CHARACTERIZATION AND IMMUNE TARGETING OF A NOVEL TUMOR ANTIGEN, EPHA2

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Abstract

In order to generate and monitor effective and specific immune responses against tumors, a clear understanding of relevant tumor antigens and their derivative epitopes recognized by T lymphocytes is warranted. The characterization of tumor antigen epitopes recognized by T lymphocytes has been a major focus of study over the past decade. Both CD8+ and CD4+ T lymphocytes contribute to the immune response against tumors, and the determination of the epitopes they recognize is necessary for their incorporation into immunotherapy protocols for cancer. The tumor antigens recognized by T lymphocytes fall into 3 major categories: Tumor-specific (TSA), Cancer Testis (CT), and Tumor Associated Antigens (TAA). Our goal in the following studies was to characterize a novel TAA, EphA2, since this protein has been linked to metastasis in numerous cancer settings.

The definition of epitopes seen by T lymphocytes will assist in vaccine strategies for immunotherapy protocols against EphA2+ tumors. In the following studies, I have defined 8 novel EphA2 T cell epitopes (5 HLA-A2 restricted and 3 HLA-DR4 restricted) recognized by CD8+ and CD4+ T lymphocytes, respectively. The anti-EphA2 CD4+ functional response was skewed based on the presence of disease or increased staging of RCC disease, with patients with active disease exhibiting a Th2-biased CD4+ response. I have also linked the expression of EphA2 in primary RCC tumors to the time to recurrence in patients affected with RCC.

Furthermore, I have demonstrated that the cell surface expression of EphA2 on tumors can be modulated using EphA2 agonists. This agonist treatment results in the enhanced recognition of EphA2+ tumors by specific CTLs. With reports of the overexpression of protein phosphatases (PPs) in several cancer settings, we discovered the EphA2 was constitutively underphosphorylated in certain cancer cell lines, likely as the consequence of overexpressed PP activity. Finally, I have shown that by neutralizing the activity of cellular phosphatases utilizing phosphatase inhibitors, that we can induce the phosphorylation of EphA2 and its subsequent degradation via a largely proteasome-dependent pathway. As a result, this thesis has defined a novel tumor antigen, EphA2, and demonstrated the possibility that modulation of its expression in tumor cells may result in increased recognition by specific T effector cells that may be germane to the design of improved and efficacious therapies for the treatment of patients with EphA2+ tumors.

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PREFACE

I would first like to thank my mentor, Dr. Walter Storkus. I came to Pittsburgh to work for him and will be forever thankful that I did. I feel extremely fortunate to have spent my graduation under his guidance under such a gifted scientist and patient and trusted friend. I would like to the Storkus Lab, who have always been supportive and offered their help and suggestions whenever I needed it. I would next like to thank my Thesis Committee for their suggestions and guidance throughout my time here in Pittsburgh. To all my friends in Pittsburgh, believe me it does come to an end. Finally, I would like to thank my family for their love and support over the past 5 years. I would like to extend a special thank you to my parents, John and Deborah Herrem. Without you, I would have never made it this far. I will be forever grateful for all of your support during the last year of my time here and could not have asked for more loving and supportive parents. I only hope I can be half the parents to my children as you are to me. You instilled the discipline, and work ethic it took for me to do this, and for that, I dedicate this thesis to you.

1. INTRODUCTION

The Eph family of molecules contains 14 members, comprising the largest known cohort of receptor tyrosine kinases (RTK) (1). Based on sequence similarity and binding affinities, two classes of receptors have been defined, EphA and EphB. There are 8 members of the EphA receptor family (EphA1 – EphA8) that primarily bind 5 ephrin-A ligands (ephrinA-1 – ephrinA-5). Ephrin-A ligands are attached to the cell surface via glycosylphosphotidylinositol (GPI) anchors and tend to bind only EphA receptors. There are 6 members of the EphB receptor family (EphB1 – EphB6). The EphB receptors primarily bind 3 ephrin-B ligands (ephrinB1 – ephrinB3) which are transmembrane proteins. However, Eph-ephrin binding is promiscuous in some respects, as there is some crossover between Eph receptors and ephrin ligands. Similarly to other RTK, Eph receptors are type 1 transmembrane proteins (2). The extracellular N-terminus consists of a highly conserved ligand binding region, followed by a cysteine-rich region, and two membrane-proximal fibronectin Type-III repeats (Figure 1). The intracellular region of Eph receptors starts with a juxtamembrane segment with contains two major auto-phosphorylation sites, followed by a conserved kinase domain, which phosphorylates secondary adapter proteins and can phosphorylate the Eph receptors themselves (3). The most C-terminal region contains a sterile a motif (SAM), and a PDZ binding domain. The PDZ domain may be involved in receptor oligomerization (4), facilitating the binding of adapter proteins , or mediating receptor dimerization (5). The binding of Eph receptors to their ephrin ligands occurs at a 1:1 stoichiometric ratio, with the complex forming a circularized heterotetramer (6).

1.1. Eph:Ephrin Signaling

The sequence of signals that are propagated when ephrins and Eph receptors interact has been studied extensively over the past several years. In their inactive conformations, Eph receptors contain an inhibitory loop in the juxtamembrane segment that inhibits the kinase domain. Upon binding their respective ligands, two key tyrosine residues in the juxtamembrane region are phosphorylated, which allows for the kinase domain of Eph receptors to adopt their active conformation (7). Up to 10 tyrosine residues in an Eph receptor are phosphorylated upon ligand binding (8). These provide docking sites for secondary messenger molecules containing SH2 domains to associate with the newly phosphorylated tyrosines on Eph receptors. Signaling between Eph receptors and ephrin ligands is bi-directional. Upon binding its Eph receptor, ephrin-B ligands become phosphorylated on tyrosine residues (9). This allows for the consequent association of Grb4, a SH2 containing secondary messenger, which has been shown to play a role in cytoskeletal regulation (10). Similar to Eph receptors, ephrin-B ligands also contain a PDZ binding domain, that upon tyrosine phosphorylation provides a molecular framework for the construction of larger multi-molecular complexes (11). Upon binding their respective receptors, ephrin-A ligands activate the mitogen associated protein kinase (MAPK) pathway, resulting in alterations in cellular structure and adhesive properties (12). Although their interaction results in the activation of erk-1, which is associated with cellular proliferation, the binding of Eph receptors to ephrins does not necessarily promote cell proliferation and differentiation (13). In fact, certain evidence suggests that signaling either indirectly or directly through Eph receptors may actually suppress cell growth.

1.2. Eph Receptors and Development

Eph receptors were originally defined through their involvement in various embryonic developmental processes and tissue formation pathways. Antagonism (blocking) of the interaction of Eph receptors and ephrin-B ligands in the developing presomitic mesoderm in *Zebrafish* results in the improper formation of somatic boundaries (14). These interactions are also important in mediating axon guidance, regulating and directing nascent nerve networks during development (15). In *Xenopus* embryos, EphA4 and EphB4 in addition with ephrin-B2, are involved in developing the boundary between the third-arch neural crest cells and the second branchial arch. Eph receptors also play a role in the development of tissue "topography" based on gradients of Eph/ephrin expression (16). EphA3 and EphA4, along with ephrin-A2 and ephrin-A5, in particular, are involved in the developmental guidance of axons that attach the retina to the tectum (17) in the eye.

The organization of the vascular system is yet another area where the Eph/ephrins system plays a vital role during development. The mRNA for ephrin-A1 is detected in multiple developmental vascular tissues, including the limb buds, the dorsal aorta, and primary head veins in mice (18, 19). Both ephrin-B2 and EphB4 are expressed predominantly on the murine venous endothelium (20). If either of these genes are knocked out, mid-gestational lethality and major vascular defects are observed in the mouse embryo (21, 22), demonstrating the importance of these molecules in mammalian development. *In vitro*, the addition of ephrin-B2 and ephrin-B1 induces angiogenic vessel sprouting as efficiently as either VEGF or angiopoetin-1 (21). Finally, the Eph/ephrin system has also been reported to play a key role in the developing cardiac vasculature as well. EphB2, EphB4, and ephrin-B2 are expressed in the embryonic cardiac tissue, suggesting they play key roles in the developing embryo (21, 22).

1.3. Eph Receptors and Cytoskeleton

The developing embryo is not the only area where the Eph/ephrin system exerts its biologic influence. Eph/ephrin binding and subsequent signaling has been found to induce processes that modify the cytoskeleton of affected cells. This occurs via the recruitment and/or activation of signaling proteins directly involved in cytoskeletal reorganization. (23-27). EphB2 when activated can phosphorylate R-Ras, which inhibits the ability of R-Ras to mediate integrin signaling (28). EphB2 and EphB3 have been found to play a role in the migration of epithelial cells in the crypt/villus in the colon (29). When these genes are knocked out in mice, the structure of the intestinal epithelium is disrupted and does not form properly.

1.4. EphA2 Background

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 130kDa (Type-1) glyco-protein that is expressed at low levels on numerous epithelial tissues (30). Along with other Eph/ephrin family members, EphA2 is involved in the organization of the developing nervous system (31) and vasculature (32), and is even expressed in embryonic stem cells (33). In normal adult tissues, EphA2 is localized at epithelial cell-to-cell contacts, and is believed to play a role in contact inhibition (34). This function involves inhibiting the growth of epithelial cells while they are in contact with other cells, and is critical for the organization and formation of epithelial layers in EphA2+ tissues. Activation of EphA2 results in the de-phosphorylation of focal adhesion kinase (FAK), which in turn suppresses integrin function (35). EphA2 expression appears to be regulated by E-cadherin (36, 37). A major protein involved in cell adhesion, E-cadherin is expressed in embryonic and

virtually all adult epithelial cells (38). E-cadherin is dependent on Ca^{+2} binding for proper function, and is located at the adherens junctions between cells (39). When E-cadherin expression and its phosphorylation is down-regulated via improper cytoplasmic expression, EphA2 protein expression becomes increased and its cellular localization is disrupted (36) This demonstrates a key inverse relationship between these two proteins that may major roles in cellular adhesion.

1.5. EphA2 and Cancer

The role of RTKs and in carcinogenesis have been increasingly characterized over the last decade (Table 1)(40-55). In particular, growing attention has been given to EphA2 in carcinogenesis with research demonstrating that EphA2 is correlated with the progression to metastasis. Metastasis is the process whereby cancerous cells leave their tissue of origin; invade the extracellular matrix and traffick via either the lymphatics or the bloodstream to distal tissue sites. Metastatic disease is the most advanced and deadly type of cancer as it tends to be lethal and is extremely hard to treat.

EphA2 protein has been reported to be overexpressed 10-100 times greater in metastatic prostate carcinoma cells when compared to non-invasive prostate epithelial cells (56). EphA2 protein is also expressed in greater amounts in malignant mammary tissue when compared to benign mammary epithelia (57, 58). Zelinski, et. al. have further reported that forced overexpression of EphA2 via transfection was sufficient to confer malignant transformation on MCF-10, a benign human mammary epithelial cell line. In melanoma, EphA2 is vastly overexpressed in aggressive MUM-2B melanoma cells when compared to poorly invasive MUM-2C melanoma cells (59). PANC1, a poorly differentiated pancreatic adenocarcinoma that

is highly invasive, overexpresses EphA2 when compared to the non-invasive Capan-2 pancreatic cancer cell line (60). Given this evidence demonstrating the association of EphA2 overexpression and dysregulation with carcinogenesis, along with its correlation with metastasis, one would predict that the degree and distribution of EphA2 protein expression in primary tumors may prove predictive of clinical outcome in patients with EphA2+ tumors.

1.6. Mechanism of EphA2 Overexpression

The 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 (Figure 2).

In order to mediate proper signaling, EphA2 needs to bind its corresponding ligand on adjacent epithelial cells and in the cancer setting, this ability appears to be disrupted. Additionally, in EphA2+ tumors that lose expression of E-cadherin, EphA2 expression is increased and it is no longer found at sites of cell-to-cell contact, but in a perinuclear location (36). When this occurs, EphA2 is no longer found at cell-to-cell contacts, exists in a non-phosphorylated (inactive) state, and instead adopts a diffuse expression pattern throughout the cell. As a result, cell-to-cell contacts become weakened and cell-to-ECM attachments are enhanced (61). This theory has been strengthened by the finding that the addition of exogenous ligand can restore appropriate tyrosine phosphorylation of EphA2 in tumor cells (62). Consequently, ubiquitin-dependent EphA2 protein degradation is enhanced; normalized EphA2

"metabolism" is restored and metastatic characteristics, including decreased ECM attachments, decreased motility, and increased cell-to-cell adhesions result. Therefore, the dysregulated over-expression of EphA2 may represent a first critical step in the progression to metastasis.

1.7. EphA2 and Tyrosine Phosphorylation

Upon ligand stimulation, autophosphorylation of the EphA2 receptor occurs which promotes the association of EphA2 with c-Cbl (62). C-cbl contains an internal SH2 domain, which allows for it to bind to phosphorylated RTKs (63). It also contains an ubiquitin E3-ligase which is responsible for the addition of ubiquitin molecules in order to target proteins for degradation via the proteasome and/or for sorting within the endocytic/lysosomal pathway (64). This is a major pathway in the recycling of RTK after they have signaled as a consequence of engaging their ligands. In this pathway, internalized, activated EphA2 molecules that become de-phosphorylated are recycled to the cell membrane, while those that remain phosphorylated undergo proteasome-dependent degradation (65).

As a consequence, cellular phosphatases play major regulatory roles in dictating the cellular fate of activated RTK. In particular, protein tyrosine phosphatases (PTP) are responsible for the removal of phosphate groups added to RTK when they have been appropriately triggered by binding their respective ligands (66). Of these, the low molecular weight-protein tyrosine phosphatase (LMW-PTP) has been reported to play a major role in acting on phospho-EphA2 (pEphA2) as a substrate (67). Under normal conditions, LMW-PTP removes the phosphate groups on tyrosine residues that are added to RTKs upon ligand stimulation (68, 69). This removal of phosphate groups is necessary for the regulation of secondary signals that are generated upon ligand binding to RTKs. However, the balance of phosphotyrosine content is

critical. In order for RTKs to be properly recycled, they need to be phosphorylated. Any disruption of this process could lead to an accumulation of RTKs on the surface of the cells that express them. Recently, it has been reported that LMW-PTP is overexpressed in several cancers (70), giving credibility to its possible role in mediating tumor growth by dysregulating RTK expression/function. This hypothesis has been further supported that low molecular weight-protein tyrosine phosphatase (LMW-PTP) serves as an EphA2 PTP, and when overexpressed, LMW-PTP is sufficient to transform normal epithelial cells *in vitro* (67). Overexpression of LMW-PTP decreases the phosphotyrosine content of EphA2 and likely plays a role in enhancing the stability and overexpression of EphA2 in cancer cells. The increased stability of EphA2 on the surface of tumor cells may be an additional mechanism through which EphA2+ cancers acquire metastatic capabilities.

1.8. EphA2 and Angiogenesis

As tumors increase in size, they require more nutrients and oxygen for their continued and sustained growth. The inability of 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. Tumors can create a neovasculature system using a process called angiogenesis that will provide them the necessary nutritional requirements that are needed for progressive growth (71). Establishment of neovessels also provides primary solid tumors portals through which they may metastasize and eventually colonize distant sites. Indeed, neoangiogenesis, the process in which new blood vessels are created from the existing vasculature, has been correlated with metastasis and poor prognosis in several cancers, including breast and pancreatic carcinomas (72, 73). Many different factors, including EphA2 and its ligands, are implicated in the process of

angiogenesis. EphA2, along with one its ligands ephrin-A1, are expressed in growing neovessels of breast and sarcoma tumors (74). Soluble EphA2 receptors have been found to inhibit VEGF-mediated angiogenesis (75), and can successfully serve as antagonists in inhibiting tumor angiogenesis and growth in several animal models, including pancreatic cancer (76, 77) and melanoma (78). This additional role for EphA2 in the angiogenesis process further strengthens the argument that EphA2 is a key player in the functional promotion of metastasis *in situ*. Indeed, along with its proposed roles in maintaining cell-to-cell contacts and inhibiting integrin function, the overexpression of EphA2 protein and dysregulation of its signaling implicate EphA2 as a major oncogenic factor.

1.9. Cancer Overview

Approximately 1,368,030 people in the United States will be diagnosed with some form of cancer along with approximately 563,000 that will die from cancer in the year 2004. There are many biological processes where cells need to grow and expand (e.g. wound healing), however cancer arises when the checkpoints that cells employ to regulate their growth are disrupted. Cancer is believed to starts out as a local, clonal outgrowth of cells. As the primary tumor continues to successfully grow in its original location, it necessarily promotes the process of neo-angiogenesis that is required to provide a supportive blood supply carrying oxygen and nutrients. Some tumors acquire the ability to leave their original location and colonize distant sites in the body, a process called metastasis. Once this process has occurred, cancer becomes increasingly difficult to treat and has an increased mortality. Historically, there have been three major traditional modalities for the clinical management of cancer: surgery, radiation, and chemotherapy. Surgery is the process where the existing tumor is physically removed from the

body. Radiation therapy is another method clinicians have used to battle cancer. Considered a local treatment, radio-therapy involves using various forms of radiation to cause DNA damage to cancer cells or the supportive vascular bed, forcing these tissues to undergo apoptosis and to shrink in size. Chemotherapy is the last of the three major therapies utilized against cancer. Chemotherapy involves the administration of chemicals that preferentially inhibit the ability of cancer cells to survive and replicate. These drugs are generally applied systemically, and can be taken either in the form of a pill, or by injections provided s.c. or i.v. These three methods have proved useful in many situations and have saved many lives, although their long-term efficacy is generally perceived as limited, with recurrent disease frequently found to be refractory to the original treatment modality.

1.10. Immunotherapy Basics

One of the more recent types of therapies that have been utilized to treat cancer is immunotherapy. Immunotherapy involves using various components of the immune system to treat cancer. The first evidence suggesting that manipulation of the immune system of cancer patients could be beneficial towards treating cancer involved the administration of interleukin-2 (IL-2) (79). IL-2 is a cytokine that is produced by T-cells that has the ability to induce proliferation of T cells that have recognized their specific antigen (80). The administration of IL-2 in patients with metastatic renal carcinoma or metastatic melanoma was able to induce clinical regression at a rate of 15-20%. These findings demonstrate that immunotherapy may hold promise, given that relative success noted for cytokine-based therapies and the anticipation that combinational immunotherapy approaches may further enhance the frequencies for objective clinical responses in prospective clinical trials.

One of the focal points of cancer immunotherapy has been in activating and sustaining the cell-mediated arm of the anti-tumor immune system, with the majority of attention being directed towards to tumor-specific T-lymphocytes. T-lymphocytes are divided into two primary groups based on their expression of a cell surface molecule that aids in their binding to human leukocyte antigen (HLA) molecules. Termed CD8+ or CD4+ T cells, both sets of T-lymphocytes recognize antigens that are presented to them via HLA molecules. CD4+ T-lymphocytes recognize antigens presented by HLA Class II proteins, while CD8+ T-lymphocytes typically recognize tumor antigens in the context (as peptides that bind) of HLA Class I molecules.

1.11. HLA Class I Antigen Processing

HLA Class I proteins are highly polymorphic and form non-covalent, heterotrimeric complexes with the non-MHC encoded β2-microglobulin "light chain" and short peptides derived from intracellular degradative pathways. Such complexes are presented to, and recognized by, CD8+ T cells (81). These complexes are assembled in the ER as the culmination of a series of sequential protein-protein interactions, the first of which is the cotranslational insertion of class I "heavy chain" (i.e. α-chain) containing three Ig-like domains into the endoplasmic reticulum (ER; (82). This chain is bound in the ER by calnexin until it associates with ß2-microglobulin (83). The addition of ß2-microglobulin promotes the disassociation of calnexin, and the HLA- ß2-microglobulin complex is then bound by the ER chaperones calreticulin and tapasin (83). These proteins retain the HLA/ß2-microglobulin complex in the ER until it becomes bound by a peptide with sufficient affinity for the class I binding groove, ensuring sufficient stability with the HLA/ß2-microglobulin complex to allow for transport of such complexes to the cell surface where they may be recognized by T cells (84). Peptides that

bind HLA Class I proteins are actively transported into the ER by the transporter of antigenic peptides (TAP) in an ATP (energy)-dependent process (85). HLA Class I molecules bind peptides of approximately 8-10 amino acids in length, and are primarily derived from endogenous proteins that have been degraded by the 26S proteasome (86, 87). Upon entrance into the ER, peptides are then loaded onto HLA Class I molecules. This allows for the disassociation of the HLA/ß2-microglobulin/peptide complex from the tapasin and calreticulin chaperones. From this point, the HLA/peptide trimeric complex is transported through the Golgi apparatus to the cells surface for presentation to CD8+ T cells. Similar to CD4+ Th cells, CD8+ T cells recognize peptides bound to HLA proteins via their TCR. The cell surface molecule CD8 binds to the α3 domain of the HLA protein, which stabilizes and "restricts" the interaction of the CD8+ T cell TCR with HLA class I molecules.

1.11.1. 26S proteasome and degradation of cellular proteins

When proteins become tagged with the polypeptide ubiquitin, they may become targeted to the proteasome for degradation (88). The 26S proteasome is a 2000 kiloDalton (kDa) multimeric complex responsible for intracellular protein turnover (89, 90). It is composed of 2 major subunits: the 19S regulatory complex and the 20S core particle. The 20S core is made up of 28 subunits and is arranged in 4 stacked rings (91). Within these subunits are active enzymatic sites for proteolytic degradation. The conformational structure of the intact proteasome is crucial, given that when these proteolytic subunits are isolated individually, they lack protease activity (87). Overall, these subunits display 3 types of peptidase activity. The first, type of specificity is "chymotrypsin-like" due to preferential cleavage of peptide sequences after large hydrophobic residues. The second is a "trypsin-like" activity; i.e. cleavage of peptide sequences after basic

residues. Lastly, a "caspase-like" activity has been reported that mediates cleavage after acidic amino acid residues.

Two 19S regulatory complexes cap both ends of the 20S proteasome. The 19S regulatory complex is made up of approximately 20 subunits and is 700-kD in size (87). This complex enhances the peptidase activity of the 20S core complex, and is able to bind ubiquitinated proteins, thereby facilitating the funneling of proteins into the 20S core catalytic particle. Upon insertion into the active proteasome, protein substrates are broken down in short, polypeptides that range in length from 4 to 24 residues.

1.12. HLA Class II Antigen Processing

Traditionally known as T-helper (Th) cells, CD4+ T-lymphocytes recognize antigens presented by HLA Class II proteins via their clonotypic T cell receptors (TCR) (92). The TCR contains complementary determining regions (CDR) that bind to the HLA Class II proteins to facilitate the recognition of antigens by Th cells. This interaction is stabilized by the T cell surface molecule CD4, which binds to the ß2 region of the HLA Class II protein. HLA Class II proteins are heterotrimeric in structure, consisting of one monomorphic α-chain, one polymorphic ß-chain and one antigenic peptide. Both the α and β chains contain two Ig-like domains, dimerize to form a peptide-binding groove, and are responsible for presenting peptides to CD4+ Th cells. HLA Class II proteins are assembled in the endoplasmic reticulum (ER), and interact with the invariant chain (Ii), a protein which occupies the binding groove of HLA Class II proteins in this cellular compartment (93). This prevents "premature" HLA loading of Class II α/β dimers with autologous antigenic peptides. From the ER, Class II complexes are selectively targeted to compartments called MIIC by protein sequence sorting motifs located in the cytoplasmic domain of the co-associated Ii chain. During its journey to the MIIC compartment,

the Ii chain bound to HLA Class II is trimmed down to yield class II-associated Ii-chain peptides (CLIP) (93). The peptides CD4+ Th cells recognize are processed by the endocytic pathway of antigen processing. Exogenous antigens are internalized by antigen presenting cells (APC) into endosomal vesicles (92). These endosomes then fuse with the specialized lysosomal compartment MIIC, where exogenous antigens are broken down into short peptides. Upon fusion of the antigen containing endosome and MIIC, the molecule HLA-DM removes CLIP, and edits HLA Class II binding peptides generated from exogenous antigens to ensure the stability of HLA Class II protein. These peptides are approximately 15-20 amino acids in length. Once loaded, HLA Class II molecules are transported to the cell surface for presentation to CD4+ Th cells. CD4+ T lymphocytes recognize antigens via their T cell receptor (TCR)

1.13. Tumor Antigens

Many biological changes that cancerous cells undergo result in the expression of tumor antigens. Some of the antigens enhance the tumorigenicity of cancer, where others become expressed as a result of carcinogenesis, but may play no critical and beneficial role in the developing tumor microenvironment. Most "clinically interesting" tumor antigens are proteins that tumor cells express abnormally and can be potentially targeted by the immune system. There are three major classes of tumors antigens: cancer testis (CT) antigens, tumor specific antigens (TSA), and tumor associated antigens (TAA). During the course of carcinogenesis, many types of cancer undergo de-differentiation, that is, they revert to an earlier differentiative form of tissue (94, 95). During this process, proteins that are not expressed in adult somatic tissues are now present in these cancerous cells. The CT family of tumor antigens are normally expressed in fetal testicular tissue during development (96), and have been extensively

characterized and utilized for cancer immunotherapy studies (97-99). Members of this family include GAGE, RAGE, and MAGE (100). Tumor specific antigens are proteins that are only expressed on a specific tumor type and no others, with arguably the best examples being the clonotypic BCR and TCR expressed by B and T cell leukemias/lymphomas, respectively. While less prototypical, gp100, MART-1, and tyrosinase are also three such antigens that are expressed only on melanoma and each encodes T cell epitopes that have been defined and used in immunotherapeutic vaccine protocols (100). Tumor Associated Antigens (TAA) are typically proteins that are expressed on a wide range of tumors. They are not specific with regard to the histology of cancer and are the most common type of tumor antigen. In many cases, TAA are expressed by both normal tissues and cancer cells, although cancer cells may overexpress these proteins (vs. normal tissues) resulting in differential recognition by specific T cells. Given normal cell expression of these antigens and both central and peripheral tolerance mechanisms, TAA-specific T cells are commonly of moderate-to-low avidity to avoid autoimmune pathology.

1.14. CD8+ T Cell-Mediated Immunity

Based on decades of in vivo animal modeling and recent vaccine trials in humans, it is believed that in order for tumors to be rejected by the immune system a tumor-specific CD8+ T lymphocyte response must be promoted and sustained in treated patients with cancer. Such responses appear best initiated through a process termed “cross-presentation”, in which host antigen presenting cells (APCs) acquire exogenous tumor antigens from dead or dying tumor cells and after processing, present these to CD8+ T cells (101). Alternatively, specific CD8+ T cells may encounter MHC class I peptide complexes that are directly presented by the tumor cell. Upon recognition of their specific peptide, anti-tumor CD8+ T cells undergo several hallmark

responses. They undergo a developmental transformation to become effector cytotoxic T lymphocytes (CTL) and acquire the ability to kill their target cells after specific antigen recognition (102). They may also secrete important pro-inflammatory cytokines (i.e. γ-interferon (γ-IFN) and TNF-α) in response to specific TCR stimulation.

CTL are able to kill target cells they recognize through several different mechanisms. Upon antigen recognition of a potential target cell, CTL may secrete perforin and/or granulysin, both of which are stored in pre-formed cytoplasmic granules of the CTL and aid in the killing of target cells. Monomers of perforin are secreted from CTL and then insert themselves into the plasma membrane of target cells, aggregate to form a pore, and allow for other apoptosis-mediating factors to enter the tumor cell more effectively (103). Granulysin is a membrane perturbing polypeptide that most likely aids in the delivery of the cytotoxic mediators of CD8+ T cell-mediated immunity (104). The major family of proteins that mediate CTL induced apoptosis are granzymes of which granzyme-B has been reported to be the most potent (105). Granzymes are serine proteases that induce apoptosis of targets cells via several mechanisms. Granzyme B is able to stimulate the caspase pathway of apoptosis with its ability to cleave procaspase-3. This results in the formation of mature caspase-3, which in turn mediates the degradation of iCAD (inhibitor of caspase-activated DNase). No longer bound by iCAD, CAD is then free to induce DNA fragmentation which ultimately results in the induction of apoptosis and cellular death (106). In addition to caspase-3 activation, Granzyme B is also able to alter the membrane integrity of mitochondrial in a caspase-3 independent mechanism (107). Granzyme B binds BID (BH3 interacting death domain), which results in the production of tBID (truncated BID). tBID is then able to disrupt the outer membrane integrity of mitochondria. This results in

the release of several pro-apoptotic factors from mitochondria, including cytochrome C, endonuclease G, and Htr2/OMI, all of which aid in the promotion of apoptosis.

The tumor necrosis factor (TNF) receptor gene superfamily represents an additional pathway through which CTL are able to induce the death of their target cells. Although all TNFR family members vary in primary sequence, they all contain a homologous intracellular sequence called a death domain (108). Perhaps the most well-known receptor/ligand pair in this family is Fas and FasL (CD95/CD178). When the TCR of CTL are engaged and activated by interactions with HLA Class I proteins, the T cells upregulate their expression level of FasL (109). FasL is a homotrimeric protein that binds to 3 Fas receptors on CTL target cells (110). Upon binding, the death domains of the 3 Fas receptors are clustered and recruit pro-apoptotic adaptor proteins (e.g. FADD) via interactions with the death domains on the adaptor proteins The secondary adaptor proteins then are able to induce apoptosis in a caspase-8 dependent manner (111).

1.14.1. Immunoregulatory Actions of γ-IFN

γ-IFN is perhaps the hallmark cytokine of "clinically preferred" cell-mediated immunity. γ-IFN induces several key changes in the cells to which it binds and activates. First, it is able to induce an increase in the surface expression of HLA molecules on treated target cells (112), in many cases making them more easily recognized by specific CD8+ T cells. Secondly, γ-IFN induces the expression of several conditional proteasomal subunits: LMP2, LMP7, and MECL-1 (113). These subunits alter protease activity in the proteasome so that the peptides generated are more likely to bind HLA Class I molecules. Thirdly, γ-IFN is able to induce the expression of PA-28, a proteasome activator that is able to increase the proteolytic efficiency of the 20S

proteasome (114). Other additional immunoregulatory effects of γ-IFN include, but are not limited to, increases in TAP, ß2-microglobulin, and tapasin expression by treated cells. All of these proteins are involved in enhancing HLA Class I antigen processing and increase the efficiency of CD8+ T cell mediated immunity.

1.15. CD4+ T Cell-Mediated Immunity

Traditionally known as T-helper (Th) cells, CD4+ lymphocytes are believed to exert their influence on the adaptive immune response primarily by secreting cytokines in response to antigen recognition. Cytokines are secreted proteins that have many immunomodulating effects on the cells they interact with. The types of cytokines that CD4+ Th cells secrete allows for them to be functionally discriminated into three major groups; namely Th1, Th2, and Th3/Tregulatory (Treg) (115). A Th1-type cytokine profile involves Th production of the cytokines γ-IFN and IL-2 (116), with both of these cytokines known to promote the cell-mediated arm of the immune system. γ-IFN and IL-2 upregulate HLA expression (112, 117, 118), promote CTL proliferation and differentiation, enhance HLA Class I antigen processing, and suppress Th2-type immune responses that may be sub-optimal to the regulation of cancer *in situ*. A Th2-type cytokine profile includes the production of IL-4, IL-5 and IL-13 (115). These cytokines are best known for promoting the humoral arm of the immune response by enhancing the ability of B lymphocytes to produce antibodies and suppression of the development of Th1-type immune responses. A Th3 cytokine profile is characterized by the production of IL-10 and TGF-ß. A Th3-biased immune response may also be called a T-regulatory (Treg) response due to the ability of IL-10 and TGF-ß to suppress the activity of Th1- and Th2-type biased CD4+ T lymphocytes (119). Although not generally known for their ability to induce cell death of their

target cells, CD4+ Th cells can express FasL, affording effector CD4+ T cells the capacity to kill tumor cells through an apoptotic pathway (120). In contrast, the ability to mediate the perforin/granzyme pathway of target cell death appears biased towards effector CD8+ CTLs.

1.16. Dendritic Cells

Dendritic cells (DC), B cells, and macrophages comprise a group of cells termed "professional" antigen presenting cells (APC). Professional APCs play a critical role in the presentation of antigens and activation of T cells. Of these 3, DC are recognized as the only APC that is reproducibly able to activate naïve T cells (121), and they are the most potent activators of antigen-specific memory T cells (122). This is in part due to their constitutive or inducible expression of T cell co-stimulatory molecules (e.g. CD80, CD86, CD40), HLA Class I and II molecules, and their production of cytokines, such as interleukin-12, 15, and 18 (122). IL-12 is a key cytokine secreted by DC given its ability to polarize the immune response towards a Th1 phenotype (123) and induce the production of γ-IFN by T lymphocytes (124). In the periphery, DC express the chemokine receptor CCR6 (125) and exist in an immature state which is characterized by their ability to capture antigens through a variety of mechanisms. Such mechanisms include macropinocytosis (126), receptor-mediated endocytosis (127), and phagocytosis (128). Immature DC also express low surface levels of HLA molecules, CD80, CD86, and CD40. Upon antigen capture, DC begin to mature, and they lose their ability to capture antigens and acquire the ability to stimulate T lymphocytes. DC maturation is characterized by their upregulation of HLA molecules, increased expression of T lymphocytes co-stimulator molecules (e.g. CD86, CD80, CD40) and the expression of CD83 (122). Upon maturation DC acquire the ability to migrate towards the secondary lymphoid organs (e.g.

spleen, lymph nodes) due to the downregulation of CCR6 and the upregulation of CCR7 (129). The expression of CCR7 sensitizes DC to the effects of SLC which is expressed in the secondary lymphoid tissues (130). When they arrive at the secondary lymphoid organs, DC encounter $CD4^+$ T cells and induce their activation. This activation is dependent on the interaction of CD40 on the DC and CD40L on the T-helper cell (131). This contact enables the DC to become a more potent activator of $CD8^+$ T cells and induce their differentiation into effector CTLs (122). This interaction is crucial for the induction of anti-tumor immunity that is sought for immunotherapy protocols in the treatment of cancer. Although much focus has been placed on the role of CTL regarding the fight against cancer, the role of T-helper cells should not be forgotten. Th1-type T-helper cells can produce pro-inflammatory cytokines that can promote and recruit anti-tumor immunity into tumor sites (78), whereas Th2-type responses have generally not proven to be beneficial for eliciting CTL anti-tumor activity and promoting anti-tumor immunity *in situ*.

1.17. T lymphocytes and Cancer Immunotherapy

With the identification and characterization of tumor antigens expressed by various types of cancer, researchers quickly set out to define which derivative peptide epitopes could be recognized by anti-tumor T lymphocytes. The underlying question was that if T cell epitopes could be defined, would they prove capable of generating tumor antigen-specific T lymphocytes that could also recognize cancer cells and possibly mediate rejection when employed in clinical vaccines? One of the popular methods to generate T cell responses against tumor antigens is the use of dendritic cells (DC) and other antigen presenting cells (APC) (132). Through the utilization of DC, tumor antigens pulsed DC have been used to generate anti-tumor immune

responses against a diverse array of tumor antigens. There are several popular methods to determine whether or not a tumor antigen derived epitope can be recognized by T lymphocytes. These methods include, but are not limited to, RNA transfection (133), DNA transfection (134), and viral infection (135). The most popular method is the use predictive peptide binding algorithms that have been developed to determine which peptide sequences from cloned tumor antigens would be able to bind to certain HLA allelic molecules (99). The entire sequence of a tumor antigen is analyzed, and peptides with a predicted high affinity for binding to specified HLA molecules are synthesized. From there, peptides can be pulsed onto DC, with the antigen-loaded DC then co-incubated with autologous T responder lymphocytes in a process called in-vitro stimulation (IVS. After 7-10 days of *in vitro* priming/boosting, responder T lymphocytes are then analyzed for their ability to recognize the specific antigens they were stimulated with. These T cells can also be co-incubated with tumor cells that express the antigen and HLA allele of interest and tested for their ability to recognize tumors that naturally process and present the candidate epitope. These experiments can assess whether T lymphocytes can recognize naturally-occurring MHC/peptide complexes presented on tumor cells and whether or not those antigens may be relevant for tumor immunotherapy. Significant numbers of ongoing studies are being performed to determine how, when and where to administer tumor antigen-based vaccines to afford the greatest degree of clinical efficacy, or whether ex vivo primed anti-tumor T cells should be adoptive transferred to yield a high rate of prevention or cures in these patients.

1.18. Monoclonal Antibodies

One set of tools studied for application as cancer immunotherapeutic agents are monoclonal antibodies (mAb). mAb are proteins that are created by the fusion of a plasma cell with a tumor

cell and allow for the continued production of a clonal antibody. mAb specific for a given tumor antigen may bind to that antigen expressed by tumor cells in situ, thereby promoting a cytolytic anti-tumor immune response via the recruitment of complement and/or Fc receptor+ natural killer (NK) cells (136). Anti-tumor mAbs may also be therapeutic in the cancer setting if they are able to mediate the functional neutralization (e.g. blocking binding sites or functional signaling) of tumor antigens (such as RTKs). For example, the mAb Herceptin binds to the Her2/neu tumor antigen, which is a member of the EGFR family of growth factor receptors. This binding induces the internalization of Her2/neu, thereby inhibiting the proliferation of Her2/neu positive tumors (137).

mAbs are unique tools for use as cancer immunotherapy agents due to the fact that a patients HLA type is not a key factor for accrual into protocols administrating the mAb (i.e. passive transfer as a therapy). All that is necessary is that a tumor must express the tumor antigen the mAb is specific for. This increases the fraction of cancer patients that can be treated with this single modality of immunotherapy and opens the door for combinational therapies that may combine antibodies and vaccines or adoptive strategies focused on anti-tumor T cells.

1.19. Summary

With increasing evidence demonstrating that the receptor tyrosine kinase EphA2 is overexpressed in a number of epithelial based cancers, with even higher expression in metastatic disease, I wanted to investigate whether the EphA2 protein contained peptide epitopes recognized by T lymphocytes, in hopes of defining an appropriate treatment modality for patients with EphA2+ tumor types, such as melanoma and a diverse array of carcinomas. I used EphA2 peptide-pulsed DC as *in vitro* stimulators to ascertain to whether EphA2 peptides could elicit

specific T cell responses and an agonist monoclonal antibody (specific for EphA2 that has been generated that is able to bind overexpressed EphA2 proteins on the tumor cell surface and not bind EphA2 on normal tissues) to induce the internalization and degradation of EphA2 to conditionally facilitate the presentation of EphA2-derived CD8+ T cell epitopes. This latter treatment as well as our strategies to prevent the activity of LMW-PTP result in an acute increase in EphA2 epitopes presented in MHC class I complexes and in the enhanced ability of EphA2-specific CD8+ T lymphocytes to recognize EphA2+ tumors. We believe that this may define a novel combinational immunotherapy design involving DC vaccination with a consequent delivery of agonist anti-EphA2 mAb or PTP inhibitors to promote the CTL-mediated (conditional) eradication of EphA2+ tumor lesions in patients. These studies are significant because they have determined the immunogenecity of a new tumor antigen that is overexpressed by multiple cancer types and serve as the basis for the development of novel prospective immunotherapy protocols designed to elicit, sustain and enhance the in vivo efficacy of EphA2-specific T cells.

Scope of This Thesis

We have characterized EphA2 as a tumor-associated antigen in the setting of renal cell carcinomas (RCC). We have observed that a higher expression of EphA2 in primary RCC specimens was associated with a shorter (<1yr) time to recurrence of disease, where a low expression of EphA2 was associated with a more prolonged disease-free interval. We have also noted that metastatic cell lines express higher levels of EphA2 than do primary RCC cell lines, which is consistent with reports of EphA2 playing a role in metastasis in other forms of cancer. We then identified immunogenic EphA2-derived peptide epitopes and characterized the magnitude and functional nature of CD4+ and CD8+ T cell immune responses against EphA2 in HLA-A2+ and/or HLA-DR4+ normal donors and RCC patients. While both normal donors and RCC patients were able to recognize EphA2 epitopes, the functional anti-EphA2 CD4+ T cell responses in patients with RCC was disease status-dependent, with patients with either active disease or a higher staging of RCC displayed a Type-2 (i.e. IL-5) -biased functional phenotype. Patients with NED or early Stage I or II disease primarily displayed a Type-1-biased (i.e. IFN-γ) functional phenotype. Interestingly, we observed that treatment of EphA2+ RCC lines with EphA2 agonists restored proper phosphorylation of EphA2 protein and its consequent degradation via a proteasome-dependent pathway. Importantly, this conditional manipulation resulted in increased recognition of EphA2+ tumors by EphA2 specific CD8+ T cell clone, consistent with the enhanced loading of EphA2 epitopes into tumor cell expressed MHC class I complexes. Finally, given the constitutive underphosphorylation of EphA2 on EphA2+ tumors, we were also able to promote EphA2 phosphorylation and proteasomal processing by treating tumor cells with PTP inhibitors. Hence we have characterized EphA2 as a carcinoma-associated antigen and determined pharmacologic means by which to enhance tumor cell recognition by

anti-EphA2 T cells. We believe that combinational vaccine + conditional tumor processing strategies should be explored as novel immunotherapeutic strategies for patients with EphA2+ tumors.

Preface Chapter 2

Using an immunohistochemistry approach and archived patient tissues, our goal was to determine whether the level of EphA2 protein overexpression in resected primary RCC tumor specimens was predictive of the time to disease recurrence (TTR) in patients rendered free of disease post-surgery. We analyzed 3 groups of patients; i.e. those who recurred in: (1)< 1yr, (2) between 1 and 5 years and (3) more than 5 years post-surgery. We discovered that high relative expression of EphA2 in primary tumor lesions was associated with a shorter TTR (<1yr), higher tumor volume, and higher Factor VIII+ blood vessels indicative of tumor neoangiogenesis. We also found that primary tumors with high expression levels of EphA2 were less structurally organized and displayed dysregulated intracellular EphA2 expression patterns when compared to primary tumors expressing lower levels of EphA2 protein.

The studies in **Chapter 2** support the relevancy of EphA2 as a novel tumor antigen that is linked with tumor lesions that may be better positioned to progress and metastasize.

2. Resected Tumor Expression of EphA2 is Prognostic of Disease-Free Interval in Surgically-Cured Patients with Renal Cell Carcinoma

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2.1. ABSTRACT

While normally expressed at sites of cell-to-cell contact in adult epithelial tissues, recent studies have shown that the receptor tyrosine kinase (RTK) EphA2 is overexpressed in numerous epithelial-type carcinomas, with the greatest level of EphA2 expression observed in metastatic lesions. Using specific anti-EphA2 mAb 208 and immunohistochemistry, we evaluated EphA2 protein expression levels in renal cell carcinoma (RCC) specimens surgically resected from patients resulting in clinical cures. RCC lesions expressing higher levels of EphA2 tended to be larger, more-highly-vascularized tumors. Perhaps most notable, the degree of EphA2 overexpression (vs. normal matched autologous kidney tissue) appeared predictive of short (< 1 Yr) vs. longer (≥ 1 Yr)-term disease-free interval among the RCC patients evaluated. These data suggest that EphA2 expression level may serve as a useful prognostic tool in the clinical management of patients who have been successfully treated with surgery, but who may be at a greater risk for accelerated disease recurrence.

2.2. INTRODUCTION

The Eph family of molecules constitutes the largest known family of receptor tyrosine kinases (RTKs), consisting of two classes of receptors, EphA and EphB, and two classes of corresponding ligands, ephrin-A and ephrin-B, respectively. Ephrin-A ligands are attached to the cell surface via GPI-anchors and preferentially bind only EphA receptors. Ephrin-B ligands are transmembrane proteins and primarily bind EphB receptors. Largely known for their role(s) in neuronal development and tissue remodeling (138, 139), recent reports suggest that Eph receptors also play a role(s) in oncogenesis (36, 56-58, 60, 140-143).

EphA2 is of particular interest due to evidence suggesting its involvement in developing malignancies. EphA2 is a 130kDa protein normally localized to sites of cell-to-cell contact, where it plays a role in contact growth inhibition (36). However cellular overexpression of EphA2, either as a result of its constitutive dysregulation or ectopic gene insertion, results in the disruption of cell-to-cell contacts, and enhancement of cell-to-extracellular matrix (ECM) attachments (36). As a result, tumor cells that overexpress EphA2 exhibit increased motility and invasive properties, consistent with a pro-metastatic phenotype (36). Overexpression of EphA2 has been observed in numerous cancer types (140), including melanoma (141, 142) and carcinomas of the breast (57, 58, 143), lung (144), pancreas (60) and prostate (56).

It has been recently suggested that the quantitative levels of EphA2 expressed by cancer cells might provide useful prognostic information with regard to the metastatic potential of human tumors *in situ* and the clinical disease course of patients with cancer. Kinch et. al. (145, 146) reported that high level EphA2 expression in primary lung tumors is predictive of brain

metastases, where lower levels predict either extended disease-free status or locoregional (contralateral) lung metastasis. Likewise, Miyazaki et. al. (147) reported a significant correlation between EphA2 overexpression in esophageal squamous cell carcinoma with the presence of lymph node metastasis, the number of lymph node metastasis, and less well differentiated tumors. Five-year survival rates were also reported to be lower for patients with EphA2+ tumors vs. those patients with EphA2-negative tumors (147). In light of this evidence, and our previous report supporting the prevalent overexpression of EphA2 in RCC (148), we performed immunohistochemical analyses to determine whether the relative level of EphA2 expressed by resected RCC tumors was indicative of the disease-free interval observed for a given surgically-cured patient.

2.3. MATERIALS AND METHODS

2.3.1. Patients and Tissues

RCC tumor and adjacent normal kidney specimens were collected from a total of 34 patients with consent under IRB-approved protocols. All patients were treated by radical or partial nephrectomy and rendered disease-free. Patient and tumor characteristics and time-to-recurrence are indicated in Table 2. Among these patients, a subset of 8 individuals, remain clinically-free of disease for more than 13 years post-surgery. For these patients, the time since surgery is reported as their "disease-free interval". All the resected tissues were fixed in paraformaldehyde (Sigma Chemical Co., St. Louis, MO) and embedded in paraffin.

2.3.2. Construction of RCC Tissue Microarray (TMA)

Twelve RCC that recurred within 1 year or surgery, 16 RCC that recurred within 1-10 years after surgery and 13 RCC that remained disease-free 10 years after surgery were selected for construction of the RCC TMA. All cases were retrieved from the surgical pathology file at the Department of Anatomic Pathology of the Cleveland Clinic Foundation, and were reviewed and the diagnoses confirmed. One representative paraffin block from each case was used to construct a tissue microarray. Briefly, areas of interest (tumor or normal adjacent tissue) were traced with a marker pen on an H&E stained slide. The corresponding areas were then marked on the corresponding paraffin blocks. The TMA was made on a manual TMA arrayer (Beecher Instruments, Sun Prairie, WI). Three 1.5 mm-thick tissue cores were taken from each block to build the TMA. After construction, 4-micron sections were cut, mounted on Superfrost Plus

glass slides and stored for future use. An H&E staining was performed on the initial slide to verify the histologic diagnosis, with a map of case number and tissue diagnosis maintained in a Microsoft Excel spreadsheet.

2.3.3. Immunohistochemistry

Tissue microarray slides were de-paraffinized and stained with a murine anti-hEphA2 mAb 208 (1:100 dilution; MedImmune, Inc., Gaithersburg, MD), rabbit anti-human Factor VIII mAb (1:400 dilution; Dako Corp., Carpinteria, CA), or normal mouse/rabbit IgG control Ab (1:200 dilution; Sigma) in immunohistochemical analyses. Detection was determined by using Vectastain anti-IgG and NovaRed kits (Vector Labs, Burlingame, CA). Three independent evaluators analyzed slides under 40x magnification and scored EphA2 expression using an arbitrary scale of 0 (low) – 5 (high). Three serial sections from each specimen were evaluated in order to reduce intra- and inter-specimen scoring variations. The mean (+/- S.D.) value of the nine aggregate determinations (3 readers x 3 sections/lesion) of EphA2 expression intensity for each tumor and matched normal adjacent kidney specimen were calculated for each patient. EphA2 staining results are reported as a ratio of EphA2 expression in tumor sections vs. matched normal adjacent kidney.

2.3.4. Statistical Analyses

Statistical differences between groups were evaluated using a two-tailed Student's T test, with p values < 0.05 considered significant.

2.4. RESULTS

2.4.1. RCC lesions express variable levels of EphA2.

Curative surgery was performed on 34 RCC patients (Table 2). The resected tissues were dissected into tumor and uninvolved "normal" kidney specimens, prior to being formalin-fixed, assembled into tissue arrays, and sectioned as described in Materials and Methods. Each tissue array contained 3 "cores" derived from a given patient's tumor and normal adjacent kidney specimen, such that each slide analyzed contained 3 replicate sections of each tumor/normal kidney tissue resected. After de-paraffinization, slides were subjected to immunohistochemical analyses for EphA2 expression using the specific anti-EphA2 mAb208 vs. normal IgG control Abs.

RCC tumor specimens exhibited profound variability in their level of EphA2 expression. For example, patient #19's tumor overexpressed EphA2 relative to their normal adjacent kidney tissue, and the pattern of expression appeared dysregulated, with EphA2 protein located throughout the cytoplasm and on the tumor cell membrane (Fig. 3A). This was vastly different from the pattern of distribution in adjacent normal renal tissue (Fig. 3C), where EphA2 expression was restricted to the epithelial cells lining the tubules within the kidney. In marked contrast, RCC tumor resected from patient #16 exhibited a weaker EphA2 staining pattern (Fig 3e), with the intensity of EphA2 expression by the tumor indistinguishable from that of the normal, matched kidney tissue (Fig. 3G).

2.4.2. Larger, more vascularized tumors tend to exhibit higher levels of EphA2 expression.

Recent reports in breast carcinoma and melanoma models suggest that EphA2 expression *in situ* may be associated with larger, better vascularized tumors (74, 149). Furthermore, EphA2 blockade limits murine breast tumor growth *in vivo* (77), suggesting that EphA2 plays a role in tumor progression. We therefore analyzed whether the relative EphA2 expression levels observed in RCC lesions was correlated with resected tumor volume and the degree of tumor vascularity. For these studies, the relative expression level of EphA2 was determined in tumor vs. normal adjacent kidney tissue in a blinded manner by 3 individuals utilizing a 0-5 scoring scale, with 5 representing strongest expression and 0 representing lack of expression by the stained tissue. This value was then plotted versus the volume of the resected tumor (from Table 2). As shown in Figure 4A, we observed a trend between increased RCC tumor expression of EphA2 and tumor volume, although this correlation only provided an R^2 value of 0.33 and would not be considered statistically significant (p=.093). Similarly, an immunohistochemical analysis of blood vessels in RCC lesions using anti-Factor VIIII antibodies (Figure 4B) revealed that a trend between the degree of EphA2 expression and the number of blood vessels per tumor section (R^2 = 0.32, p=.005).

2.4.3. High levels of EphA2 Expression by EphA2+ Tumors Appear Predictive of Acute Relapse

Given previous reports supporting a prognostic potential for EphA2 expression in lung and esophageal cancer (145, 146), we next evaluated whether the relative level of EphA2 expressed

by resected RCC tumors was predictive of the disease-free interval of a given patient cured by therapeutic surgery. For these studies, patient materials were divided into 3 cohorts for prospective analyses; a) patients who recurred within 1 year of surgery (n = 10), b) patients who recurred between 1 and 5 years of surgery (n = 13), and c) patients who recurred or who remain disease-free 5 or more years post-surgery (n = 11).

When data from all 34 patients was evaluated (Figure 5), we observed that the disease-free interval for a given patient was inversely related to the relative intensity of EphA2 expressed by the patient's resected RCC tumor (vs. the matched normal kidney tissue). This correlation was most striking ($p = 0.0003$) when considering patients who recurred in less than 1 year from the time of their curative surgery (relative EphA2 expression = 3.81 (mean) $\pm$ 0.77 (S.D.)) vs. patients who remain disease free now more than 5 years post-treatment (relative EphA2 expression = 1.27 $\pm$ 0.37). A p value < 0.001 was still obtained when considering patients who recurred within their first year of surgery vs. those patients who relapsed between 1-5 years post-surgery (relative EphA2 expression = 1.60 $\pm$ 0.57). However, a comparison of relative tumor cell expression of EphA2 amongst patients recurring between 1-5 vs. > 5 years post-surgical cure did not support any statistically-significant difference (i.e. $p = 0.43$). This suggests that the relative level of EphA2 expressed by RCC tumors resected from patients that are rendered disease-free after surgery provides an index that may be predictive of how quickly a treated patient will recur.

2.5. DISCUSSION

The key discovery of this study is that the expression of EphA2 in resected RCC tumors may serve as a prognostic indicator of disease-free interval in patients that are cured by therapeutic surgery. RCC lesions exhibiting the highest expression levels of EphA2 protein displayed the most abbreviated time to disease recurrence (i.e. < 1 year), while patients whose tumors expressed levels of EphA2 that were indistinguishable from normal adjacent kidney tissue remained disease-free for extended periods of time post-surgery. When combined with recent studies in lung (146) and renal (148) carcinoma, these results support EphA2 expression as an important index related to disease progression, metastatic spread and durability of therapeutic efficacy in multiple tumor histologies. As EphA2 can be overexpressed by a broad range of cancer types (56-58, 60, 140-144), the prognostic utility of this marker may prove important in the monitoring and clinical management of a large cohort of cancer patients.

Our finding that EphA2 expression tended to be directly associated with tumor volume (i.e. tumor burden) and the number of tumor-associated Factor VIII+ vessels may relate to the involvement of EphA2 and its primary ligand Ephrin-A1 in the formation of neovessels in progressor tumor lesions (74). When the EphA2 signaling pathway is blocked, VEGF-mediated angiogenesis is inhibited (149). Furthermore, recent studies have also shown that the expression of EphA2 is associated with increased tumor cell proliferation and increased tumor thickness in the melanoma setting (150). Overall, these data suggest that overexpression of EphA2 by tumor or endothelial cells within the tumor microenvironment provide a growth advantage to the neoplastic cells, favoring a progressor lesion *in situ.*

The reason(s) why the EphA2 protein becomes overexpressed in aggressive cancers remains incompletely understood, but likely involves both transcriptional and post-transcriptional mechanisms. cDNA array analyses provide strong support for elevation of EphA2 mRNA in the setting of melanoma (140, 149), and a similar finding would be anticipated for at least a subset of renal cell carcinomas. Alternatively, or additionally, it has been recently suggested that the steady-state level of "accumulated" EphA2 in cancer cells may be regulated by: 1) defective ligand-induced EphA2 activation and/or 2) the activity of the low molecular weight-protein tyrosine phosphatase (LMW-PTP) which prevents the ubiquitination and degradation of the phosphorylated EphA2 RTK generated after receptor crosslinking (67, 151). Given these findings, clinical approaches invoking specific RNAi, agonist antibodies (151) or PTP inhibitors may serve to reduce EphA2 expression within tumor lesions, resulting in less aggressive tumor cells, prevention of tumor neoangiogenesis and improved clinical outcomes.

Preface Chapter 3

Using DC-based *in vitro* vaccinations as a model system, our goal was to define EphA2-derived epitopes recognized by CD8+ and CD4+ T-lymphocytes. We analyzed T cell responses to 5 putative HLA-A2-presented and 3 putative HLA-DR4-presented EphA2 epitopes in both normal donor and patients with RCC. All 5 HLA-A2 binding peptides ($EphA2_{162-70}$, $EphA2_{391-99}$, $EphA2_{546-54}$, $EphA2_{883-91}$, and $EphA2_{961-99}$) and 3 HLA DR4 binding peptides ($EphA2_{53-68}$, $EphA2_{63-75}$, and $EphA2_{663-78}$ were recognized by both normal donors and RCC patients. EphA2-specific T cells were able to recognize EphA2+ tumors in an HLA-A2 (in the case of SLR24) and HLA-DR4 (in the case of PC3.DR4) restricted manner. The functionality of CD4+ Th cells was biased in a manner dependent on the patient's disease status, with a Th2-bias most notable among patients with active, advanced stage disease. Patients who were disease-free at the time of analysis or who had limited locoregional Stage I disease tended to exhibitTh1-biased immunity to EphA2 peptide epitopes.

The studies in **Chapter 3** support the immunogenicity of EphA2 epitopes and that these are naturally processed and presented in MHC complexes by EphA2+ tumor cells. This data was reported in *Cancer Research* in 2003, in which I was a co-first author. Further studies in **Chapters 4** and **5** will address whether conditional modulation of EphA2 expression can enhance the immune response to EphA2+ tumors.

3. Disease Stage Variation in CD4+ and CD8+ T cell Reactivity to the Receptor Tyrosine Kinase EphA2 in Patients with Renal Cell Carcinoma

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Running title: T cell responses against EphA2

3.1. ABSTRACT

We have evaluated CD8+ and CD4+ T cell responses against a new tumor-associated antigen, the receptor tyrosine kinase EphA2, which is broadly expressed in diverse cancer histologies and is frequently overexpressed in advanced stage/metastatic disease. We report herein that EphA2 is overexpressed in renal cell carcinoma (RCC) cell lines and clinical specimens of RCC and find that the highest levels of EphA2 are consistently found in the most advanced stages of the disease. We identified and synthesized five putative HLA class I-binding and three class II-binding peptides derived from EphA2 that might serve as targets for immune reactivity. Each peptide induced specific, tumor-reactive CD8+ or CD4+T cell responses as measured using IFN-γ ELISPOT assays. The EphA2 peptides elicited relatively weak responses from CD8+ T cells derived from normal healthy volunteers or from RCC patients with active disease. In marked contrast, immune reactivity to EphA2-derived epitopes was greatly enhanced in CD8+ T cells that had been isolated from patients who were rendered disease-free, after surgery. Furthermore, ELISPOT analyses demonstrated prominent EphA2-restricted Th1-type CD4+ T cell activity in patients with early-stage disease, whereas Th2-type and Tr-type responses predominated in patients with more advanced forms of renal cell carcinoma. These data suggest that the immune system of cancer patients actively monitors EphA2-derived epitopes and that the magnitude and character of T cell responses to EphA2 epitopes may convey much-needed predictive information about disease stage and outcome.

3.2. INTRODUCTION

The molecular identification of tumor antigens recognized by the immune system has paved the way for the development of new immunotherapeutic strategies for the treatment of cancer (152-162). While many cytotoxic T lymphocyte (CTL)-defined tumor-associated epitopes have been applied clinically in cancer vaccinations (163-167), comparatively few class II-restricted epitopes recognized by CD4+ T cells have been identified and clinically integrated to date (98, 168-175). Current paradigms suggest that CD4+ T cells (at least Th1-type) play critical roles in the optimal induction and maintenance of clinically beneficial tumor immunity (168, 176). Hence, CD4+ and CD8+ T cell epitopes derived from antigens that are unique to, or overexpressed on, tumor cells may provide effective vaccine components.

The Eph family of molecules constitutes the largest family of receptor tyrosine kinases in the human genome. Eph kinases are comprised of two major classes (EphA and EphB), which are distinguished by their specificities for ligand (ephrin-A and ephrin-B, respectively, (1)). Largely known for their role in neuronal development (16, 139, 177), recent reports suggest that Eph receptors play a role in carcinogenesis (35, 178-180). For example, EphA2 is overexpressed and functionally altered in a large number of different cancers, where it appears to promote the development of disseminated disease. In normal cells, EphA2 localizes to sites of cell-to-cell contact (35), where it may play a role as a negative regulator of cell growth. In contrast, EphA2 is frequently overexpressed and often functionally dysregulated in advanced cancers, where it contributes to many different aspects of malignant character. Overexpression of EphA2 has been observed in a wide array of solid tumors, including melanoma (141, 142) and prostate (56), breast (61) and lung (144) carcinomas. Indeed, the highest degree of EphA2 expression among

tumors is most commonly observed in metastatic lesions (56, 179). Given this pattern of expression in tumor cells, immunologic targeting of EphA2 in situ could prove effective at eliminating disseminated disease. Furthermore, with relevance to diagnosis and immune monitoring of patients, the frequency and functional status of T cells reactive against EphA2 may serve as an appropriate index of "clinically important" anti-tumor immunity in patients with diverse forms of cancer.

In the clinical setting, several findings suggest that T cell-mediated immunity provides a safeguard against the development and progression of RCC and may effectively mediate the regression of established lesions. RCC lesions are typically infiltrated with large numbers of lymphocytes (181, 182), though the benefits of leukocytic infiltration upon clinical outcome remain unknown (183). While this may reflect variance in the functional subsets of CD4+ and CD8+ T cells in these infiltrates, data addressing the prognostic benefit of Th1/Tc1- vs. Th2/Tc2-biased immunity in RCC patients has been equivocal (184, 185). A better understanding of the constitutive nature and specificity of CD8+ and CD4+ T cell responses in RCC patients will likely provide insights necessary to design, implement, and monitor more effective treatments.

In the present study, we demonstrate that high levels of EphA2 expression are observed in the setting of RCC and that some patients with this disease exhibit both CD8+ and CD4+ T cell responses to novel EphA2-derived epitopes. More importantly, our findings suggest that the reactivity of T cells against EphA2 may be useful in distinguishing disease status and outcome.

3.3. MATERIALS AND METHODS

3.3.1. Cell Lines and Media

The T2.DR4 (HLA-A2+/-DRB1*0401+; (175)) cell line (kindly provided by Dr. Janice Blum, Indiana University School of Medicine, Indianapolis, IN) was used as the peptide-presenting cell in ELISPOT assay. The following SLR20-SLR26 clear-cell RCC lines were evaluated in Western Blot analyses. The normal, human proximal tubular epithelial kidney cell line HK-2 (American Type Tissue Collection, ATCC, Rockville, MD) was also evaluated in Western Blot analyses. Hypothetically, HK-2 represents a normal control cell line, although it has been transformed by transfection with the HPV-16 E6/E7 genes (186). The EphA2+ PC-3 and PC-3.DR4 prostate carcinoma cell line was included as positive controls for Western Blot analysis of EphA2 protein expression (56) and were also used as targets in ELISPOT assays. All cell lines were maintained in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 10mM L-glutamine (all reagents from GIBCO/Life Technologies, Grand Island, New York) in a humidified atmosphere under 5% CO_2 tension at 37°C.

3.3.2. Peripheral Blood and Tumor Specimens

Peripheral blood was obtained by venipuncture from 40 patients diagnosed with RCC and 14 normal individuals and was collected into heparinized tubes. Peripheral blood lymphocytes (PBLs) were isolated by centrifugation on a Ficoll-Hypaque gradient (LSM, Organon-Teknika, Durham, NC). RCC tumor lesions and matched normal kidney tissue were surgically-resected

and paraffin-embedded. Informed consent, under an IRB-approved protocol, was obtained from all patients prior to sample acquisition. Patient and normal donor information is provided in Table 3. All individuals included were HLA-A2+ or/and HLA-DR4+, as determined by fluorescence-activated cell sorter analysis using the HLA-A2-specific antibodies (BB7.2 and MA2.1) and HLA-DR4-specific antibody (anti-HLA-DR4 monoclonal antibody clone 359-13F10, IgG, kindly provided by Dr. Janice Blum, Indiana University School of Medicine, Indianapolis, IN). Among the RCC patients and normal individuals, 9 patients and 6 normal individuals expressed both the HLA-A2 and HLA-DR4 major histocompatibility antigens.

3.3.3. Western Blot Analyses

Tumor cells (5-10 x 106) were analyzed for EphA2 expression via Western blots using the anti-human EphA2 polyclonal antibody (clone: C-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Cell pellets were lysed using 200µl of 1% NP-40 in PBS containing protease inhibitors (Complete, Boehringer Mannheim, Indianapolis, IN) for 1 hour on ice. After centrifugation at 13,500 x g for 30 minutes, the supernatant was mixed 1:1 with SDS-PAGE running buffer and proteins separated on 10% PAGE gels, prior to electro-blotting onto nitrocellulose membranes (Millipore, Bedford, MA). Blots were imaged on Kodak X-Omat Blue XB-1 film (NEN Life Science Products, Boston, MA) using horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (Biorad, Hercules, CA) and the ECL chemilluminescence detection kit (NEN Life Science Products).

3.3.4. Immunohistochemistry for EphA2 in RCC tissue

RCC tumor specimens were obtained surgically under an IRB-approved protocol and paraffin-embedded. Five-micron sections were de-paraffinized and rehydrated in double-distilled H20 and then PBS. Anti-EphA2 mAb (Ab 208; mIgG1) or isotype-matched control mAb was incubated on sections for 1h at RT. After PBS washing, sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 20 min at RT, and after washing, were then incubated with avidin-biotin-complex peroxidase (Vectastain ABC kits, Vector Laboratories). After a subsequent wash, reaction products were developed using a Nova Red substrate kit (Vector Laboratories), and nuclei were counterstained with hematoxylin. The expression of EphA2 was evaluated independently by two investigators with a microscope under 40X magnification.

3.3.5. Peptides selection and synthesis

The protein sequence of EphA2 protein was obtained from GENBANK (accession number XP 048780) and analyzed for HLA-A0201 and HLA-DRB1*0401 binding peptides using neural network algorithms (187, 188). The top ten candidate HLA-A2 binding peptides were then analyzed for their ability to be generated by proteasomal cleavage using the PAProC prediction algorithm (www.uni-tuebingen.de/uni/kxi/), with only those peptides capable of being processed by the proteasome selected for synthesis. All peptides were synthesized by Fmoc chemistry by the University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Facility. Peptides were >96% pure based on high-performance liquid chromatography profile and mass spectrometric analysis performed by the UPCI Protein Sequencing Facility. In total, we evaluated reactivity to

five HLA-0201 and three HLA-DR0401 predicted binding peptides that received high binding scores (Table 4).

3.3.6. Antigen Stimulation of PBLs

PBLs were resuspended at 10^7/ml in AIM-V medium (GIBCO/Life Technologies) and were incubated for 60 min at 37°C in a humidified 5% CO_2 incubator. Non-adherent (T cell-enriched) cells were gently washed out with PBS and subsequently frozen. The plastic adherent cells were cultured in AIM-V medium supplemented with 1000 units/ml rhGM-CSF (Immunex Corporation, Seattle, WA) and 1000 units/ml rhIL-4 (Schering-Plough, Kenilworth, NJ). Seven days later, dendritic cells (DCs) were harvested and used to stimulate autologous CD8+ or CD4+ T cells. Non-adherent autologous cells were used as "enriched" sources of T cell responders. CD8+ T cells (in HLA-A2-positive patients and healthy donors) or CD4+ T cells (in HLA-DR4-positive patients and healthy donors) were positively isolated to >98% purity using specific magnetic beads (MACS; Miltenyi Biotec, Auburn, CA). Two hundred thousand DCs were cocultured with 2 x 10^6 CD8+ or CD4+ T cells with 10μg/ml peptide for 1 week. On day 7 of *in vitro* stimulation, the responder CD8+ T cells or $CD4^+$ T cells were harvested and analyzed in ELISPOT assays.

3.3.7. IFN-γ and IL-5 ELISPOT assays for Peptide-Reactive CD8+ T cells and CD4+ T Cell Responses

To evaluate the frequencies of peripheral blood T cells recognizing peptide epitopes, ELISPOT assays for IFN-γ and IL-5 were performed as previously described (189). CD8+ T cell responses were evaluated by IFN-γ ELISPOT assays only, while CD4+ T cell responses were

evaluated by both IFN-γ (Th1) and IL-5 (Th2) ELISPOT assays. For ELISPOT assays, 96-well multiscreen hemagglutinin antigen plates (Millipore, Bedford, MA) were coated with 10 μg/ml of anti-human IFN-γ mAb (1-D1K; Mabtech, Stockholm, Sweden) or 5 μg/ml of anti-human IL-5 (Pharmingen-BD, San Diego, CA) in PBS (GIBCO/Life Technologies) overnight at 4°C. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI-1640/10% human serum (1hr at 37°C), 10^5 CD8+ T cells or CD4+ T cells and T2.DR4 cells (2 x 10^4 cells) pulsed with 10 μg/ml synthetic peptides were seeded in triplicate in multi-screen hemagglutinin antigen plates. Control wells contained CD8+ or CD4+ T cells with T2.DR4 cells pulsed with HIV-$nef_{190-198}$ peptide (AFHHVAREL) or Malaria-$CS_{326-345}$ peptide (EYLNKIQNSLSTEWSPCSVT), or T2.DR4 cells alone. Culture medium (AIM-V; GIBCO/Life Technologies) was added to yield a final volume of 200μl/well. To validate the HLA-A2 or -DR4-restricted nature of T cell reactivity, 5 μg of blocking anti-HLA-A2 (BB7.2) or anti-HLA-DR4 (359-13F10) were added to some ELISPOT wells. The plates were incubated at 37°C in 5% CO2 for 24 hr for IFN-γ assessments, and 40 hr for IL-5 assessments. After incubation, the supernatants of the culture wells were harvested for analyses by ELISA. Cells were removed from the ELISPOT wells by washing with PBS/0.05% Tween 20 (PBS/T). Captured cytokines were detected at sites of their secretion by incubation for 2 hr with biotinylated mAb anti-human IFN-γ (7-B6-1; Mabtec) at 2 μg/ml in PBS/0.5%BSA or biotinylated mAb anti-human IL-5 (Pharmingen) at 2 μg/ml in PBS/0.5%BSA. Plates were washed six times using PBS/T, and avidin-peroxidase complex (diluted 1:100; Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) was added for 1 hr. Unbound complex was removed by three successive washes using PBS/T, then with three rinses with PBS alone. AEC substrate (3-Amino-9-ethylcarbazol; Sigma, St. Louis, MO) was added and incubated for 5 min for the IFN-γ

ELISPOT assay and the TMB substrate for peroxidase (3,3',5,5'-Tetramethylbenzidine; Vector Laboratories) was added and incubated for 10 min for the IL-5 ELISPOT assay. Spots were imaged using the Zeiss AutoImager (and statistical comparisons made using a two-tailed Student's T-test). The data are represented as mean IFN-γ or IL-5 spots per 100,000 T cells analyzed.

3.3.8. ELISAs

The supernatants harvested from CD4+ T cell ELISPOT plates were also analyzed for TGF-β and IL-10 content by ELISAs. Supernatants were isolated from ELISPOT plates at the endpoint of the culture period and frozen at –20°C until analyses in specific cytokine ELISAs. Cytokine capture and detection antibodies and recombinant cytokines were purchased from BD-Pharmingen (San Diego, CA) and used in ELISA assays per the manufacturer's instructions. The limit of detection for the TGF-β and IL-10 assays were 60 pg/ml and 7 pg/ml, respectively.

3.3.9. Statistical Analyses

Statistical significance of differences between the two groups was determined by applying Student's t test or two sample t test with Welch correction after each group had been tested for equal variance. We defined statistical significance as a p value of less than 0.05.

3.4. RESULTS

3.4.1. Expression of EphA2 in tumor cell lines and in RCC tissues

EphA2 was overexpressed in malignant renal epithelial cell and Western blot analyses were used to verify EphA2 protein levels in RCC cell lines (Figure 6A). Metastatic RCC lines expressed EphA2 at greater levels than primary RCC lines; and were similar to the levels of staining previously noted for the prostate carcinoma PC-3 (56). While used as a model for normal proximal kidney endothelial cells, the HK-2 cell line is HPV-16 E6/E7-transformed and expresses levels of EphA2 consistent with those observed for primary RCC lines. Normal PBLs expressed undetectable levels of EphA2 protein. Consistent with these findings, immunohistochemical analyses performed on paraffin-embedded RCC specimens (Figure 6B, Table 3) verified intense expression of EphA2 in 13 of 14 evaluable patient tumor biopsies.

3.4.2. Identification of EphA2 epitopes recognized by T cells

To identify potential T cell epitopes, the EphA2 protein sequence was subjected to algorithms designed to identify putative HLA-A2 binding motifs and sites of proteasomal cleavage. Similarly, a neural network algorithm was used to identify EphA2 peptide sequences that would be predicted to bind HLA-DR4 and have the potential to serve as CD4+ T cell-recognized epitopes (187). In aggregate, 8 peptides were synthesized for subsequent analyses; and among these, 5 peptides were predicted to serve as CD8+ T cell epitopes and 3 peptides were predicted to serve as Th epitopes (Table 4).

Peripheral blood T cells were isolated from normal HLA-A2+ and/or -DR4+ donors and stimulated with autologous DCs that had be previously loaded with relevant synthetic peptides.

Responder T cells were subsequently evaluated for specific reactivity against peptide-pulsed T2.DR4 (HLA-A2+/DR4+) antigen-presenting cells and renal cell carcinoma cell lines that expressed the EphA2 antigen and HLA-A2 and/or HLA-DR4. The IFN-γ ELISPOT assay was used to evaluate 8 HLA-A2+ donor CD8+ T cell responses to the 5 putative CTL epitopes as well as 7 HLA-DR4+ donor CD4+ T cell reactivities against the 3 potential Th epitopes.

Each peptide was recognized by at least one normal donor, and only one donor (HLA-DR4+) failed to respond to any of the EphA2 (Th) epitopes (Table 5). Among the HLA-A2 donors, the $EphA2_{546\text{-}554}$ and $EphA2_{883\text{-}891}$ peptides were most commonly reacted against (6/8 donors evaluated), with the responses to $EphA2_{883\text{-}891}$ typically being of a higher frequency. Among the HLA-DR4+ donors evaluated, 6/7 donors responded against at least one predicted EphA2-derived Th epitope, with responses against the $EphA_{63\text{-}75}$ and $EphA2_{663\text{-}677}$ being most prevalent. Bulk and cloned peptide-reactive T cell lines derived from HLA-A2+ or -DR4+ donors recognized EphA2+ tumor cell lines in the appropriate HLA class I- or class II- (HLA-A2 or -DR4) restricted manner (Figure 7)

3.4.3. Analysis of peptide-specific IFN-γ release by peripheral blood CD8+ T cells in ELISPOT assays

We next assessed peripheral blood CD8+ T cells responses against these sequences in 29 HLA-A2+ RCC patients (Table 3) and 10 HLA-A2+ normal donors. CD8+ T cells were enriched to 98% purity for all experiments. Responses were evaluated using IFN-γ ELISPOT assays after 7 day "primary" *in vitro* stimulations. As shown in Figure 8 , the number of IFN-γ spots (per 100,000 CD8+ T cells) observed for T cell responses against EphA2 peptides in HLA-A2+ patients prior to surgery (Pre-Op) or patients with residual disease after surgery (Post-RD)

were as low as those observed in normal HLA-A2+ donors. In contrast, elevated ELISPOT reactivity to EphA2 epitopes was observed in RCC patients who were categorized as disease-free (no-evidence of disease: NED) after surgery (Post-NED). Interestingly, CD8+ T cells from RCC patients exhibiting long-term survival (Post-LTS; > 2 year survival post-surgery) despite having some degree of active disease also showed elevated ELISPOT reactivity to EphA2 CTL epitopes. There were no significant differences in anti-EphA2 CD8+ T cell responses when comparing patients with Stage I vs. Stage IV, if the patient had active disease (Figure 9). Only patients that were analyzed at a time when they were disease-free (i.e. no evidence of disease, NED) or if they were long-term survivors, exhibited CD8+ T cells with elevated reactivity to EphA2 epitopes (Figure 9).

We evaluated the change of CD8+ T cell reactivity against EphA2 peptides pre- and post-therapy in 4 HLA-A2+ patients (Figure 10). Three of these individuals were Stage I patients who had local disease prior to surgical intervention, while the remaining patient had Stage IV disease. Notably, CD8+ T cell reactivity against EphA2 peptides was very low prior to surgery in all four RCC patients. After being rendered free of disease, CD8+ T cell reactivity against EphA2-derived CTL epitopes was significantly increased in each of the three Stage I patients. In marked contrast, the single evaluable Stage IV RCC patient, who had residual tumor burden after surgery, remained poorly responsive to EphA2 peptides (Figure 10).

3.4.4. Peptide-specific IFN-γ and IL-5 release by CD4+ T cells in ELISPOT assay

IFN-γ (Th1-type) and IL-5 (Th2-type) ELISPOT assays were used to discern the altered frequency and functional bias of patient-derived Th cells against EphA2 peptides. Peripheral blood T cells were stimulated for one week with peptide-pulsed immature autologous DC (which

do not appear to skew the Th1/Th2 balance, (189) prior to CD4+ T cell isolation and ELISPOT analyses. The frequencies of CD4+ T cell responders against EphA2 peptides were evaluated in 19 HLA-DR4+ RCC patients (Table 3).

The functional nature of T cell reactivity towards EphA2 related to disease progression. Patients with Stage I disease patients displayed strongly Th1-polarized reactivity against EphA2 peptides whereas patients with more advanced stages of the disease polarized towards strong Th2 reactivity (Figure 11). Not every patient reacted against each peptide, but their responses were consistently polarized in accordance with the patient's disease stage.

We had access to one set of matched blood samples from an HLA-DR4+ patient pre- and post-therapy. This individual had been rendered free of disease after surgery. While the CD4+ T cells from this donor were Th1-biased before and after surgery, the frequency of IFN-γ spots associated with T cell responses against the $EphA2_{53-68}$ and $EphA2_{63-75}$ (but not the $EphA2_{663-677}$) epitopes increased post-treatment (Figure 12). This donor was also HLA-A2 and we observed that increased Th1-type CD4+ T cell-mediated immunity to EphA2 occurred in concert with increased frequencies of circulating IFN-γ secreting anti-EphA2 CD8+ T cells in this patient (i.e. Figure 9; filled circles).

3.4.5. TGF-β and IL-10 production from RCC patient CD4+ T cells against EphA2 peptides

To evaluate whether Th3/Tr1 CD4+ T cells were present in the peripheral blood of RCC patients, we measured TGF-β and IL-10 production following *in vitro* peptide-stimulation. TGFβ1 production by responder CD4+ T cells was only observed in a subset (i.e. 3 of 8) of Stage IV patients (Figure 13) and notably, these same patients displayed coordinately weak Th1-

or Th2-type (IFN-γ and IL-5 ELISPOT) CD4+ T cell reactivity against EphA2 peptides. IL-10 production (above the detection limit of the ELISA) was not observed for any specimen tested.

3.5. DISCUSSION

The molecular definition of tumor-associated antigens has facilitated the development of immunotherapies designed to prime and boost tumor-specific T cell responses in cancer patients. In concert with these advances, cytokine release assays provide a powerful means to monitor the specificity and magnitude of evolving anti-tumor CD8+ and CD4+ T cell responses in the peripheral blood of patients before, during and after treatment (190).

The major finding of our present study is a demonstration that patients with RCC exhibit detectable CD4+ and CD8+ T cell reactivity towards the receptor tyrosine kinase EphA2 that is aberrantly expressed at a high frequency in RCC tumors. EphA2-specific CD8+ T cell activity is inversely proportional to the presence of active disease in these patients and is increased within 6 weeks following therapeutic intervention that result in disease-free status. Interestingly, two HLA-A2+ patients with Stage IV disease were identified who were long-term survivors (> 40 months) post-surgery. Both of these individuals displayed elevated peripheral blood frequencies of IFN-γ-secreting CD8+ T cells reactive against EphA2-derived epitopes. It is tempting to hypothesize that the continued maintenance of high anti-EphA2 CD8+ T cell activity in these patients relates to their continued survival with active disease.

Somewhat in contrast with the CD8+ T cell results, we have also shown that a fine balance of patient Th1-type versus Th2-type CD4+ T cell responses to EphA2 peptides can distinguish between disease-grades. In particular, the most advanced forms of RCC tend to polarize towards Th2- or Tr-type anti-EphA2 responses. This polarization in functional CD4+ T cell responsiveness, combined with the potential suppressive activity mediated by T regulatory cells in patients with Stage IV disease, may play facilitating roles in disease progression.

Our findings are unique in part because they indicate that EphA2 may provide a much-needed target antigen for the design of immunotherapies for RCC. First, EphA2 is strongly expressed by a large number of RCC specimens, including 22 of 24 (92%) RCC cell lines and 29 of 30 (97%) RCC biopsy samples that we have evaluated to date (current report and data not shown). These findings are consistent with evidence emerging from studies of other tumor types, which indicate that high levels of EphA2 expression are characteristic of many forms of cancers, including melanoma and breast, colon, esophageal, head and neck, prostate and lung carcinomas, among others (56, 61, 74, 141, 142, 144, 147). If our present studies can be extended to these other clinical indications, EphA2-specific T cell activity could provide an opportunity for a broadly applicable therapeutic intervention for cancer.

Interestingly, CD8+ T cell reactivity against EphA2 peptides (as determined in IFN-γ ELISPOT assays) differed greatly between RCC patients with active disease and those patients rendered free of disease. Yet, anti-EphA2 CD8+ T cell reactivity did not distinguish RCC disease stage. One potential explanation for this finding is that RCC tumors may suppress the generation, functionality and durability of CD8+ T cell responses against EphA2 *in situ*. This hypothesis is consistent with general tumor-associated immune suppression of peripheral CTL and NK cell activity, as has been previously reported (191). Notably, CD8+ T cell reactivity against EphA2-derived epitopes significantly increased in the peripheral blood of three HLA-A2+ patients with Stage I RCC after curative surgery. In contrast, in a Stage IV patient, surgical intervention without "cure" did not change the low frequency of CD8+ T cell reactivity towards EphA2 peptides. These results are consistent with the requirement for tumor clearance *in situ* (i.e. termination of chronic (tumor) antigenic stimulation) to allow for elevation in functional Tc1-like anti-tumor CD8+ T cell responses (192, 193). An alternative explanation is that

expansion or maintenance of EphA2-specific CD8+ T cell activity may require the concerted support of specific Th1-type responses or a shift of existing patient Th2-type or T suppressor-type to Th1-type immunity, particularly in the advanced cancer setting (189).

Th1-type biased CD4+ T cell response could only be observed in a subset of Stage I RCC patients, and Th2- or Tr-type biased CD4+ T cell responses were almost always observed in Stage IV RCC patients. It is important to stress that polarization away from Th1-type immunity in patients with advanced stage disease was tumor-specific, since individuals with Stage IV disease responded to influenza- and EBV-derived T helper epitopes in a "normal" Th1-biased manner ((189) and data not shown).

While longitudinal data was available for only one HLA-DR4+ patient with Stage I disease, Th1-type immunity against at least some EphA2 epitopes was strengthened and EphA2-specific, Th2-type responses lessened after surgical resection of the patient's tumor. These results are consistent with previous reports that in most cancers, the immune response is believed to be suppressed (or deviated) in advanced stage cancer patients. Our results also suggest that the nature of CD4+ T cell responses against "late-stage" EphA2 peptides correlates with RCC disease stage. This finding contrasts with our previous observations for CD4+ T cell responses against the "early-stage" MAGE-6 epitopes where disease-state, but not disease-stage correlations were noted (189).

Th3/Tr CD4+ T cell subsets may play dominant roles as antigen-specific T "suppressor" cells, in part due to secretion of immunosuppressive cytokines such as TGF-β and/or IL-10 (54). Based on our detection of TGF-β (but not IL-10) production in 3 of 8 (38%) patients with Stage IV disease, it is tempting to speculate that the population of human CD4+CD25+ T suppressor cells may hinder the patient's ability to productively eliminate EphA2-overexpressing tumors

(194). These same patients failed to exhibit discernable Th1-type or Th2-type reactivity to EphA2 peptides, supporting the overall suppressive dominance of EphA2-specific T suppressor-type immunity over specific Th1- or Th2-type responses. These results suggest that Th2- or T suppressor-type responses are prevalent against EphA2 epitopes in advanced Stage RCC patients and likely contribute to the hyporeactivity of tumor-specific cellular immunity noted in these individuals. Future studies could test this hypothesis using flow cytometric analyses to detect HLA-DR4/EphA2 peptide tetramer binding and co-expression of CD25, CTLA-4 or the glucocorticoid-induced tumor necrosis factor receptor (as markers of T suppressor cells, (194)).

EphA2-derived epitopes have potential to serve as components of a cancer vaccine. Unlike MAGE-6 reactive T cells, which are skewed toward Th2-type responses in early-stage disease (189), the imbalance in Th reactivity associated with EphA2 does not appear to occur until later-stage disease. Hence, EphA2-based adjuvant vaccination of Stage I patients could have utility for eliciting protective immunity in patients at high risk for disease recurrence or to prevent prospective metastases. Vaccination with both EphA2-derived CD4+ and CD8+ T cell epitopes may prompt high frequency anti-EphA2 CTL induction that is stabilized by the concurrent activation of specific Th1-type CD4+ T cells. Alternatively under appropriate re-polarizing or activating conditions (195), dendritic cell-based vaccines incorporating EphA2 peptides may allow for previously muted Th1-type immunity to be functionally "resurrected" in patients with advanced stage disease, yielding potential therapeutic benefit. We are currently developing autologous DC/EphA2-based vaccines for the treatment of patients with renal, melanoma, prostate, head and neck or pancreatic cancer at the University of Pittsburgh Cancer Institute and the Cleveland Clinic Foundation.

Preface Chapter 4

Given that EphA2 epitopes are recognized by both CD8+ and CD4+ T lymphocytes, we next investigated whether or not we could modulate the expression of EphA2 protein in EphA2+ tumor cell lines, and whether this modulation could enhance the recognition of these tumor cells by anti-EphA2 CTLs. We utilized two EphA2 agonists, B61.Ig and mAb208, to induce the degradation of overexpressed EphA2 protein in several EphA2+ tumors cell lines. This agonist treatment resulted in the conditional induction of EphA2 phosphorylation and consequent degradation of EphA2 protein. EphA2 degradation was determined to be proteasome-dependent, as the addition of MG132 inhibited EphA2 destruction. Notably, the triggering of EphA2 degradation by B61.Ig or Ab208 resulted in enhanced recognition by a CD8+ T-cell clone specific for $EphA2_{883\text{-}91}$, supporting the ability of this conditional therapeutic strategy to sensitize EphA2 overexpressing tumor cells to immune surveillance.

The studies in **Chapter 4** demonstrate our ability to modulate the proteasome-dependent processing of EphA2 protein in tumor cells, thereby supporting such manipulation as a potential immunotherapeutic protocol for the treatment of patients harboring EphA2+ tumors.

4. Conditional Triggering of Specific CD8+ T cell Recognition of EphA2+ Tumors After Treatment with Ligand Agonists

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Running title: RTK-Triggering Enhances CTL Recognition

Key Words: Renal Cell Carcinoma, EphA2, Receptor Tyrosine Kinase, Proteasome, CD8+ T Lymphocyte

4.1. ABSTRACT

EphA2 is a receptor tyrosine kinase (RTK) that is overexpress at the protein level in a broad range of cancer types, where the degree of overexpression has been correlated with clinical prognosis. We have previously reported that the EphA2 protein contains multiple peptide epitopes that can be recognized by effector CD4+ and CD8+ T cells isolated from normal donors and patients with cancer. We have now evaluated whether the triggering of EphA2 internalization using ligand agonists (B61-Ig and anti-EphA2 mAb 208) results in the proteasomal degradation of EphA2 and an increased T cell recognition as a result of peptide loading into HLA class I molecules. We report that both ligand agonists promote EphA2 phosphorylation and consequent degradation that could be blocked by the proteasome inhibitor MG-132, but not by chloroquine. While ligand agonist treatment had no effect on MHC class I expression, treated tumor cells were recognized significantly better than control, untreated tumor cells by a CD8+ T cell clone specific for the HLA-A2-presented $EphA2_{883-891}$ epitope. These results suggest that agonist treatments that promote EphA2 degradation may not only result in decreased proliferation and tumorigenic potential of EphA2+ tumors, but also their increased recognition by the EphA2-specific T cells.

4.2. INTRODUCTION

The Eph family of molecules contains 14 members, comprising the largest known family of receptor tyrosine kinases (RTK) (1). Based on sequence similarity and binding affinities, two classes of receptors have been defined, EphA and EphB. There are 8 members of the EphA receptor family (EphA1 – EphA8) that primarily bind 5 ephrin-A ligands (ephrinA-1 – ephrinA-5). Ephrin-A ligands are attached to the cell surface via glycosylphosphotidylinositol (GPI) anchors and generally bind only to EphA receptors (196). There are 6 members of the EphB receptor family (i.e. EphB1 – EphB6). The EphB receptors primarily bind ephrin-B ligands (i.e. ephrinB1 – ephrinB3) which are transmembrane proteins (11).

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. EphA2 is a 130kDa (Type-1) glycoprotein that is expressed at low levels on many normal epithelial tissues (30). In normal adult tissues, EphA2 is localized at epithelial cell-to-cell contacts, and is believed to play a role in contact inhibition of cell growth and motility (36, 57). Hence not surprisingly, dysregulation in EphA2 expression and function has been linked to tumor progression and metastasis (56, 60, 144, 146, 147, 197, 198). EphA2 protein has been reported to be overexpressed 10-100 fold in metastatic prostate carcinoma cells when compared to non-invasive prostate epithelial cells (56), and is also expressed in greater amounts in malignant mammary tissue when compared to benign mammary epithelia (57, 58) Zelinski, et. al. have further reported that forced overexpression of EphA2 cDNA via transfection was sufficient to confer malignant transformation on MCF-10, a benign human mammary epithelial cell line. Given its overexpression on multiple epithelial based tumors, EphA2 can be considered a general, shared tumor associated antigen (TAA).

Previously, we have characterized EphA2 epitopes recognized by CD8+ and CD4+ T cells isolated from normal HLA-A2+ and/or –DR4+ donors and patients with renal cell carcinoma (RCC) (148). We also documented that EphA2 is expressed in greater amounts in higher stage RCC (Stage IV vs. Stage I) and in metastatic RCC cell lines versus primary RCC lines. Given this cumulative evidence demonstrating the association of EphA2 overexpression and dysregulation with carcinogenesis, its correlation with metastasis and its ability to be recognized by T-lymphocytes, EphA2 may represent a clinically important tumor antigen that should be targeted in immunotherapy applications designed for the treatment of patients with EphA2+ tumors.

Since ligand agonists have been previously reported to promote receptor tyrosine kinase internalization (199, 200), we analyzed whether Ephrin-A1(B61)-Ig and anti-EphA2 mAb (Ab208) would promote the degradation of overexpressed tumor cell EphA2 via a mechanism leading to enhanced recognition of the treated tumor cell by EphA2-specific T cells. We show that both B61-Ig and Ab208 promote rapid phosphorylation and consequent proteasome-dependent degradation of EphA2 protein and that tumor cells treated in this manner elicit stronger responses from an anti-EphA2 specific CD8+ T cell clone. Such conditional augmentation of tumor cell recognition by ligand agonist treatment in concert with active immunization against EphA2 epitopes or the adoptive transfer of EphA2-specific T cells may represent a novel and effective clinical strategy for the treatment of patients with EphA2+ malignancies.

4.3. MATERIALS AND METHODS

4.3.1. Cell Lines and Media

The T2.DR4 (HLA-A2+/-DRB1*0401+; (175)) cell line (kindly provided by Dr. Janice Blum, Indiana University School of Medicine, Indianapolis, IN) was used as the peptide-presenting cell in ELISPOT assay. The EphA2+ HLA-A2- PC-3 prostate carcinoma cell line was used as positive control for Western Blot analysis of EphA2 protein expression (56) and was also used as a negative control target in ELISPOT assays. SLR24, an EphA2+ HLA-A2+ cell line was tested in western blot and ELISPOT assays. All cell lines were maintained in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 10mM L-glutamine (all reagents from GIBCO/Life Technologies, Grand Island, New York) in a humidified atmosphere under 5% CO_2 tension at 37°C.

4.3.2. Western Blot Analyses

Tumor cells were grown to 80-90% confluency, serum starved overnight, then treated with agonists where indicated. Samples were analyzed for EphA2 expression via Western blots using the rabbit anti-human EphA2 polyclonal antibody (clone: C-20), Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Samples were also analyzed for Axl (clone C-20, Santa Cruz Biotechnology, Santa Cruz, CA) protein content. Confluent flasks were lysed using 500μl lysis buffer (1% Triton –X, 150nM NaCl, 10mM Tris pH7.4, 1mM EDTA, .2mM SOV 0.5% NP-40) in PBS containing protease inhibitors (Complete, Roche Diagnostic, Mannheim, Germany) for 30min at 4°C. After centrifugation at 13,500 x g for 20 minutes, the supernatant was mixed 1:1 with SDS-

PAGE running buffer and proteins separated on 7.5% PAGE gels, prior to electro-blotting onto nitrocellulose membranes (Millipore, Bedford, MA). Blots were imaged on Kodak X-Omat Blue XB-1 film (NEN Life Science Products, Boston, MA) after using horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (Biorad, Hercules, CA) and the Western Lighting™ chemilluminescence kit (PerkinElmer, Boston, MA). Immunoprecipitation for EphA2 were performed using the anti-EphA2 antibody D7 (Upstate Biotech, Inc.). Anti-phosphotyrosine antibodies (Clone pY99, Santa Cruz Biotechnology were used to assess pEphA2 content. Mouse anti-β-actin antibodies (clone AC-15, Abcam, Cambridge, MA) were used as a loading control.

4.3.3. EphA2 Agonists

B61.Ig and mAb208 were obtained from Dr. Michael Kinch (MedImmune, Gaithersburg, MD). B61.Ig is a chimeric protein consisting of the ligand binding domain of ephrin A-1 fused with the Fc portion of a mouse IgG antibody and was used at 30μg/ml where indicated. mAb208 is a mouse monoclonal antibody specific for EphA2 and was used at 8μg/ml where indicated.

4.3.4. ELISPOT Assays

ELISPOT assays for IFN-γ were performed as previously described (148). CL.142, an HLA-A2+ CD8+ T cell clone specific for $EphA2_{883\text{-}91,}$ was generated as previously described (201). CL.142 responses were evaluated by IFN-γ ELISPOT assays. 96-well multiscreen hemagglutinin antigen plates (Millipore, Bedford, MA) were coated with 10 μg/ml of anti-human IFN-γ mAb (1-D1K; Mabtech, Stockholm, Sweden) in PBS (GIBCO/Life Technologies) overnight at 4^oC. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI-1640/10% human serum (1hr at 37^oC), 10^5 CD8+ T cells and

T2.DR4 cells (2 x 10^4 cells) pulsed with 10 μg/ml $EphA2_{883-91}$ peptide (TLADFDPRV) or SLR24 +/- treatment overnight with B61.Ig were seeded in triplicate in multi-screen hemagglutinin antigen plates. Control wells contained CL.142 with T2.DR4 cells pulsed with HIV-$nef_{190-198}$ peptide (AFHHVAREL) or PC3, an HLA-A2- EphA2+ tumor cell line, or T2.DR4 cells alone. Culture medium (AIM-V; GIBCO/Life Technologies) was added to yield a final volume of 200μl/well. The plates were incubated at 37°C in 5% CO_2 for 24 hr for IFN-γ assessments. Cells were removed from the ELISPOT wells by washing with PBS/0.05% Tween 20 (PBS/T). Captured cytokines were detected at sites of their secretion by incubation for 2 hr with biotinylated mAb anti-human IFN-γ (7-B6-1; Mabtec) at 2 μg/ml in PBS/0.5%BSA. Plates were washed six times using PBS/T, and avidin-peroxidase complex (diluted 1:100; Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) was added for 1 hr. Unbound complex was removed by three successive washes using PBS/T, then with three rinses with PBS alone. AEC substrate (3-Amino-9-ethylcarbazol; Sigma, St. Louis, MO) was added and incubated for 5 min for the IFN-γ ELISPOT assay. Spots were imaged using the Zeiss AutoImager (and statistical comparisons made using a two-tailed Student's T-test. We defined significance as a p value of less than or equal to 0.05.

4.4. RESULTS

4.4.1. B61.Ig and mAb208 Induce EphA2 Phosphorylation in Tumor Cell Lines.

Proper EphA2 metabolism is dependent on the ability of the EphA2 to become activated and phosphorylated on cytoplasmic domain tyrosine residues, (i.e., Y588, Y594, Y772, Y921, as determined by GenBank). This allows for the RTK to become ubiquitinated and internalized, prior to being recycled to the cell surface or degraded via the proteasome. In cancerous cells, this regulation can be disrupted resulting in RTK hypophosphorylation (65). Indeed, the EphA2 protein expressed in PC3 prostate carcinoma cells is constitutively underphosphorylated (Fig. 14). In order to determine whether treatment of PC3 with ligand agonists induces normalization in phosphorylation of EphA2, PC3 were treated with either B61.Ig or mAb208, and the phosphorylated EphA2 protein content was analyzed in Western Blots after immunoprecipitation of EphA2 protein. As depicted in Figure 14, both ligands induced the rapid phosphorylation of EphA2 within 10-30 minutes of treatment.

4.4.2. B61.Ig and mAb208 Induce the Proteasome-Dependent Degradation of Tumor Cell Expressed EphA2 Protein

To determine whether agonist ligands also promote EphA2 degradation, the EphA2+ prostate carcinoma cell line PC3 (56) and the RCC cell line SLR24 were each treated with B61.Ig or mAb208 for 6 hrs at 37°C, lysed, and EphA2 protein levels were assessed in Western Blots. The B61.Ig and mAb 208 treatment regimens were able to induce EphA2 degradation in both the PC3 and SLR24 cell lines (Figs. 15A, 15B, and data not shown), as well as additional EphA2+ carcinomas (including the pancreatic carcinoma PANC-3.27; data not shown),

suggesting the generality of these findings. EphA2 agonist effects were specific for EphA2, since protein expression levels of an alternate RTK overexpressed by these cell lines, AXL, remained unaltered in its protein expression level after treatment with these ligands (Figs. 15A, 15B).

To determine whether ligand-induced degradation of EphA2 was dependent on the proteasome, we repeated these assays in the presence of the 26S proteasome inhibitor MG-132. While MG-132 effectively blocked EphA2 degradation promoted by addition of B61-Ig or mAb208 (Fig. 16), the addition of the endosomal/lysosomal inhibitor chloroquine had no effect, supporting the proteasome-dependency of this event.

4.4.3. EphA2 Agonist Treatment Enhances CD8+ T Cell Recognition of EphA2+ Tumors

Given that EphA2 can be triggered to be degraded using EphA2 agonists on EphA2+ tumors via the proteasome, this could theoretically lead to an increase in EphA2 epitope presentation on MHC Class I molecules expressed by EphA2+ tumors and to tumor cell recognition by EphA2-specific CD8+ T cells. To address this issue, we initially evaluated the effects of ligand agonist treatment on the expression of MHC Class I molecules on EphA2 agonist treated EphA2+ tumor cells, using the SLR24 RCC cell line as a model. Neither HLA Class I nor control antigen CD40 expression on SLR24 cells was affected by either treatment (Table 6).

To determine whether ligand agonist treatment of EphA2+ tumors results in enhanced recognition by specific CD8+ T cells, non-treated or B61-Ig treated SLR24 cells were analyzed

as stimulators for the $EphA2_{883\text{-}91}$ specific CTL clone 142 (CL.142) in IFN-γ ELISPOT (Fig. 17). Pretreatment of SLR24 with B61.Ig significantly enhanced CL.142 recognition of SLR24 relative to untreated control cells.

4.5. DISCUSSION

In this study, we report that the administration of specific ligand agonists to EphA2 overexpressing pancreatic, prostate and RCC cell lines induces the phosphorylation and proteasome-dependent degradation of EphA2 This treatment regimen did not affect the quantitative level of MHC Class I or CD40 expression on EphA2+ tumor cell lines. However, treated tumor cell lines elicited increased reactivity from cloned EphA2-specific CD8+ T-cells. These findings suggest that the use of EphA2 agonists may represent a new modality for consideration as a single agent or a component in combinational immunotherapies due to their capacity to conditionally sensitize EphA2+ tumor cells to specific CD8+ T cell reactivity.

The activation of EphA2 and other RTK is necessary for their proper function and metabolism. In normal epithelia, EphA2 is located at cell-to-cell boundaries, and it is generally believed that one of the major functions of this RTK is to maintain cell-to-cell contacts and to limit epithelial cell growth. This is critical for the organization and formation of epithelial layers in EphA2+ tissues. Normal activation of EphA2 results in the de-phosphorylation of focal adhesion kinase (FAK), which in turn suppresses integrin function (35), and limits the ability of EphA2+ epithelial cells to make contacts with the extracellular matrix (ECM). However, when EphA2 expression becomes dysregulated, affected cells become more invasive, at least in part, due to an increase in cellular contacts formed with the ECM (61). This appears to be a common event in tumor metastases, where EphA2 is frequently dysregulated and overexpressed (197). Under such conditions, EphA2 ligand agonists can restore a normalized pattern of contact inhibited growth and reduce the invasiveness of EphA2+ tumor cells (199).

The phosphorylation of the EphA2 after interaction with ligand agonists promotes the association of EphA2 with c-Cbl (62). C-cbl contains an internal SH2 domain, which allows for

it to bind to phosphorylated RTKs. It also contains an ubiquitin-E3 ligase which is responsible for the addition of ubiquitin molecules to the respective RTK. This targets proteins for degradation via the proteasome and/or for sorting within the endosomal/lysosomal pathway (202). This is a major pathway in the recycling of EphA2 and RTK after they have signaled as a consequence of engaging their ligands. In this pathway, internalized, activated EphA2 molecules that become dephosphorylated are recycled to the cell membrane, while those that remain phosphorylated undergo proteasome-dependent degradation. In the context of our studies, this results in the consequent MHC class I presentation of EphA2-derived peptides, such as $EphA_{883-891}$ to specific CD8+ T cells.

The finding that dysregulated tumor-overexpressed EphA2 is hypophosphorylated is consistent with reports of frequent co-ordinate overexpression of regulatory protein tyrosine phosphatases in cancer cells that may serve to inactivate phospho-EphA2 species quickly after they are formed. Recently, it has been reported that low molecular weight-protein tyrosine phosphatase (LMW-PTP) is overexpressed in many cancer types (70, 203), and that EphA2 is a major substrate for this enzyme (67). This increased expression and function of LMW-PTP, along with other intracellular phosphatases, i.e. PTP-1B (204, 205), may contribute to the accumulated overexpression of EphA2, as well as other tumor-associated RTKs. The use of phosphatase inhibitors on EphA2+ tumors could possibly result in restoration of EphA2 phosphorylation, proteasomal processing and MHC-presentation. This theory is currently being evaluated in our laboratory.

In summary, the ability to conditionally trigger EphA2 degradation and the presentation of its derivative peptide epitopes in MHC complexes offers new hope for the improved efficacy of combinational immunotherapy approaches. Ideally, such therapies would take the form of an

EphA2-based vaccine that can activate specific CD8+ T cells that could then be directed to more effectively recognize and eradicate EphA2+ tumor cells *in situ* after the administration of EphA2 ligand agonists. Alternatively, *ex vivo* expanded EphA2-specific CD8+ T cells could be adoptively transferred into patients that are co-treated with EphA2 ligand agonists.

Preface Chapter 5

Since overexpression of protein tyrosine phosphatases has been reported in certain cancer settings, and these enzymes appear to regulate the magnitude of RTK phosphorylation and consequent proteolytic processing, we next explored whether phosphatase inhibitors augmented EphA2 degradation in RCC cell lines *in vitro*. We observed that EphA2 was constitutively underphosphorylated in EphA2+ tumors, but that the addition of protein tyrosine phosphatase inhibitor-2 (PTP I-2) rapidly reversed this condition and induced the phosphorylation of EphA2 in the PC3 prostate carcinoma cell line. Furthermore, we observed that treatment of tumor cells with PTP I-2 (or a range of other phosphatase inhibitors) also induced the co-ordinate degradation of additional overexpressed RTKs including the epidermal growth factor receptor EGFR (erbB-1) and Axl.

The results in **Chapter 5**, in conjunction with the results in **Chapter 4**, provide the foundation for the development of novel combinatorial therapies implementing RTK agonists and phosphatase inhibitors to promote the conditional processing and MHC presentation of RTK-derived peptide epitopes for immune cell recognition. Furthermore, by reducing EphA2 (and other RTK) protein expression, the immune system will be targeting less aggressive tumors in situ, which may further accentuate the efficacy of this approach.

5. PTP Inhibitors Promote Enhanced Tumor Cell Processing of Receptor Tyrosine Kinases

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5.1. ABSTRACT

EphA2 is a novel oncoprotein that is overexpressed in a wide range of cancer types, where it is linked to metastatic potential and poor clinical prognosis. Cancer cell overexpression of EphA2 appears linked to the inability of this receptor tyrosine kinase to become phosphorylated for a sufficient period of time to allow for its consequent degradation in neoplastic cells. Since instability in phosphorylated EphA2 species may be regulated by cellular phosphatases that have also been reported to be overexpressed in cancer, we studied whether the inhibition of cellular phosphatases would result in the conditional augmentation in EphA2 phosphorylation and proteasome-dependent degradation in EphA2+ tumor cell lines *in vitro.* Treatment of tumor cells with the nitric oxide donor NO-PAPA, or the PTP-inhibitors sodium orthovanadate and protein tyrosine phosphatase inhibitor-2, but not the protein serine/threonine phosphatase inhibitor okadaic acid, resulted in the efficient degradation of EphA2 protein. Interestingly, this affect was not restricted to EphA2, as two additional tumor cell expressed RTKs, Axl and EGFR/erb1, were also induced by these PTP inhibitors to undergo degradation. . Furthermore, by treating tumor cells with the anti-EphA2 agonist mAb208 (that promotes phosphorylation of EphA2) and NO-PAPA (to block PTPs), we observed an additive effect on conditional EphA2 degradation. These results confirm the dependency of EphA2 (and other RTK) degradation on PTPs and suggests that clinical application of combinatorial therapies using RTK agonists and PTP inhibitors may exhibit efficacy by limiting the oncogenic effects of RTKs in cancer cells.

5.2. INTRODUCTION

We have previously identified EphA2 as a novel tumor antigen in the setting of renal cell carcinoma (148). Although EphA2 overexpression had been reported by others in prostate and lung cancers, the mechanism(s) underlying the overexpression/accumulation of this protein had not been resolved. EphA2 belongs to the largest known family of receptor tyrosine kinases (RTK, (1)), and is believed to play a major role in the contact growth inhibition of epithelial cells (36). The regulation of RTKs has been studied extensively given their reported effects in carcinogeneis (65, 148, 200, 204). In normal cells, upon RTK interaction with a multivalent ligand, the RTK becomes autophosphorylated on cytoplasmic tyrosine residues. This phosphorylation promotes the association of pRTKs with c-Cbl, an ubiquitin E3-ligase. Ubiquitinated pRTKs are then internalized and sorted in intracellular endosomal/lysosomal compartments, with a fraction of RTKs recycled to the plasma membrane and a fraction targeted for proteasome-dependent degradation (202). The sorting decision for recycling vs. degradation appears related to both the stability of the state of RTK phosphorylation and the degree of polyubiquitination (202). Perturbation of c-Cbl function or RTK phosphorylation each result in dysregulation in RTK homeostasis and overexpression/accumulation of these proteins in affected cells (206).

A major regulator of RTK metabolism is the family of protein tyrosine phosphatases (PTPs; (204, 207). PTP play major regulatory roles in dictating the cellular fate of activated pRTK. PTP are responsible for the removal of phosphate groups added to pRTK when they have been appropriately activated via physiologic ligands binding to the RTK extracellular domain (69, 70, 205, 208). The balance of phosphotyrosine content in activated RTK species is critical in determining the fate of these molecules. For instance, if the pRTK is de-phosphorylated,

interactions with c-Cbl are prevented and the RTK is not ubiquitinated or internalized, resulting in its accumulation on the cell surface. Recently, several groups have reported that PTPs are frequently overexpressed in a diverse array of cancer types (70, 207). In particular, it has been reported that the low molecular weight-protein tyrosine phosphatase (LMW-PTP) is overexpressed in several cancers (70) and that EphA2 is a substrate for the LMW-PTP, giving credibility to the potential role of LMW-PTP in indirectly regulating tumor cell growth and invasiveness by dysregulating RTK expression/function

Given our existing knowledge that agonist (ligand) induction promotes the phosphorylation and subsequent degradation of EphA2 (**Chapter 4**), this chapter explores the hypothesis that the inhibition of cellular phosphatases may restore normal phosphorylation and metabolism of EphA2 in tumor cell lines. We show that the inhibition of PTP, but not protein serine/threonine phosphatases through a variety of pharmacologic inhibitors results in increased EphA2 phosphorylation and consequent degradation. Furthermore, we noted that ligand agonists and PTP inhibitors acted additively in promoting EphA2 proteolysis. These results confirm the notion that the dysregulation of EphA2 phosphorylation is a major factor in EphA2 overexpression and provide a model for developing novel therapeutics that may block the malignant characteristics of RTK overexpression in cancer cells.

5.3. MATERIALS AND METHODS

5.3.1. Cell Lines and Media

The EphA2+ HLA-A2- PC-3 prostate carcinoma cell line was used as positive control for Western Blot analysis of EphA2 protein expression (56). SLR24, an EphA2+ HLA-A2+ cell line and Panc 3.27, a pancreatic carcinoma cell line were also tested in PTP inhibition studies. All cell lines were maintained in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 10mM L-glutamine (all reagents from GIBCO/Life Technologies, Grand Island, New York) in a humidified atmosphere under 5% CO_2 tension at 37°C.

5.3.2. Pharmacologic PTP Inhibitors

NO-PAPA was obtained by a generous gift from Dr. Lawrence Keefer (National Cancer Institute, Bethesda, MD). Sodium orthovanadate, phenylarsine oxide, levamisole, and okadaic acid were all obtained from Sigma-Aldrich (St. Louis, MO). PTP Inhibitor-2 was obtained from EMD Biosciences (San Diego, CA). All pharmacologic agents were used per the manufacturer's instructions and applied as indicated in figure legends.

5.3.3. Western Blot Analyses

Tumor cells were grown to 80-90% confluency, serum starved overnight, then treated with agonists where indicated. Samples were analyzed for EphA2 expression via Western blots using the rabbit anti-human EphA2 polyclonal antibody (clone: C-20), Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Samples were also analyzed for Axl (clone C-20, Santa Cruz Biotechnology, Santa Cruz, CA) and EGFR (clone 1005, Santa Cruz Biotechnology, Santa Cruz, CA).

Confluent flasks were lysed using 500µl lysis buffer (1% Triton-X100, 150nM NaCl, 10mM Tris pH7.4, 1mM EDTA, .2mM SOV 0.5% NP-40) in PBS containing protease inhibitors (Complete, Roche Diagnostic, Mannheim, Germany) for 30min at 4°C with agitation. After centrifugation at 13,500 x g for 20 minutes, the supernatant was mixed 1:1 with SDS-PAGE running buffer and proteins separated on 7.5% PAGE gels, prior to electro-blotting onto nitrocellulose membranes (Millipore, Bedford, MA). Blots were imaged on Kodak X-Omat Blue XB-1 film (NEN Life Science Products, Boston, MA) after using horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (Biorad, Hercules, CA) and the Western Lighting™ chemilluminescence kit (PerkinElmer, Boston, MA). Immunoprecipitation for EphA2 were performed using the anti-EphA2 antibody D7 (Upstate Biotech, Inc.). Anti-phosphotyrosine antibodies (Clone pY99, Santa Cruz Biotechnology were used to assess pEphA2 content. Mouse anti-β-actin antibodies (clone AC-15, Abcam, Cambridge, MA) were used as a loading control. Densitometric visualization was done using a White/UV Transilluminator (UVP Products, Upland, CA) and analysis performed using Labworks (UVP) software. Statistical comparisons of protein content were made using a two-tailed Student's T-test. We defined significance as a p value of less than or equal to 0.05.

5.4. RESULTS

5.4.1. NO-PAPA Induces EphA2 Degradation

Amid reports that nitric oxide (NO) metabolites (e.g. peroxynitrites) are able to inhibit LMW-PTP (209), and that LMW-PTP can use EphA2 as a substrate, we initially chose to evaluate whether the addition of NO to EphA2+ tumors could induce EphA2 degradation. We used NO-PAPA, a nucleophilic salt that upon addition to aqueous media releases NO (210), to test whether we could conditionally induce the degradation of EphA2. PC3, an EphA2+ prostate carcinoma cell line, was treated for 6 hours, lysed, and EphA2 protein expression was assayed via western blot. As shown in Figure 18, NO-PAPA was able to induce the degradation of EphA2 in a dose-dependent and non-toxic manner. These results suggest that a known LMW-PTP inhibitor (NO) can induce EphA2 degradation in tumor cell lines *in vitro.*

We have reported previously (**Chapter 4**) that the addition of EphA2 agonists could induce the phosphorylation and subsequent degradation of EphA2. We next tested whether or not the co-administration of both NO-PAPA and mAb208 could induce EphA2 degradation more efficiently than either reagent alone. PC3 cells were treated with either NO-PAPA, mAb208, or both for 6hr, with EphA2 protein expression consequently analyzed via western blot. Co-treatment of tumor cells with NO-PAPA and mAb208 was able to promote significantly more EphA2 degradation than either NO-PAPA of mAb208 alone (Fig.19A; vs. NO-PAPA, $p = .05$; vs. mAb208, $p = .015$). Interestingly, EphA2 was not the only RTK affected in this manner by NO-PAPA treatment. Axl, another RTK family member that has also been implicated in cancer, was similarly induced to undergo degradation after treatment with NO-PAPA (Fig 19B). These results raise the possibility that the inactivation of PTP may induce the coordinate degradation of

multiple, overexpressed RTKs in cancer cells, a process that may be further accentuated by inclusion of RTK agonist signals.

5.4.2. PTP I-2 Induces the Phosphorylation of EphA2

These findings suggested that inactivation of PTP may represent a relevant means through which RTK expression may be profoundly down-modulated. However, since the effects of NO are pleiotropic, we wished to further clarify the relevancy of PPs in the observed effects on RTK expression. Central to our hypothesis of PTP inhibition leading to RTK degradation is the restoration of tyrosine phosphorylation on RTK. We tested a specific PTP inhibitor, protein tyrosine phosphatase inhibitor-2 (PTP I-2), for its ability to allow for EphA2 phosphorylation to occur in PC3 cells. As shown in Fig. 20 addition of PTP I-2 to PC-3 cultured cells resulted in the rapid phosphorylation of EphA2 protein. This same effect was also demonstrated in EphA2+ RCC (SLR24) and pancreatic carcinoma (PANC-3.27) cell lines, demonstrating the generality of these results in cancer cells of diverse histologic types (data not shown).

5.4.3. PTP Inhibitors Induce RTK Degradation

Given the result that PTP I-2 is able to induce EphA2 phosphorylation/degradation, we next wished to determine whether tumor cell treatment with other pharmacologic PTP inhibitors produced similar results. We tested numerous PTP inhibitors (e.g. phenylarsine oxide (PAO), levamisole, Table 7) and focused our comparative analyses on sodium orthovanadate (SOV) vs. PTP I-2, since the other agents exhibited significant cellular toxicity. As described above, PC3 cells were treated for 6 hr with our candidate PTP inhibitors, lysed, and lysates were resolved via SDS-PAGE. Using western blot assays (Fig. 21) both SOV and PTP I-2 were able to induce the

degradation of EphA2 (Fig. 21A). Consistent our results in Fig 21A, these PTP inhibitors also induced the degradation of additional tumor cell-expressed RTKs, including Axl (Fig. 21B) and EGF-R (erbB-1) (Fig. 21C). This process was clearly dependent on PTP and not general PP inhibition, as the addition of okadaic acid (OKA), a serine-threonine phosphatase inhibitor, had no effect on EphA2 protein expression (Fig. 22). EphA2 induced degradation by PTP I-2 was able to be induced by lactacystin and chloroquine (Fig. 23) The inhibition by chloroquine is interesting due to that PTP I-2 induced RTK degradation may increase RTK epitope presentation to CD4+ T cells. These results as a whole are significant since they support a novel and translatable strategy for the conditional promotion of RTK protein down-regulation and processing through the use of PTP inhibitors. This provides clinicians with a novel therapeutic tool for potential implementation in the treatment of numerous types of cancers.

5.5. DISCUSSION

We have shown that PTP inhibitors restore EphA2 phosphorylation in EphA2+ tumor cell lines and induce its degradation. Interestingly, this observation was true not only of EphA2, but two other RTK (Axl, EGFR/erb1) family members that were more effectively degraded in the presence of PTP inhibitors. This is consistent with phosphorylation being a rate-limiting requirement for proper RTK metabolism. The inhibition of PTP and restoration of EphA2 phosphorylation supports our hypothesis that the lack of proper EphA2 phosphorylation is a major mechanism for EphA2 overexpression in cancer cells.

As we have previously demonstrated in **Chapter 4,** EphA2+ tumors are sensitized to recognition by CTL after treatment with EphA2 ligand agonists. Hence, it is possible that the treatment of RTK+ tumor cells with PTP inhibitors (alone or with ligand agonists) will similarly lead to enhanced RTK-specific immune recognition of these conditionally manipulate target cells. If validated, this would provide a compelling strategy to enhance tumor cell sensitivity to a broad repertoire of tumor-reactive T effector cells that would be restricted by multiple patient MHC molecules. As such, this approach would circumvent current limitations in immunotherapies using synthetic tumor antigen-derived peptide epitopes that are relevant in only a subset of patients with cancer. We are currently assessing this hypothesis in our laboratory.

Currently, PTP inhibitors are being used clinically (i.e. nitroprusside, orthovanadate) for the treatment of hypertension. Pending dose-limiting toxicities, the use of PTP inhibitors could be readily translated into cancer therapeutic protocols. In conjunction with EphA2 agonists, the use of PTP inhibitors could provide a novel combinatorial therapeutic approach to the treatment of EphA2+ tumors. Our demonstration that PTP inhibitors induce the degradation of additional RTKs (Axl, EGFR), and our and others reports that EGFR/erb1 and HER2/neu undergo

degradation after treatment with anti-RTK mAbs (Appendix A), suggest that the coordinate application of RTK agonists and PTP inhibitors could prove clinically beneficial to patients presenting with a wide range of tumors the overexpress RTKs.

6. SUMMARY AND CONCLUSIONS

While vaccines based on whole tumor cell vaccines have been applied clinically for the past several decades, this approach has proven marginally effective in the treatment of cancer patients and provided minimal mechanistic insight into either the specificity of clinically-important T cells or biochemical pathways in tumor cells that may be exploited for therapeutic gain. We believe that the molecular characterization of tumor antigens and their derivative epitopes is necessary for optimal therapy benefit to be derived from vaccine strategies designed to elicit specific CD4+ and CD8+ T cells in the setting of cancer. In these studies, we have focused our efforts on the characterization of a novel tumor antigen, EphA2. EphA2 overexpression has been repeatedly reported in numerous cancer settings, and is also correlated with metastatic disease and tumor neoangiogenesis. We set out to define T cell epitopes recognized by both CD8+ and CD4+ T lymphocytes. In addition, we were also interested in determining the functional CD4+ T cell response to EphA2 epitopes as it relates to cancer staging. We had the good fortune of having archived tissue microarrays (TMA) that also allowed us to analyze the expression of EphA2 in resected RCC biopsy specimens in order to determine if the relative expression of EphA2 was predictive of the time to disease recurrence in RCC patients. Finally, we studied whether or not the modulation of EphA2 degradation in tumor cell lines that overexpress this antigen resulted in improved proteasome-dependent processing of this protein and improved recognition of treated tumor cells by specific CTLs. In these studies, we have efficiently defined and characterized a novel tumor antigen that we believe should now be targeted by new treatment strategies for the more effective management of EphA2+ tumors.

The initial phase of my studies involved analyzing the expression of EphA2 protein in tumor biopsies excised from patients with RCC. We were interested in determining whether the

level of EphA2 protein expression on primary tissue samples was predictive of the time to recurrence in the evaluated patients. Tissue microarrays (TMA) containing patients with recurrence intervals of months to more than 17 years were assessed for relative EphA2 expression levels by immunohistochemistry. We noted a strong correlation between the degree of expression of EphA2 protein in the excised lesions and the time to recurrence of RCC disease. Patients that exhibited a high protein expression of EphA2 (ave. score = 3.81) were more likely to experience disease recurrence in less than 1 year. This was significant when they were compared to patients who exhibited lower levels of EphA2 protein expression. These latter patients experienced RCC disease recurrence only after 1 year of resection. While the average score for EphA2 expression was lower for the cohort of patients who recurred 5 or more years (ave score = 1.27) after surgery versus patients who recurred between 1 and 5 years (ave score = 1.6) post-treatment, this difference was not statistically significant. We further noted that tumors displaying higher levels of EphA2 expression tended to be more vascularized as determined by the density of Factor VIII+ vessel staining. This suggests the relative level of EphA2 expression may be associated with, or represent a surrogate marker for tumor neoangiogenesis in RCC, as has been recently intimated in the setting of colorectal carcinoma. RCC tumors expressing high levels of EphA2 protein also tended to be larger in volume when compared to low EphA2 expression RCC tumors. These results support the hypothesis that EphA2 is a major factor in tumor progression and may promote the metastatic capacity of tumor by enhancing access to the circulatory system and promoting a more invasive/aggressive form of the disease. With respect to RCC, EphA2 overexpression should be considered as a relevant negative prognostic indicator and should be contemplated for adoption in the clinical management of patients with RCC (i.e. the more aggressive treatment of patients with lesions overexpressing EphA2 and/or more

frequent follow-up of surgically cured patients whose resected lesions were high EphA2 expressors).

Next, we wished to analyze the protein expression of various primary and metastatic RCC cell lines available in our laboratory to test the hypothesis that EphA2 expression is correlated with metastatic disease. We confirmed this hypothesis by using immunohistochemistry and western blot analysis demonstrating the positive correlation between RCC overexpression and metastatic phenotype. Metastatic RCC cell lines expressed more EphA2 compared to primary RCC cell lines, and both of these groups expressed more EphA2 compared than the HK2, a normal human kidney cell line. This correlation with metastasis was also observed in RCC tissue samples where EphA2 protein expression was assessed immunohistochemically. Stage IV RCC samples expressed considerably more EphA2 protein when compared to Stage I RCC tissues.

The next phase of our studies was to define T cell epitopes contained within EphA2, and to assess whether those epitopes were differentially recognized by T cells isolated from normal donors and patients with RCC. In order to evaluate T cell specificity for EphA2 epitopes, we chose to utilize a DC *in vitro* based method to assess T cell specificity for EphA2 peptides using cytokine-specific ELISPOT assays. T lymphocytes isolated from both normal donors and patients with RCC were able to recognize both the (5) HLA-A2- and (3) HLA-DR4-presented epitopes that we identified based on motif analyses performed using net-based predictive algorithms. Of critical importance, *in vitro* generated EphA2-specific T cells were also able to recognize EphA2+ tumors in both HLA-A2- and HLA-DR4-restricted manners, confirming that our candidate epitopes were naturally processed and MHC-presented and are therefore, biologically relevant.

Given recent reports from our group, among others, suggesting a polarization in anti-tumor T cell responses away from the Tc1/Th1-type in cancer bearing individuals, we were keen to assess the functionality of anti-EphA2 T cells and whether any Type-1 vs. Type-2 or T-regulatory biases would be observed and whether these would be correlated with the presence of disease or disease stage in patients with RCC. With respect to CD8+ T cell functionality, patients who had no evidence of disease (NED) after tumor excision or who were long term survivors (LTS) exhibited a Tc1 response (IFN-γ production) to HLA-A2-presented EphA2 epitopes. Patients who still had residual disease after tumor excision or who had active RCC disease did not produce detectable levels of IFN-γ in response to EphA2 epitopes. Interestingly, we were able to test several RCC patients before and after their surgical treatment for RCC and noticed that after surgery (within 6 weeks post-surgery), patients who had previously demonstrated Type-2 immunity against EphA2 epitopes, were now able to produce Type-1 CD8+ T cell responses to this antigen. This data supports the notion that EphA2+ tumors may adversely affect the immune system. This may be due to the production of Th1-inhibiting cytokines (e.g. IL-5, IL-10, TGF-β) by RCC. The recognition of EphA2+ TCC tumors in this tumor microenvironment would skew the T cell away from producing the necessary cytokines (i.e. IFN-γ) required for cell-mediated immune clearance of EphA2+ tumors. The (at least temporary) restoration of Type-1 immune responses against EphA2 epitopes after the surgical removal of RCC supports this hypothesis.

Similar results were obtained when CD4+ T cell functionality was assessed in patients with RCC disease. The CD4+ T cell immune response in patients with Stage IV RCC disease was skewed towards Th2-type immune responses, where patients with Stage I disease exhibited Th1-type immunity against EphA2. For those patients where pre- and post- surgery samples were

available, the ratio of Th1(IFN-γ):Th2(IL-5) cytokines production in response to EphA2 epitopes was significantly increased when RCC tumors were excised from diseased patients.

We also tested the supernatants of cultured CD4+ T cells from RCC patients in order to assay for the presence of Th3/Treg-type suppressor CD4+ T cells. Th3- and Treg-type "suppressor" T cells produce immunoregulatory cytokines (e.g. IL-10, TGF- β) in order to temper the immune response by preventing overactive inflammatory immune responses. Hypothetically, tumors may impact the immune response by influencing CD4+ T cells to become functionally Th3-like. By using cytokine-specific ELISA assays, we confirmed this hypothesis by detecting TGF-β production in the supernatants of CD4+ T cells stimulated with EphA2 peptides, although these responses were observed in only a minority of Stage IV RCC patients. Thus, in addition to lacking Type-1 immune responses against EphA2 epitopes and exhibiting prevalent (and ineffective) anti-EphA2 Type-2 immunity, advanced stage RCC patients also may be clinically hampered due to the presence of Treg-type immunity against tumor antigens, such as EphA2. If generalizable to most/all tumor antigen-specific responses, this active skewing in Th responses would provide the tumor a degree of "protection", allowing for tumor growth to continue unchecked by the T cell immune response. Given such subterfuge observed in specific T cell responsiveness in RCC patients, these results suggest that EphA2 is an important tumor antigen in the setting of RCC.

After demonstrating that T cells (of moderate avidity) are able to recognize EphA2 epitopes expressed naturally by tumor cells, we explored whether or not we could modulate T cell recognition by conditional regulation of EphA2 processing in EphA2+ tumor cell lines. With the positive correlation of EphA2 expression with metastasis, tumor neoangiogenesis, and a poor prognosis in certain cancer settings, a reduction in EphA2 protein expression levels on tumors

may prove therapeutically beneficial by inhibiting EphA2-dependent tumor vascularization and the development of metastases. In our initial series of experiments, we utilized two EphA2 agonists, B61.Ig and the monoclonal antibody 208 (mAb 208) to assess whether or not EphA2 crosslinking/activation could conditionally induce the degradation of EphA2. Agonist addition to several different EphA2+ tumors induced the degradation of EphA2, while it did not affect the expression levels of other RTK family members (such as Axl, EGF-R, etc.). EphA2 agonist treatment also had no effect on tumor cell expression of HLA Class I molecules or T-cell costimulatory molecules (e.g. CD40). As is the case for other RTKs, the internalization of EphA2 is dependent upon the autophosphorylation of its intracellular domain. As a possible mechanism underlying its overexpression in cancer cells, we hypothesized that the lack of proper tyrosine phosphorylation of EphA2 could be a major contributing factor. We analyzed the phosphorylation content of EphA2 on PC3 prostate carcinoma cells and observed that EphA2 is constitutively underphosphorylated in this cell line. Upon agonist treatment, however, EphA2 rapidly became phosphorylated on at least one tyrosine residue, which was subsequently followed by degradation of the activated pEphA2 species.

We next wanted to study whether such modulation of EphA2 protein expression would result in enhanced T cell immune responsiveness against EphA2+ tumors. Indeed, the induction of EphA2 degradation would hypothetically result in the increased frequency of EphA2 epitopes presented on HLA Class I molecules by EphA2+ tumors, which could possibly enhance the recognition of EphA2+ tumors by CD8+ T cells. With HLA Class I antigen processing being proteasome-dependent, we tested whether the inhibition of the proteasome would inhibit the degradation of EphA2 protein in conditionally triggered tumor cells. By using the peptide

aldehyde proteasome inhibitor MG132, we were able to effectively inhibit EphA2 degradation induced by EphA2 agonists, demonstrating the proteasome dependency of this process.

In order to test whether EphA2 agonist treatment could increase EphA2+ tumor cell recognition by CD8+ CTLs, we utilized an HLA-A2-restricted CTL clone specific for the $EphA2_{883\text{-}91}$ (CL.142) epitope as an effector cell population. By using ELISPOT assays, we show that the treatment of HLA-A2+/EphA2+ SLR24 cells with B61.Ig significantly increased the ability of CL.142 to recognize and react against the tumor cell line. This suggests that the incorporation of EphA2 agonists into combinational immunotherapeutic protocols might prove clinically effective in promoting CTL targeting of EphA2+ tumors in vivo. This might prove most compelling in adoptive (CTL) transfer or active vaccination (i.e. EphA2 protein/peptide-pulsed DC) regimens, where the use of EphA2 agonist would not only be envisioned to inhibit the tumorigenicity of EphA2+ cancers, but also to result in increased *in vivo* recognition of EphA2+ tumors by autologous CTLs in patients with active or residual disease. While immunotherapies involving the adoptive transfer of immune cells has been utilized in the clinic, with some benefits reported (with several complete responses reported in the setting of melanoma, (211), there has not been a great deal of success associated with this single mode of therapy. By combining this approach along with a conditioning regimen making tumor cells more easily recognizable by transferred effector T cells, response rates may be improved upon.

As mentioned previously, EphA2 is constitutively underphosphorylated in the PC3 prostate carcinoma cell line. We explored whether the reversal of the underphosphorylation of EphA2 in cancer cells might also result in the restoration of proper EphA2 metabolism and in improved CTL recognition of tumor cells. With reports suggesting that the LMW-PTP is overexpressed in many cancers, and that this enzyme acts on EphA2 as a substrate, we studied whether or not

pharmacologic inhibition of LMW-PTP activity would induce the degradation of EphA2. Similarly, nitric oxide (NO) metabolites have been reported to irreversibly inhibit LMW-PTP. The addition of NO-PAPA, a nucleophilic salt that releases NO into media, was able to induce EphA2 degradation in a dose-dependent manner. Of major interest, EphA2 was not the only RTK affected by NO-PAPA. Axl, another RTK implicated in carcinogenesis, was also induced (within hours) to degrade by the addition of NO-PAPA to tumor cell cultures.

Based on our previous results using EphA2 agonists to induce EphA2 degradation, we next evaluated whether or not a combinatorial treatment of tumor cells with both and EphA2 agonist along with a LMW-PTP inhibitor (i.e. NO-PAPA) would lead to additive synergistic degradation of EphA2 protein in treated tumor cells. As assessed by Western blot, we found that the addition of mAb208 in conjunction with NO-PAPA was more efficient in inducing EphA2 degradation than either single modality treatment ($p = .015$). This supports the possibility of using both EphA2 agonist induction and phosphatase inhibitors as a bimodal treatment for EphA2+ tumors. As interesting as these findings were, the effects of NO are known to be rather pleiotropic, so we wished to further clarify that specific inhibition of phosphatases was responsible for the induction of EphA2 degradation that we had observed.

We approached this by performing analogous experiments using additional, more-refined phosphatase inhibitors (e.g. sodium orthovanadate, protein tyrosine phosphatase inhibitor-2). We observed that while different phosphatase inhibitors worked better than others, they were each capable of inducing the degradation of EphA2 protein in treated tumor cell lines *in vitro*. Interestingly, these treatments also coordinately induced the degradation of two additional RTKs, Axl and EGFR (erb-B1). We further showed that protein tyrosine phosphatase inhibitor-2 (PTP I-2) treatment led to a dramatic upregulation of EphA2 phosphorylation. These findings confirm

that a lack of tyrosine phosphorylation is a major mechanism underlying tumor cell overexpression of EphA2 protein. This is most likely due to overexpression or overactivity of cellular PTPs in EphA2+ tumors, a theory that we are currently evaluating in our laboratory. We believe that the dysregulation of EphA2 phosphorylation represents a major mechanism of EphA2 overexpression (Fig. 24).

With PTP inhibition resulting in the degradation of EphA2 protein in EphA2+ tumors, we are currently exploring the possibility that this treatment may enhance the recognition of EphA2+ tumors by CTL, similar to our results using EphA2 agonists. In addition to CTL studies, the role of CD4+ T cell should also be explored, since we observed some degree of suppression in EphA2 triggered degradation by chloroquine. The ability of chloroquine, a known inhibitor of phago/lyzosome fusion, to inhibit EphA2 degradation suggests that EphA2 processing may also occur within the MHC class II biosynthetic pathway, with peptides targeted into HLA Class II antigen complexes. Theoretically, this would results in an increase of HLA Class II presented EphA2 epitopes, and ultimate presentation to, and reactivity by, specific CD4+ T cells.

A major hurdle to overcome in the possible clinical application of PTP inhibitors is dosing and toxicity. Although RTK degradation is a consequence of PTP inhibition, other antigen processing proteins/complexes may also be negatively affected (e.g. proteasome, TAP) which could limit the generation and presentation of HLA complexes containing EphA2 epitopes, and consequently improved immunoreactivity to treated tumor cells. We also note that PTP inhibitors display increased toxicity over time, providing a potentially substantial obstacle in the clinical translation of these PTP inhibitor-based studies.

Having said this, there are several PTP inhibitors (e.g. orthovanadate and nitroprusside) currently being employed in clinical applications, in the setting of hypertension. We have reported here that orthovanadate induces the degradation of several RTKs, including EphA2, and believe the nitroprusside will have a similar effect. Interestingly, many clinically-used dyes have also been reported to serve as PTP inhibitors (212). For example, Evans Blue, commonly used for sentinel node biopsies in cancer patients, is a potent inhibitor of PTP-1B and YPTP1. These compounds could be readily applied in combinational approaches to induce RTK degradation and enhanced immune recognition in the cancer setting.

A potential preferred approach to circumventing the possible detrimental affects of previously-applied PTP inhibitors, however, is the use of RNA inhibition (RNAi) to tactically "knock out" specific PTPs implicated in EphA2 dysregulation. RNAi involves using dsRNA specific for a given protein to inhibit the translation of the target gene by inducing the destruction of the specific mRNA. This approach allows for the specific targeting of PTPs without the deleterious pleiotropic effects of conventional pharmacologic PTP inhibitors. RNAi targeting PTP-1B is currently being used clinically for the treatment of Type-2 diabetes, and should be considered for implementation in cancer clinical trials in the near future.

Not only could RNAi limit the potential harmful side effects of PTP inhibitors, they could also be used to specifically establish which PTPs are dominantly involved in tumor cell overexpression of EphA2 protein. As mentioned previously, LMW-PTP is reported to use EphA2 as a substrate, making it a prime candidate for RNAi studies. However, additional PTPs have also been reported to be involved in carcinogenesis (204, 205, 207).

The work performed in this thesis has defined a novel tumor antigen, EphA2, which is overexpressed in numerous cancer cell types of epithelial origin (i.e. carcinomas). The EphA2

protein contains epitopes that are recognized by T-lymphocytes and appear therapeutically-relevant. Our results demonstrate the ability to target EphA2 for conditional degradation, leading to improved recognition by specific CTLs allows for development of novel immunotherapeutic approaches that may be applied to numerous types of cancer. Combined with our findings that (PTP) phosphatase inhibitors also induce the degradation of EphA2, this thesis introduces the possibility of using a combinatorial therapeutic approach for the treatment of EphA2+ tumors. Along with reports illustrating the efficacy of several other monoclonal antibodies used clinically for the treatment of other tumors (e.g. Herceptin, Erbitux), this type of therapy could be effective against a wide range of tumor types, and would not be restricted to a patients HLA type (as would be the case for peptide-based vaccines). This therapy would not only enhance CTL recognition of EphA2+ tumors, it would possibly also inhibit tumor neoangiogenesis and limit the ability of tumors to metastasize. There are a plethora of RTKs that appear to be overexpressed in a broad range of cancer histologies, and given the availability of PTP inhibitors and specific agonist anti-RTK monoclonal antibodies, these results may support a general combinational therapy platform for the majority of cancers.

RTK	Overexpressed in cancer?	Reference
EphA2	melanoma, breast, colon, lung, pancreatic, prostate	34,55, 56, 59, 143, 148
Axl	breast, lung, esophageal,	40, 41
EGFR/erb1	colorectal, breast, ovarian, prostate	42-45
Her2/neu	breast, lung, prostate, cervical,	42, 46-48
PDGFR	brain, breast	53, 54
KIT	colorectal, RCC, ovarian, breast	49-52

Table 1. RTK and Overexpression in Cancer

Table 2. RCC Patient Characteristics

Patient	Sex	Age*	Date Treated	Date Recurred	DFI (Yrs)	Survival (Yrs)	Disease Staging: T	Grade	Tumor Size (cm^3)
1	M	64	1-21-86	3-5-87	1.1	3.0	3B	NG	322
2	M	63	3-11-86	3-6-91	5.0	6.5	1	NG	NA
3	M	59	4-25-86	5-28-87	1.1	1.1	2	NG	560
4	M	56	2-2-87	2-27-00	13.0	13.0	3a	NG	4
5	M	50	3-26-87	1-3-90	2.7	7.5	3b	NG	269
6	M	71	4-1-87	11-4-91	4.6	11.9	3b	NG	180
7	F	65	4-20-87	NED	16.8	16.8	3b	NG	14
8	F	59	5-21-87	7-13-90	3.2	4.0	3a	NG	864
9	M	68	6-18-87	NED	16.7	16.7	1	NG	14
10	M	52	12-21-87	NED	16.3	16.3	2	NG	434
11	F	74	2-10-88	9-15-92	4.6	5.2	1	NG	8
12	M	48	2-24-88	9-10-91	3.5	8.3	1	II	113
13	M	51	8-8-88	4-24-89	0.7	4.3	1	NG	NA
14	M	87	8-10-88	9-25-90	2.1	6.1	2	NG	158
15	M	40	9-1-88	NED	15.4	15.4	4	III	66
16	M	37	9-28-88	NED	15.3	15.3	1	NG	87
17	F	52	11-21-88	11-29-90	2.1	2.1	2	IV	288
18	M	45	3-5-89	5-5-93	4.2	6.2	2	IV	383
19	M	61	4-21-89	8-18-89	0.3	0.7	3B	IV	384
20	F	63	11-27-89	10-19-90	0.9	1.3	2	III	168
21	M	58	1-22-90	11-15-95	5.8	11.5	3b	II	NA
22	F	55	3-20-90	10-18-90	0.6	1.3	2	IV	270
23	F	57	3-26-90	8-2-93	3.3	3.5	2	II	NA
24	M	46	5-1-90	7-23-91	1.2	14.0	1	II	NA
25	M	65	7-5-90	8-1-90	0.1	8.8	1	IV	36
26	M	54	12-5-90	NED	13.2	13.2	3a	NG	NA
27	F	50	2-1-91	9-29-93	2.7	12.2	1	II	NA
28	M	58	4-19-91	6-28-91	0.2	2.4	2	III	525.
29	F	69	8-28-91	9-20-91	0.1	0.3	3b	IV	NA
30	F	76	5-14-92	9-14-92	0.3	8.0	1	II	16
31	F	50	6-1-93	11-29-93	0.5	3.9	3b	II	19
32	M	66	4-19-94	6-13-96	2.2	6.7	2	III	525
33	M	56	6-28-95	7-18-96	1.1	9.0	3a	II	34
34	M	55	11-27-95	8-20-96	0.8	4.0	3b	III	6916

All patients were diagnosed with clear cell RCC with N0, M-. *Patient's age at the time of tumor resection. Abbreviations: DFI = Disease-free interval; NA = Not available; NG = Not graded; NED = No evidence of disease.

Table 3 HLA-A2 and/or DR4 positive RCC patients evaluated in this study.

RCC Patient	Age	Sex	Stage	Treatment	Disease status at time of evaluation (Months)	HLA Typing: A2 (+/-)/DR4 (+/-)		Tumor Expression EphA2
SLR30-pre	63	F	I	none	Local Dis.	+	-	NA
SLR31	66	M	I	S	Local Dis.	+	-	2+
SLR32	62	F	I	S	Local Dis.	+	-	2+
SLR33	54	F	I	S	Local Dis	+	-	3+
SLR34	71	M	I	none	Local Dis.	+	+	NA
SLR35	75	F	I	none	Local Dis.	+	+	NA
SLR36-pre	60	M	I	none	Local Dis.	+	+	NA
SLR37	52	M	I	none	Local Dis.	+	-	NA
SLR38-pre	69	M	I	none	Local Dis.	+	-	NA
SLR39	65	M	I	S	NED (3)	+	-	3+
SLR30-post	63	F	I	S	NED (1.5)	+	-	NA
SLR40	53	M	I	S	NED (3)	+	-	NA
SLR36-post	60	M	I	S	NED (2)	+	+	NA
SLR41	64	F	I	S	NED(2)	+	-	2+
SLR38-post	69	M	I	S	NED (2)	+	-	3+
SLR42	58	F	I	S	Local Dis. (3)	+	-	3+
SLR43	53	F	I	S	Local Dis. (1.5)	+	-	3+
SLR44-pre	69	M	IV	none	Mets.	+	-	NA
SLR45	65	M	IV	S	Mets	+	-	4+
SLR46	45	F	IV	S	Mets	+	-	0
SLR47	53	F	IV	S	NED (1.5)	+	-	NA
SLR48	54	M	IV	S	Mets. (61)	+	-	NA
SLR49	52	F	IV	S, R, IFN-α, IL-2	Mets. (41)	+	-	2+
SLR44-post	69	M	IV	S	Mets (2)	+	-	4+
SLR50	54	M	IV	S,R,C	Mets (21)	+	-	NA
SLR51	41	M	IV	S,R,IL-2	Mets	+	+	NA
SLR52	58	M	IV	S,R,IFN-α	Mets	+	+	NA
SLR53	52	M	IV	S	Mets	+	-	NA
SLR54	49	F	IV	C,IL-2	Mets	+	+	NA
SLR55	79	M	IV	C,IFN-α	Mets	+	+	NA
SLR56	56	M	IV	R,C,IFN-α, IL-2	Mets	+	-	NA
SLR57	68	F	IV	S	Mets	+	-	3+
SLR58	55	F	IV	none	Mets	+	+	NA
SLR59	52	F	I	none	Local Dis.	-	+	NA
SLR60-pre	58	M	I	none	Local Dis.	-	+	NA
SLR61	60	M	I	S	Local Dis.	-	+	2+
SLR62	64	M	I	S	NED (3)	-	+	NA
SLR63	53	F	I	S	NED (1.5)	-	+	NA
SLR60-post	58	M	I	S	NED (2)	-	+	NA
SLR64	65	M	I	S	NED (10)	-	+	NA
SLR65	53	M	II	S	Local Dis.	-	+	NA

SLR66	45	M	IV	none	Mets.	-	+	NA
SLR67	57	M	IV	C,R	Mets	-	+	NA
SLR68	69	M	IV	S,R,C	Mets	-	+	NA
SLR69	49	M	IV	S,C,R,IFNα, IL-2	Mets	-	+	NA

Individual SLR designations reflect specimen number based on date harvested. In 5 cases, both pre- and (6 weeks) post-therapy blood specimens were available for analysis, as indicated. Where indicated, the time of peripheral blood isolation (in months) post-therapy is provided. Abbreviations used: C, Chemotherapy; IFN-α, recombinant Interferon-alpha therapy; IL-2, recombinant Interleukin-2 therapy; Mets, Metastatic Disease; NA, Not available for evaluation; NED, No evidence of disease; R, Radiotherapy; S, Surgery. HLA-A2 and -DR4 status was determined using allele-specific monoclonal antibodies and flow cytometry gating on peripheral blood monocytes, as described in Materials and Methods. IHC stained tumor biopsies were available from 14 patients and were stained for EphA2 expression as outlined in Materials and Methods. EphA2 expression is indicated on an arbitrary 0 to 4+ scale.

Table 4. Selection of EphA2 Peptides for Analysis.

Selected HLA-A2 Presented EphA2 Peptides:

Sequence Start Amino Acid #	AA Sequence of Nonamer	Binding Score*	Peptide Generated By Proteasome	Peptide Synthesized For Analysis
883	TLADFDPRV	1084	YES	YES
546	VLLLVLAGV	1006	YES	YES
550	VLAGVGFFI	556	NO	NO
58	IMNDMPIYM	138	NO	NO
961	SLLGLKDQV	127	YES	YES
253	WLVPIGQCL	98	NO	NO
12	LLWGCALAA	71	NO	NO
391	GLTRTSVTV	70	YES	YES
120	NLYYAESDL	68	NO	NO
162	KLNVEERSV	49	YES	YES

Selected HLA-DR4 Presented EphA2 Peptides:

Sequence Start Core AA#	AA Sequence of Nonamer Core	Binding Score*	Peptide Synthesized For In Vitro Analysis
666	IMGQFSHHN	577	$_{663}$EAGIMGQFSHHNIIR$_{677}$
67	YSVCNVMSG	95	$_{63}$PIYMYSVCNVMSG$_{75}$
55	MQNIMNDMP	39	$_{53}$DLMQNIMNDMPIYMYS$_{68}$

*The higher the binding score, the greater the stability of the predicted peptide-MHC complex. Binding scores and qualitative determination of proteasomal processing were predicted using on-line algorithms as described in Materials and Methods.

Table 5. Normal donor T cell responses to putative EphA2-derived peptide epitopes.

HLA-A2-Presented EphA2 Peptides:

CD8+ T Cell Response to Peptide on T2.DR4[a]:

(IFN-γ Spots/10^5 CD8+ T Cells)

Normal Donor #	**162**	**391**	**546**	**883**	**961**
A2-1	9	0[b]	31	0	2
A2-2	40	81	14	85	21
A2-3	3	14	10	0	21
A2-4	2	0	11	58	0
A2-5	11	0	14	172	4
A2-6	0	91	76	145	13
A2-7	132	0	0	37	0
A2-8	15	0	0	165	0
Total Responses:	**5/8**	**3/8**	**6/8**	**6/8**	**3/8**

HLA-DR4-Presented EphA2 Peptides:

CD4+ T Cell Response to Peptide on T2.DR4[a]:

(IFN-γ Spots/10^5 CD4+ T Cells)

Normal Donor #	**53**	**63**	**663**
DR4-1	43	11	21
DR4-2	38	36	57
DR4-3	4	7	14
DR4-4	0	0	0
DR4-5	0	156	41
DR4-6	0	121	67
DR4-7	54	48	72
Total Responses:	**3/7**	**6/7**	**6/7**

Responder CD4+ or CD8+ T cells were analyzed for reactivity against the HLA-A2+/DR4+ target cell line T2.DR4 pulsed with no peptides, or pulsed with irrelevant or EphA2-derived peptides. T cell reactivity against T2.DR4 cells pulsed with the HLA-A2-presented HIV-$nef_{190-198}$ epitope served as the CD8+ T cell negative control, while HLA-DR4-presented Malarial circumsporozooite $(CS)_{326-345}$ epitope served as the CD4+ T cell negative control. These control values were subtracted from experimental determinations in order to determine EphA2-specific T cell responder spot numbers[a] per 100,000 T cells. [b]A value of "0" reflects a frequency < 1/100,000 T cells. The appropriate HLA-A2 or –DR4 restricted nature of specific T cell recognition of peptides was validated by inclusion of anti-HLA-A2 or -DR4 mAb in replicate ELISPOT wells, respectively, with >90% inhibition of EphA2-specific recognition observed (data not shown). Values significantly ($p < 0.05$) elevated over T2.DR4 + control peptide values are <u>underlined</u>.

Table 6. EphA2 Agonists Do Not Inhibit MHC Class I or CD40 Expression on SLR24 tumor cells.

The SLR24 RCC cell line was either not treated or treated with MG132 (50 μM) and/or mAb208 (10 μg/ml) as outlined in the legend to Figure 3. Treated cells were then analyzed for expression of MHC class I and CD40 molecules by flow cytometry as described in Materials and Methods. Data presented is the mean fluorescence intensity of expression for the indicated markers.

		Mean Fluorescence Intensity:		
Treatment	MG-132 (+/-)	Control	W6/32	CD40
Untreated	-	0.5	124.7	14.8
"	+	0.6	127.4	16.6
mAb208	-	5.5	116.5	19.8
"	+	7.2	123.7	20.9

Table 7. ED50 Dosage of Pharmacologic Inhibitors.

The indicated pharmacologic PTP inhibitors were assessed for their ability to induced EphA2 degradation. The dosage required for 50% reduction in EphA2 protein content (after 6h treatment) is indicated below.

P.I.	$ED_{[50]}$
NO-PAPA	2mg/ml
Sodium Orthovanadate	100mM
PTP Inhibitor-2	100μM
Levamisole	500μM
Phenylarsine Oxide	1μM

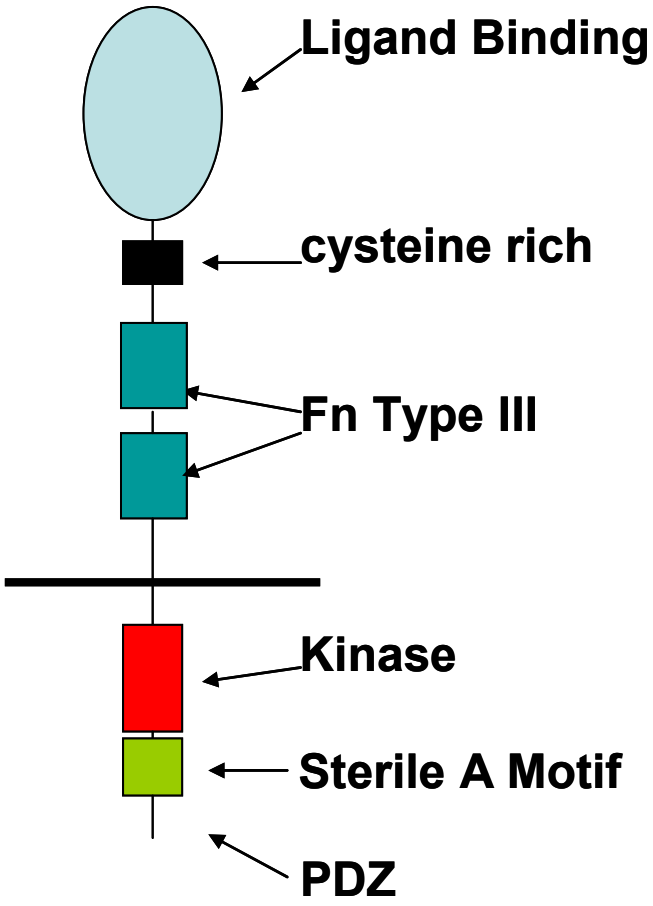


Figure 1. Schematic Diagram of EphA receptor protein structure.

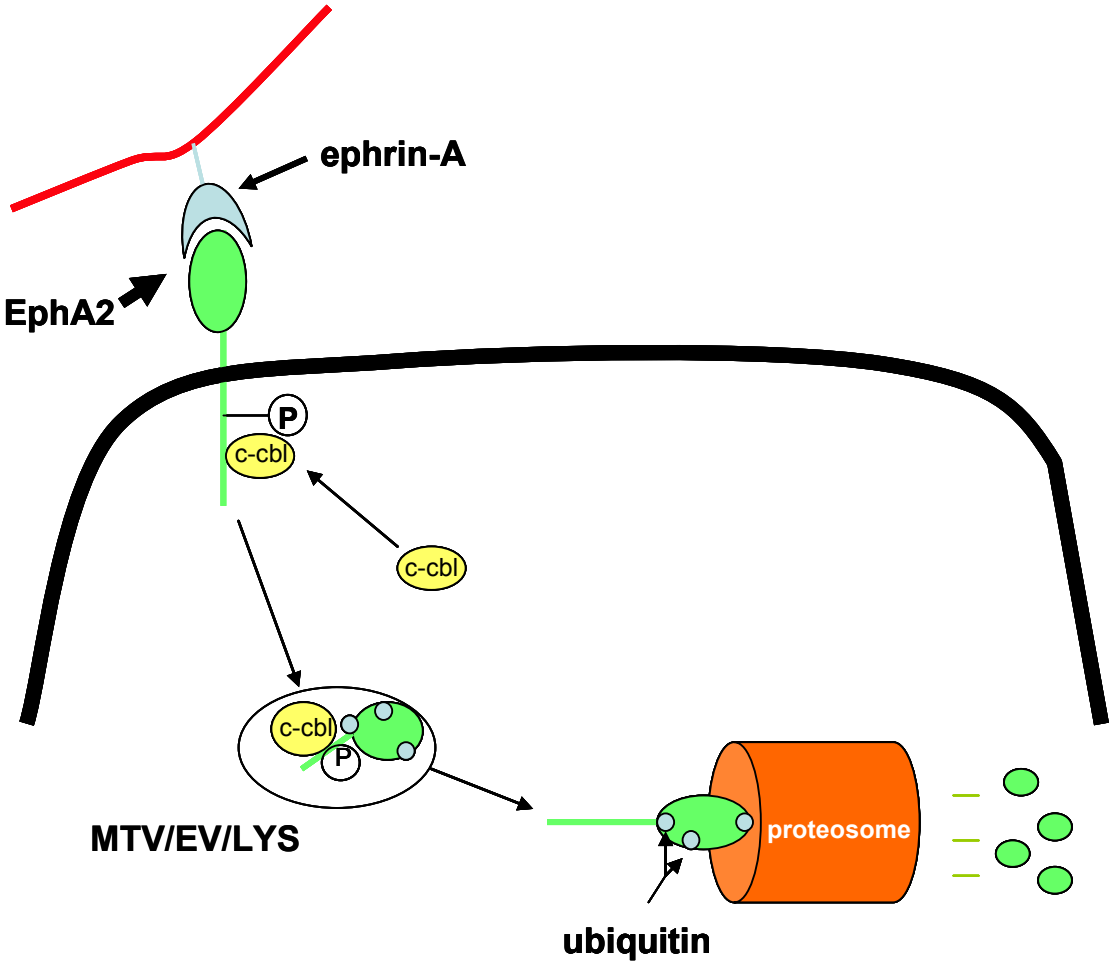


Figure 2. Schematic Diagram of EphA2 Normal Metabolism.

MTV – multi-tubular vesicle. EV – endosomal vesicle. LYS – lysosome.

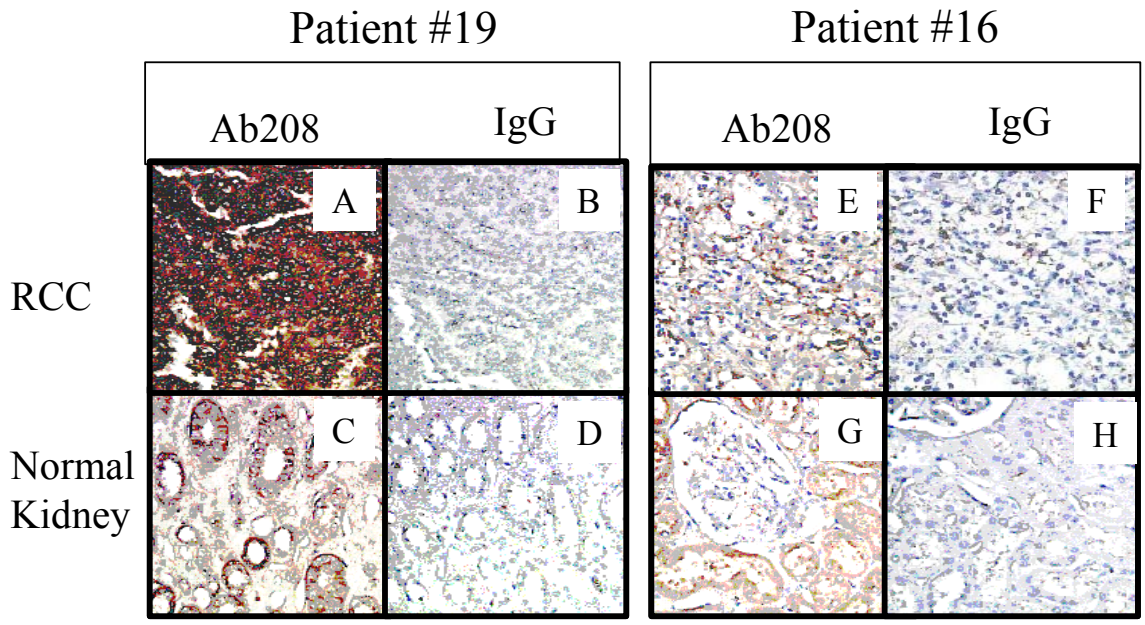


Figure 3. Immunohistochemical analysis of RCC Specimens.

Resected tumor and adjacent normal kidney were obtained from RCC patients #19 (panels A-D) and #16 (panels E-H) and stained using anti-EphA2 (panels A, C, E, G) or control IgG (panels B, D, F, H) Abs, as outlined in Materials and Methods. Depicted images were prepared under 40X magnification.

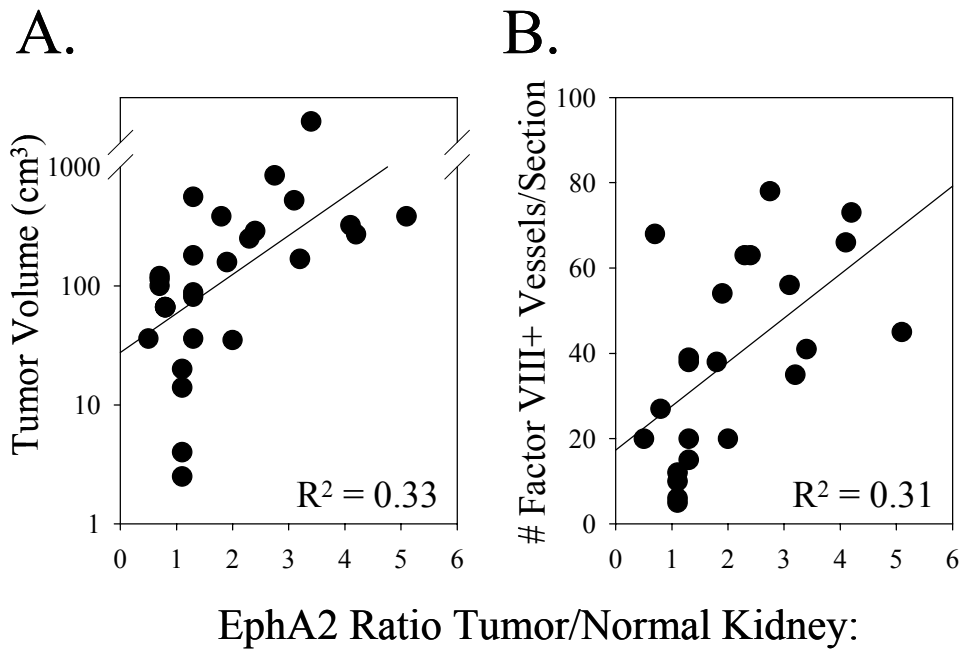


Figure 4. Relative EphA2 expression is higher in larger, more vascularized RCC lesions.

In panel A, the relative tumor expression of EphA2 was determined (as outlined in Materials and Methods) and plotted against the calculated volume of the resected RCC lesion (Table 1). Sequential tissue sections were also stained with anti-Factor VIII antibodies in order to assess the number of tumor blood vessels, with the relative level of vascularity then plotted against EphA2 expression level in panel B. Each symbol within a panel reflects an individual patient evaluated.

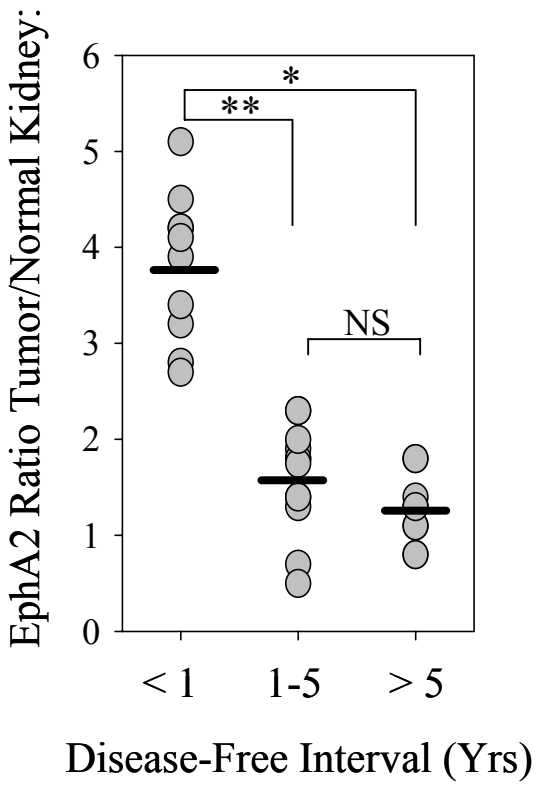


Figure 5. Relative EphA2 expression in resected RCC is prognostic of disease-free interval in surgically-cured patients.

The relative tumor expression of EphA2 was determined (as outlined in Materials and Methods) for each patient and the data plotted based on the disease-free interval observed for that patient (i.e. < 1 year (n = 10), between 1 and 5 years (n = 13), or > 5 years (n = 11)) after curative surgery. Each symbol within a panel reflects an individual patient evaluated. *p = 0.0003, **p = 0.001, NS = not significant.

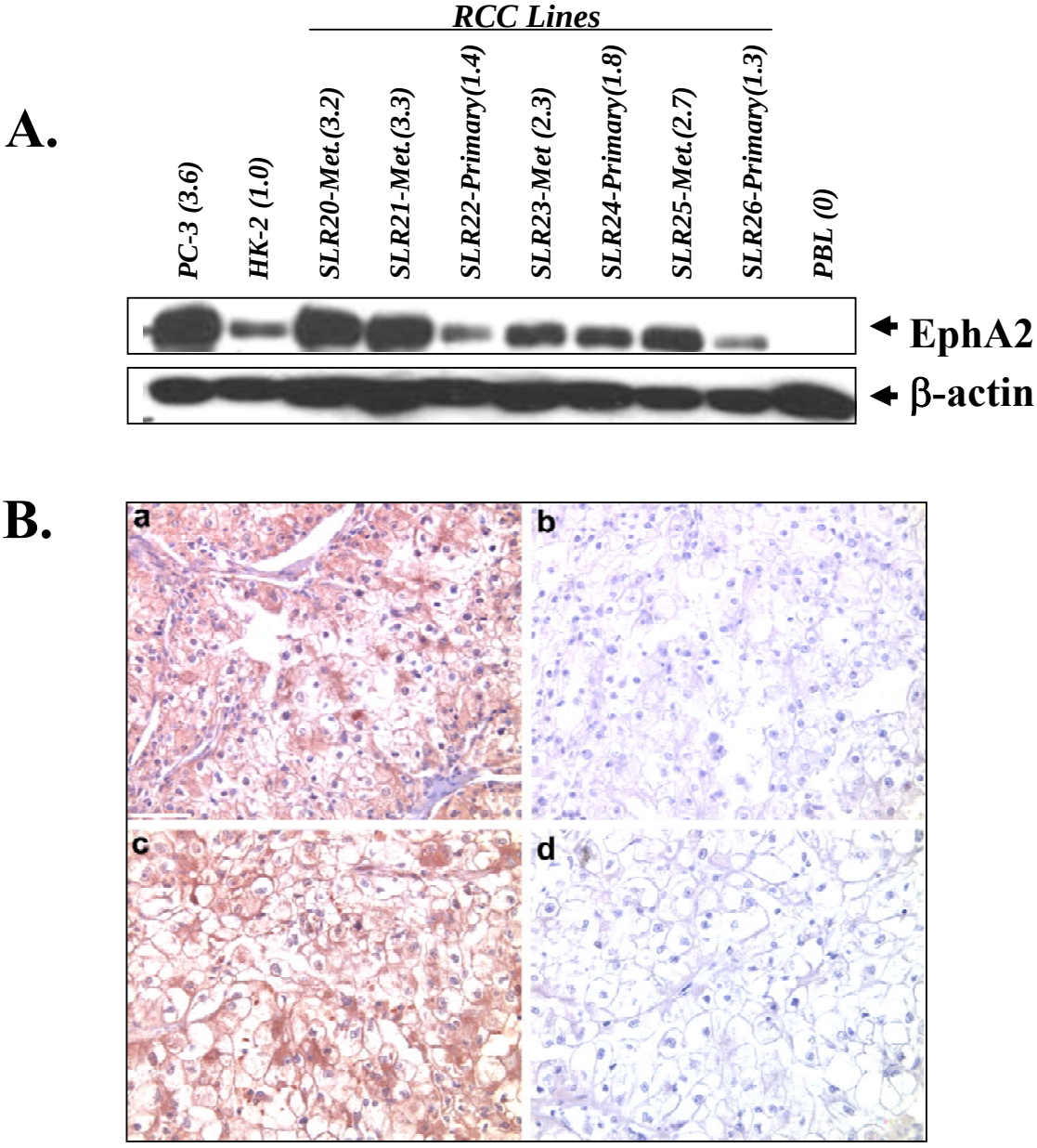


Figure 6. EphA2 is frequently overexpressed in renal cell carcinoma (RCC) cell lines and RCC lesions.

Anti-EphA2 and control anti-β-actin antibodies were used in performing Western Blot analyses of lysates generated from the indicated RCC cell lines, the normal kidney tubular epithelial cell line HK2 and normal peripheral blood lymphocytes (negative control), panel A. Primary and metastatic clear cell RCC lines were assessed as indicated. The PC3 prostate cell line and normal donor PBLs served as positive and negative controls, respectively. Densitometry levels of EphA2 expression (normalized to β-actin levels) are indicated in parentheses and are reported relative to HK2 expression of EphA2 assigned an arbitrary value of 1. In panel B, primary (Patient SLR33; panels a and b) and metastatic (Patient SLR45; panels c and d) RCC paraffin-embedded tissue sections were stained using anti-EphA2 antibody (Ab 208; panels a and c) or isotype control antibody (panels b and d) in immunohistochemical analyses (40X magnification).

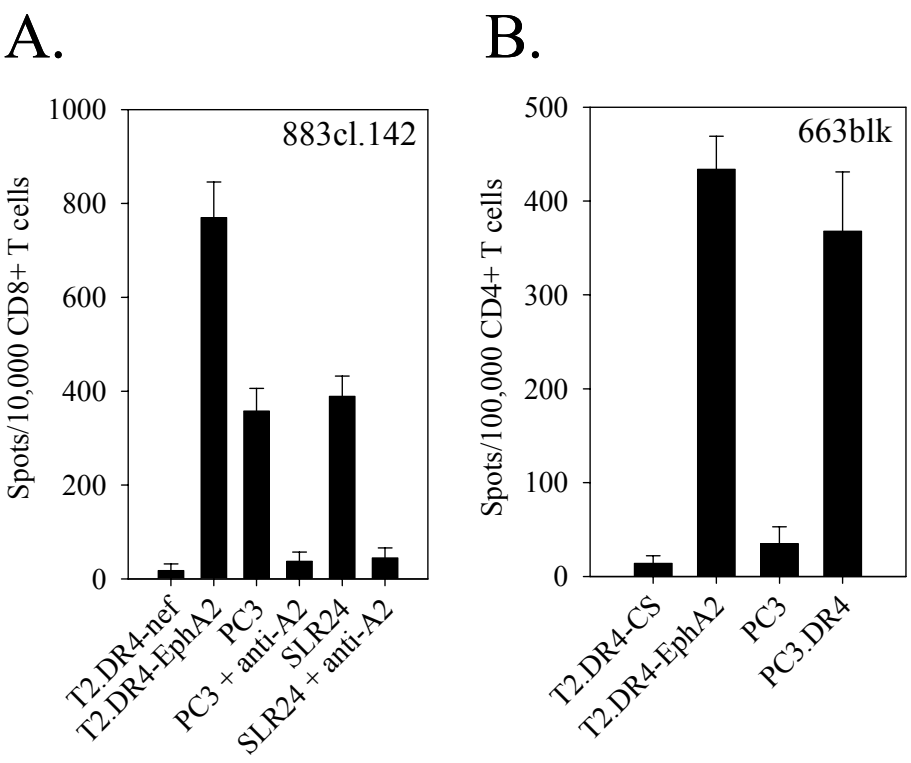


Figure 7. Anti-EphA2 T cells recognize HLA-matched, EphA2+ RCC tumor cell lines.

CD8+ and CD4+ T cell lines were expanded from normal HLA-A2+ or –DR4+ donors using in vitro stimulations with specific EphA2 peptides and evaluated for reactivity against HLA-matched, EphA2+ tumor target cell lines in IFN-γ ELISPOT assays. Depicted are examples of data generated from a CD8+ T cell clone reactive against the $EphA2_{883\text{-}891}$ epitope (panel A) and a bulk CD4+ T cell line after 3 rounds of in vitro stimulation with the $EphA2_{663\text{-}677}$ epitope (panel B). Target cells included HLA-A2+/DR4+ T2.DR4 cells pulsed with irrelevant (HIV-nef or Malarial CS) or relevant EphA2-derived peptides, the HLA-A2+/EphA2+ RCC line SLR24, the HLA-DR4-negative/EphA2+ PC3 cell line and the HLA-DR4+/EphA2+ PC3.DR4 tumor cell line. The HLA-A2 restricted nature of 883cl.142 reactivity to tumor cell line targets was validated by inclusion of the blocking anti-HLA-A2 mAb BB7.2. Data are reported as spots per 10,000 (panel A) or 100,000 (panel B) T cells analyzed, are based on the mean +/- SD of triplicate determinations and are reflective of at least 3 independent experiments in all cases.

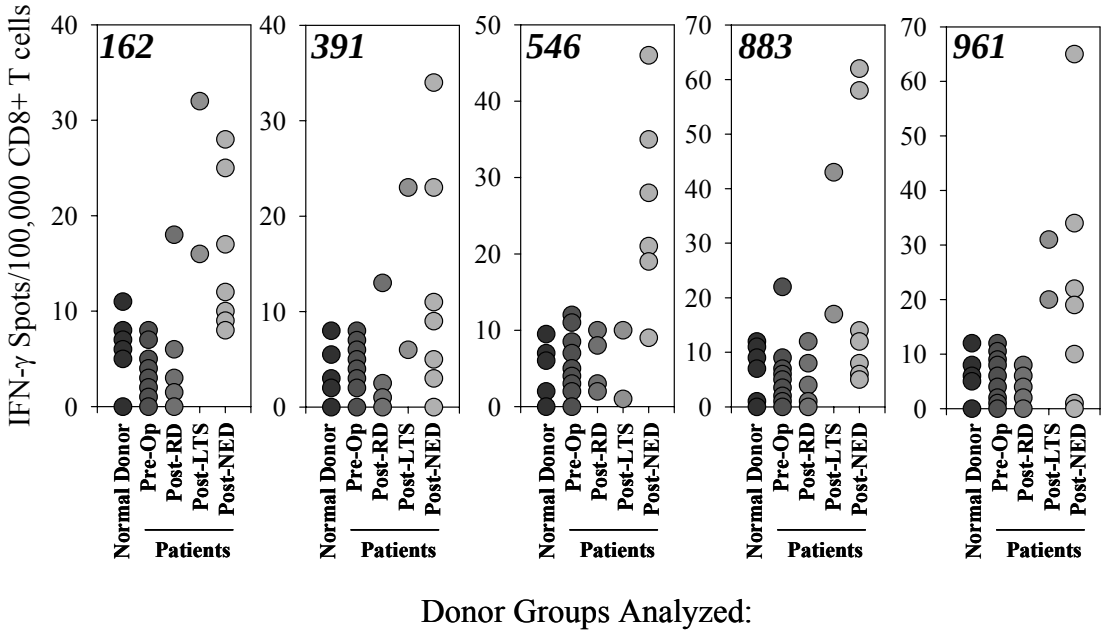


Figure 8. IFN-γ ELISPOT analyses of RCC patient CD8+ T cell responses to EphA2-derived epitopes versus disease status.

Peripheral blood CD8+ T cells were isolated from HLA-A2+ normal donors or patients with RCC and stimulated with immature, autologous dendritic cells pre-pulsed with the individual EphA2-derived epitopes, as outlined in Materials and Methods. After one week, responder T cells were analyzed in IFN-γ ELISPOT assays for reactivity against T2.DR4 (HLA-A2+) cells pulsed with the indicated EphA2 epitope. Data are reported as IFN-γ spots/100,000 CD8+ T cells and represent the mean of triplicate determinations. T cell reactivity against T2.DR4 cells pulsed with the HLA-A2-presented HIV-nef$_{190\text{-}198}$ epitope served as the negative control in all cases, and this value was subtracted from all experimental determinations in order to determine EphA2-specific spot numbers. Each symbol within a panel represents an individual donor's response to the indicated HLA-A2 presented EphA2 peptides. Abbreviations used: Pre-Op, pre-operative patients; Post-RD, patients post-therapy (< 21 months) but with residual disease; Post-LTS, patients post-therapy (> 41 months) with residual disease; Post-NED, patients post-therapy with no evidence of disease.

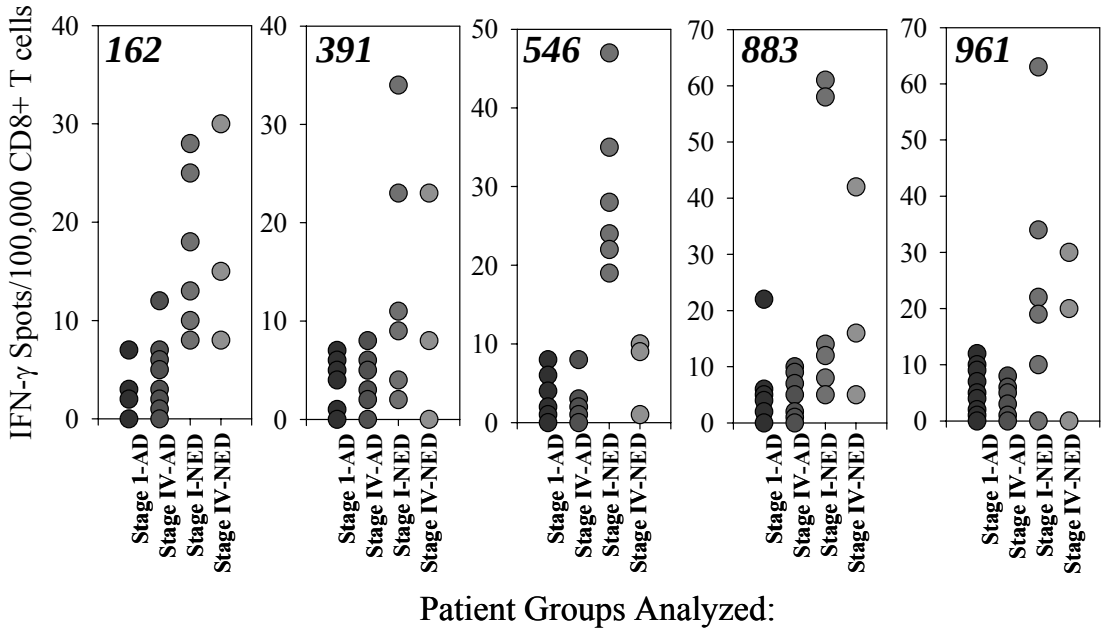


Figure 9. IFN-γ ELISPOT analysis of RCC patient CD8+ T cell responses to EphA2-derived epitopes versus disease stage.

Data reported in Figure 8 have been re-plotted as a function of disease-stage. Abbreviations used: AD, patients with active disease; NED, patients with no evidence of disease.

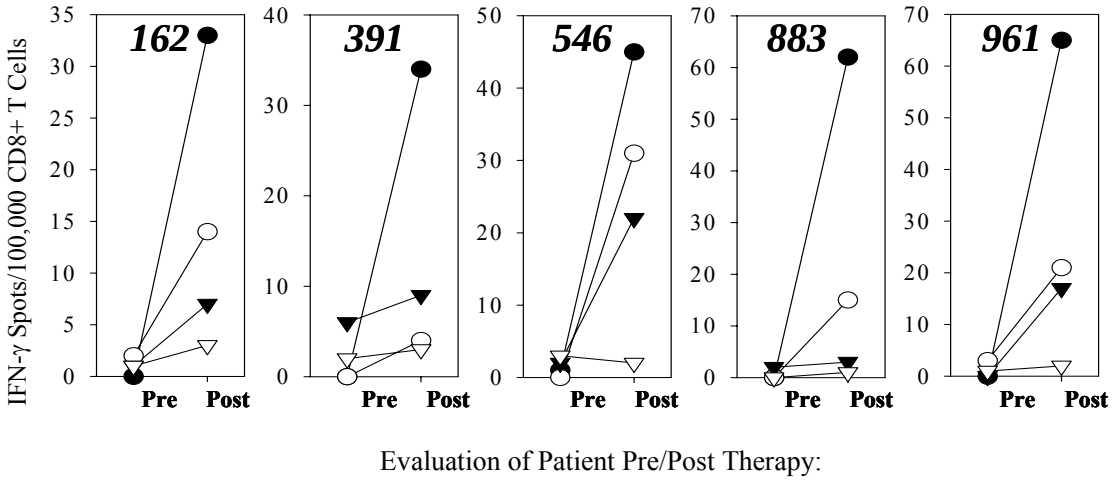


Figure 10. Observed changes in peripheral blood CD8+ T cell responses to EphA2 epitopes pre- versus post-surgery in 4 HLA-A2+ patients with RCC.

Peripheral blood CD8+ T cells were isolated pre- and (6 week) post-surgery from patients with RCC, and evaluated for reactivity to EphA2 epitopes in IFN-γ ELISPOT assays, as outlined in the Figure 3 legend. The three Stage I RCC patients (●, ○,τ) were rendered free of disease as a result of surgical intervention, while the single Stage IV RCC patient (∇) had residual disease after surgery. Each symbol within a panel represents an individual patient's response.

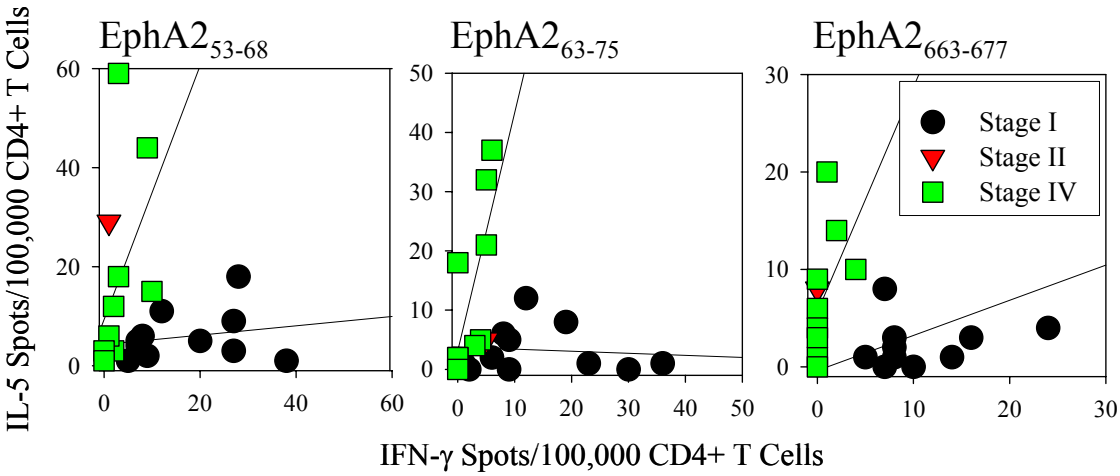


Figure 11. Disease-stage skewing of functional CD4+ T cell responses to EphA2 Th epitopes in HLA-DR4+ RCC patients with active disease.

Peripheral blood was obtained from 19 HLA-DR4+ patients (Table 1) and CD4+ T cells isolated by positive MACS™-bead selection as described in Materials and Methods. After a one-week in vitro stimulation with EphA2 Th peptide-pulsed, autologous DCs, responder CD4+ T cells were evaluated against T2.DR4 cells pulsed with the indicated EphA2 epitopes in IFN-γ and IL-5 ELISPOT assays. Data are reported as IFN-γ spots/100,000 CD4+ T cells and represent the mean of triplicate determinations. T cell reactivity against T2.DR4 cells pulsed with the HLA-DR4-presented Malarial circumsporozooite $(CS)_{326-345}$ epitope served as the negative control in all cases, and this value was subtracted from all experimental determinations in order to determine EphA2-specific spot numbers. Each symbol within a panel represents an individual patient's response. Linear regression lines for Stage I and Stage IV patient data is indicated for each peptide.

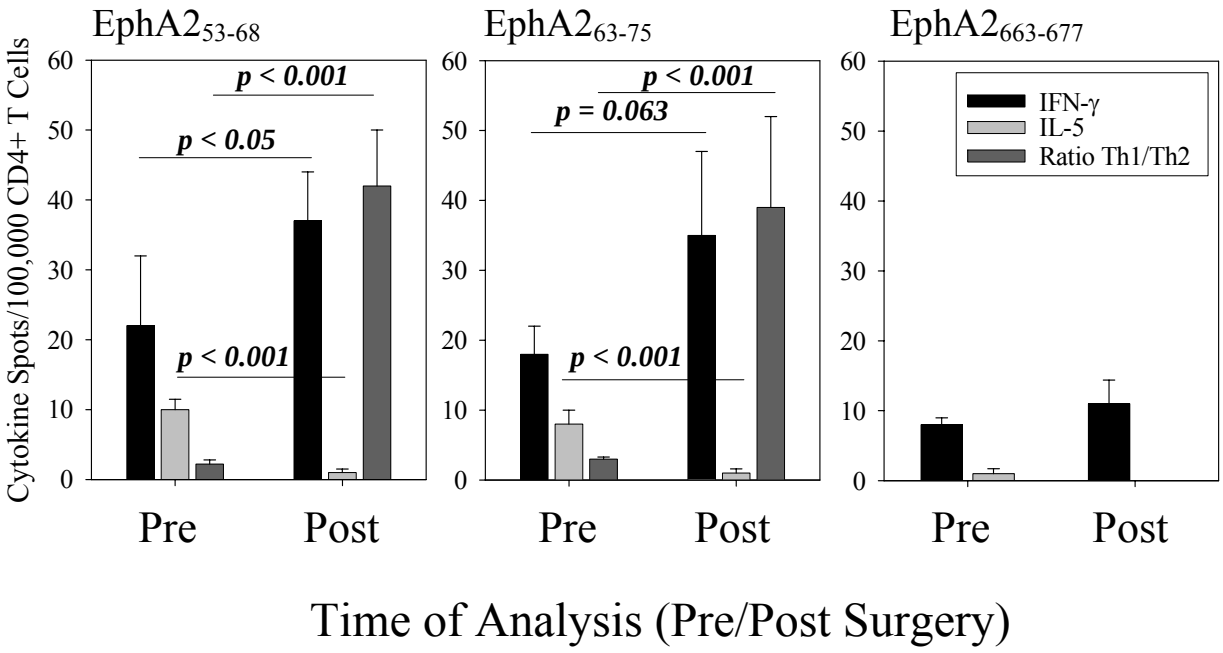


Figure 12. Therapy-associated enhancement of Th1-type, and reduction in Th2-type, CD4+ T cell responses to EphA2 in an HLA-A2+/DR4+ patient with Stage I RCC.

Pre- and post-surgery peripheral blood was available for a single RCC patient with Stage I disease. CD4+ T cells were isolated and analyzed for reactivity to EphA2 Th epitopes, as outlined in the Figure 5 legend. A statistically-significant increase in Th1-type (IFN-γ) and decrease in Th2-type (IL-5) CD4+ T cell response post-surgery was noted for the EphA2$_{53-68}$ epitope. Therapy-induced changes in CD4+ T cell response to the EphA2$_{63-75}$ epitope were similar, with the IFN-γ results approaching a p value of 0.05 and the significant reductions in IL-5 responses noted ($p < 0.001$). T cell responses to the EphA2$_{663-677}$ epitope pre-/post-surgery were not significantly different. The ratio of Th1/Th2-type responses pre- and post-therapy is also indicated for peptides EphA2$_{53-68}$ and EphA2$_{63-75}$. p values for significant differences are indicated.

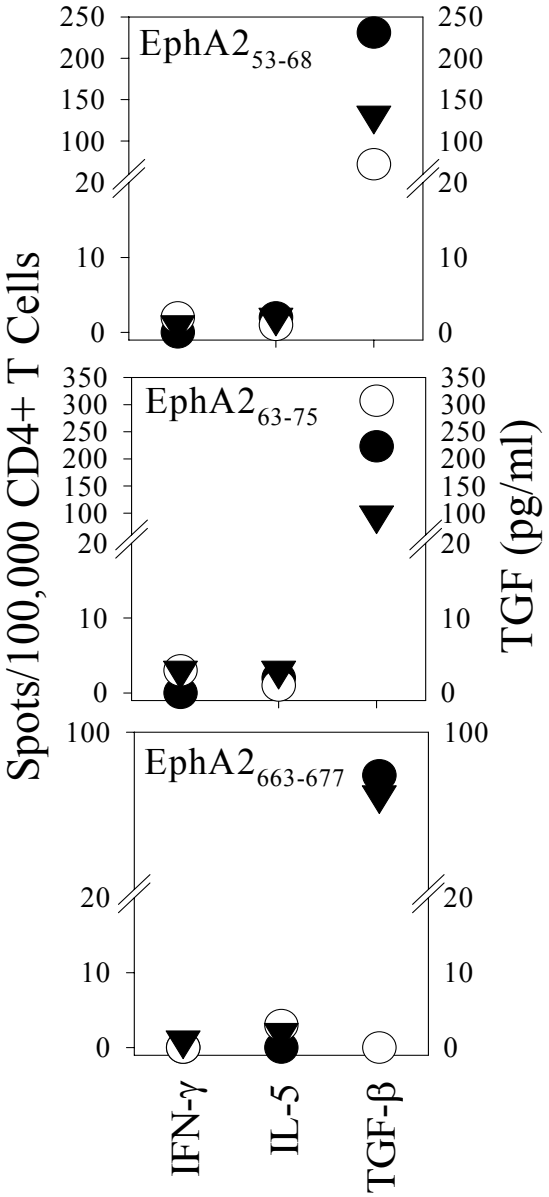


Figure 13. Suppressor CD4+ T cell responses to EphA2 Th epitopes in HLA-DR4+ patients with advanced Stage IV RCC.

Supernatants were harvested from the culture wells of IFN-γ ELISPOT assays and analyzed for levels of TGF-β1 using a commercial ELISA kit. TGF-β1 secretion in response to EphA2 peptides was only detectable in the supernatants of 3 (of 8 evaluated) patients with Stage IV RCC. The corresponding IFN-γ and IL-5 ELISPOT data for these individual patients' CD4+ T cell responses to EphA2 peptides is also provided. Each symbol within a panel represents an individual patient's response.

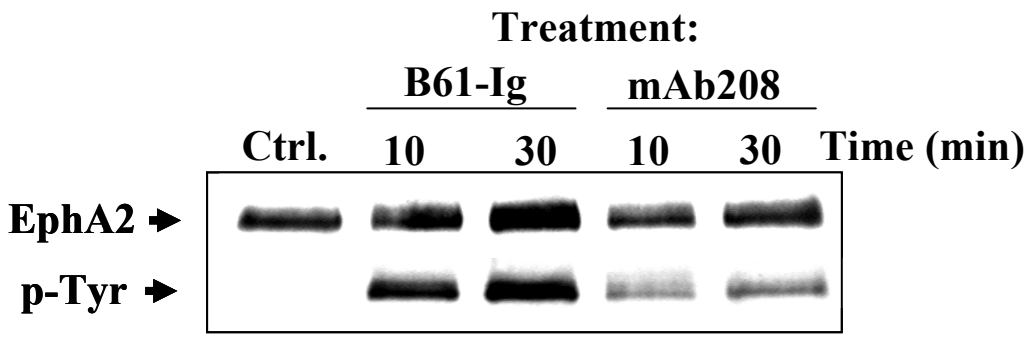


Figure 14. EphA2 Agonists Induce the Phosphorylation of EphA2.

PC3 (2-4 x 10^6) cells were treated at the indicated time points (in min) with either B61.Ig (30 ug/ml) or mAb208 (8 ug/ml). B61.Ig is a fusion protein consisting of the EphA2 binding domain of ephrin-A1 (a major ligand of EphA2) fused to a human Fc region. Cellular lysates were resolved by SDS-PAGE and EphA2 protein was immunoprecipitated using the anti-EphA2 antibodies D7 in pull-down assays. Western blot analyses were then performed using anti-EphA2 and anti-phosphotyrosine antibodies, respectively. Data are representative of 3 independent experiments performed.

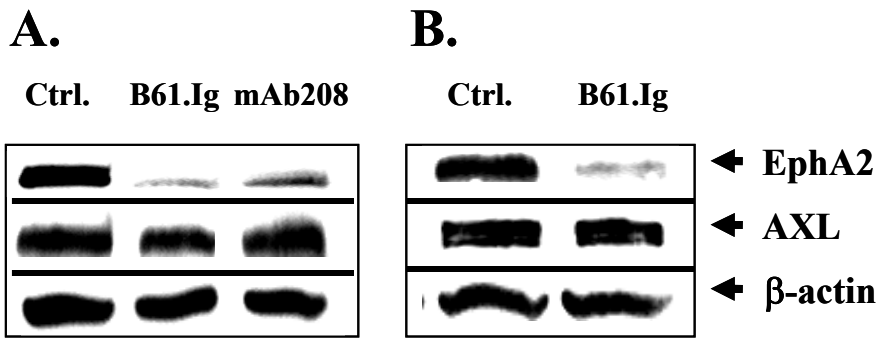


Figure 15. EphA2 Agonists Induce the Degradation of EphA2.

PC3 (A) and SLR24 (B) were treated for 6 hours with either B61.Ig (30 μg/ml) or mAb208 (8 μg/ml) at 37°C. Cell lysates were resolved by 12.5% SDS-PAGE and Western blot analyses were performed using Anti-EphA2 and control anti-β-actin antibodies. Anti-Axl antibodies were used to image identically-prepared lysates as a specificity control in these experiments. Data are representative of 3 independent experiments performed on each tumor cell line.

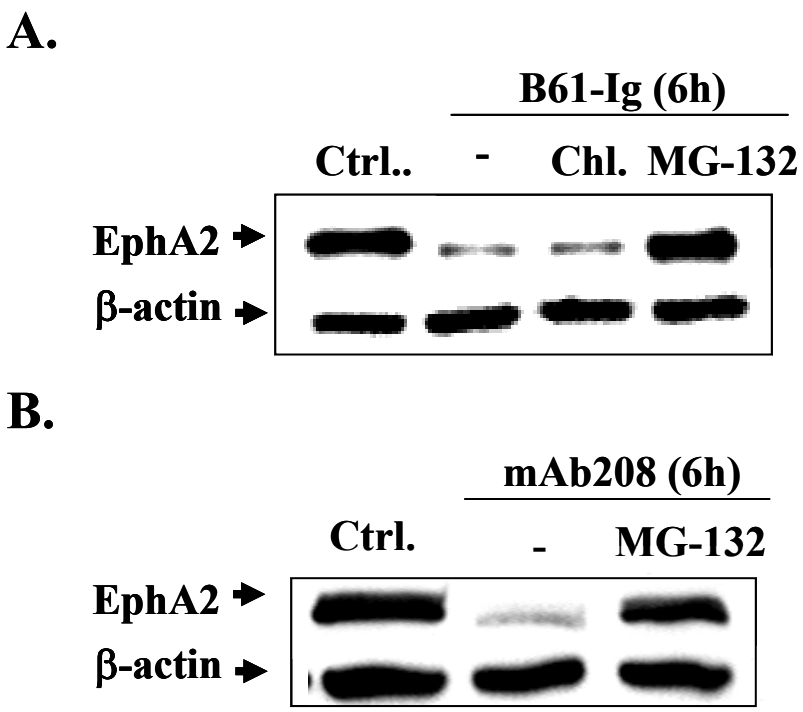


Figure 16. EphA2 Agonists Induced Degradation is Inhibited by MG132, but not by Chloroquine.

PC3 cells were either treated with B61.Ig (A) or mAb208 (B) as described previously in the legend of Figure 1. MG132 (50μM, Sigma, St. Louis, MO) and Chloroquine (100μM, Sigma, St. Louis, MO) were also added to cultures where indicated 30 min. prior to the addition of EphA2 agonists and remained in the cultures for the duration of the 24h experiment. Cell lysates were generated and resolved using SDS-PAGE. Western blot analyses were then performed using anti-EphA2 antibodies and negative control anti-β-actin antibodies. Data are representative of 3 independent experiments performed.

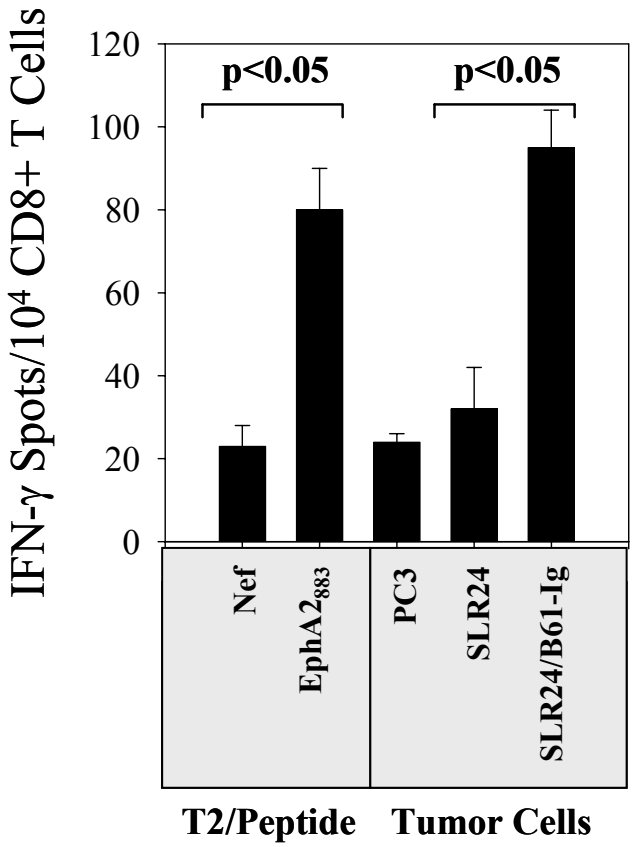


Figure 17. EphA2 Agonists Sensitize SLR24 to EphA2-specific HLA-A2 Restricted CTL CL.142.

The anti-EphA2 CTL clone CL142 (148) was analyzed for reactivity against T2 (A2+) cells pulsed with the $EphA2_{883-91}$ peptide epitope and untreated or agonist-triggered HLA-A2+ EphA2+ RCC line SLR24 in IFN-γ ELISPOT assays. Control target cells include: T2 pulsed with HIV-$nef_{190-198}$ (negative control for peptide specificity) and the PC3 (HLA-$A2^-$ EphA2+) prostate carcinoma cell line. B61.Ig treatment (30μg/ml) was applied overnight to ensure EphA2 degradation and HLA antigen processing and presentation of EphA2 epitopes). Data are reported as IFN-γ specific spots/ 50,000 CL.142 cells and are derived from one representative experiment of 3 performed.

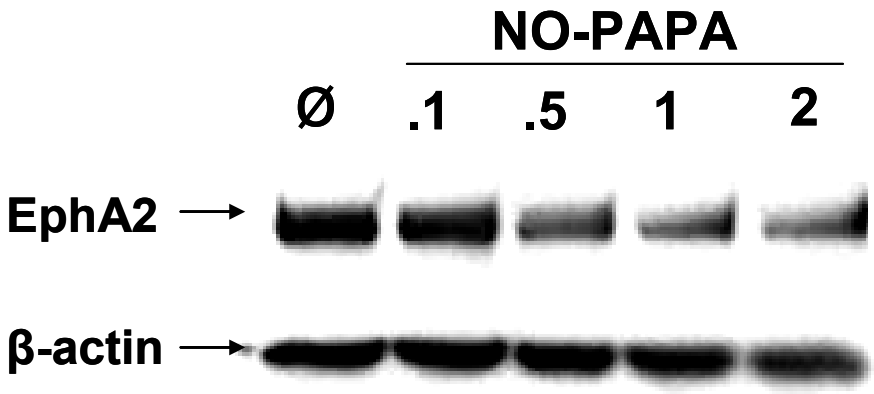


Figure 18. NO-PAPA Induces EphA2 Degradation.

PC3 cells (2-4 x 10^6) were treated for 6 hr with NO-PAPA at the indicated concentrations (mg/ml). Cells were then lysed, with lysates being resolved by SDS-PAGE. Western blot analyses were then performed using anti-EphA2 and anti-β-actin (control) antibodies. Data are representative of 3 independent experiments performed.

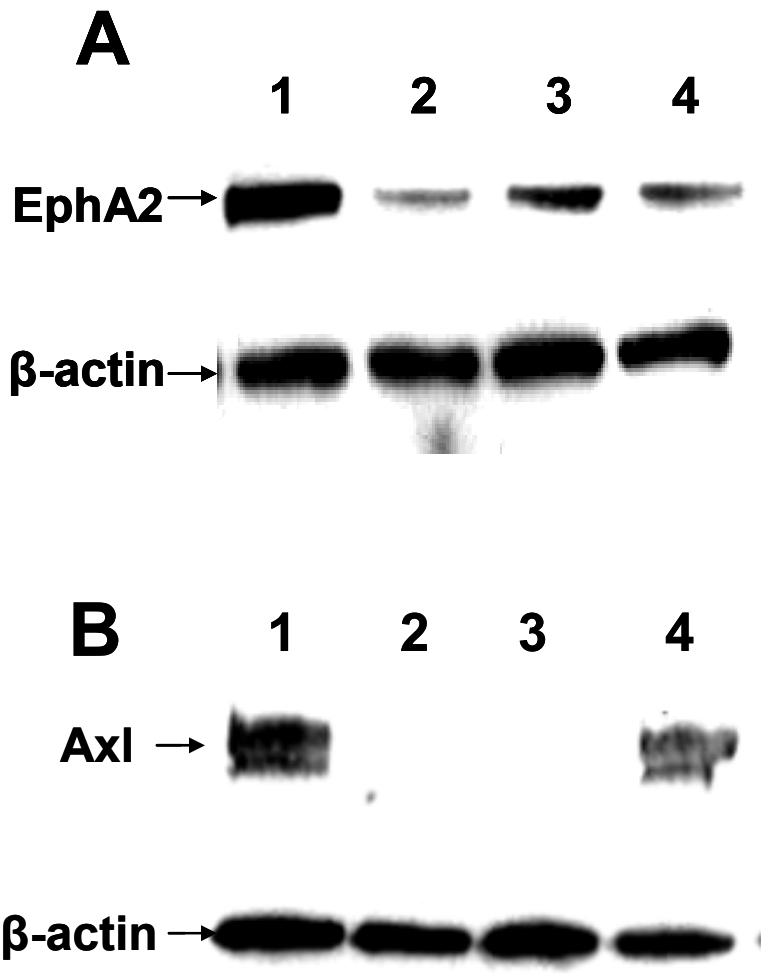


Figure 19. Co-Treatment of tumor cells with NO-PAPA and mAb 208 promote enhanced EphA2 degradation.

PC3 cells (2-4 x 10^6) were treated for 6 hr with either NO-PAPA (Lane 4, 2mg/ml), mAb208 (**Chapter 4,** Lane 3, 8ug/ml) or both (Lane 2). Cells were then lysed, with lysates being resolved by SDS-PAGE. Western blot analyses were then performed using anti-EphA2, anti-Axl antibodies and anti-β-actin antibodies. Data are representative of 3 independent experiments performed.

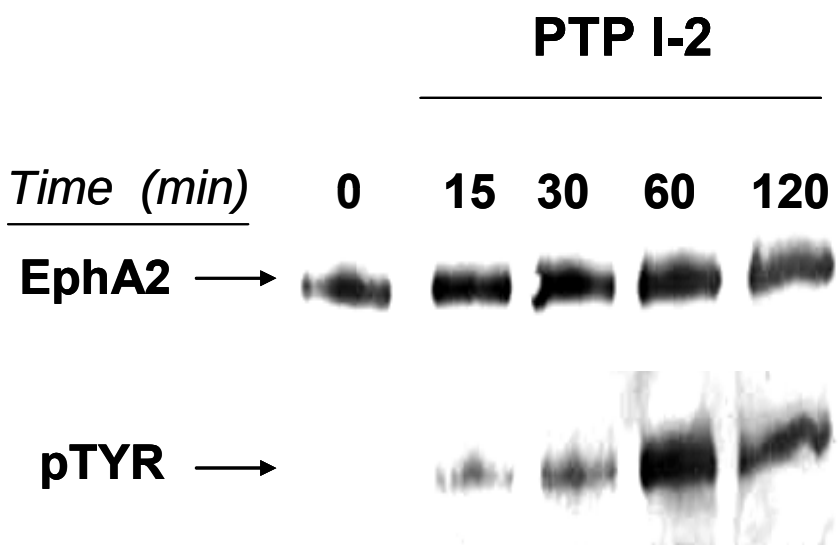


Figure 20. Protein Tyrosine Phosphatase Inhibitor-2 (PTP I-2) Promotes EphA2 Phosphorylation.

PC3 cells (2-4 x 10^6) were treated at the indicated time points with PTP I-2 (100uM). Cells were then lysed, with lysates being resolved by SDS-PAGE. EphA2 protein was immunoprecipitated using the anti-EphA2 antibodies D7 in pull-down assays. Western blot analysis was then performed using anti-EphA2 and anti-phosphotyrosine antibodies, respectively. Data are representative of 3 independent experiments performed.

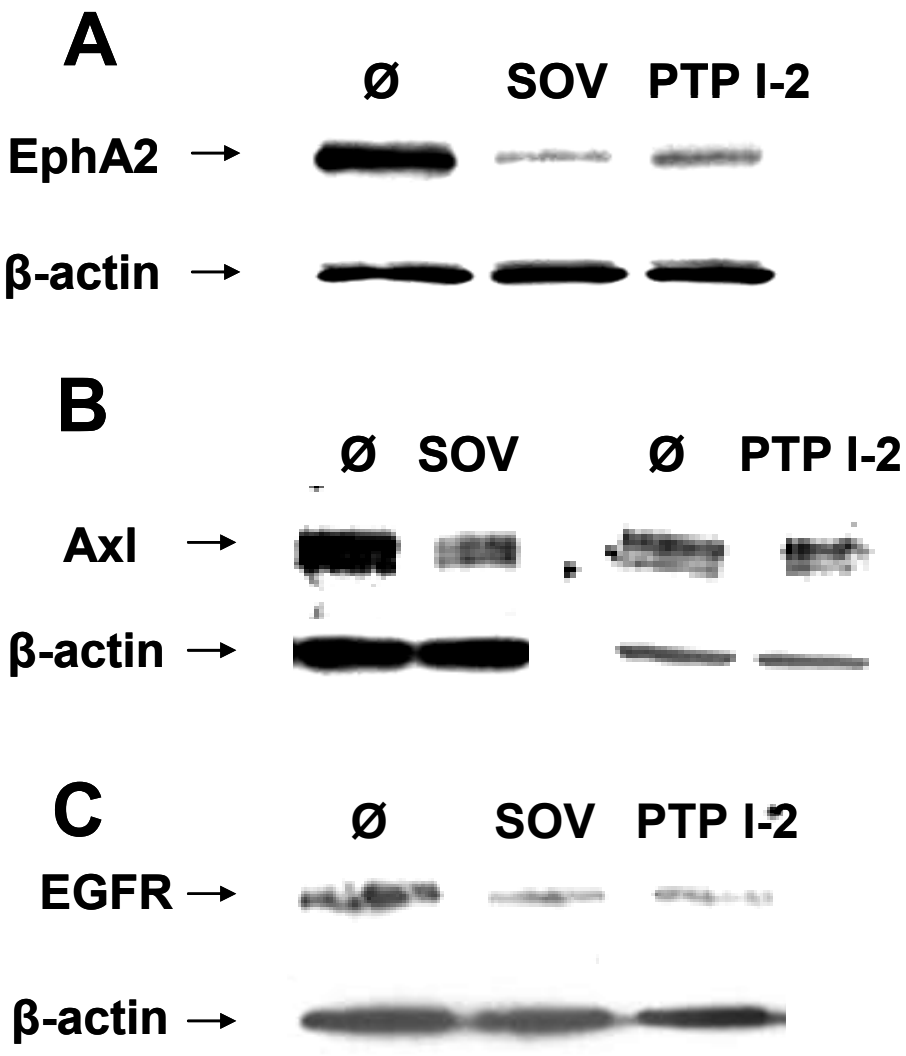


Figure 21. Pharmacologic PTP inhibitors Induced the Degradation of Multiple RTKs.

PC3 cells (2-4 x 10^6) were treated for 6 hr with PTP I-2 (100μM) and SOV (100mM), respectively. Cells were then lysed, with lysates being resolved by SDS-PAGE. Western blot analyses were then performed using anti-EphA2, anti-Axl, or anti-EGFR antibodies, respectively. Anti-β-actin blotting was performed as a control. Data are representative of 3 independent experiments performed.

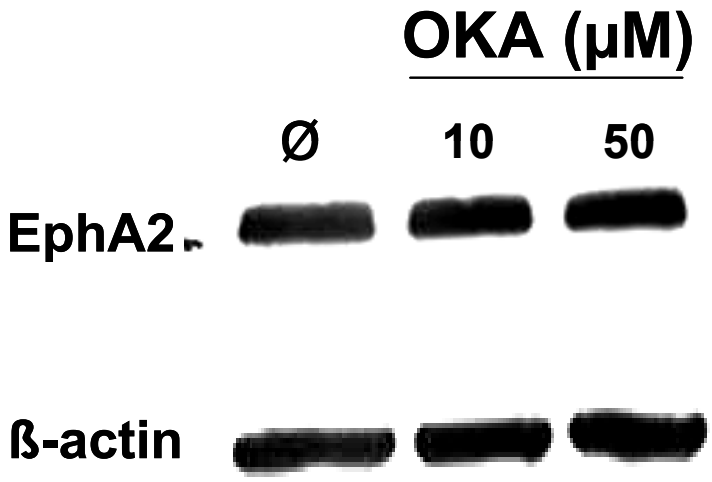


Figure 22. Unlike PTP inhibitors, Okadaic Acid Does Not Modulate EphA2 Degradation in the PC3 Cell Line.

PC3 cells (2-4 x 10^6) were treated for 6 hr with OKA at the indicated concentrations. Cells were then lysed, with lysates being resolved by SDS-PAGE. Western blot analyses were then performed using anti-EphA2 and anti-β-actin antibodies. Data are representative of 3 independent experiments performed.

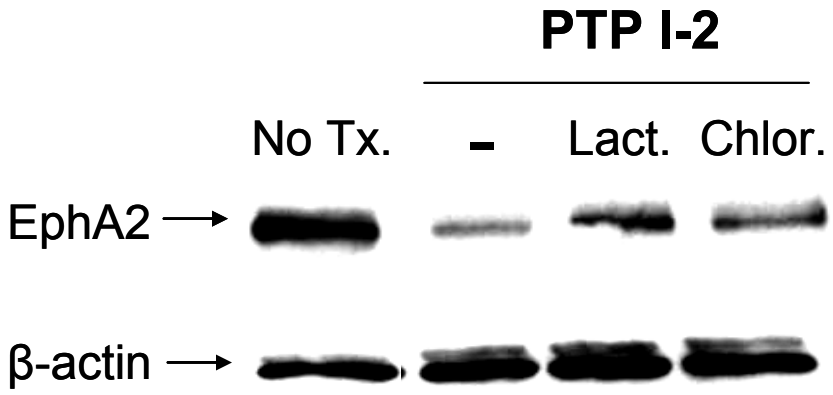


Figure 23. Lactacystin and Chloroquine Inhibit PTP I-2 Induced EphA2 Degradation.

PC3 cells (2-4 x 10^6) were treated for 6 hr with PTP I-2 (100uM). Certain groups were pre-treated for 30min with lactacystin (20uM, Sigma, St. Louis, MO) or chloroquine (100uM, Sigma, St. Louis, MO). Cells were then lysed, with lysates being resolved by SDS-PAGE. Western blot analyses were then performed using anti-EphA2 and anti-β-actin antibodies. Data are representative of 3 independent experiments performed.

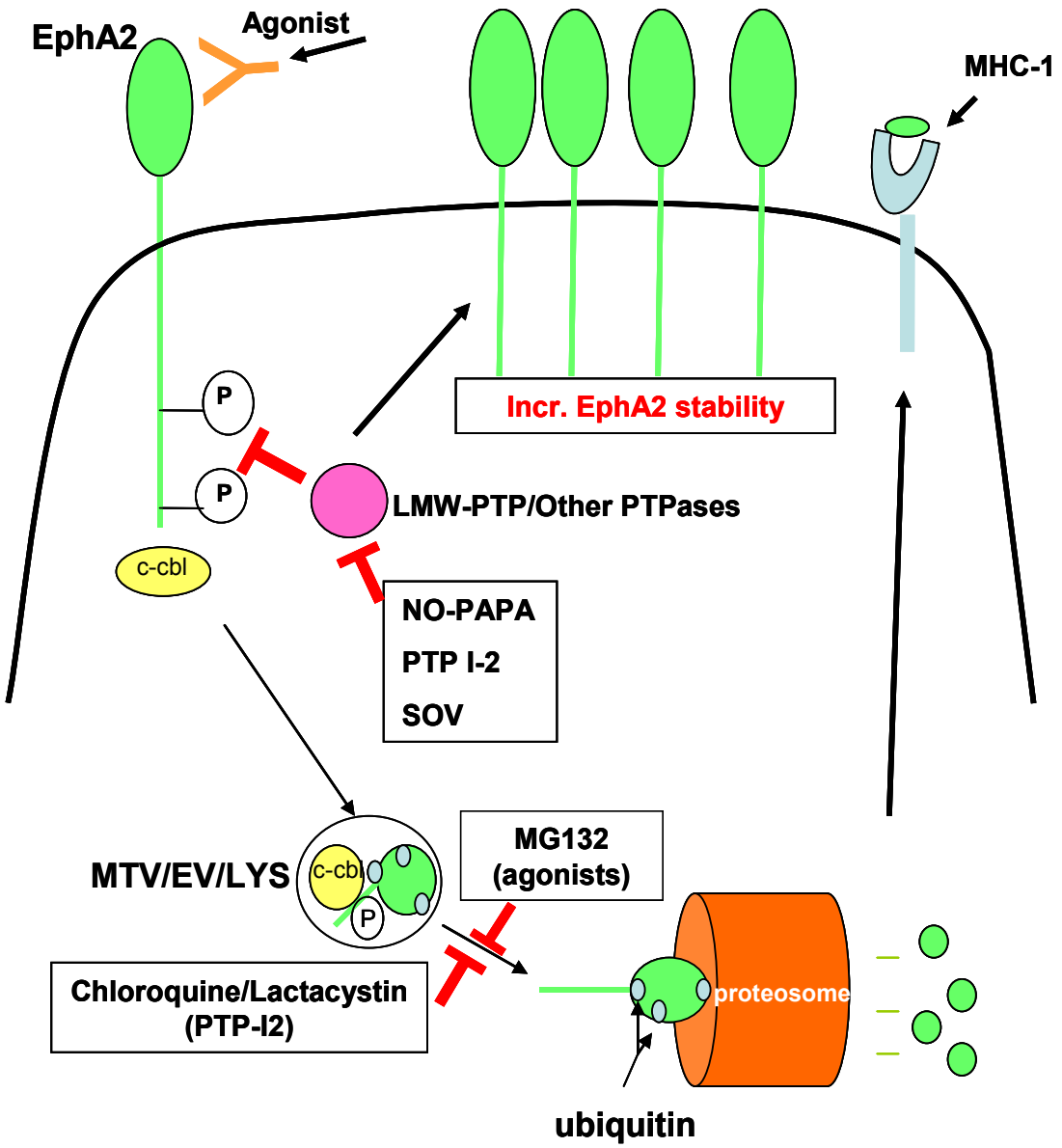


Figure 24. Proposed Mechanism of EphA2 Overexpression.

Overexpression of protein tyrosine phosphatases (PTP) represents a major mechanism of EphA2 overexpression in cancer. This can be reversed by the addition of EphA2 agonist and/or PTP inhibitors which restore the effects of proper phosphorylation of EphA2 which ultimately leads to its proteolytic destruction. This results in the increased recognition by EphA2-specific CTL, which is a proteasome dependent process as the effects of both treatments can be inhibited using proteasome inhibitors (e.g. lactacystin, MG132). EphA2 degradation via PTP inhibitors can be inhibited using chloroquine as well; potentially leading an increase in peptides presented on MHC Class II molecules for presentation to CD4+ T cells, in EphA2+, MHC Class II+ tumors. MTV – multi-tubular vesicle. EV – endosomal vesicle. LYS – lysosome.

APPENDIX A

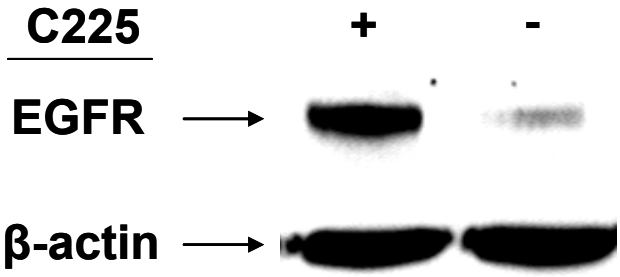
C225 Agonist Addition Induces EGFR Degradation

Purpose – Similar to our results demonstrating the induction of EphA2 upon agonist administration (**Chapter 4)**, we tested whether the addition of C225, a mAb for EGFR1, could induce the degradation of EGF-R (erbB-1).

Methods -- PC3 cells were treated as previously described (Chapter 4) using 10ug/ml C225. Cells were lysed after 6 hr and lysates were resolved via SDS-PAGE. EGFR1 protein was assayed by Western blot and detected using anti-EGFR antibodies.

Results – Similar to mAb208's effects on EphA2, the addition of C225 (10μg/ml) was able to induce the degradation of EGFR1. This affect was inhibited with MG132, confirming the proteasome dependency of this process (data not shown).

Conclusions – Combined with our results in **Chapter 4**, these findings demonstrate the generality of the ability induce RTK degradation using agonists and PTP inhibitors.



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