cells were stained with FITC-

conjugated anti-mouse IgG (MP Biomedicals, Solon, OH) for 30 min at 4°C. Cells were washed twice, then analyzed using an LSRII flow cytometer (BD Biosciences, San Jose, CA). Total HLA class I complexes, HLA-A2 complexes and empty HLA-A2 molecules were monitored by W6/32 (Serotec Inc., Raleigh, NC), BB7.2 (American Type Culture Collection) and HC-A2 (the kind gift of Dr. H. Ploegh, Massachusetts Institute of Technology, [259]) respectively. Purified $mIgG_1$ (Sigma-Aldrich) was served as isotype control mAb for those antibodies. B7 family molecules were analyzed by PE conjugated mAbs against CD80, CD86, B7-H1, B7-H2 and B7-H4 along with PE conjugated isotype control (All eBiosciences, CA).

3.3.5. Proteasome Function Analysis

SLR20 cells were transfected with the proteasome sensor vector (PSV; BD Biosciences) using lipofectamine 2000 (Invitrogen) and selected in cultures containing G418 (Invitrogen), thus generating SLR20.PSV cells. PSV expresses a fluorescent substrate for the proteasome [269], which accumulates in the cytoplasm of cells if proteasome function is inhibited. SLR20.PSV cells were grown to 80-90% confluency, before being cultured in the absence or presence of 17-DMAG or the proteasome inhibitors MG-132 (Sigma-Aldrich) or PS-341 (Bortezomib; kindly provided by Dr. Ram Ganapathi**,** Cleveland Clinic Foundation) at the indicated concentrations for 24h at 37°C and 5% CO_2 tension. Fluorescence was detected in the FITC bandwidth (i.e. 488nm) by flow cytometry.

3.3.6. Analysis of EphA2 Proteins in Cytosol and Membrane Fractions

SLR20.A2 cells were treated with 17-DMAG (0-1000 nM) and/or mAb208 for 24h and harvested with Trypsin-EDTA (Invitrogen). Half of cells were incubated with lysis buffer for 30 min at 4°C, centrifuged at 13,500 x g for 10 min as described in Western blot section, then both of obtained supernatant and pellet fractions were mixed with Laemmli Sample buffer with 2% β-Mercaptoethanol. The other half of cells were subjected to repeated freeze-thaw cycle, centrifugation at 17,000 x g for 5h, then both of supernatant (cytosolic fraction) and pellet fractions (crude membrane fraction) were mixed with Laemmli Sample buffer with 2% β-Mercaptoethanol. All samples were then analyzed for EphA2 protein by Western blot.

3.3.7. T cell lines and Clones

Bulk $CD8^+$ T cell lines and clones specific for $EphA2_{58-66}$ or $EphA2_{883-891}$ were generated as previously described [8]. Briefly, mature dendritic cells (DC) were developed from peripheral blood mononuclear cells (PBMC; obtained with written consent under an IRB-approved protocol) isolated from normal HLA-$A2^+$ donors in 7 day cultures containing rhGM-CSF (Sargramostim; Amgen, Thousand Oaks, CA) and rhIL-4 (Peprotech, Rocky Hill, NJ), then pulsed with either the $EphA2_{58-66}$ or $EphA2_{883-891}$ peptides (10 μM) for 4h at 37°C at 5% CO_2 tension. Autologous $CD8^+$ T cells were stimulated on a weekly basis with peptide-pulsed autologous DC or PBMC for 3-4 cycles (at a T:DC ratio of 10:1, or at a T:PBMC ratio of 1:1) to generate a bulk population of peptide-specific $CD8^+$ T cells. These T cells were used in tumor recognition assays and for cloning via limiting dilution assays [260]. T cells were maintained in IMDM media supplemented with 10% human AB serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM L-glutamine and MEM Non-essential amino acids (all reagents from Invitrogen, except human AB serum that was purchased from Sigma-Aldrich). After their initial isolation, T cell clones were expanded *in vitro* in IMDM media supplemented with 1 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich) and 200 U/ml rhIL-2 (Peprotech).

3.3.8. Tumor Recognition Assays

Tumor recognition by anti-EphA2 T cells was evaluated by IFN-γ ELISPOT assays as described before [8, 260] or using a commercial hIFN- γ ELISA (BD-Biosciences). For both the ELISPOT and ELISA protocols, tumor cells were treated with 100-500 nM 17-DMAG and/or 10 μg/ml anti-EphA2 mAb208 (agonistic mAb, kindly provided by MedImmune, [66]) for 24-48h, prior to their harvest using Trypsin-EDTA (Invitrogen). MOPC21 mAb (mouse IgG; Sigma-Aldrich) was served as specificity controls of mAb208. After washing with PBS (Invitrogen), tumor cells were co-cultured with anti-EphA2 T cell lines/clones at an effector:target cell ratio of 2:1 for 24h at 37°C and 5% CO_2 tension. In some assays, where indicated, the class I-restricted nature of $CD8^+$ T cell recognition of tumor cells was assessed by inclusion of 10 μg/well W6/32 (pan HLA-class I) mAb. To assess the impact of proteasome function, TAP function, endosomal acidification and retrotranslocation on 17-DMAG-treated tumor cells recognition by anti-EphA2

$CD8^+$ T cells, MG-132 (10 μM), $ICP47_{1-35}$ peptide (10 μg/ml), chloroquine (100 μM) or *Pseudomonas aeruginosa* exotoxin A (10-50 μg/ml), respectively, were added to tumor cells during the 24h treatment period. As a negative control for the $ICP47_{1-35}$ peptide in these studies, the "reverse", scrambled $ICP47_{35-1}$ peptide [265] was used at a concentration of 10 μg/ml. After harvest, tumor cells were washed twice with PBS, prior to using these cells as targets for T cell recognition.

3.3.9. Statistical Analyses

Two-tailed Student's t tests were used to evaluate the difference between groups, with p values < 0.05 considered significant.

3.4. RESULTS

3.4.1. The HSP90 inhibitor 17-DMAG induces EphA2 degradation that is blocked by inhibitors of proteasome function, but not endosomal acidification.

The EphA2 (over)expressing RCC cell line SLR20 was incubated in the absence or presence of 17-DMAG (0-1000 nM) for 24-48h. The resultant cells were then analyzed for EphA2 protein levels by Western blotting (i.e. total protein; Figure 12A) and flow cytometry (i.e. cell surface protein; Figure 12B). In both cases, tumor EphA2 levels were reduced at both 24h and 48h post-treatment with 17-DMAG treatment (IC_{50} approximately 250 nM). Inclusion of the proteasome inhibitor MG-132 blocked the ability of 17-DMAG to promote EphA2 protein loss (Figure 12C), suggesting HSP90 effects on EphA2 are at least partially proteasome-dependent. In contrast, inclusion of chloroquine, which disrupts endosomal acidification [270], failed to impact 17-DMAG-induced degradation of EphA2 protein (Figure 12C). Single and combinational (17-DMAG + inhibitors) drug treatments did not lead to tumor cell death based on retention of control β-actin signal in the Western Blot experiments and appropriate forward/side scatter gating profiles in the flow cytometry assays (Figure 12 and data not shown).

Unexpectedly, a greater degree of EphA2 reduction was noted in Western blot analyses than in flow cytometry analyses. To address the pool of EphA2 protein most sensitive to 17-DMAG effects, SLR20 was treated with 17-DMAG (0-1000 nM) and/or EphA2 agonistic antibody mAb208 for 24h. Cell lysate and cell lysate pellet obtained from regular cell lysate preparation (Figure 13A) and intracellular fraction and crude membrane fraction obtained by freeze-thaw cycle (Figure 13B) were analyzed by Western blotting. Comparable patterns of EphA2 reduction were observed regardless of the sample preparation and regardless of the reagents used to trigger EphA2 degradation. Twenty-four hour treatment with 17-DMAG was effective in reducing EphA2 levels in both the cytoplasmic and cell membrane fractions of tumor lysates. The reason for the discrepancy between Western blot analysis and flow cytometry analyses conceivably might be attributed to the high back ground staining of certain cells by mAb (B2D6) used in the flow cytometry analyses.

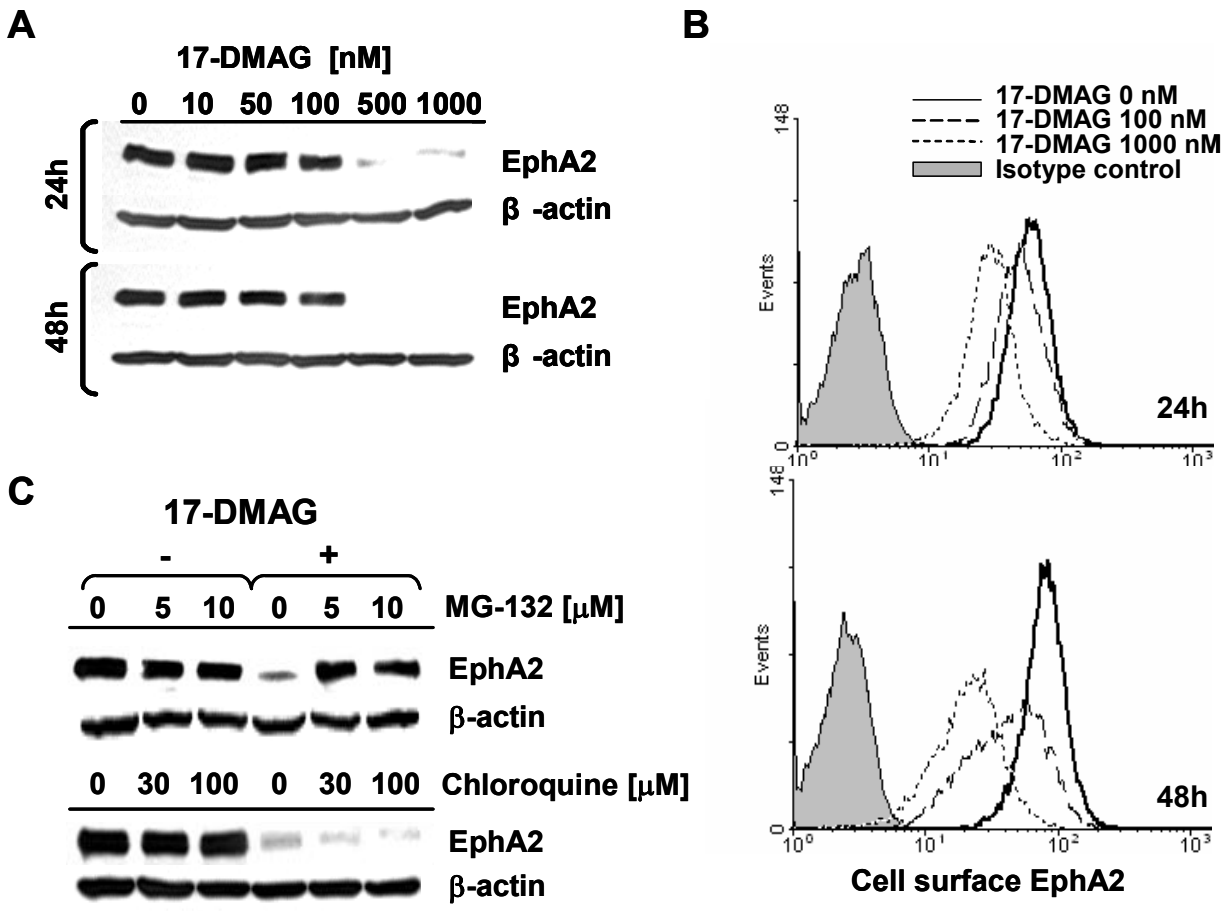

Figure 12. HSP90 inhibitor 17-DMAG promotes the loss of tumor EphA2 protein (via degradation) in a dose-, time- and proteasome-dependent manner.
A. The EphA2$^+$ SLR20 RCC line was incubated in the absence or presence of 17-DMAG (10-1000 nM) for 24h or 48h at 37^{o}C, before generation of cell lysates and Western blot analysis to determine levels of EphA2 protein expression. β-actin was monitored as an internal control protein. **B.** SLR20 cells were treated as above, with cell surface expression of EphA2 protein monitored by flow cytometry. Differences in tumor cell MFI expression of EphA2 were significant for 17-DMAG-treated versus control, untreated tumor cells evaluated in flow-based assays (i.e. p = 0.008 at 24h for 500 nM 17-DMAG treated (MFI = 28 +/- 13) versus untreated (MFI = 60 +/- 12). **C.** SLR20 cells were treated with 500 nM 17-DMAG in the absence or presence of MG-132 (10 μM) or chloroquine (100 μM) prior to Western blot analysis in order to analyze the dependency of EphA2 (versus control β-actin) protein loss on proteasome function or endosomal acidification, respectively. Data in each panel are representative of 4 independent experiments performed.

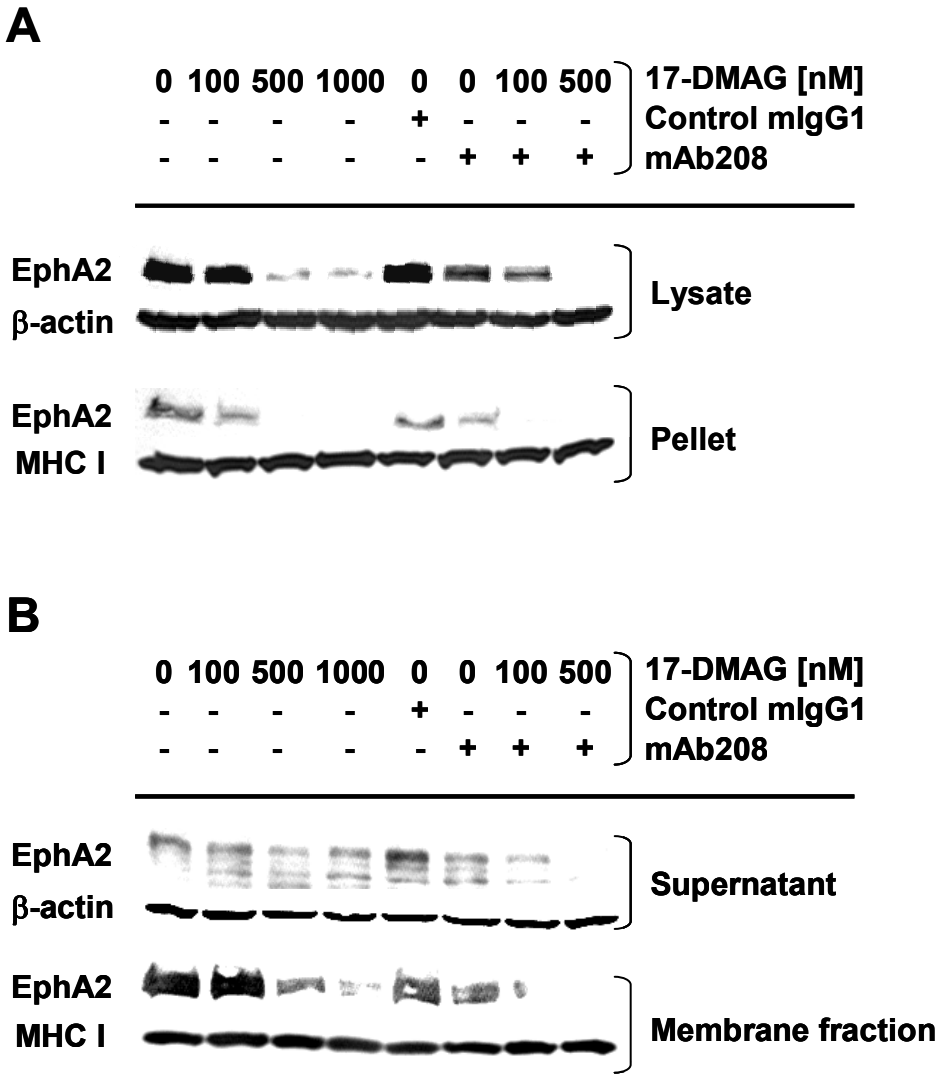

Figure 13. 17-DMAG-induced changes in EphA2 protein are similarly reflected in the membrane-associated pool of proteins.
SLR20.A2 cells were treated with 17-DMAG (0-1000 nM) and/or mAb208 for 24h and harvested with Trypsin-EDTA. Half of the cells were incubated with lysis buffer for 30 minutes at 4^{o}C, centrifuged at 13,500 x g for 10 min, and the obtained supernatant and pellet fractions were mixed with Laemmli Sample buffer containing 2% β-Mercaptoethanol, After separation of proteins by 7.5% SDS-PAGE gel electrophoresis, levels of EphA2 protein expression were determined by Western blotting (**A**). The other half of cells were subjected to repeated freeze-thaw cycle, centrifugation at 17,000 x g for 5h, and both the supernatant (cytosolic fraction) and pellet fractions (crude membrane fraction) were mixed with Laemmli Sample buffer with 2% β-Mercaptoethanol, and levels of EphA2 protein determined by Western blotting (**B**). β-actin or MHC class I heavy chain was monitored as internal control proteins, as indicated.

I believe this to be unlikely, since this mAb did not stain EphA2 negative cell lines, B16 (mouse melanoma), Colo38, LNCap (Appendix Figure 3A). Since no other mAb for flow cytometric detection of EphA2 was available, we tried to detect EphA2 by flow cytometry using the ephrin-A1 Fc fusion protein and FITC conjugated anti-human Fc antibody as a "sandwich stain" (Appendix Figure 3B). Like the B2D6 results, I was still unable to reduce the level of EphA2 reduction to the level suggested by Western blotting. Finally, the cell harvesting procedure did not appear to introduce differences in the perceived results, since I noted no change in EphA2 staining intensity when comparing results obtained using cell harvested using Trypsin/EDTA versus EDTA alone (Appendix Figure 3C). Although Western blot analyses of the crude membrane fraction of SLR20.A2 cells did not reveal the existence of 17-DMAG resistant membrane associated EphA2, we observed quantitative variance between flow cytometry and Western blotting analyses.

To address the generality of reduction in EphA protein expression levels as a consequence of 17-DMAG treatment, I repeated these initial experiments using a panel of tumor cell lines, including RCC (SLRs) and melanoma (Mel) cell lines, as well as the breast carcinoma (SKBR3) and ovarian carcinoma (SKOV3) cell lines. Overall, over a broad range of 17-DMAG concentrations (0-2000 nM), I observed losses in EphA2 expression post-17-DMAG treatment versus control, regardless of the cell line evaluated (Figure 14). SKBR3 and SKOV3 were very sensitive to 17-DMAG treatment with regard to EphA2 protein reduction, while the RCC line SLR24 was somewhat "resistant". This difference in sensitivity could have simply been the result of differential expression levels of HSP90 in these cell lines, however, both SLR20 (17-DMAG sensitive) and SLR24 (less sensitive) exhibited comparable levels of HSP90 protein expression even after 17-DMAG treatment (Appendix Figure 4). Differential surface features of the EphA2 protein, such as charge, hydrophobicity, and phosphorylation may underlie why these cell lines respond somewhat variably to 17-DMAG, and clearly more studies are warranted to provide an answer to this question.

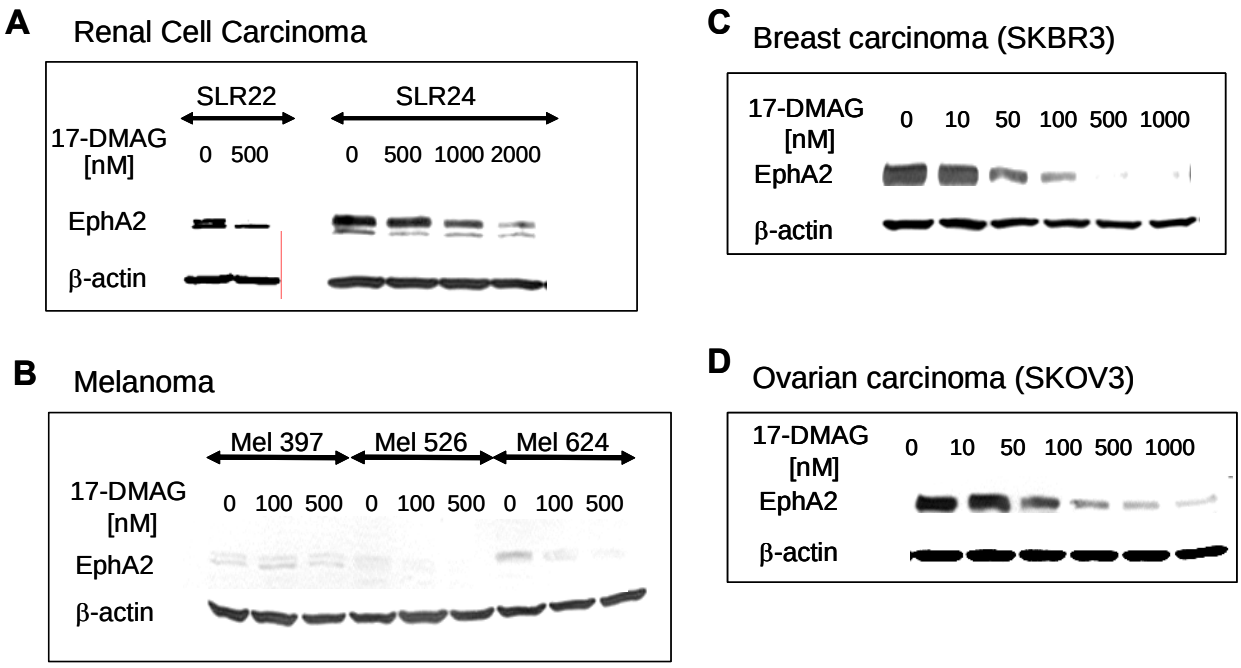

Figure 14. RCC, Melanoma, Breast and Ovarian tumors are all sensitive to 17-DMAG-induced reduction in EphA2 protein levels.
The SLR22, SLR24 (RCC, **A**), Mel397, Mel526, Mel624 (melanoma, **B**), SKBR3 (breast carcinoma, **C**) and SKOV3 (ovarian carcinoma, **D**) cell lines were incubated in the absence or presence of 17-DMAG (10-2000 nM) for 24h at 37^{o}C, before the generation of cell lysates and the performance of Western blot analyses to determine levels of EphA2 protein expression. β-actin was monitored as an internal control protein.

Although 17-DMAG reduced EphA2 protein levels on/in tumor cells of various histological types at a concentration of 500 nM, such treatment did not affect control (non-HSP client) protein expression levels (i.e. CAIX in RCC or MART-1 in melanoma cell lines; Figure 15).

In addition to 17-DMAG, another Geldanamycin derivative HSP90 inhibitor, 17-AAG, was tested for its efficacy in reducing tumor cell EphA2 protein expression levels. A strong reduction in EphA2 protein expression was observed by Western blotting, as well as, flow cytometry (Appendix Figure 5). This result is consistent with those obtained using 17-DMAG. However, higher concentration of 17-AAG versus 17-DMAG were required in order to obtain comparable reduction levels. Importantly, the presence of serum in culture medium impaired the efficacy of

17-AAG to reduce EphA2 level, while 17-DMAG was only minimally affected (Appendix Figure 6), thus with translation of this work in mind, I employed 17-DMAG alone for all further studies.

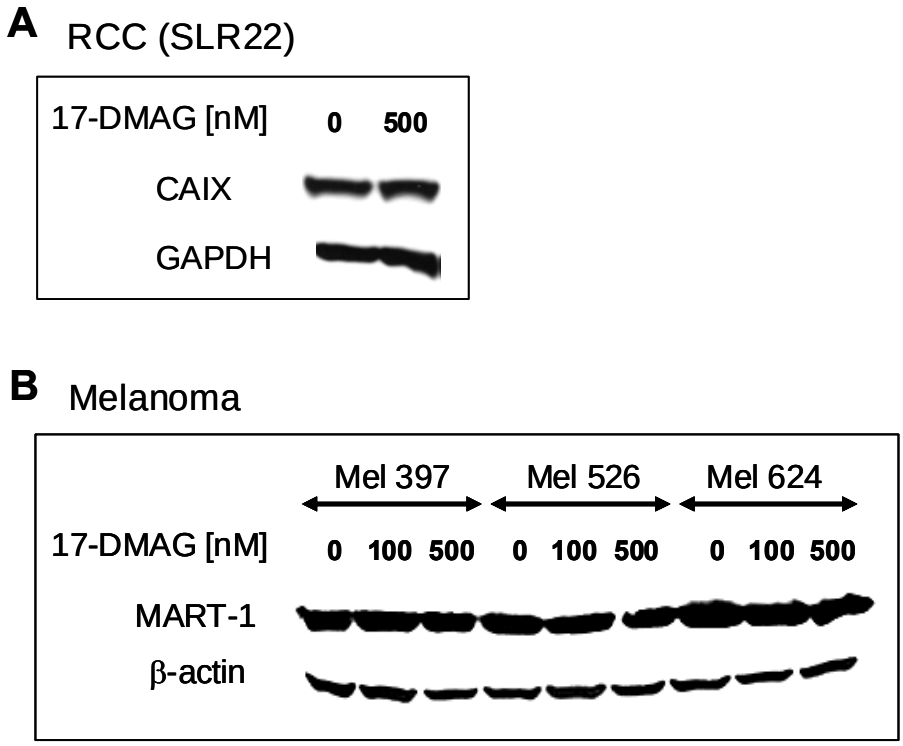

Figure 15. Tumor cell CAIX and MART-1 protein levels are not affected by 17-DMAG treatment.
The SLR22 (RCC, **A**) and Mel397, Mel526, Mel624 (melanoma, **B**) cell lines were incubated in the absence or presence of 17-DMAG (100 or 500 nM) for 24h at 37^{o}C, before the generation of cell lysates and the performance of Western blot analyses to determine levels of CAIX or MART-1 proteins expression. β-actin was monitored as an internal control protein.

3.4.2. Treatment of tumor cells with HSP90 inhibitor does not significantly alter major components of the tumor MHC class I antigen processing machinery (APM)

Since my ultimate goal was to discern potential augmentation in immune recognition of EphA2^{+} tumor cells after treatment with 17-DMAG, we next assessed the impact of drug treatment on the expression and/or function of components of tumor cell MHC class I APM. Proteasome activity was analyzed using a model employing SLR20 cells transfected with a proteasome sensor vector (i.e. SLR20.PSV cells), in which intracellular fluorescent protein accumulates when proteasome function is inhibited. As depicted in Figure 16A, the proteasome inhibitors MG-132 and PS-341 both increased the fluorescence of SLR20.PSV cells, while 17-DMAG had minimum effect even after 48h at concentrations in excess of that required to promote EphA2 degradation (as shown in Figure 12). Transporter associated protein (TAP) levels (both TAP-1 and TAP2) were next analyzed by Western blot, and were also shown to be unaffected by 17-DMAG treatment (Figure 16B). Finally, tumor cell surface MHC class I levels were shown to be unaltered by 17-DMAG as assessed by flow cytometry using a pan-class I-reactive mAb (Figure 16C). These results suggest that 17-DMAG does not have a deleterious effect on tumor cell MHC class I/peptide complex generation and corollary expression of such complexes on the tumor cell surface.

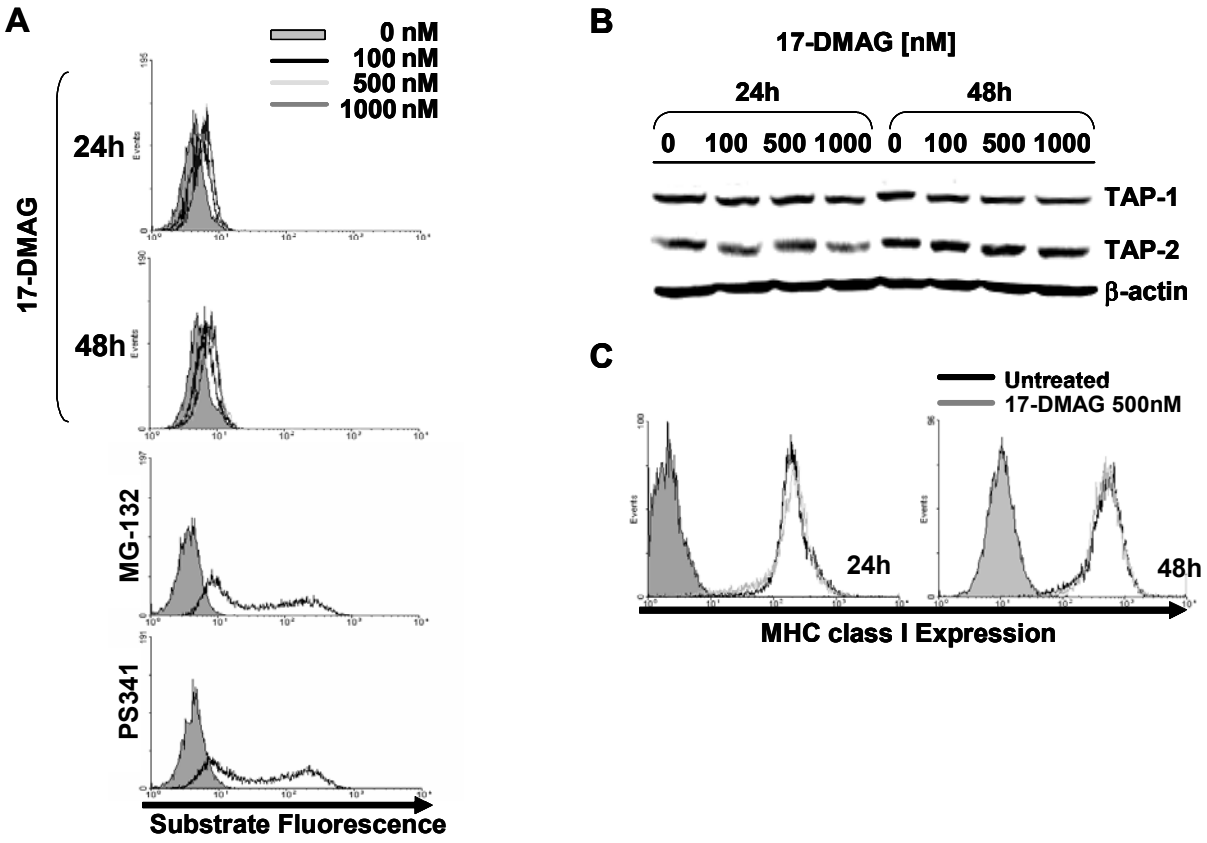

Figure 16. The tumor cell MHC class I antigen processing machinery (APM) is not impaired by 17-DMAG treatment at doses capable of promoting EphA2 degradation.
A. Proteasome sensor vector transfected SLR20 (SLR20.PSV) cells were treated with the indicated concentrations of 17-DMAG for 24h or 48h, or they were incubated with the proteasome inhibitors MG-132 (10 μM) or PS-341 (10 μM) for 24h. Fluorescence intensity of control versus treated tumor cells was then monitored by flow cytometry (i.e. fluorescence intensity increases as a function of inhibiting proteasome-mediated degradation of the fluorescent transgene product). **B.** SLR20 cells were incubated with 17-DMAG at the indicated concentrations for 24h or 48h, with subsequent whole cell lysates subjected to Western blot analyses for TAP-1 and TAP-2 protein content. **C.** SLR20 cells were treated with 17-DMAG (500 nM) either for 24h or 48h and cell surface MHC class I levels determined using W6/32 (a pan-class I reactive mAb) and flow cytometry. Isotype control mAb staining = filled histograms; W6/32 staining = empty histograms. Representative data from 1 of 3 independent experiments performed is presented in each case.

3.4.3. 17-DMAG treatment of tumor cells transiently enhances tumor recognition by bulk and cloned $CD8^+$ T cells specific for EphA2 in a proteasome dependent manner

Bulk $CD8^+$ T cell lines and clones reactive against EphA2 were generated from normal HLA-$A2^+$ donors using an *in vitro* stimulation protocol employing autologous DC pulsed with either the $EphA2_{58-66}$ or $EphA2_{883-891}$ (HLA-A2-presented) peptide epitopes as stimulator cells. After 2-3 rounds of identical restimulation, responder $CD8^+$ T cells were assessed for recognition of RCC cell lines (control or 17-DMAG pre-treated) in IFN-γ ELISPOT assays. As shown in Figure 17, bulk (Figure 17, A and B) or cloned (Figure 17C) peptide-primed $CD8^+$ T cells recognized $EphA2^+$, HLA-$A2^+$ RCC lines (SLR22 (Figure 17A) and SLR20.A2 (Figure 17, B and C)) in a manner that was class I-restricted (i.e. inhibited by W6/32 mAb; Figure 17A). These same T cell lines or clone reacted poorly against the $EphA2^+$, but HLA-$A2^{neg}$ SLR20 cell line (Figure 17, B and C). When SLR20.A2 was pretreated with 17-DMAG for 24h prior to co-culture with bulk or cloned T cells reactive against either of the EphA2 peptides, tumor cell recognition by T cells was significantly enhanced ($p < 0.05$; Figure 17, B and C). However, this enhanced immune recognition was transient in nature, since tumor cells pre-treated for 48h with 17-DMAG were recognized to a degree that was comparable with untreated tumor cells (Figure 17C).

To address the question of whether the reduction of T cell recognition at 48h versus 24h is related to tumor cell expression of co-stimulatory/co-inhibitory molecules, SLR20.A2 cells were untreated or treated for 24-48h with 17-DMAG at doses of 100-1000 nM. Cells were then analyzed for cell surface expression of the co-stimulatory CD80 (B7.1) and CD86 (B7.2) versus co-inhibitory molecules B7-H1, B7-H2 and B7-H4 by flow cytometry (Figure 18). While SLR20.A2 cells expressed strong levels of B7-H1, expression levels for CD80, CD86, B7-H2 and B7-H4 were weak to negative. There might be a slight increase of B7-H2 staining of SLR20.A2 cells after 17-DMAG treatment, however, an increase in isotype staining intensity was also noted under these conditions. No significant differences were noted in expression profiles for any of these co-inhibitory receptors +/- 17-DMAG treatment and 24h versus 48h incubation time. Data are representative of 2 independent assays performed.

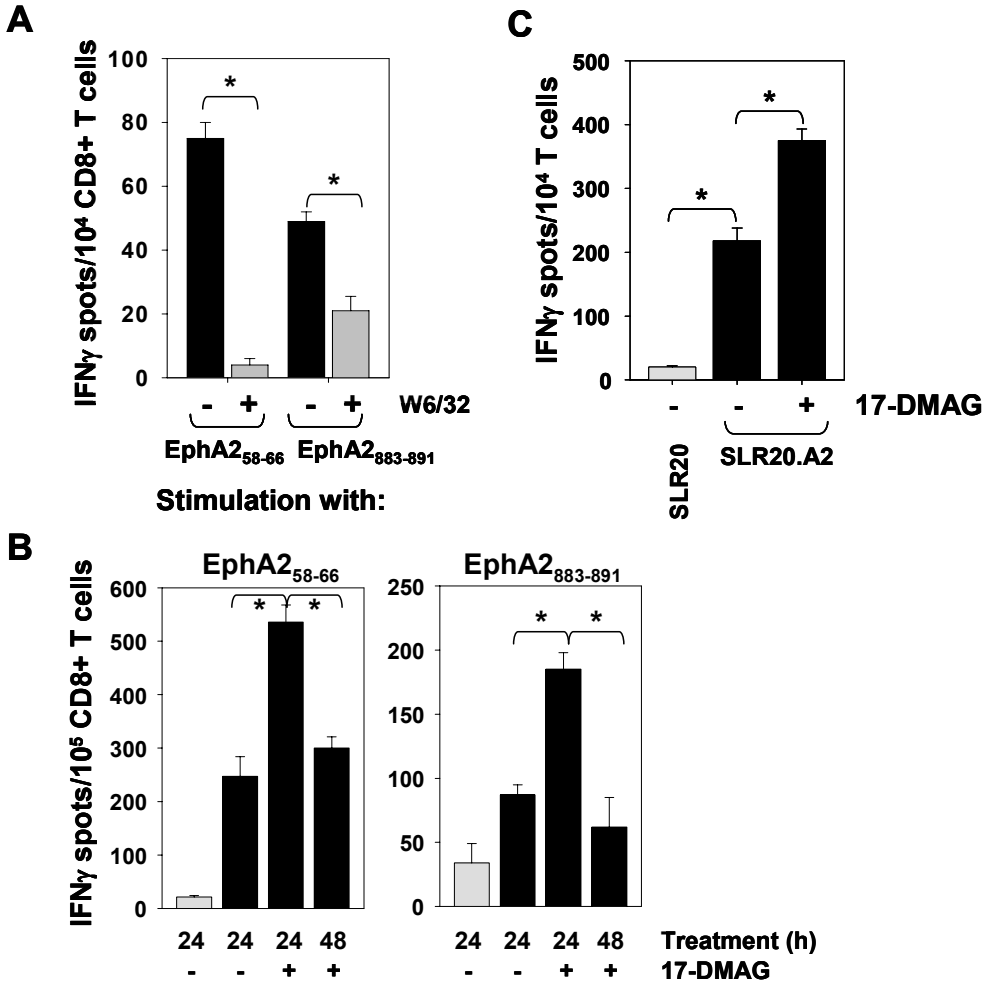

Figure 17. Recognition of EphA2$^+$ SLR20.A2 tumor cells by bulk anti-EphA2 CD8$^+$ T cells is enhanced after treatment with 17-DMAG.

A. HLA-A2-restricted CD8$^+$ T cells were generated *in vitro* by repeated stimulation with autologous DC pulsed with the EphA2$_{58-66}$ or EphA2$_{883-891}$ peptide epitopes. These bulk CD8$^+$ T cells were then co-cultured with the EphA2$^+$, HLA-A2$^+$ SLR22 RCC line alone (black bars) or in the presence of blocking anti-class I mAb W6/32 (gray bars) for 24h at 37^{o}C in IFN-γ ELISPOT assays. **B.** SLR20 (EphA2$^+$, HLA-A2^{neg}; light gray bars) or SLR20.A2 (EphA2$^+$, HLA-A2$^+$; black bars) cells were cultured in the absence or presence of 500 nM 17-DMAG for 24h or 48h, prior to analysis of these target cells in IFN- γ ELISPOT assays. Bulk anti-EphA2$_{58-66}$ or anti-EphA2$_{883-891}$ CD8$^+$ T cell lines were used as responder cells. **C.** An HLA-A2-restricted, EphA2$_{58-66}$ peptide-specific CD8$^+$ T cell clone was established from bulk CD8$^+$ T cell cultures in limiting dilution cultures. This T cell clone (15/9) exhibited low functional avidity based on recognition of T2 (HLA-A2$^+$) cells pulsed with titrations of the EphA2$_{58-66}$ peptide (i.e. half-maximal T cell reactivity at a peptide concentration of approximately 1 μM; Figure 8A.). SLR20 (EphA2$^+$, HLA-A2^{neg}; light gray bars) or SLR20.A2 (EphA2$^+$, HLA-A2$^+$; black bars) cells were cultured in the absence or presence of 500 nM 17-DMAG for 24h, prior to analysis of these target cells in IFN- γ ELISPOT assays. In all panels, data are reported as the mean +/- SD of triplicate determinations from a single representative IFN- γ ELISPOT assay, with 3 independent assays performed in each case. *$p < 0.05$ for all indicated comparisons.

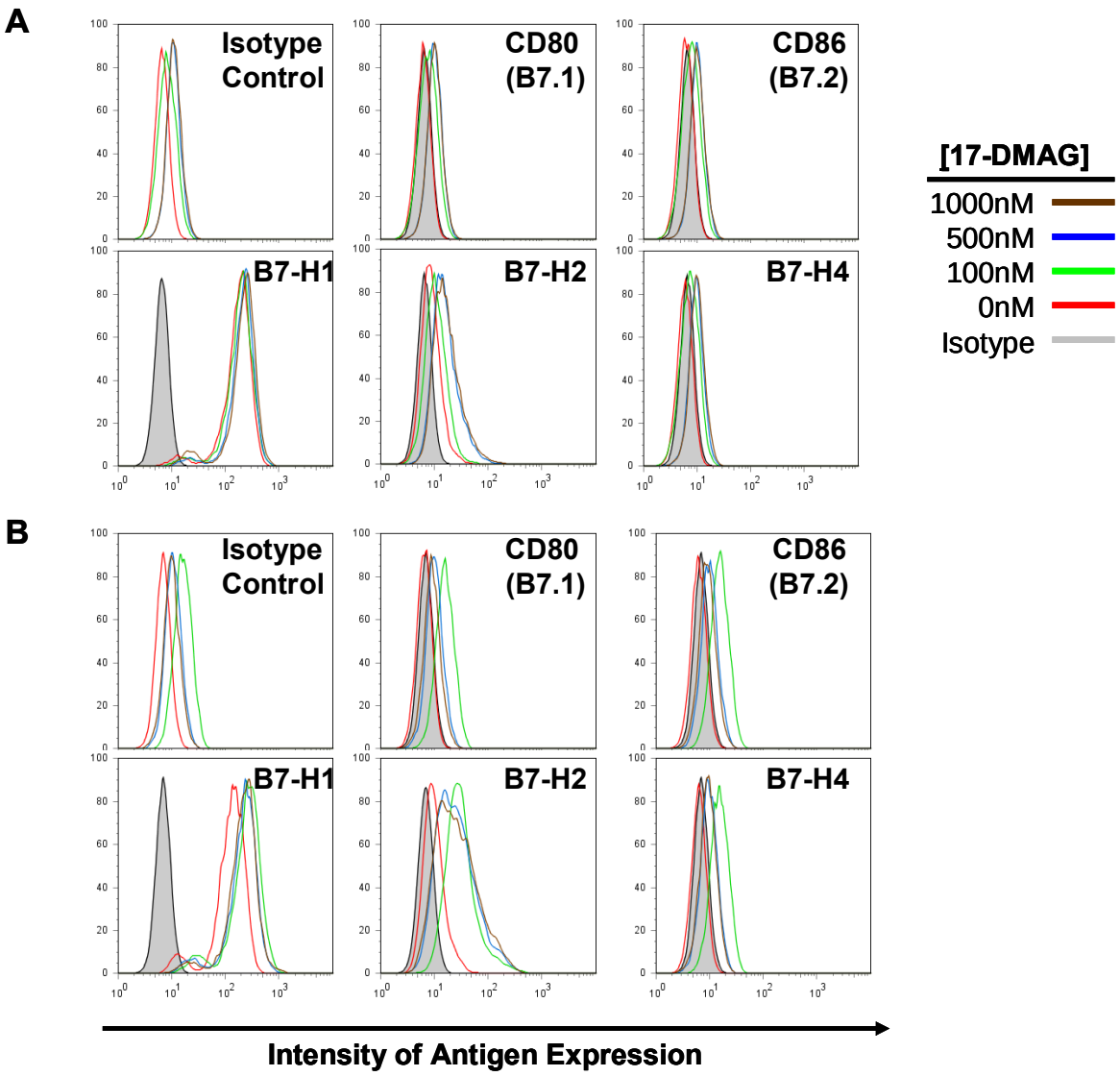

Figure 18. 17-DMAG treatment has minimal impact on tumor cell expression of co-inhibitory receptors.

SLR20.A2 cells were untreated or treated for 24h (**A**) or 48h (**B**) with 17-DMAG at doses of 100-1000 nM. Cells were then analyzed for cell surface expression of the co-stimulatory CD80 (B7.1) and CD86 (B7.2) versus co-inhibitory molecules B7-H1, B7-H2 and B7-H4 by flow cytometry. While SLR20.A2 cells expressed strong levels of B7-H1, expression levels for CD80, CD86, B7-H2 and B7-H4 were weak to negative. No significant differences were noted in expression profiles for any of these co-inhibitory receptors +/- 17-DMAG treatment and 24h versus 48h incubation time. Data are representative of 2 independent assays performed.

I also assessed whether the loading ability of exogenous peptide by tumor cells is differential at 48h (Figure 19). SLR20.A2 tumor cells were cultured in the absence or in the presence of 500 nM 17-DMAG for 24h or 48h. Tumor cells were then loaded with the indicated concentrations of synthetic $EphA2_{58-66}$ peptide and analyzed for their ability to be recognized by bulk T cells using the IFN-γ content of culture supernatants as an indicator. The ability of 17-DMAG treated tumor cells to be loaded by exogenous peptide was somewhat diminished when compared with untreated tumor cells (Figure 19A), but the loading ability of these target cells was not different at the 24h versus 48h time points post-DMAG application. Alternatively, untreated or 17-DMAG-treated SLR20.A2 cells were analyzed in flow cytometry assays using the mAb HC-A2 which reacts against "empty" HLA-A2 complexes [271]. In these studies, a cohort of tumor cells treated briefly with citrate buffer (pH 3.0 for 1 minute) was used as a positive control; i.e. such treatment denatures cell surface MHC class I complexes, leading to the enhanced expression of "empty" HLA-A2 complexes reactive with the HC-A2 mAb. As shown in Figure 19B, there was no discernable change in the prevalence of "empty" HLA-A2 complexes on the tumor cell surface at 24h or 48h. All of these data indicate that the reduction in the ability of 17-DMAG to enhance T cell reactivity at 48h versus 24h post-treatment is not due to a defect in the ability of tumor cell MHC class I molecules to functionally present peptide epitopes.

To determine whether 17-DMAG could sensitize tumor cells of an alternate lineage to anti-EphA2 $CD8^+$ T cell recognition, we treated SKOV3.A2 ovarian carcinoma cells ($EphA2^+$, $HLA\text{-}A2^+$; Figure 20A) with 17-DMAG for 24h *in vitro*. As previously shown in Figure 14D, 17-DMAG (500 nM)-treated SKOV3.A2 cells displayed reduced EphA2 protein expression (versus control untreated tumor cells). Anti-$EphA2_{58-66}$ bulk $CD8^+$ T cells exhibited enhanced recognition of 17-DMAG-treated SKOV3.A2, but not parental SKOV3 ($EphA2^+$, $HLA\text{-}A2^{neg}$), tumor cells (Figure 20, B and C). Such recognition was 17-DMAG dose-dependent (Figure 20B), as well as, MHC class I-restricted in nature based on the inhibitory effects associated with inclusion of mAb W6/32 during the co-culture period (Figure 20C). Since the ability of 17-DMAG to promote EphA2 degradation in tumor cells was inhibited by MG-132, but not by chloroquine (Figure 12), we next evaluated the impact of these agents on anti-EphA2 $CD8^+$ T cell recognition of HSP90 inhibitor-treated tumor cells. As shown in Figure 20D, enhanced T cell recognition of 17-DMAG-treated SKOV3.A2 cells was effectively ablated by the

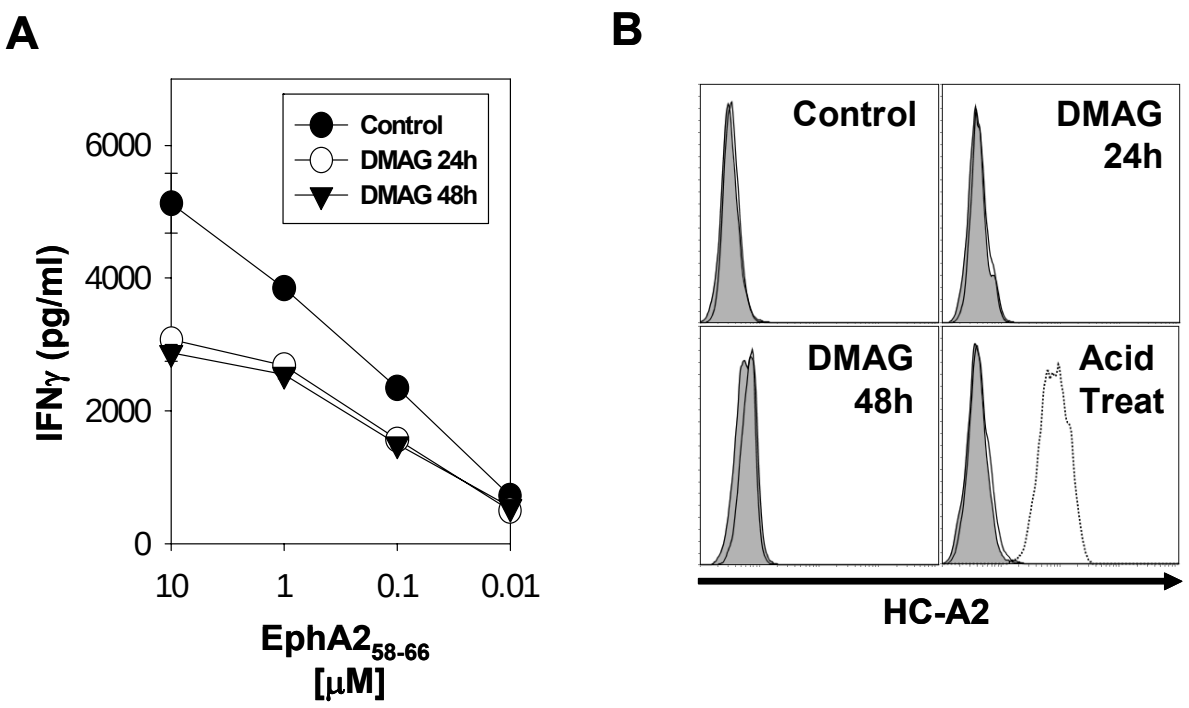

Figure 19. The ability of anti-EphA2$_{58-66}$ T cells to recognize peptide-pulsed SLR20.A2 tumor cells is reduced equitably after 24h and 48h treatment with 17-DMAG, however, this appears unrelated to the level of "empty" HLA-A2 complexes expressed on the tumor cell membrane.

SLR20.A2 tumor cells were untreated or treated with 500 nM 17-DMAG for 24h or 48h. **A.** Tumor cells were then loaded with the indicated concentrations of synthetic EphA2$_{58-66}$ peptide and analyzed for their ability to be recognized by clone 15/9 T cells. After 24h incubation, culture supernatants were harvested and analyzed for IFN-γ content using a specific ELISA. Results are reported as the mean +/- SD of triplicate determinations. Alternatively, in **B.**, untreated or 17-DMAG-treated SLR20.A2 cells were analyzed in flow cytometry assays using the mAb HC-A2 which reacts against "empty" HLA-A2 complexes [271]. In these studies, a cohort of tumor cells treated briefly with citrate buffer (pH 3.0 for 1 minute) was used as a positive control; i.e. such treatment denatures cell surface MHC class I complexes, leading to the enhanced expression of "empty" HLA-A2 complexes reactive with the HC-A2 mAb. Isotype control mAb staining = filled histograms; HC-A2 staining = empty histograms. Two independent assays were performed in each case.

proteasome inhibitor, but not by the lysosomotropic drug (chloroquine). Notably, MG-132 also reduced basal recognition of SKOV3.A2 cells by anti-EphA2 T cells (Figure 20D), supporting the normal loading of tumor cell class I complexes with EphA2 peptides occurs via a proteasome-dependent pathway.

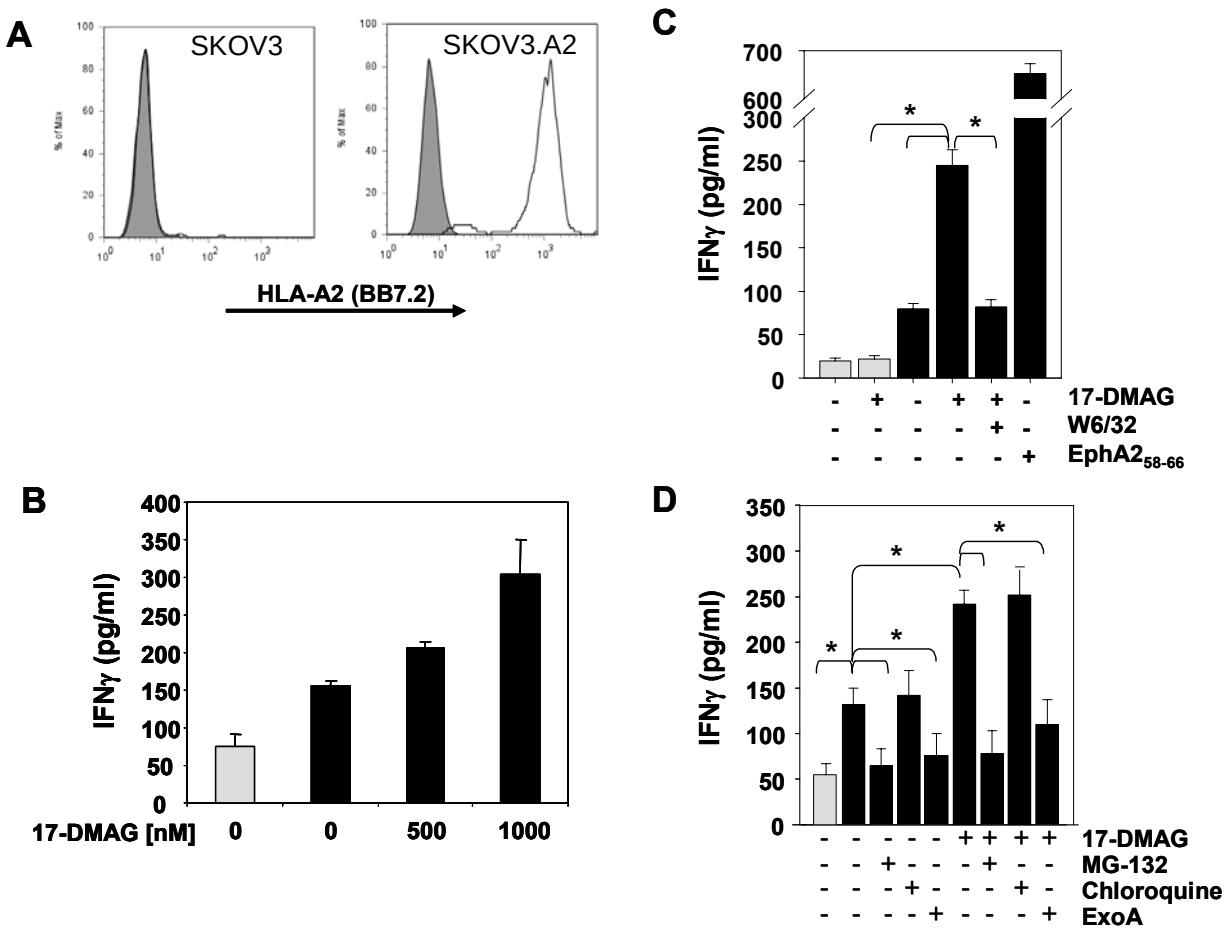

Figure 20. 17-DMAG promotes enhanced recognition of the EphA2$^+$, HLA-A2$^+$ ovarian carcinoma cell line SKOV3.A2 *in vitro* via a mechanism likely involving EphA2 retrotranslocation and proteasome-mediated degradation, but not lysosomal acidification.
A. SKOV3.A2 tumor cells were analyzed for expression of cell surface HLA-A2 protein by flow cytometry using BB7.2 mAb (open profiles) versus isotype control mAbs (filled profiles). **B.** SKOV3.A2 tumor cells were untreated or treated with 17-DMAG (500 or1000 nM) for 24h. Cell-free supernatants were harvested after a 24h co-culture of bulk anti-EphA2$_{58\text{-}66}$ T cells (10^5) and tumor cells ($0.5x10^5$; +/- 17-DMAG pretreatment) and analyzed for IFN- γ content by specific ELISA. Light gray bars indicate untreated SKOV3, and black bars indicate SKOV3.A2. **C.** SKOV3.A2 tumor cells were untreated or treated with 17-DMAG (500 nM) for 24h and co-cultured with bulk anti-EphA2$_{58\text{-}66}$ T cells in the absence or in the presence of W6/32 or EphA2$_{58\text{-}66}$ peptide (T cell 10^5, tumor cell $0.5x10^5$). Cell-free supernatants were harvested and analyzed for IFN-γ content by specific ELISA. **D.** SKOV3 or SKOV3.A2 tumor cells were untreated or treated with 500 nM 17-DMAG +/- MG-132 (10 μM) or chloroquine (100 μM) or exotoxin A (50 μg/ml) for 24h, before being used as targets for bulk anti-EphA2$_{58\text{-}66}$ CD8$^+$ T cells (10^6) responses monitored using IFN- γ ELISA. All ELISA data is reported as the mean +/- SD of triplicate assay determinations; *$p < 0.05$ for all indicated comparisons. Data in all panels are from 1 representative experiment of 3 performed.

3.4.4. 17-DMAG treatment of tumor cells enhances tumor recognition by a low avidity $CD8^+$ T cell clone specific for EphA2 via an ERAD- and TAP-dependent mechanism.

17-DMAG promotes enhanced recognition by $CD8^+$ T cells capable of recognizing peptides derived from both the extracellular (i.e. $EphA2_{58-66}$) and the intracellular (i.e. $EphA2_{883-891}$) domains of this RTK (Figure 17B). This suggests that both domains of this transmembrane protein must become accessible for proteasomal processing into peptides that are then conveyed via TAP into the ER for loading in nascent HLA-A2 complexes in tumor cells. Current paradigms [207, 266, 267] suggest that cytosolic access for the extracellular domains of transmembrane proteins may be accomplished through a retrotranslocation process involving Sec61-dependent "ratcheting" of the target protein into the cytoplasm, where it may become a substrate for the proteasome. To test this hypothesis, we added the ERAD inhibitor exotoxin A [265] to tumor cells during culture with 17-DMAG, before their analysis in Western blotting and T cell assays. As shown in Figure 21A, exotoxin A inhibits 17-DMAG-induced degradation of tumor cell EphA2 protein. Notably, exotoxin A also prevents enhanced recognition of SLR20.A2 tumor cells by anti-EphA2 (clone 15/9) $CD8^+$ T cells after 24h treatment with 17-DMAG (Figure 21, B and C), but does not negatively impact the recognition of EphA2 peptide-pulsed tumor cells (Figure 21B). Similar results were obtained using a bulk anti-$EphA2_{58-66}$ $CD8^+$ T cell line and the SKOV3.A2 tumor cell line (Figure 20D).

The dependence of the anti-EphA2 T cell sensitizing effects of 17-DMAG on tumor cell TAP function was next addressed. We observed that co-treatment of tumor cells with 17-DMAG and an N-terminal fragment of the ICP47 protein ($ICP47_{1-35}$), a (*Herpes Simplex*) viral inhibitor of TAP [265], ablated enhanced T cell recognition when compared to tumor cells treated with 17-DMAG alone ($p < 0.05$). This effect was specific, as inclusion of a scrambled peptide ICP_{35-1} (bearing the reverse AA sequence of $ICP47_{1-35}$) exhibited no such inhibitory effect (Figure 21C).

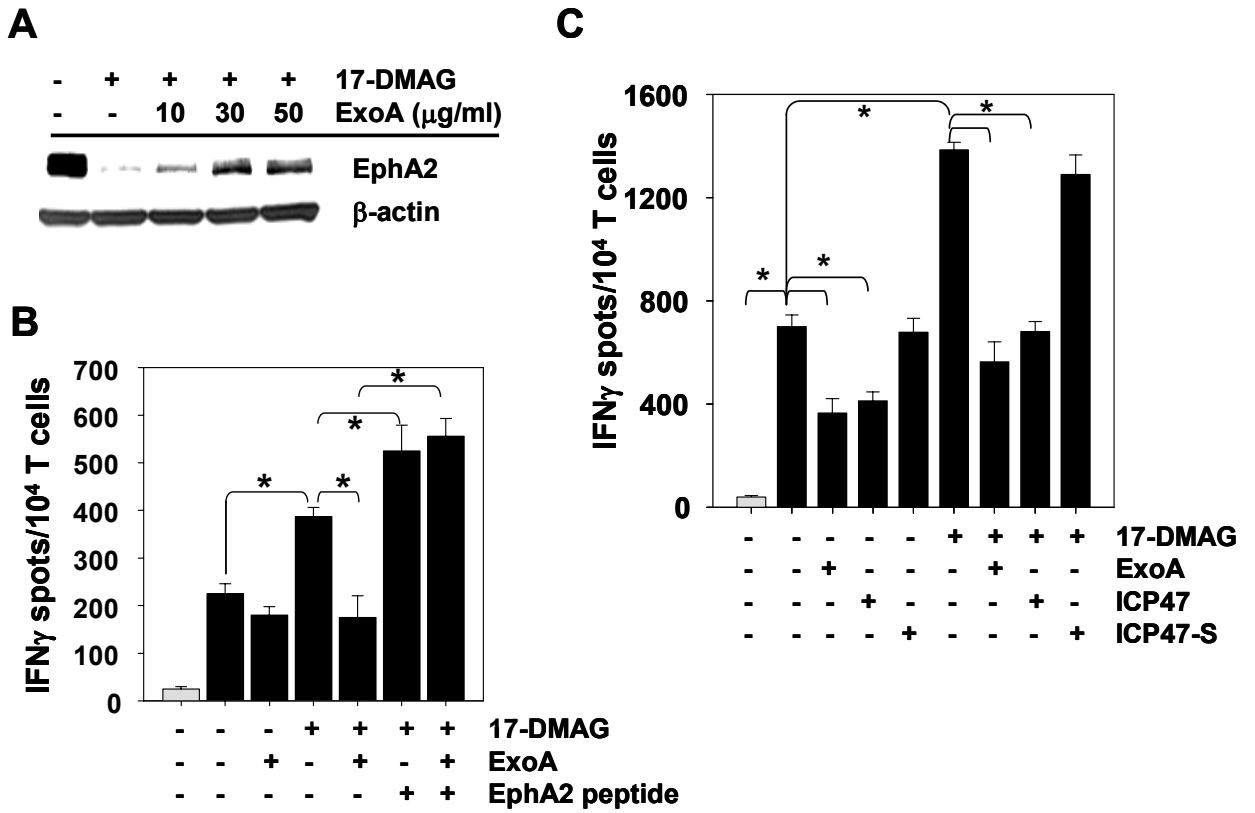

Figure 21. 17-DMAG treatment improves recognition of the SLR20.A2 RCC cell line by a low avidity, anti-EphA2$_{58-66}$ CD8$^+$ T cell clone in a TAP- and ERAD-dependent manner. **A.** SLR20.A2 tumor cells were untreated or treated for 24h with 500 nM 17-DMAG +/- 10, 30 or 50 μg/ml exotoxin A, then analyzed for expression of EphA2 versus control β-actin protein by Western blotting. **B.** Untreated SLR20 cells (light gray bars) or SL20.A2 cells (black bars) pretreated as in panel **A** using 10 μg/ml exotoxin A were used as targets for recognition by anti-EphA2$_{58-66}$ CD8$^+$ T cell clone 15/9 in IFN- γ ELISPOT assays. Drug-treated tumor cells pulsed with exogenous EphA2$_{58-66}$ peptide (10 μg/ml) to clone 15/9 was included as a positive control. **C.** SLR20 (light gray bars) and SLR20.A2 (black bars) tumor cells were untreated or treated with 500 nM 17-DMAG +/- 50 μg/ml exotoxin A, 10 μg/ml ICP47$_{1-35}$ peptide or 10 μg/ml ICP47$_{35-1}$ scrambled peptide for 24h, prior to use as target cells for clone 15/9 T cell recognition in IFN- γ ELISPOT assays. All ELISPOT data are reported as the mean +/- SD of triplicate determinations. In all cases, data are representative of that obtained in 3 independent assays performed. * $p < 0.05$ for all indicated comparisons.

3.4.5. Combined treatment of EphA2$^+$, HLA-A2$^+$ tumor cells with both 17-DMAG and agonist (anti-EphA2) mAb208 results in superior recognition by a low avidity CD8$^+$ T cells specific for EphA2.

As I previously reported (in Chapter 2) that EphA2 agonist mAb208 promotes the proteasomal destruction of tumor EphA2 protein and enhances specific CD8$^+$ T cell recognition of treated tumor cells [260], I next investigated whether the combined use of both 17-DMAG and mAb208 would result in an even greater degree of tumor cell recognition by specific T cells when compared to either treatment modality alone. SLR20.A2 cells were cultured for 24h in the absence or presence of 17-DMAG +/- mAb208 or control IgG, with IFN-γ ELISPOT assays subsequently performed using the 15/9 CD8$^+$ T cell clone and a bulk anti-EphA2$_{883\text{-}891}$ CD8$^+$ T cell line. As depicted in Figure 22, A and B, while both 17-DMAG and mAb208 treatment resulted in reduced EphA2 expression on SLR20.A2 cells, combined treatment with both reagents yielded an even greater degree of EphA2 protein loss. T cell assays similarly supported the greatest degree of tumor cell recognition by CD8$^+$ T cells (targeting either of the EphA2 epitopes) after SLR20.A2 cells were pretreated with both 17-DMAG and mAb208 versus either reagent alone ($p < 0.05$; Figure 22, C and D).

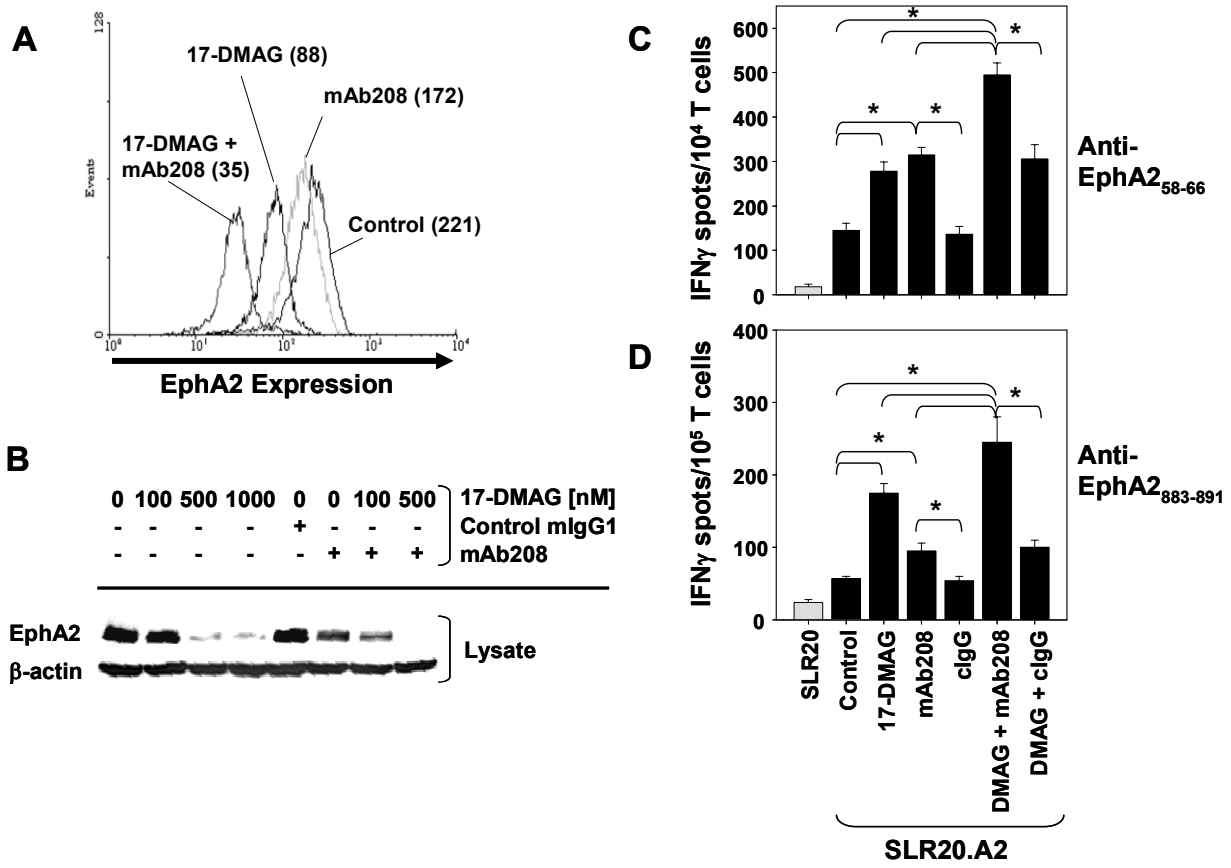

Figure 22. Superior *in vitro* recognition of SLR20.A2 tumor cells by anti-EphA2 CD8$^+$ T cells after combined treatment with 17-DMAG and agonistic mAb208.

A. SLR20.A2 cells were untreated or treated for 24h with 17-DMAG (100 nM) +/- 10 μg/ml of (anti-EphA2) mAb208 or control IgG (cIgG), after which, cells were washed and analyzed by flow cytometry for expression of cell surface EphA2 molecules. Data in parentheses represents the mean fluorescence intensity value for each cell population analyzed and are representative of 4 independent assays performed. $p < 0.05$ for differences in the MFI between all cohorts in pair-wise comparisons. In **B.**, SLR20.A2 cells were untreated or treated with 17-DMAG (100-1000 nM) +/- control IgG (10 μg/ml) or agonist anti-EphA2 mAb208 (10 μg/ml) for 24h. After harvest, cells were lysed in TritonX-100-containing buffer. After centrifugation, the soluble lysate was recovered and analyzed via Western blotting for comparative levels of EphA2. β-actin served as a control protein. In panels **C**. and **D.**, SLR20 control cells (light gray bars) or SLR20.A2 (black bars) tumor cells pre-treated for 24h (as indicated) were used as targets for recognition by anti-EphA2$_{58-66}$ clone 15/9 or bulk anti-EphA2$_{883-891}$ T cells, respectively, in IFN-γ ELISPOT assays. *$p < 0.05$ for all indicated comparisons. Representative data are provided from 1 of 3 independent experiments performed in each case.

3.5. DISCUSSION

In 2004, Rosenberg *et al.* reported that the objective clinical response rates for cancer patients treated with immunotherapies was less than 10 % [177], despite the majority of reported studies claiming that tumor-specific T cells were increased in peripheral blood of treated patients [257, 264]. One of the reasons for this discrepancy could be that therapy-induced T cells are only able to respond to high levels of specific MHC class I-peptide complexes expressed on professional APC versus tumor cells. Such differential recognition could be attributed to the clearance of high-avidity T cells capable of reacting against "self"(tumor) antigens as a consequence of negative selection. I, therefore, hypothesized that improved clinical benefits might be gained by conditionally and selectively enhancing tumor antigen processing and presentation in order to allow for improved tumor recognition by tumor antigen-specific $CD8^+$ T cells that exhibit only low-to-moderate intrinsic avidity [3].

Indeed, in the current study I have determined that; 1) EphA2, which is commonly overexpressed in a broad range of cancer types, is a novel client protein of HSP90 and that HSP90 inhibitors, such as 17-DMAG, promote tumor cell EphA2 degradation; 2) 17-DMAG treatment of $EphA2^+$ tumor (RCC and ovarian carcinoma) cells improves recognition by low avidity anti-EphA2 $CD8^+$ T cells; 3) enhanced T cell recognition of 17-DMAG-treated tumor cells is MHC-dependent and appears unrelated to tumor cell expression of co-stimulatory/co-inhibitory molecules which remains unchanged upon HSP90 antagonism, and 4) $EphA2^+$ tumor cell recognition by specific $CD8^+$ T cells may be further enhanced by combined treatment with 17-DMAG and EphA2 agonists (versus treatment with either single modality).

Notably, tumor recognition by T cells reactive against peptides found in the extracellular (i.e. $EphA2_{58\text{-}66}$) as well as intracellular (i.e. $EphA2_{883\text{-}891}$) domains of the target protein was improved by treatment with 17-DMAG. This suggests the superior processing/presentation of peptides derived from the full-length, transmembrane protein may be anticipated as a consequence of drug application. Mechanistically, I observed that enhanced T cell recognition of 17-DMAG-conditioned tumor cells was ablated upon inclusion of proteasome (MG-132), TAP ($ICP47_{1\text{-}35}$) or Sec61 (*Pseudomonas aeruginosa* exotoxin A) inhibitors. In contrast, there was

minimal impact associated with the inclusion of the lysosomotropic agent chloroquine, which interferes with endosomal acidification and lysosomal processing of protein antigens. These data suggest that the major pool of EphA2 protein undergoing (constitutive as well as) enhanced proteasomal processing as a consequence of 17-DMAG inhibition likely enters the tumor cytosol via a retrotranslocation event [207, 265-267].

However, at present, I cannot exclude the possibility that the observed ablation of $CD8^+$ T cell recognition of tumor cells after treatment with exotoxin A is also, at least partly, due to secondary effects on the ER stress response or to protein synthesis inhibition. My rationale to use exotoxin A as an inhibitor of retrotranslocation was based on the study reported by Koopmann *et al.* [272], in which this drug was applied to a free microsome model system and shown to inhibit Sec61-mediated retrotranslocation of antigenic peptides. In the Koopmann paper, it was found that exotoxin A molecules block the Sec61p channel from the ER-luminal side and thereby inhibit peptide export (i.e. into the cytosol). However the effective concentration of exotoxin A required in the ER for this affect appeared much higher than which one might approach via the treatment of a whole cells with exogenous exotoxin A. This leads one to question whether exogenous "loading" with exotoxin A can effectively inhibit retrotranslocation in the absence of confounding secondary effects. However, in defense of the selectivity of exotoxin A on the retrotranslocation pathway in intact cells, Ackerman et al. [265] showed that the treatment of myelogenous KG1.K^b cells with exotoxin A effectively blocked the cross-presentation of exogenous OVA to specific $CD8^+$ T cells without limiting protein synthesis or the ability of treated cells to process and present antigenic peptides derived from proteins in the cell cytoplasm (i.e. conventional, direct presentation). Nevertheless, additional studies will be required in my model to strictly implicate the mechanism(s) of action associated with exotoxin A-mediated antagonism of the beneficial effects of HSP90 inhibitors on tumor cell recognition by $CD8^+$ T cells.

At present, we also cannot distinguish whether this pool of EphA2 protein affected derives from an early endosomal compartment (i.e. internalized after interaction with the EphA2 ligands co-expressed by adjacent tumor cells that is unaffected by chloroquine) and/or from newly-synthesized, miss-folded EphA2 proteins within the exocytic pathway. However, given the

observed quantitative variance in 17-DMAG-induced EphA2 degradation as imaged using flow cytometry versus Western blotting analyses, and the apparent synergy of agonist mAb208 and 17-DMAG in promoting EphA2 protein loss and enhanced T cell recognition, it could be suggested that mAb208 primarily affects the membrane pool of EphA2 protein, while 17-DMAG primarily affects the intracellular this protein. In either case, derivative EphA2 peptides would then appear to be integrated into "empty" class I complexes after TAP-dependent transfer into the MHC class I loading compartment.

While several HSP90 inhibitors (including geldanamycin, 17-AAG and 17-DMAG) have now entered advanced clinical trials, to our knowledge, very few papers have addressed the potential effects of HSP90 inhibitors on tumor cell recognition by T cells. In this regard, Castilleja *et al.* reported that the HSP90 inhibitor geldanamycin induced degradation of non-mutated Her2/neu in a treated ovarian carcinoma cell line, resulting in enhanced recognition by an HLA-A2-restricted CTL line reactive against the Her2/neu$_{369\text{-}377}$ epitope [273]. They also noted that the level of HLA-A2 class I molecules expressed by their tumor cell line was increased after treatment [273]. Even though this latter result is somewhat in contrast to our current results, the mechanistic paradigm(s) for HSP90 inhibitor effects on immune augmentation appear consistent in the two studies. In marked contrast, Callahan *et al.* reported that H-2L^d expression was reduced on cells treated with HSP90 inhibitors radicicol, geldanamycin or 17-AAG due to the inhibited loading of peptides into MHC class I complexes, and that radicicol treatment of SV40 large T-antigen (TAg)-expressing SVB6 cells impaired their recognition by an anti-TAg T cell clone (K11; [274]). However the doses of HSP90 inhibitors used by these investigators were high compared to those used (50x and 16x) in our study and by Castilleja *et al.* [273], respectively. Such drug excess could result in adverse effects at the level of the tumor cell APM or in the expression of alternate integrins/adhesion molecules required for cognate T cell recognition. Moreover, while TAg may interact with HSP90, its degradation is only modestly induced by HSP90 inhibitors [275], which could limit the pool of derivative TAg peptides for the consequent loading of MHC class I complexes required for specific T cell reactivity to occur.

My data suggest that 17-DMAG enhances tumor cell recognition by EphA2-specific CD8$^+$ T cells in a transient manner, with improved recognition observed 24h, but not 48h, after drug

treatment. This contrasts slightly with the inhibitory effects of 17-DMAG on tumor EphA2 expression levels which were comparable or somewhat greater at 48h versus 24h. One possible explanation for this dichotomy may involve differential rates at which the diverse array of HSP90 client proteins undergo proteasomal destruction upon application of 17-DMAG (Kawabe *et al*., unpublished results). As a result, the competitor substrates for proteasomal processing of EphA2 protein may vary over time after drug treatment. If this hypothesis is correct, EphA2 may be more efficiently processed and/or be less effectively competed for loading MHC class I complexes during the first 24h of HSP90 inhibitor administration. We noted that the ability of 17-DMAG treated tumor cells to be loaded by exogenous peptide was somewhat diminished when compared with untreated tumor cells, but that this was not differential at the 24h versus 48h time points post-DMAG application and that this did not appear to reflect any change in the prevalence of "empty" HLA-A2 complexes on the tumor cell surface as a consequence of drug treatment. I interpret these results to suggest that the average affinity of presented peptides (including EphA2 epitopes) in tumor cell HLA-A2 (and corollary stability of MHC complexes that limit loading of exogenous peptides) may be increased as a consequence of 17-DMAG treatment (for 24 or 48h). These findings appear to mitigate concerns that the class I APM is substantially altered in tumor cells after extended treatment (i.e. 48h versus 24h) with HSP inhibitors, which could have logistically prevented the sustained MHC presentation of EphA2 peptides.

It is also possible that "chronic" treatment of tumor cells with 17-DMAG suppresses the production of EphA2 based on direct (or indirect) effects on EphA2 transcription, etc. In this context, activation of the Ras-Raf-MAPK signaling pathway has been shown to enhance EphA2 transcription [81], however, Raf is a client protein of HSP90 (see http://www.picard.ch/downloads/Hsp90interactors.pdf). Hence, HSP90 inhibitors may antagonize this signaling cascade [276] and consequently limit EphA2 transcription by promoting Raf degradation. We are currently in the process of analyzing such possibilities in order to establish an optimal treatment regimen capable of improving tumor cell presentation of EphA2 epitopes to the immune system.

Although my data supports the increased presentation of EphA2 peptides in tumor cell HLA-A2 complexes after treatment with 17-DMAG, there is some concern for the mechanistic implications of the cross-reactivity of 17-DMAG with another HSP90 family member, gp96 (GRP94). Gp96 is now known to play important roles in antigen (cross)presentation based on its ability to deliver chaperoned, antigenic peptides to nascent MHC class I and class II molecules [277, 278]. This has resulted in intense research designed to utilized this protein as an "adjuvant" in cancer vaccines [279]. A preferred binding capacity of 17-AAG and 17-DMAG for HSP90 versus gp96 has been previously reported by Chandarlapaty *et al.* [251], however, these results may be controversial and I cannot formally exclude a role for HSP90 inhibitors on tumor gp96 in the results obtained in my model system.

Prospectively, we will perform *in vivo* experiments in which EphA2$^+$ tumor-bearing mice will be treated with orally administered 17-DMAG in hopes of enhancing tumor elimination by adoptively-transferred or vaccine-induced anti-EphA2 CD8$^+$ T cells. However, it may still be difficult to discern the specific impact of improved MHC class I/EphA2 peptide complex presentation on therapy outcome given the pleiotropic effects of 17-DMAG on tumor cells *in vivo* [242]. Additionally, tumor growth could be substantially inhibited as a consequence of 17-DMAG's effects on the tumor vasculature, since EphA2, VEGFR1, VEGFR2, PDGFR and c-KIT are molecules important for tumor neoangiogenesis and also known client proteins of HSP90 (see http://www.picard.ch/downloads/Hsp90interactors.pdf). In addition, overexpression of STAT-3 (an HSP90 client protein) has been implicated in more aggressive tumor cells and has been suggested to be involved in the tumor-induced accumulation of regulatory T cells [280], that may limit therapeutic efficacy. Hence, the accentuated and sustained degradation of STAT-3 that could occur under the influence of 17-DMAG [281] may lead to a reduction in regulatory T cells found in the tumor microenvironment and improved tumor eradication mediated by anti-tumor CD8$^+$ T cells. We believe the ability to discriminate between the underlying influences of these various mechanisms will be optimized via the study of "pulse" (acute) versus chronic administration treatment with 17-DMAG. In particular, enhancement in tumor cell RTK antigen presentation may be best interpreted in the context of short 24-48h treatment cycles, where the effects of 17-DMAG on the tumor vasculature and Treg may require extended periods of drug administration.

In conclusion, my results suggest that an effective combinational immunotherapy for clinical translation [3] may be defined by short-term 17-DMAG administration to improve EphA2$^+$ tumor cell recognition by CD8$^+$ T cells that have been previously elicited in response to EphA2-based vaccination or that are provided via adoptive transfer. Such combinational approaches might be further improved by inclusion of Type-I or -II interferon co-administration to further improve tumor cell APM function and the MHC class I (and II) presentation of targeted epitopes by tumor cells *in vivo* [149, 282].

Preface Chapter 4

My goal was to determine whether treatment of tumor cells with the HSP90 inhibitor 17-DMAG results in the coordinate degradation of multiple (overexpressed) RTK, leading to improved recognition by RTK-specific $CD8^+$ T cells *in vitro*. As in the case of studies outlined in Chapter 3, Western Blot and ELISA analyses were performed to monitor Her2/neu (ErbB2) and EGFR (ErbB1) degradation and corollary MHC class I-presentation of RTK-derived peptides on tumor cells, respectively. I observed that 17-DMAG concomitantly reduced tumor cell expression of the Her2/neu and EGFR oncoproteins on diverse types of solid cancers. I also showed that tumor cell treatment with 17-DMAG enhanced specific recognition by anti-Her2/neu reactive $CD8^+$ T cells (as was the case of EphA2 specific T cells in Chapter 3). Together with the studies detailed in **Chapter 3**, the current data suggest that HSP90 inhibitors are capable of conditionally promoting the enhanced processing and presentation of RTK-derived epitopes allowing for superior immune recognition, based on these findings, I believe that drugs, such as 17-DMAG have potential as conditional "sensitizers" of tumor cells to immune intervention and may be used as a co-therapy to vaccines or adoptive T cell transfer modalities.

4. HSP90 Inhibitor Coordinately Enhance Recognition of RTK$^+$ Tumor Cells by Specific CD8$^+$ T cells

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All the results reported in this study were obtained by Mayumi Kawabe.

4.1. ABSTRACT

Published reports, including our own, have documented the coordinate overexpression of multiple receptor tyrosine kinases (RTKs) by tumor cells [1], which may contribute to the proliferative or metastatic potential of cancer cells and to the typically poorer clinical prognosis for patients harboring such cancer lesions. Many current therapies have focused on the silencing or neutralization of RTK expression or their associated signaling pathways. Based on results that I presented in Chapter 3, as well as published works, we know that many (if not all) RTKs represent client proteins for salvage by HSP90. Hence, I believe that my previous findings that HSP90 inhibitor 17-DMAG promotes tumor cell EphA2 degradation and conditional enhancement in tumor cell recognition by anti-EphA2 $CD8^+$ T cells, may be generalized as a paradigm for alternate RTKs, such as EGFR and Her2/neu. In the current chapter, I demonstrate multiple RTKs that are concomitantly overexpressed by RCC tumor cell lines may be driven into the proteasomal degradation pathway by HSP90 inhibitor 17-DMAG, leading to improved tumor cell recognition by specific anti-RTK T cells *in vitro*. When taken together with the data previously reported in Chapter 3, I believe this suggests the likely efficacy of a generalized combinational therapy for RTK^+ tumors consisting of specific vaccination or adoptive T cell transfer and cyclical 1-2 day administrations of an HSP90 inhibitor to maximize the therapeutic potential of the anti-RTK immune response.

4.2. INTRODUCTION

Receptor tyrosine kinases (RTKs) are comprised of 58 known members (Table 1) and regulate diverse cellular functions including proliferation, differentiation, migration, and survival [1]. Slightly more than half of the known RTKs have been reported to be either mutated or overexpressed in cancer cells, including EGFR, ErbB2-4, PDGFR-α/β, VEGFR1-3, FGFR1-4, MET, RON, EphA2, EphB2/4, AXL, TIE, TEK [1, 7, 16-18, 20-23].

Among these, Her2/neu (ErbB2) and EGFR (ErbB1) have been the most intensively studied, and arguably, the most clinically-relevant RTKs to date. These receptors trigger downstream signaling pathways that are not linear but consist of a rich multilayered network, which allows for horizontal interactions and permits multiple combinatorial responses [283-285]. An interesting feature of this subfamily is that they form homo- or heterodimeric complexes among the four ErbB (ErbB1-4) family members, thereby providing signal amplification and diversification under physiologic conditions. In tumor cells, these receptors may be activated by additional mechanisms such as ligand-independent receptor dimerization due to their overexpression and signaling pathways may be disrupted due to mutations.

Her2/neu is a "ligand-less" receptor, but it represents the preferred heterodimerization partner for all other ErbB subfamily members. Hence, it exerts its function through a co-receptor function, with Her2/neu-containing heterodimers providing stronger intracellular signaling than those originating from other ErbB complex receptors [286]. Overexpression of Her2/neu is observed in 30% of breast and prostate cancers and its overexpression has been linked with poor clinical prognosis [287-289]. One of the immunotherapeutic approaches targeting Her2/neu is passive immunotherapy using the anti-Her2/neu antibody Trastuzumab, which represents a standard of care for patients with Her2 overexpressing, metastatic breast cancer. The clinical efficacy of this Ab appears clear, especially when administered in conjunction with chemotherapy. However not all patients with Her2/neu overexpressing tumors display benefits after treatment with Traszumab, and the provision of this drug is associated with profound cardiotoxicity (particularly in combinational protocols with chemotherapy agents). Another developing therapy strategy involves patient vaccination with MHC class I- and class II-restricted Her2/neu-derived peptides, or recombinant Her2/neu protein or cDNA [283, 290]. Among several Her2/neu peptide

epitopes, the Her2/neu$_{369-377}$ peptide (also known as E75) is a naturally-processed and MHC class I-presented peptide which has been extensively evaluated in pre-clinical studies and clinical trials. This epitope is now in Phase II clinical trials after Phase I trials demonstrated that it promotes specific CD8$^+$ T cell responses *in vivo* in patients [291, 292].

In contrast to Her2/neu, EGFR has a series of well-known ligands, including epidermal growth factor (EGF) and transforming growth factor-alpha (TGFα) [286], which are also frequently overexpressed in tumors [293]. Specific ligand binding to the receptor triggers both homo- and heterodimer formation, and dysregulation of EGFR has been reported to be induced by its overexpression and/or mutation of tumor cell [286]. Overexpression promotes homodimer formation in the absence of ligand binding. EGFR has been reported to be overexpressed in wide range of human cancers including carcinomas of the bladder, breast, colon, head-and-neck, lung, ovary, prostate and glioblastoma, as well as, in glioma, where overexpression is linked to poorer clinical outcome [294-297]. Treatment strategies targeting the EGFR pathway involve antibody-based immunotherapy and small molecule RTK inhibitors (e.g. Gefitinib). Two antibodies (Cetuximab, Pantitumumab) have recently been FDA-approved and have become standard care for metastatic colorectal cancer, where they have been associated with 10-20% objective response rates based on RECIST criteria [298, 299]. On the contrary and somewhat surprisingly, only one study to my knowledge, has reported the ability of EGFR to serve as a target for T cell recognition.

I showed in **Chapter 3** that the MHC class I-presentation of peptides derived from EphA2 (a novel client protein of HSP90) could be conditionally increased by tumor treatment with the HSP90 inhibitor 17-DMAG. Since both Her2/neu and EGFR are also known to represent HSP90 client proteins [242, 300-302], the treatment of tumor cells with 17-DMAG would be hypothesized to result in the coordinate, facilitated generation of epitopes derived from multiple RTKs (ErbB family members as well as EphA2). In this chapter, I studied how 17-DMAG (at concentrations that do not disrupt the MHC class I APM) affects EGFR and Her2/neu protein levels expressed by various types of solid cancer cell lines, as well as, how this drug augments specific CD8$^+$ T cell recognition of treated tumor cells *in vitro*.

4.3. MATERIALS AND METHODS

4.3.1. Cell lines and Media

SKOV3 (Her2/neu^{+}, HLA-A2^{neg}; kindly provided by Dr. Nora Disis, University of Washington) and SKOV3.A2 (Her2/neu^{+}, HLA-A2^{+}) ovarian carcinoma cell lines (established via transduction of the corresponding parental cell lines with a recombinant retrovirus encoding HLA-A2.1 provided by Dr. Peter Cresswell, Yale University) were used both in Western blot and ELISA analyses. RCC (SLR20, SLR22, SLR24; [8]), melanoma (Mel397, Mel526, Mel624; [268]) and breast carcinoma (SKBR3; kindly provided by Dr. Nora Disis) cell lines were used in Western blot analyses. All cell lines were maintained in RPMI-1640 media supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 10 mM L-glutamine (all reagents from Invitrogen, Carlsbad, CA, unless otherwise indicated) and were free of mycoplasma contamination.

4.3.2. HSP90 inhibitor and Peptides

HSP90 inhibitor 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG, NSC 707545) was obtained from the National Cancer Institute (Bethesda, Maryland). Lyophilized 17-DMAG was dissolved in sterile water as a stock solution and then diluted with RPMI-1640 before use. The HLA-A2-restricted, Her2/neu$_{369\text{-}377}$ (KIFGSLAFL; also known as E75, [303]) peptide epitope was synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry by University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Facility, as previously described [8]. The purity of peptides was >96% based on high performance liquid chromatography, with peptide identity validated by mass spectrometric (MS/MS) analyses performed by the UPCI Protein Sequencing Facility (a Shared Resource).

4.3.3. Western Blot Analyses

Tumor cells at 80-90% confluency were incubated with 17-DMAG (10-1000 nM) in 2% human serum supplemented RPMI-1640 media for 24-48h, as indicated in the text. Harvested cells were

then incubated with lysis buffer (1% TritonX-100, 150 mM NaCl, 10 mM Tris-HCl pH7.4, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% NP-40 in PBS; all reagents from Sigma-Aldrich) containing a protease inhibitor cocktail (Complete mini, Roche Diagnostic, Mannheim, Germany) for 30 minutes at 4°C. Lysates were cleared by centrifugation at 13,500 x g for 10 min, and proteins in the lysate resolved by SDS-PAGE before electro-blotting onto PVDF membranes (Millipore, Bedford, MA). Polyclonal antibodies against AXL, EGFR, Her2/neu, c-Met, RON-β, VEGFR1 and VEGFR2, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (all from Santa Cruz Biotechnology, Santa Cruz, CA, with the exception of the anti-Her2/neu Ab purchased from DAKO) were used to probe blot. Mouse anti-β-actin Ab (clone AC-15, Abcam) and goat anti-mouse mAb (BioRad) were used to detect β-actin as a loading control. Probed proteins were visualized by Western Lighting™ chemiluminescence detection kit (Perkin Elmer, Boston, MA) and exposed to X-Omat film (Eastman Kodak, Rochester, NY) for 1-5 min.

4.3.4. Flow Cytometry

Control or treated tumor cells were harvested with Trypsin-EDTA (Invitrogen), washed and then incubated with anti-EGFR (Santa Cruz Biotechnology) or anti-Her2/neu mAbs (Calbiochem; TA-1) for 30 minutes at 4°C. After washing with PBS/0.2% BSA/0.02% NaN3, cells were stained with FITC-conjugated anti-mouse IgG (MP Biomedicals, Solon, OH) for 30 minutes at 4°C. Cells were washed twice, then analyzed using an LSRII flow cytometer (BD Biosciences, San Jose, CA). Isotype control mAb was $mIgG_1$ (Sigma-Aldrich).

4.3.5. T cell lines

Bulk $CD8^+$ T cell lines specific for $Her2/ner_{369-377}$ were generated as previously described [8]. Briefly, mature dendritic cells (DC) were developed from peripheral blood mononuclear cells (PBMC; obtained with written consent under an IRB-approved protocol) isolated from normal $HLA\text{-}A2^+$ donors in 7 day cultures containing rhGM-CSF (Sargramostim; Amgen, Thousand Oaks, CA) and rhIL-4 (Peprotech, Rocky Hill, NJ), then pulsed with $Her2/ner_{369-377}$ peptides (10 μM) for 4h at 37°C at 5% CO_2 tension. Autologous $CD8^+$ T cells were stimulated on a weekly

basis with peptide-pulsed autologous DC or PBMC for 2-3 cycles (at a T:DC ratio of 10:1 or at a T:PBMC ratio of 1:1) to generate a bulk population of peptide-specific $CD8^+$ T cells. T cells were maintained in IMDM media supplemented with 10% human AB serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM L-glutamine and MEM Non-essential amino acids (all reagents from Invitrogen, except human AB serum that was purchased from Sigma-Aldrich).

4.3.6. Tumor Recognition Assays

Tumor recognition by Her2/neu specific bulk T cells was evaluated by commercial hIFN-γ ELISA (BD-Biosciences). SKOV3.A2 cells were treated with 500 or 1000 nM 17-DMAG for 24-48h, prior to their harvest with Trypsin-EDTA (Invitrogen). HLA-A2 null SKOV3 cells were served as control target cells. After washing with PBS (Invitrogen), tumor cells were co-cultured with anti-Her2/neu bulk T cells at an effector:target cell ratio of 2:1 for 24h at 37^{o}C and 5% CO_2 tension.

4.3.7. Statistical Analyses

Two-tailed Student's t tests were used to evaluate the difference between groups, with p values < 0.05 considered significant.

4.4. RESULTS

4.4.1. HSP90 inhibitor induces multiple RTK degradation concomitantly

The relative protein levels of various RTKs were assessed by Western blot analyses of a range of RCC cell lines (Figure 23). The RTKs AXL, EGFR, Her2/neu and c-Met were expressed at high levels by most of the RCC cell lines tested, while Ron-β and VEGFR2 were overexpressed in a more restricted subset of RCC cell lines. VEGFR1 overexpression was not observed. These data suggest that EphA2, AXL, EGFR, Her2/neu and c-Met represent attractive RTK target proteins, at least, in the setting of RCC. Of these cancer-related RTKs, I selected EGFR and Her2/neu for the further analyses, in large part, due to the availability of clinical-grade monoclonal antibodies targeting these two RTKs that have shown some degree of efficacy in phase I/II trials.

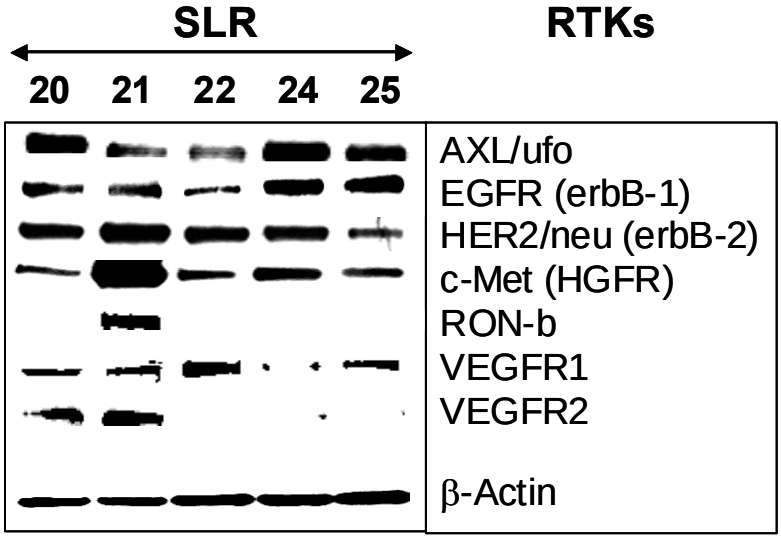


Figure 23. Multiple RTKs are simultaneously highly expressed by individual renal cell carcinoma (RCC) cell lines.
The protein expression levels of the RTKs AXL, EGFR, Her2/neu, c-Met, Ron-β, VEGFR1, VEGFR2, or control β-actin were analyzed in RCC cell lines (SLR20, 21, 22, 24, 25) by Western blotting.

I first analyzed the impact of 17-DMAG on tumor cell expression of these two RTKs initially assessing the RCC cell line SLR20 (which overexpresses both EGFR and Her2/neu). SLR20 cells were incubated for 24h in the absence of in the presence of 17-DMAG (500 nM), and analyzed for EGFR and Her2/neu protein levels by Western blotting (i.e. total protein; Figure 24A) and flow cytometry (i.e. cell surface protein; Figure 24B). The profound reductions in expression of both EGFR and Her2/neu as a result of treatment with 17-DMAG suggested an extension of the paradigm established in my former studies based on EphA2.

To address the generality of 17-DMAG's ability to promote the degradation of EGFR and Her2/neu, I repeated these assays using a diverse panel of tumor cell lines, including other RCC (SLRs), melanoma (Mel), breast carcinoma (SKBR3), and ovarian carcinoma (SKOV3) cell lines. After a 24h treatment with 17-DMAG, the levels of EGFR and Her2/neu protein expressed by tumor cells were dramatically reduced (Figure 25).

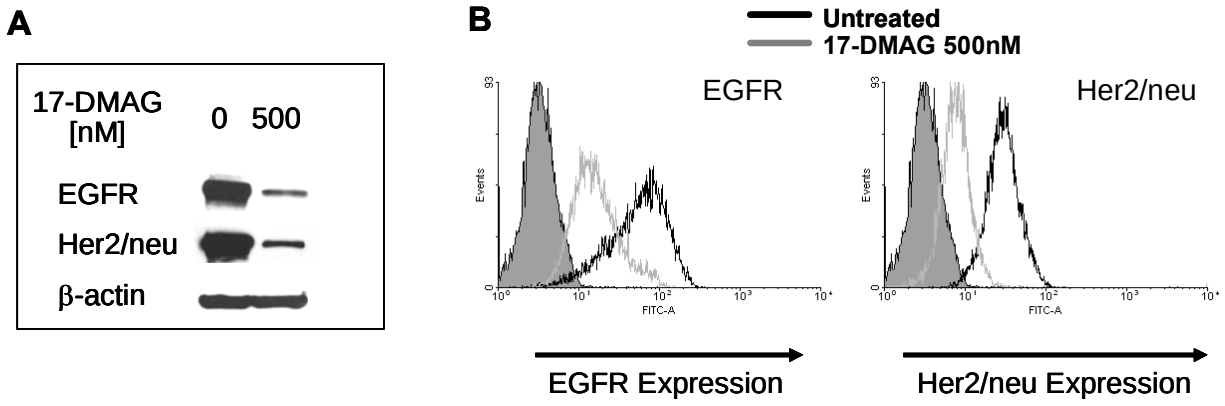


Figure 24. 17-DMAG promotes a coordinate loss in expression of multiple RTKs by the RCC cell line SLR20.
A. The RCC line SLR20 was incubated in the absence or presence of 17-DMAG (500 nM) for 24h at 37°C, before the generation of cell lysates and the performance of Western blot analyses to determine levels of EGFR and Her2/neu protein expression. β-actin was monitored as an internal control protein. **B.** SLR20 cells were treated as above, with cell surface expression of EGFR and Her2/neu protein monitored by flow cytometry using specific mAbs (open profiles) versus isotype control mAbs (filled profiles).

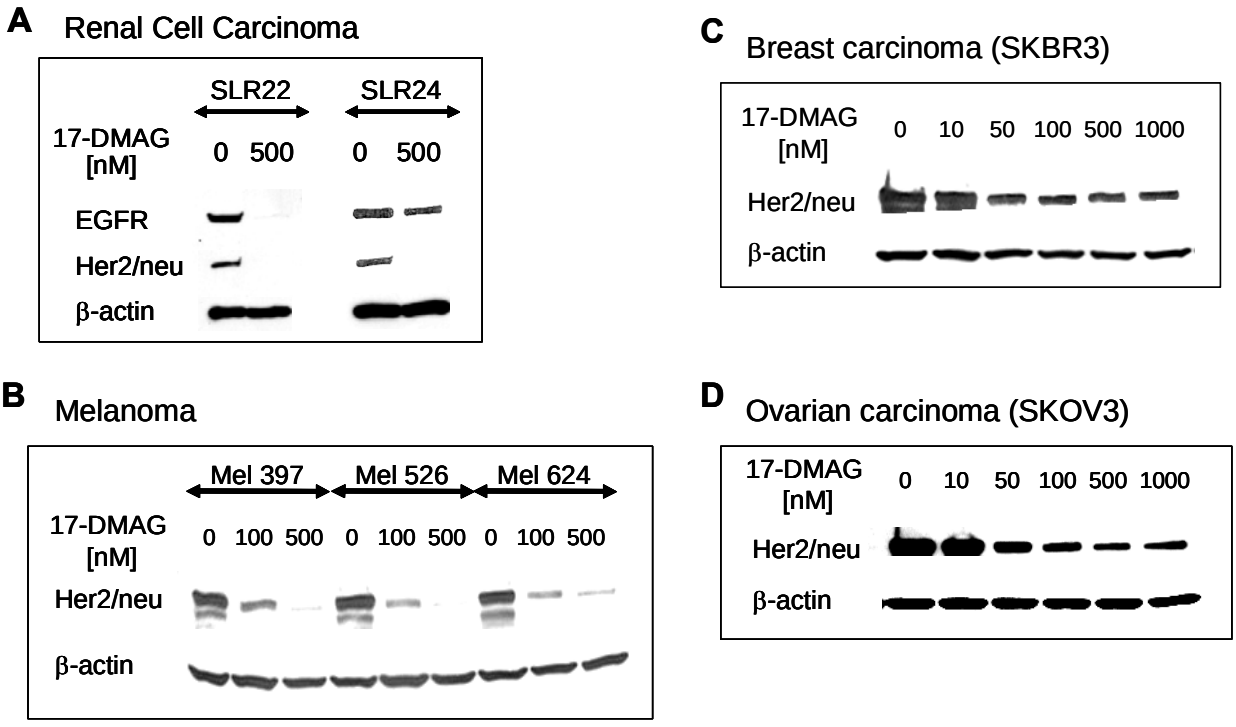


Figure 25. RCC, Melanoma, Breast and Ovarian tumors are all sensitive to 17-DMAG-mediated reduction in expression of EGFR and Her2/neu.
The SLR22, SLR24 (RCC, **A**), Mel397, Mel526, Mel624 (melanoma, **B**), SKBR3 (breast carcinoma, **C**) and SKOV3 (ovarian carcinoma, **D**) cell lines were incubated in the absence or presence of 17-DMAG (10-1000 nM) for 24h at 37^{o}C, before the generation of cell lysates and the performance of Western blot analyses to determine levels of EphA2 protein expression. β-actin was monitored as an internal control protein.

4.4.2. 17-DMAG treatment of tumor cells enhances tumor recognition by bulk CD8^{+} T cells specific for Her2/neu

Bulk CD8^{+} T cell line specific for Her2/neu was generated from normal HLA-A2^{+} donors using an *in vitro* stimulation protocol employing autologous DC pulsed with Her2/neu$_{369-377}$ (HLA-A2-presented) peptide as stimulator cells. After 2-3 rounds of restimulation, responder CD8^{+} T cells were cultured with SKOV3.A2 cells (Her2/neu^{+}, HLA-A2^{+}) which were untreated or pre-treated with 17-DMAG (500nM or 1000nM) for 24h. The content of IFNγ in the culture supernatant was analyzed by ELISA as an indicator of Her2/neu-derived peptide recognition by specific CD8^{+} T

cells. As shown in Figure 26, Her2/neu-reactive, bulk $CD8^+$ T cells recognized $Her2/neu^+$, $HLA\text{-}A2^+$ SKOV3.A2 cells, while recognition of $Her2/neu^+$, $HLA\text{-}A2^{neg}$ SKOV3 cells was observed to occur at a low, basal level. The SKOV3.A2 recognition was increased significantly after 24h treatment, and further increased after 48h treatment. The improved recognition of tumor cells at 48h over 24h was somewhat in contrast to **Chapter 3**'s results for EphA2-specific T cell reactivity, which was stronger at 24h versus 48h. I believe that this may indicate that the processing of these RTKs is kinetically differential, or that the durability of the Her2/neu peptide-class I complexes is more stable that those formed with EphA2 peptides. The improvement of SKOV3.A2 recognition was not significantly different between 500nM and 1000nM treatments, consistent with the comparable levels of Her2/neu protein reduction shown in Figure 25, and suggests that a sufficient level of the peptide epitope (exceeding the T cell activation threshold) occurs at a 17-DMAG concentration of 500nM.

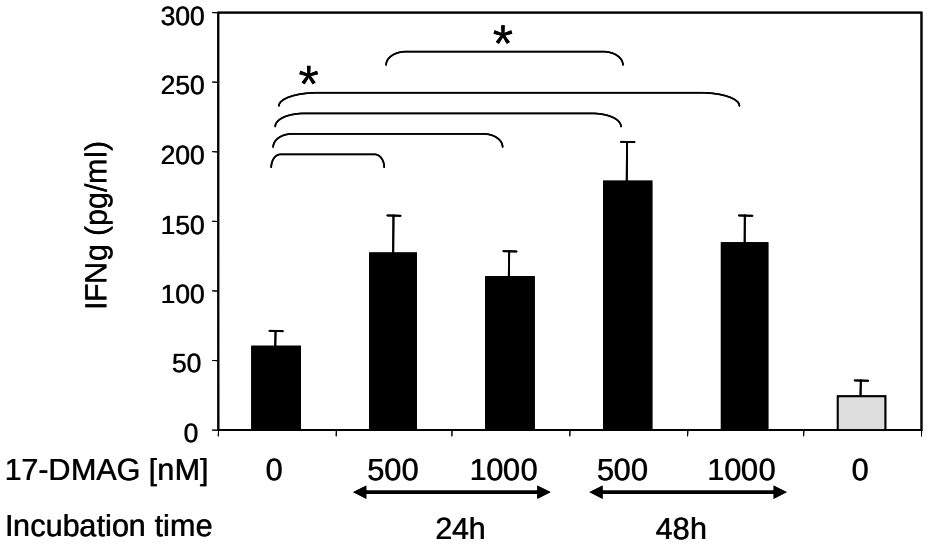


Figure 26. Recognition of $Her2/neu^+$ SKOV3.A2 tumor cells by bulk anti-EphA2 $CD8^+$ T cells is enhanced after treatment with 17-DMAG.
An HLA-A2-restricted $CD8^+$ T cell line was generated *in vitro* by repeated stimulation with autologous DC pulsed with the $Her2/neu_{369\text{-}377}$ peptide. These $CD8^+$ T cells (10^5) were then co-cultured for 24h at 37°C with the $Her2/neu^+$, $HLA\text{-}A2^+$ SKOV3.A2 ovarian carcinoma cells (0.5 x 10^5) that had be pre-cultured in the absence or presence of 17-DMAG (500 nM or 1000 nM) for 24h or 48h. IFNγ content in the cell-free culture supernatant was then analyzed by ELISA. Light gray bar indicates untreated SKOV3 cells, and black bars indicate SKOV3.A2 cells. *$p < 0.05$ for all indicated comparisons.

4.5. DISCUSSION

Since most TAA are overexpressed self antigen, tolerance selection restricts the anti-tumor $CD8^+$ T cell repertoire to a degree that limits their clinical utility. My thesis is based on the hypothesis that tumor cells might be therapeutically manipulated in order to exceed the cognate antigen threshold requirements for effective immune surveillance by normally non-self reactive T cells. In **Chapter 3** we showed that treatment of $EphA2^+$ tumor cells with the irreversible HSP90 inhibitor, 17-DMAG, enhances their recognition by EphA2-specific $CD8^+$ T cell lines and clones *in vitro*. While Her2/neu and EGFR, overexpressed in various cancer histologies is association with poor prognosis [287-289, 294-297], these proteins have been reported to serve as clients for HSP90[242, 300-302], making them targets for the action of the HSP90 inhibitor, 17-DMAG.

The major findings of this chapter are that; 1) multiple RTKs proteins are concomitantly overexpressed in a broad range of tumor cell lines; 2) 17-DMAG coordinately reduces Her2/neu and EGFR, as well as EphA2, protein levels expressed by tumor cells; and 3) 17-DMAG sensitizes Her2/neu overexpressed tumor cells for recognition by anti-Her2/neu $CD8^+$ T cells. These findings, when coupled with those reported in **Chapter 3**, strongly suggests that the treatment of tumor cells with HSP90 inhibitors has the potential to promote the coordinate proteasomal processing of multiple RTKs. My data are consistent with the study of *Castilleja et al.*, which showed another HSP90 inhibitor geldanamycin was able to induce Her2/neu degradation and improved recognition by anti-Her2/$neu_{369\text{-}377}$ $CD8^+$ T cells *in vitro* [273]. Even though there is a slight discrepancy in my report versus that of Castilleja *et al.* regarding drug effects on tumor cell HLA-A2 class I expression levels (upregulated in the Casteilleja study versus no change in my studies), the mechanistic paradigm(s) for HSP90 inhibitor effects on immune augmentation appear consistent between the two studies.

We also noted that Her2/neu protein is very sensitive for 17-DMAG treatment. A profound reduction in Her2/neu protein by tumor cells was achieved at low doses of this drug in all tumor cell lines evaluated, even the SLR24 RCC line in which EphA2 and EGFR protein expression levels were relatively resistant to the effects of 17-DMAG. It has been reported that HSP90 associates with Her2/neu intracellularly (involving the cytoplasmic kinase domain) and stabilizes

Her2/neu expression on the plasma membrane [304, 305]. HSP90 inhibitors disrupt the interaction of these two molecules, resulting in rapid degradation of Her2/neu. In addition, cell surface expression of HSP90 has recently been reported, in which the HSP was found to interact specifically with the extracellular domain of Her2/neu, leading to an enhancement in actin rearrangement and improved cell motility (involved in cancer cell invasion) [306]. HSP90 thus appears to modulate Her2/neu expression at multiple levels, and this may make this RTK particularly salient as a target for this HSP90 inhibitor.

Interestingly HSP90 inhibitor induce the MHC class I presentation of peptides derived from both EphA2 and Her2/neu, but with somewhat different kinetics; i.e. EphA2 presentation was best at 24h (**Chapter 3**), while Her2/neu presentation was best at 48h (**Chapter 4**). These findings suggest that the observed decrease of EphA2 presentation at 48h (versus 24h) was not likely due to adverse effects of 17-DMAG on the tumor cell MHC class I APM, nor due to an increase/decrease of inhibitory/activation ("co-receptor") molecules. Rather differential competition of peptides for loading MHC class I complexes and/or differential dissociation profiles for these class I complexes may represent logical reasons for the observed behavior of this drug's action in the current studies. To address this hypothesis, we are currently performing mass spectrometry analysis of extracted peptides from untreated tumors versus tumors treated with 17-DMAG for 24h versus 48h, which may reveal the relative abundance of relevant peptides over time in tumor cell MHC class I complexes.

Our finding that 17-DMAG is capable of coordinately inducing the enhanced MHC class I presentation of peptide epitopes from multiple RTKs might make pre-administration of 17-DMAG a generalized strategy to enhance the clinical efficacy of vaccines promoting specific $CD8^+$ T cell responses against HSP90 client proteins. Such poly-specific T cell targeting of tumors may have a profound clinical benefit by providing a multi-pronged attack against tumor cells. In addition, the corollary impact of "epitope spreading" that may be initiated by such a diversified initial round of immune attack against the tumor cells might be anticipated, which has been previously associated with far better treatment outcomes in the cancer therapy setting.

GENERAL DISCUSSION

RTKs are frequently overexpressed and may be constitutively activated in the majority of human cancers, hence, the silencing of their associated signaling pathways or immune targeting of these proteins may yield clinical benefit [1-3]. In particular, my thesis project has endeavored to define strategies by which the frequency of RTK-derived antigenic peptides presented in MHC class I complexes on the tumor cell surface may be transiently increased in order to improve tumor cell recognition by RTK-specific $CD8^+$ T cells that are capable of mediating tumor regression [3].

Immunotherapies (including cancer vaccines) designed to stimulate specific T cell-mediated immunity have thus far yielded rather modest objective clinical response rates, despite their ability to enhance circulating frequencies of tumor-specific T cells in the blood of many treated patients [177]. 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 [256, 257]. These T cells may become effectively activated by APCs presenting abundant levels of tumor antigen-derived peptides, but they typically fail to recognize tumor cells that naturally present low stochastic frequencies of relevant MHC/tumor peptide complexes [264]. Poor immune reactivity of tumor cells may be further exacerbated by defects in the tumor APM that limit the overall level of tumor cell expressed MHC molecules [150, 151].

Consequently interferons continue to attract attention as clinical therapeutic agents given their ability to improve antigen presentation (by APC and tumor cells) via the transcriptional up-regulation of components of the antigen processing machinery (APM). However, MHC class I-restricted T cell-mediated immune responses are typically only modestly enhanced by pre-treatment of tumor cells with interferons [258], and moreover, interferons do not selectively

increase the processing and MHC presentation of epitopes derived from a particular antigen (such as RTK). The evaluation of strategies to conditionally and selectively manipulate tumor cell presentation of T cell epitopes derived from endogenous antigens remains an understudied area of translational cancer research.

My thesis was, therefore, based on the hypothesis that tumor cells might be therapeutically manipulated in order to exceed the cognate antigen threshold requirements for effective immune surveillance by host T cells that have been (thymically and peripherally) educated to ignore "self antigens". In particular, I believe that by conditionally enhancing the proteasomal processing of tumor antigens, such as non-mutated RTKs, the level of class I/RTK peptide complexes might be (at least transiently) increased on the tumor cell surface, allowing for improved recognition by modest avidity anti-RTK $CD8^+$ T cells [3]. While I believe the core principles will likely translate to most (if not all) RTKs, I initially focused my studies on the RTK, EphA2, which is overexpressed/dysregulated in virtually all solid cancers (and where its degree of overexpression is associated with a poor clinical prognosis). Since EphA2 peptide epitopes recognized by HLA-A2-restricted $CD8^+$ T cells had been previously defined by the Storkus laboratory and others [8, 130], it was feasible to evaluated the presentation levels of EphA2 derived peptide in tumor cell-expressed MHC class I by monitoring the degree of tumor recognition by specific $CD8^+$ T cells (e.g. IFNγ production detected by ELISPOT/ELISA). Using EphA2 as a model target, I initiated a search of reagents with which to promote RTK epitope presentation selectively in tumor cell MHC class I complexes.

First, I was interested in evaluating groups of agents that had the potential to alter the life cycle of EphA2, particularly with regard to its ultimate degradation via the proteasome. I selected ephrinA1-Fc and agonistic EphA2 mAb208 as reagents to trigger the internalization of tumor cell EphA2 protein. I found that both agonists promote EphA2 autophosphorylation, rapid internalization and proteasomal degradation/processing, with a corollary improvement observed in tumor recognition by moderate-to-low functional avidity anti-EphA2 $CD8^+$ T cells *in vitro* and *in vivo*. These results suggest that the strategy of targeting the conditional proteasome-mediated destruction of tumor cell-expressed EphA2 protein may be effective for improving the anti-tumor efficacy of $CD8^+$ T effector cells reactive against EphA2-derived epitopes. Notably while single

administration of either agonist was provided into EphA2$^+$ tumor lesions in our Hu-SCID model, this approach failed to inhibit tumor progression. In marked contrast, when agonists were administered prior to the adoptive transfer of human anti-EphA2 CD8$^+$ T cells (which were also poorly therapeutic as a single modality), this combinational treatment resulted in complete tumor eradication. These results suggest that conditional proteasome-mediated destruction of tumor cell EphA2 may be therapeutically critical in order to enable the existing (or induced) EphA2-specific CD8$^+$ T cell repertoire to mediate a clinically-meaningful anti-tumor response *in vivo*,

During a search of alternative approaches to enhance EphA2 presentation on tumor cells, I became interested in the chaperone function of HSP90. Although EphA2 had not been previously reported to represent a client protein for HSP90, given that several alternate RTKs had been identified as HSP90 clients [242, 300-302], I felt this was a logical possibility. Therefore, I hypothesized that inhibition of HSP90 function might lead to the degradation of EphA2 proteins most probably via the proteasome, and that derivative peptides might gain access to nascent MHC class I molecules through the direct presentation pathway. Using a newest generation HSP90 inhibitor, 17-DMAG, I determined that EphA2 is a novel client protein of HSP90. Furthermore, treatment of tumor cells with 17-DMAG for a period of 24-48h, using a clinically well-tolerated dose of this agent, results in: 1) the proteasome-dependent degradation of tumor cell-expressed EphA2 protein and 2) the augmentation of tumor cell recognition by low avidity anti-EphA2 CD8$^+$ T cells *in vitro*. I further discerned that the mechanism underlying enhanced tumor cell recognition by T cells after 17-DMAG treatment was dependent on Sec61-mediated retrotranslocation, proteasome-mediated enzymatic cleavage and TAP-mediated delivery of EphA2 peptides into the MHC class I biosynthetic pathway. While 17-DMAG acts in a mechanistically distinct manner from EphA2 agonists, both of these modalities yield similar immunologic endpoints with regard to specific tumor cells recognition. Furthermore, I found that these 2 approaches synergize as a combinational treatment regimen in promoting EphA2 protein loss and enhanced T cell recognition was observed. Such synergy could reflect the complementation in the targeted pools of tumor cell EphA2 protein being affected by each modality, i.e. mAb208 or ephrinA1-Fc may primarily target the plasma membrane pool of EphA2 protein, while 17-DMAG may primarily affect the intracellular pool of this protein. The differential source of EphA2 may also determine the kinetics with which enhanced T cell

recognition occurs. Indeed, I noticed that the 17-DMAG induced-improvement of tumor recognition was optimal at 24h versus 48h (return to basal level), while agonists treatment yielded a more prolonged period of enhanced tumor cell recognition by anti-EphA2 $CD8^+$ T cells.

In corollary studies, I also noted that 17-DMAG treatment of tumor cells improves the MHC class I presentation of Her2/neu-derived $CD8^+$ T cell epitopes, and that the kinetics for improved recognition differed from those I previously observed for the EphA2 model. These data may indicate differential competition of specific peptides for loading into MHC class I complexes and/or differential dissociation profiles for specific MHC class I/RTK peptide complexes exists.

Interestingly, I found that both EphA2 agonist and HSP90 inhibitor treatment led to the enhanced tumor cell recognition by $CD8^+$ T cells reactive against peptides derived from the extracellular (i.e. $EphA2_{58\text{-}66}$), as well as, intracellular (i.e. $EphA2_{883\text{-}891}$) domains of the EphA2 protein. This suggests that both domains of this transmembrane protein are driven into the proteasomal processing and MHC class I presentation pathways. To date, however, I have only investigated one peptide derived from each domain, and it would be warranted to analyze the comprehensive pool of MHC class I-presented EphA2 peptides using mass spectrometric analyses. This would allow for a better understanding of the kinetics with which such epitopes are presented and how this may relate to a given treatment applied to enhance immune recognition. Furthermore, such information may be important to the selection of specific peptide species (i.e. those with the most prolonged periods of elevated presentation) for integration into vaccine formulations in order to expand specific $CD8^+$ T cells *in vivo,* or *ex vivo* for ultimate delivery via adoptive transfer.

As described above, I found that both domains of EphA2 are driven into the proteasomal processing pathway of tumor cells by either agonist or HSP90 inhibitor treatment. How both domains of the EphA2 transmembrane protein become accessible to proteasome-dependent degradation in the cytosol remains unclear. The intracellular domain (ICD) is anticipated to be naturally accessible to the action of cytosolic proteasomes, while the extracellular domain (ECD) is thought to be compartmentalized (protected) within intracellular vesicles, such as ER, Golgi, endosomes, etc. So, how is the ECD of the RTK released from such protective compartments and

delivered into cytosol where proteasome resides? For nascent and mis-folded proteins, such delivery may be achieved by protein retrotranslocation as employed during ERAD, where Sec61, Derlin-1 and/or Doa10 play pivotal roles in "ratcheting" transmembrane proteins into the cytosol in a C-terminus to N-terminus manner [207, 265-267, 307]. A similar/identical mechanism appears to underlie my observed results, since enhanced anti-EphA2 T cell recognition of 17-DMAG-conditioned tumor cells was ablated upon inclusion of the Sec61 inhibitor, *Pseudomonas aeruginosa* exotoxin A. This suggests that the major pool of EphA2 protein undergoing enhanced proteasomal processing as a consequence of HSP90 inhibition likely enters the tumor cytosol via retrotranslocation. However, at present, I cannot distinguish whether this pool of EphA2 protein derives from an early endosomal compartment (i.e. internalized after interaction with the EphA2 ligands co-expressed by adjacent tumor cells) and/or from newly-synthesized, mis-folded EphA2 proteins within the exocytic pathway. In the case of agonist-induced EphA2 peptide presentation by tumor cells, the process by which the ECD of EphA2 becomes accessible to the proteasome after internalization is more enigmatic. Possible mechanisms include retrotransportation/retrotranslocation, fusion of $EphA2^{+}$ endosomes with ER and loss of early endocytic vesicle integrity (since chloroquine does not inhibit the impact of agonists in this model), however, these possibilities will need to be addressed thoroughly in prospective experiments.

There are indeed complexed transportation pathways among early/sorting endosome, late endosome, lysosome, trans-Golgi network, Golgi and ER, that have been predominantly identified through the study of bacterial and viral toxins, since many of these pathogens have evolved diverse strategies for traversing membrane barriers in order to reach their preferred substrates in the cytosol of mammalian cells [308-310]. In fact, some reagents used in Chapter 2 and Chapter 3 investigations to assess mechanistic dependency on TAP and retrotranslocation are derived from viral or bacterial (*herpes simplex virus* ICP47 and *Pseudomonas aeruginosa* exotoxin A, respectively) sources [265]. Several strategies may be conceived to incriminate the relevant intracellular pathways taken by EphA2 proteins after agonist or HSP90 inhibitor treatment, including siRNA knockdown of critical proteins associated with vesicle transportation (e.g. Rab5, Rab9, Syn16), as well as, pharmacologic inhibition (e.g. U18666A) [265, 309, 310]. However, more direct evidence might be obtained by detailed imaging analyses of EphA2

protein translocation after agonist or HSP90 inhibitor treatment, and in the absence or presence of pharmacologic inhibitors for each pathway. We are currently considering the use of a novel tracking technology LumioTM Green (Invitrogen Life Technologies), with which one can visualize subcellular localization of FlAsH-tagged (a short amino acid tag that is less likely to negatively affect the natural behavior/function of a protein versus tagging with a prohibitively large molecule such as EGFP, etc.) proteins by fluorescence microscopy with high sensitivity and specificity [311]. We have generated multiple FlAsH-tagged EphA2 constructs that are in the initial stages of validating their utility in imaging analyses. Ultimately, a better understanding of the mechanism(s) involved in agonist- or HSP90 inhibitor-enhanced presentation of EphA2-derived T cell epitopes may allow for the therapeutic accentuation of relevant pathways, allowing for even further optimization of therapy benefits in patients harboring $EphA2^{+}$ tumors.

Furthermore, I asked whether the treatment of tumor cells with the HSP90 inhibitor 17-DMAG would result in the coordinate degradation of multiple (overexpressed) RTK, leading to improved recognition by multiple RTK-specific $CD8^{+}$ T cells *in vitro*. While not exhaustive (in screening all known RTKs), I found that 17-DMAG can coordinately promote the proteasomal degradation of, at least, Her2/neu, EGFR and EphA2 in treated tumor cells. In the cases of Her2/neu and EphA2, such proteolysis sensitizes tumor cells to recognition by specific $CD8^{+}$ T cells. Thus, my data would support a generalizeable paradigm that might be extended to additional RTKs. Interestingly, HSP90 is critical for the folding of nascent Her2/neu protein, but it also serves to stabilize mature Her2/neu on the cell membrane [304, 305]. The same is true for mutant EGFR, while HSP90 is only responsible for the folding of nascent wild-type EGFR [312, 313]. Therefore, it will be intriguing to determine whether therapy benefits observed for HSP90 inhibitors depend on HSP90 interaction with RTKs (including EphA2) on the tumor cell plasma membrane versus the same RTK proteins localized to alternative intracellular organelles.

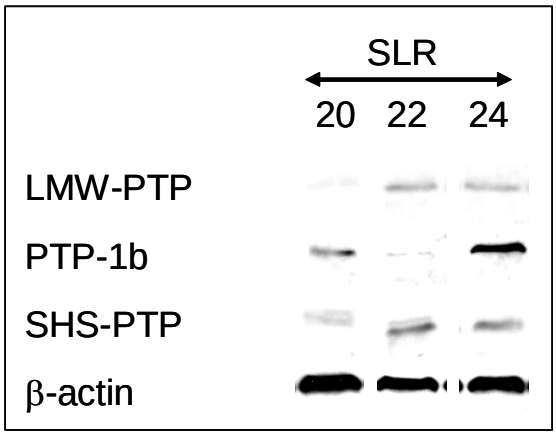
In the clinic, EphA2 agonists would be expected to be superior with regard to targeting the EphA2 protein specifically, when compared with the targeting of a broad class of client proteins (that include EphA2) in the case of HSP90 inhibitors. However, the promiscuous and coordinate effects of HSP90 inhibitors on multiple oncoprotein clients might argue for the use of these drugs in combinational immunotherapies designed to promote the concomitant immune targeting

of multiple tumor-associated antigens (such as EphA2, EGFR and Her2/neu, etc.). HSP90 inhibitors may be preferred to agonists based on their selective accumulation in tumor versus normal EphA2$^+$ tissue sites. For example, 17-AAG binds to tumor cell-derived HSP90 up to 2-logs more tightly than it does to normal cell-derived HSP90 [314]. Furthermore, in mouse models, 17-DMAG persists within tumor lesions far longer than in normal tissues, with detectable levels selectively observed in tumors 48h after a single i.v. administration of drug [249]. This suggests that drug dosing far below the MTD may be capable of modulating T cell recognition of cancer cells within the tumor microenvironment *in vivo*. However, it is also possible that the application of 17-DMAG and/or EphA2 agonists may promote increased immune recognition of normal EphA2$^+$ tissues that express a fully functional class I APM. This raises the specter of autoimmune pathology that would need to be closely monitored in prospective clinical trials applying these agents, particularly should they be combined. In this regard, no untoward, immune-mediated effects on patient EphA2$^+$ organs (i.e. lungs, kidney) have been reported in clinical trials employing HSP90 inhibitors to date [315], and EphA2 agonist therapies have yet to be investigated in phase I trials.

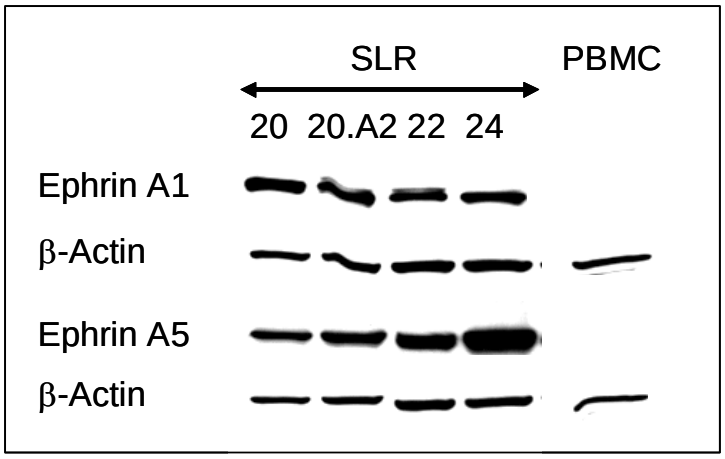
Based on the aggregate data I have presented in this thesis, I believe that it is warranted to translate my *in vitro* findings with 17-DMAG into mouse (*in vivo)* tumor models. The Hu-SCID tumor model used in Chapter 2 may be a good one to consider for this purpose, as it allows us to establish human tumors in mice and to evaluate the efficacy of oral administration of 17-DMAG, adoptive T cell transfer or a combinational treatment integrating both of these modalities. However, given severe immunodeficiency in this mouse strain, we would not be able to analyze potential autoimmune pathology resulting from therapy. Tumor-bearing, HLA-A2 Tg (HHD) mice would be alternate model to consider, as this would allow for the direct testing of combinational vaccine therapies that could be directly translated into clinical trials accruing HLA-A2$^+$ patients with solid cancers [316]. We are currently breeding HHD mice for use in the analysis of combinational EphA2 vaccine + 17-DMAG-based therapies. In such a system, we can analyze normal EphA2$^+$ tissues for inflammation and immune infiltration, and determine whether 17-DMAG exerts any adverse effects on the immune system, i.e. DC and T cell maturation/function may have concerns based on recent publications [317-319].

Overall, I have demonstrated that two reagent groups (RTK agonists, HSP90 inhibitors), that appear to mediate their effects via different mechanisms, enhance tumor cell recognition by specific $CD8^+$ T cells. Such improved immune recognition may translate into improved therapeutic benefit, particularly when combinational immunotherapies (i.e. agonists + adoptive T cell transfer) are administered to tumor-bearing animals. I have also shown that HSP90 inhibitor treatment is capable of coordinately regulating the delivery of multiple RTKs (i.e. their derivative peptides) into the direct class I presentation pathway, suggesting the generality of this approach and potential augmentation of treated tumor cell recognition by low avidity $CD8^+$ T cells capable of recognizing virtually any HSP90 client protein. Such poly-specific T cell targeting of tumors may have a profound clinical benefit by providing a multi-pronged attack against tumor cells, limiting concerns for tumor variants that fail to express a given target antigen. My data will hopefully assist in the future development of novel combinational immunotherapies employing RTK agonists and HSP90 inhibitors, which in concert with vaccines designs to elicit anti-RTK $CD8^+$ T cells or adoptively-transferred anti-RTK $CD8^+$ T cells, would be expected to provide far greater clinical benefits to RTK^+ cancer patients.

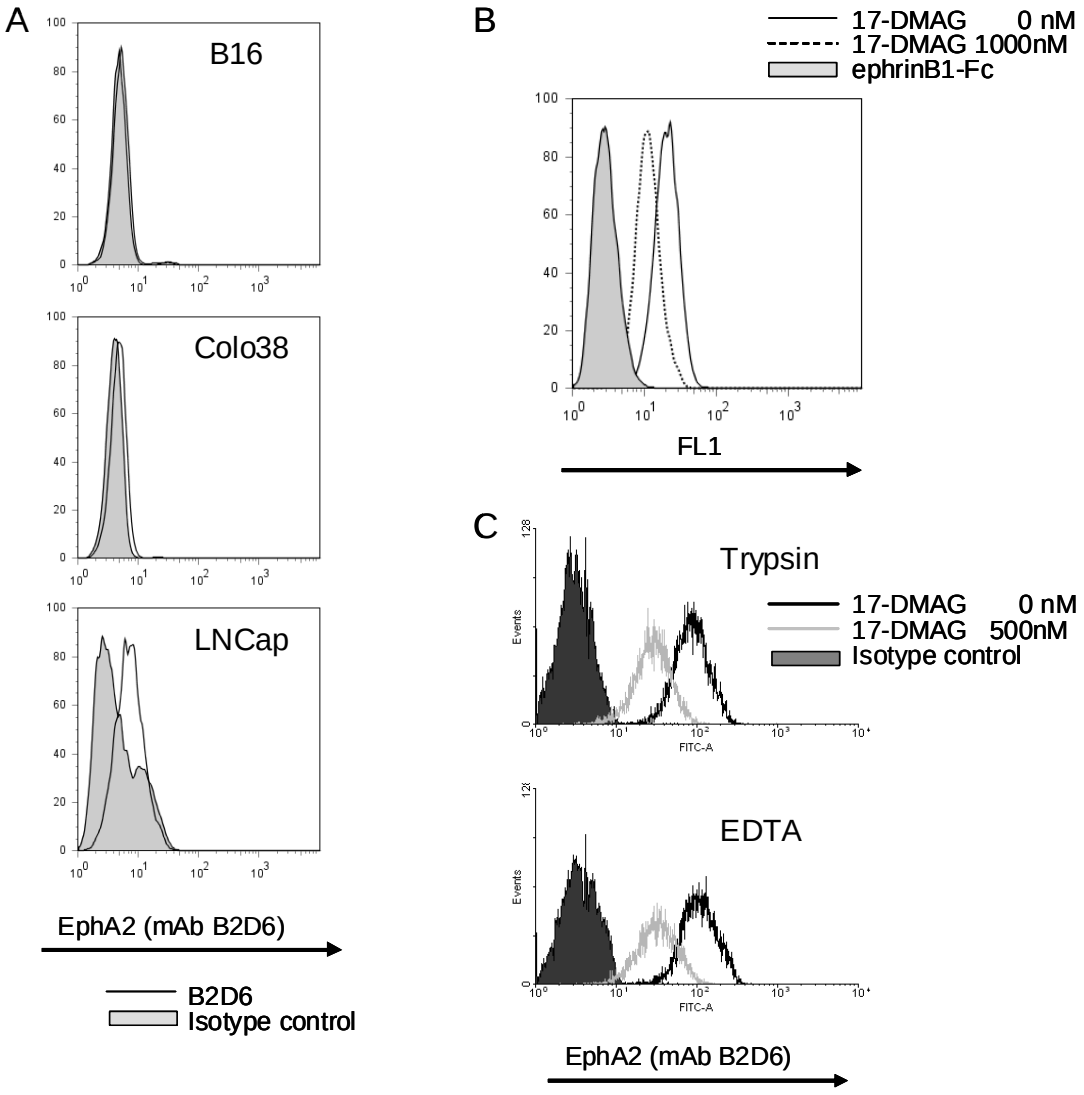
APPENDICES



Appendix Figure 1. PTP expression by RCC cell lines.
Protein expression levels of LMW-PTP, PTP-1b and SHS-PTP by RCC cell lines SLR20, 22, 24 were analyzed by Western blotting using specific antibodies (all from Santa Cruz Biotechnology). β-actin was monitored as an internal control protein.

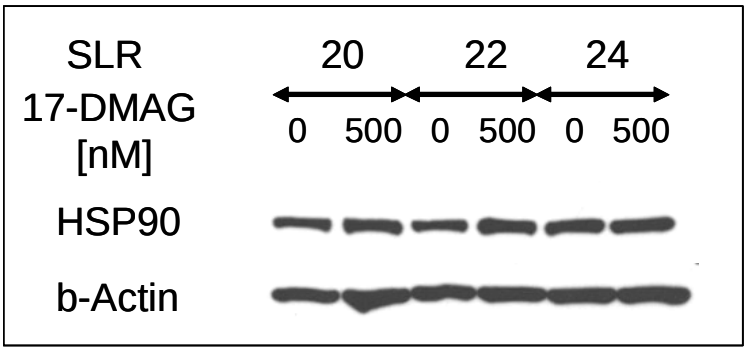


Appendix Figure 2. ephrin-A1 and ephrin-A5 protein expression by RCC cell lines.
Levels of ephrin-A1 and ephrin-A5 protein expressed by RCC cell lines SLR 20, 20.A2, 22, 24 were determined by Western blotting using specific antibodies (both from Santa Cruz Biotechnology), with PBMC serving as negative control cells. β-actin was monitored as an internal control protein.

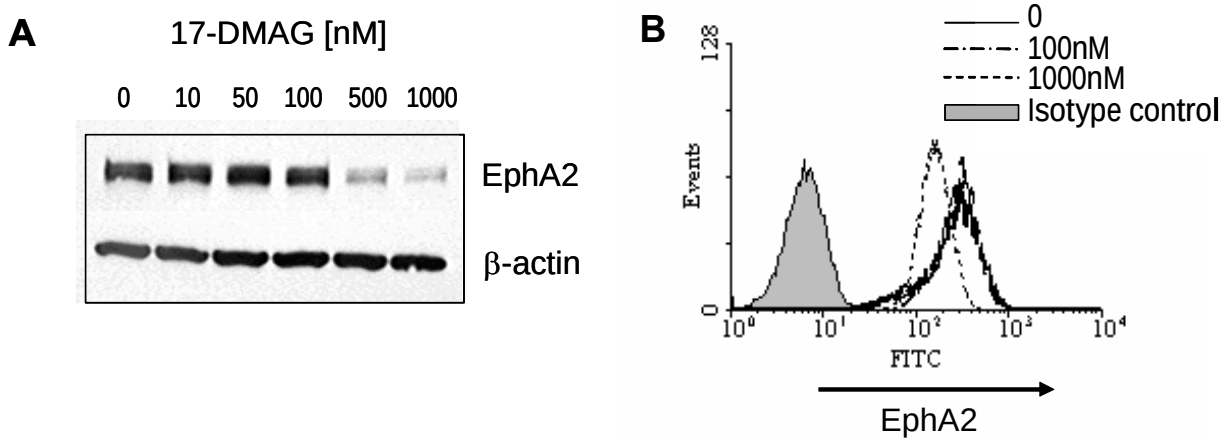


Appendix Figure 3. EphA2 negative cell lines are not recognized by anti-EphA2 mAb B2D6 and the reduction of cell surface EphA2 expression by 17-DMAG on EphA2$^+$ SLR20 tumor cells appears incomplete as monitored by flow cytometry.

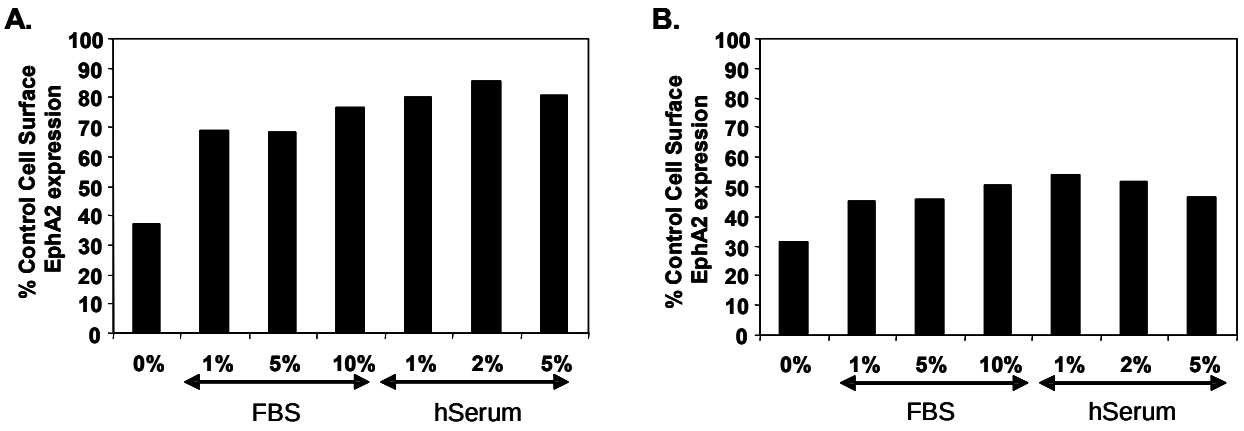
A. Melanoma cell lines B16, Colo38 and the prostate carcinoma cell line LNCap were harvested with Trypsin-EDTA, and their cell surface expression of EphA2 protein was monitored by flow cytometry by using the B2D6 anti-EphA2 mAb. **B.** SLR20 RCC cells were incubated in the absence or presence of 1000 nM 17-DMAG for 24h. Cell were harvested with Trypsin-EDTA, washed and then incubated with 10 μg/ml of ephrinA1-Fc (R&D Systems; a chimeric protein consisting of the ligand binding domain of the EphA2 ligand ephrinA1 fused with the Fc portion of a human IgG Ab) or ephrinB1-Fc (control staining; Sigma-Aldrich) for 1h at 4^{o}C. Cell-bound ephrinA1-Fc or ephrinB1-Fc was then probed with FITC-conjugated anti-human IgG (MP Biomedicals, Solon, OH) and analyzed by flow cytometry. **C.** SLR20 RCC cells were incubated in the absence or presence of 500 nM 17-DMAG for 24h. Cells were harvested with either EDTA (Invitrogen) or Trypsin-EDTA (Invitrogen) and cell surface expression of EphA2 was monitored by flow cytometry using the B2D6 mAb.



Appendix Figure 4. HSP90 protein expression is not affected by 17-DMAG.
The RCC cell lines SLR20, 22, 24 were incubated in the absence or presence of 17-DMAG (500 nM) for 24h at 37°C, before the generation of cell lysates and the performance of Western blot analyses to determine levels of HSP90 (using a specific Ab purchased from Stressgen Bioreagents Corp.) protein expression. β-actin was monitored as an internal control protein.



Appendix Figure 5. EphA2 is conditionally degraded by HSP90 inhibitor, 17-AAG, in RCC cell line SLR20.
A. SLR20 RCC cells were incubated in the absence or presence of 17-AAG (10-1000 nM) for 48h at 37°C, before the generation of cell lysates and the performance of Western blot analyses to determine levels of EphA2 protein expression. β-actin was monitored as an internal control protein. **B.** SLR20 RCC cells were incubated in the absence or presence of 17-AAG (100 or 1000 nM) for 24h at 37°C, with cell surface EphA2 expression monitored by flow cytometry.



Appendix Figure 6. Effect of serum on the EphA2 protein reduction triggered by the HSP90 inhibitors, 17-AAG and 17-DMAG

SLR20 RCC cells were untreated or treated with either 17-AAG (1000 nM, panel **A.**) or 17-DMAG (500 nM, panel **B.**) in the absence or presence of fetal bovine serum (FBS; 1-10%) or human AB serum (hSerum; 1-5%) for 24h at 37^{o}C, with cell surface expression of EphA2 protein monitored by flow cytometry. Data are reported as percentage control EphA2 cell surface expression (versus untreated cells) based on MFI values obtained.

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