[CANCER RESEARCH 63, 4481–4489, August 1, 2003]

Disease Stage Variation in CD4+ and CD8+ T-Cell Reactivity to the Receptor Tyrosine Kinase EphA2 in Patients with Renal Cell Carcinoma[1]

Tomohide Tatsumi,[2] Christopher J. Herrem,[2] Walter C. Olson, James H. Finke, Ronald M. Bukowski, Michael S. Kinch, Elena Ranieri, and Walter J. Storkus[3]

Departments of Surgery [T. T., W. C. O., W. J. S.] and Immunology [C. J. H., W. J. S.], University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213; Cleveland Clinic Foundation, Cleveland, Ohio 44195 [J. H. F., R. M. B.]; MedImmune Inc., Gaithersburg, Maryland 20878 [M. S. K.]; Department of Emergency and Organ Transplantation, Section of Nephrology, University of Bari, Bari, Italy 70124 [E. R.]; and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213 [W. J. S.]

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 enzyme-linked immunospot 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, enzyme-linked immunospot analyses demonstrated prominent EphA2-restricted T-helper 1-type CD4+ T cell activity in patients with early stage disease, whereas T-helper 2-type and T regulatory-type responses predominated in patients with more advanced forms of RCC. 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.

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 (1–11). Whereas many CTL-defined[4] tumor-associated epitopes have been applied clinically in cancer vaccinations (12–15), comparatively few class II-restricted epitopes recognized by CD4+ T cells have been identified and clinically integrated to date (16–23). 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 (24, 25). 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; Ref. 26). Largely known for their role in neuronal development (27–29), recent reports suggest that Eph receptors play a role in carcinogenesis (30–33). For example, EphA2 is overexpressed and functionally altered in a many different cancers, where it appears to promote the development of disseminated disease. In normal cells, EphA2 localizes to sites of cell-to-cell contact (32), where it may play a role as a negative regulator of cell growth. In contrast, EphA2 is overexpressed frequently 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 (34, 35), prostate (36), breast (37), and lung (38) carcinomas. Indeed, the highest degree of EphA2 expression among tumors is most commonly observed in metastatic lesions (31, 36). Given this pattern of expression in tumor cells, immunological 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" antitumor 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 (39, 40), though the benefits of leukocytic infiltration on clinical outcome remain unknown (41). Whereas 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- *versus* Th2/Tc2-biased immunity in RCC patients has been equivocal (42, 43). 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.

MATERIALS AND METHODS

Cell Lines and Media. The T2.DR4 (HLA-A2 + and − DRβ1*0401+; Ref. 23) 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

Received 3/18/03; accepted 5/14/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH grant CA57840 (to W. J. S.) and CA56937 (to J. H. F.). T. T. was supported, in part, by the Foundation for Advancement of International Science (Ibaraki, Japan).

[2] These authors contributed equally to this work and should be considered as co-first authors.

[3] To whom requests for reprints should be addressed, at Department of Surgery, University of Pittsburgh School of Medicine, L1.32e The Hillman Cancer Center, 5117 Center Avenue, Pittsburgh, PA 15213-1863. Phone: (412) 623-3240; Fax: (412) 623-7709; E-mail: storkuswj@msx.upmc.edu.

[4] The abbreviations used are: CTL, cytotoxic T lymphocyte; Th, T-helper; RCC, renal cell carcinoma; ELISPOT, enzyme-linked immunospot; PBL, peripheral blood lymphocyte; mAb, monoclonal antibody; IL, interleukin; DC, dendritic cell; PBS/T, PBS/0.05% Tween 20; TGF, transforming growth factor; Pre-Op, pre-operative patients; Post-RD, residual disease after surgery; NED, no-evidence of disease; LTS, long-term survival; Tr, T regulatory.

Downloaded from cancerres.aacrjournals.org on September 18, 2019. © 2003 American Association for Cancer Research.

epithelial kidney cell line HK-2 (American Type Tissue Collection, 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 human papillomavirus 16 E6/E7 genes (44). The EphA2+ PC-3 and PC-3.DR4 prostate carcinoma cell lines were included as positive controls for Western Blot analysis of EphA2 protein expression (36) and were also used as targets in ELISPOT assays. All of the cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all reagents from Life Technologies, Inc., Grand Island, NY) in a humidified atmosphere under 5% CO_2 tension at 37°C.

Peripheral Blood and Tumor Specimens. Peripheral blood was obtained by venipuncture from 40 patients diagnosed with RCC and 14 normal individuals, and were collected into heparinized tubes. 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 Institutional Review Board-approved protocol, was obtained from all of the patients before sample acquisition. Patient and normal donor information is provided in Table 1. All of the 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 mAb 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.

Western Blot Analyses. Tumor cells ($5–10 \times 10^6$) were analyzed for EphA2 expression via Western blots using the antihuman EphA2 polyclonal antibody (clone: H-77; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Cell pellets were lysed using 200 μl of 1% NP40 in PBS containing protease inhibitors (Complete; Boehringer Mannheim, Indianapolis, IN) for 1 h on ice. After centrifugation at $13{,}500 \times g$ for 30 min, the supernatant was mixed 1:1 with SDS-PAGE running buffer and proteins separated on 10% PAGE gels, before electroblotting 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-conjugated goat antirabbit immunoglobulin (Bio-Rad, Hercules, CA) and the ECL chemiluminescence detection kit (NEN Life Science Products).

Immunohistochemistry for EphA2 in RCC Tissue. RCC tumor specimens were obtained surgically under an Institutional Review Board-approved protocol and paraffin-embedded. Five μm sections were deparaffinized and rehydrated in double-distilled H_2O and then PBS. Anti-EphA2 mAb (Ab 208;

Table 1 *HLA-A2 and/or DR4-positive RCC patients evaluated in this study*

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. 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." Immunohistochemical 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.

RCC patient	Age	Sex	Stage	Treatment	Disease status at time of evaluation (months)	HLA typing A2 (+/−)/	HLA typing DR4 (+/−)	Tumor expression EphA2
SLR30-pre	63	F	I	None	Local Dis.	+	−	NA[a]
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

[a] C, chemotherapy; IFN-α, recombinant IFN-α therapy; IL-2, recombinant interleukin-2 therapy; Mets, metastatic disease; NA, not available for evaluation; NED, no evidence of disease; R, radiotherapy; S, surgery.

Downloaded from cancerres.aacrjournals.org on September 18, 2019. © 2003 American Association for Cancer Research.

Table 2 *Selection of EphA2 peptides for analysis*

A. Selected HLA-A2 presented EphA2 peptides

Sequence start amino acid #	AA sequence of nonamer	Binding score[a]	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

B. Selected HLA-DR4 presented EphA2 peptides

Sequence start core AA#	AA sequence of nonamer core	Binding score[a]	Peptide synthesized for *in vitro* analysis
666	IMGQFSHHN	577	$_{663}$EAGIMGQFSHHNIIR$_{677}$
67	YSVCNVMSG	95	$_{63}$PIYMYSVCNVMSG$_{75}$
55	MQNIMNDMP	39	$_{53}$DLMQNIMNDMPIYMYS$_{68}$

[a] 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."

mIgG1) or isotype-matched control mAb was incubated on sections for 1 h at room temperature. After PBS washing, sections were incubated with biotinylated goat antirabbit IgG (Vector Laboratories) for 20 min at room temperature, 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 ×40 magnification.

Peptides Selection and Synthesis. The protein sequence of EphA2 protein was obtained from GenBank (accession no. XP 048780) and analyzed for HLA-A0201 and HLA-DRβ1*0401 binding peptides using neural network algorithms (45, 46). The top 10 candidate HLA-A2 binding peptides were then analyzed for their ability to be generated by proteasomal cleavage using the PAProC prediction algorithm,[5] with only those peptides capable of being processed by the proteasome selected for synthesis. All of the peptides were synthesized by Fmoc chemistry by the University of Pittsburgh Cancer Institute's Peptide Synthesis Facility. Peptides were >96% pure based on high-performance liquid chromatography profile and mass spectrometric analysis performed by the University of Pittsburgh Cancer Institute's Protein Sequencing Facility. In total, we evaluated reactivity to five HLA-0201 and three HLA-DRB1*0401 predicted binding peptides that received high binding scores (Table 2).

Antigen Stimulation of PBLs. PBLs were resuspended at 10^7/ml in AIM-V medium (Life Technologies, Inc.) and were incubated for 60 min at 37°C in a humidified 5% CO_2 incubator. Nonadherent (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 1,000 units/ml recombinant human granulocyte macrophage colony-stimulating factor (Immunex Corporation, Seattle, WA) and 1000 units/ml recombinant human IL-4 (Schering-Plough, Kenilworth, NJ). Seven days later, DCs were harvested and used to stimulate autologous CD8+ or CD4+ T cells. Nonadherent 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). DCs (200,000) were cocultured with 2×10^6 CD8+ or CD4+ T cells with 10 μg/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.

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 described previously (47). CD8+ T-cell responses were evaluated by IFN-γ ELISPOT assays only, whereas 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) were coated with 10 μg/ml of antihuman IFNγ mAb (1-D1K; Mabtech, Stockholm, Sweden) or 5 μg/ml of antihuman IL-5 (PharMingen-BD, San Diego, CA) in PBS (Life Technologies, Inc.) overnight at 4°C. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI 1640/10% human serum (1 h at 37°C), 10^5 CD8+ T cells or CD4+ T cells and T2.DR4 cells (2×10^4 cells) pulsed with 10 μg/ml synthetic peptides were seeded in triplicate in multiscreen 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; Life Technologies, Inc.) was added to yield a final volume of 200 μl/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% CO_2 for 24 h for IFN-γ assessments, and 40 h 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/T. Captured cytokines were detected at sites of their secretion by incubation for 2 h with biotinylated mAb antihuman IFN-γ (7-B6–1; Mabtec) at 2 μg/ml in PBS/0.5%BSA or biotinylated mAb antihuman 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 h. Unbound complex was removed by three successive washes using PBS/T, then with three rinses with PBS alone. 3-Amino-9-ethylcarbazole substrate (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.

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 end point 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 and used in ELISA assays per the manufacturer's instructions. The limits of detection for the TGF-β and IL-10 assays were 60 pg/ml and 7 pg/ml, respectively.

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 $P < 0.05$.

[5] Internet address: http://www.uni-tuebingen.de/uni/kxi/.

Downloaded from cancerres.aacrjournals.org on September 18, 2019. © 2003 American Association for Cancer Research.

RESULTS

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 (Fig. 1*A*). Metastatic RCC lines expressed EphA2 at greater levels than primary RCC lines and were similar to the levels of staining noted previously for the prostate carcinoma PC-3 (36). Whereas used as a model for normal proximal kidney endothelial cells, the HK-2 cell line is human papillomavirus 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 (Fig. 1*B*; Table 1) verified intense expression of EphA2 in 13 of 14 evaluable patient tumor biopsies.

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 (45). 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 2).

Peripheral blood T cells were isolated from normal HLA-A2+ and/or -DR4+ donors, and stimulated with autologous DCs that had been loaded previously 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 RCC 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 1 normal donor, and only 1 donor (HLA-DR4+) failed to respond to any of the EphA2 (Th) epitopes. Among the HLA-A2 donors, the $EphA2_{546-554}$ and $EphA2_{883-891}$ peptides were most commonly reacted against (6 of 8 donors evaluated), with the responses to $EphA2_{883-891}$ typically being of a higher frequency. Among the HLA-DR4+ donors evaluated, 6 of 7 donors responded against at least one predicted EphA2-derived Th epitope, with responses against the $EphA_{63-75}$ and $EphA2_{663-677}$ 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 (Fig. 2; data not shown).

Analysis of Peptide-specific IFN-γ Release by Peripheral Blood CD8+ T Cells in ELISPOT Assays. We next assessed peripheral blood CD8+ T-cell responses against these sequences in 29 HLA-A2+ RCC patients (Table 1) and 10 HLA-A2+ normal donors. CD8+ T cells were enriched to 98% purity for all of the experiments.

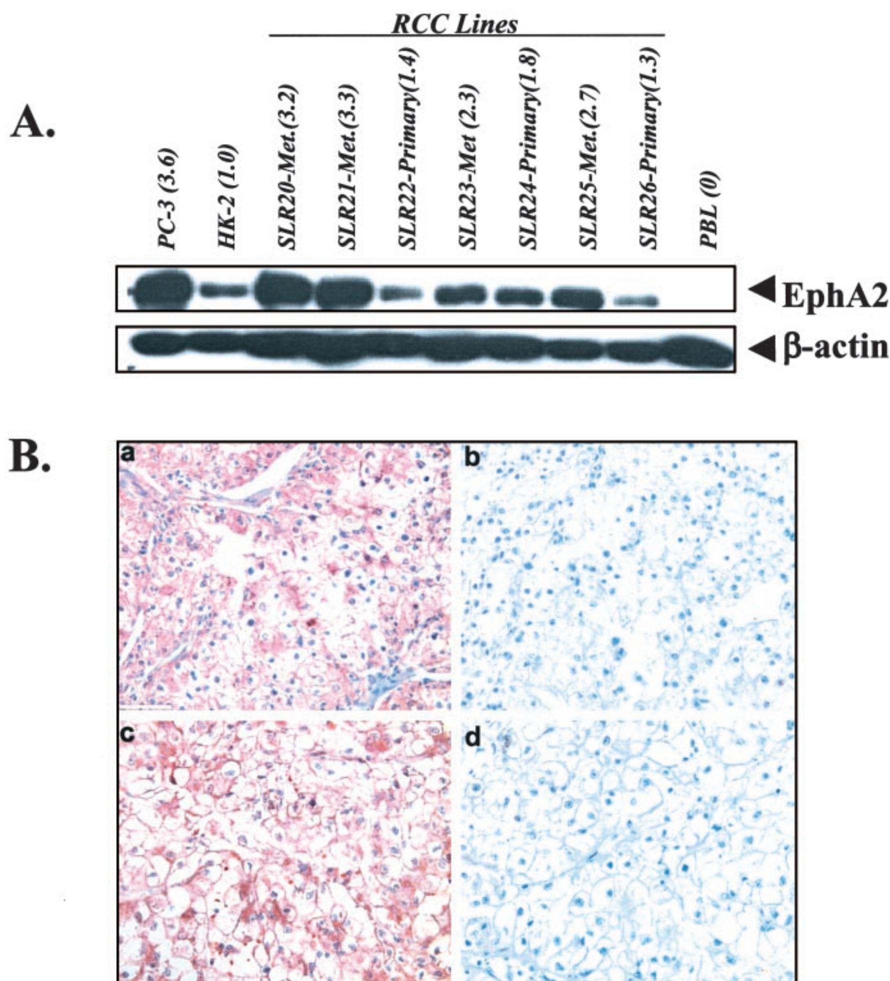

Fig. 1. EphA2 is overexpressed frequently in RCC cell lines and RCC lesions. Anti-EphA2 and control anti-β-actin antibodies were used in performing Western blot analyses of lysates generated from the indicated RCC cell lines, the normal kidney tubular epithelial cell line HK2, and normal PBLs (negative control; *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 *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 (×40 magnification).

Downloaded from cancerres.aacrjournals.org on September 18, 2019. © 2003 American Association for Cancer Research.

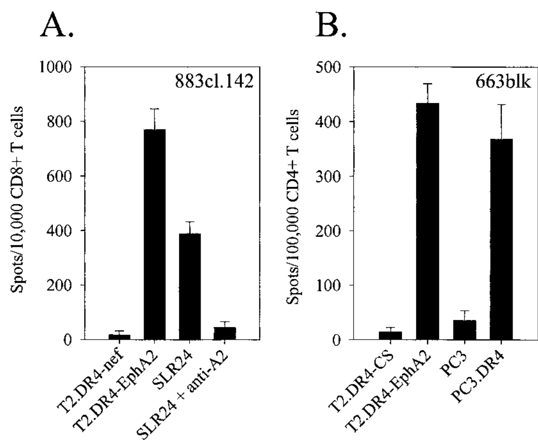

Fig. 2. Anti-EphA2 T cells recognize HLA-matched, EphA2+ RCC tumor cell lines. CD8+ and CD4+ T-cell lines were expanded from normal HLA-A2+ or –DR4+ donors using *in vitro* stimulations with specific EphA2 peptides and evaluated for reactivity against HLA-matched, EphA2+ tumor target cell lines in IFN-γ ELISPOT assays. Depicted are examples of data generated from a CD8+ T-cell clone reactive against the $EphA2_{883-891}$ epitope (*A*) and a bulk CD4+ T-cell line after three rounds of *in vitro* stimulation with the $EphA2_{663-677}$ epitope (*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+/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 reported as spots per 10,000 (*A*) or 100,000 (*B*) T cells analyzed are based on the mean of triplicate determinations and are reflective of at least three independent experiments in all cases; *bars,* ±SD.

Responses were evaluated using IFN-γ ELISPOT assays after 7-day "primary" *in vitro* stimulations. As shown in Fig. 3, the number of IFN-γ spots (per 100,000 CD8+ T cells) observed for T-cell responses against EphA2 peptides in HLA-A2+ patients Pre-Op or patients with 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 Post-NED. Interestingly, CD8+ T cells from RCC patients exhibiting 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 *versus* stage IV, if the patient had active disease (Fig. 4). Only patients that were analyzed at a time when they were disease-free (*i.e.,* NED) or if they were long-term survivors, exhibited CD8+ T cells with elevated reactivity to EphA2 epitopes (Fig. 4).

We evaluated the change of CD8+ T-cell reactivity against EphA2 peptides pre- and post-therapy in 4 HLA-A2+ patients (Fig. 5). Three of these individuals were stage I patients who had local disease before surgical intervention, whereas the remaining patient had stage IV disease. Notably, CD8+ T-cell reactivity against EphA2 peptides was very low before surgery in all 4 of the RCC patients. After being rendered free of disease, CD8+ T-cell reactivity against EphA2-derived CTL epitopes was increased significantly in each of the 3 stage I patients. In marked contrast, the single evaluable stage IV RCC

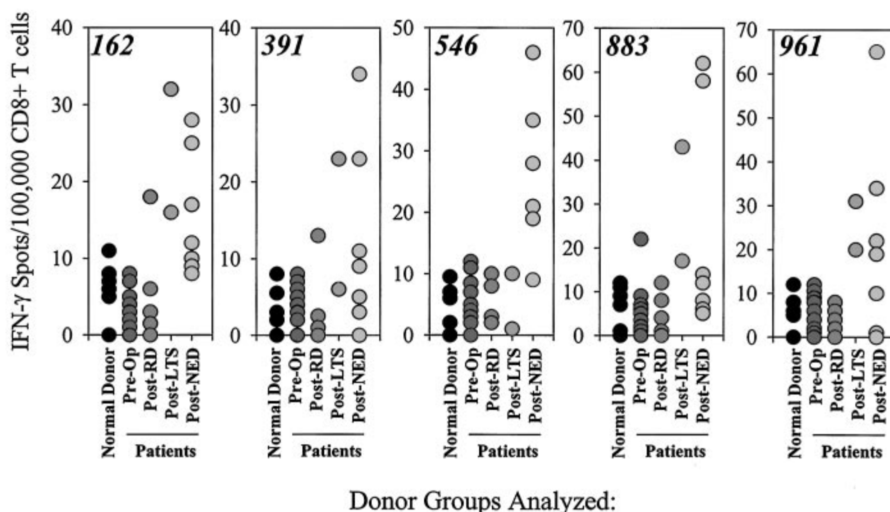

Fig. 3. IFN-γ ELISPOT analyses of RCC patient CD8+ T-cell responses to EphA2-derived epitopes *versus* disease status. Peripheral blood CD8+ T cells were isolated from HLA-A2+ normal donors or patients with RCC and stimulated with immature, autologous DCs prepulsed with the individual EphA2-derived epitopes, as outlined in "Materials and Methods." After 1 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 $HIV\text{-}nef_{190-198}$ epitope served as the negative control in all cases, and this value was subtracted from all experimental determinations to determine EphA2-specific spot numbers. Each *symbol within a panel* represents the response of an individual donor to the indicated HLA-A2 presented EphA2 peptides. *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.

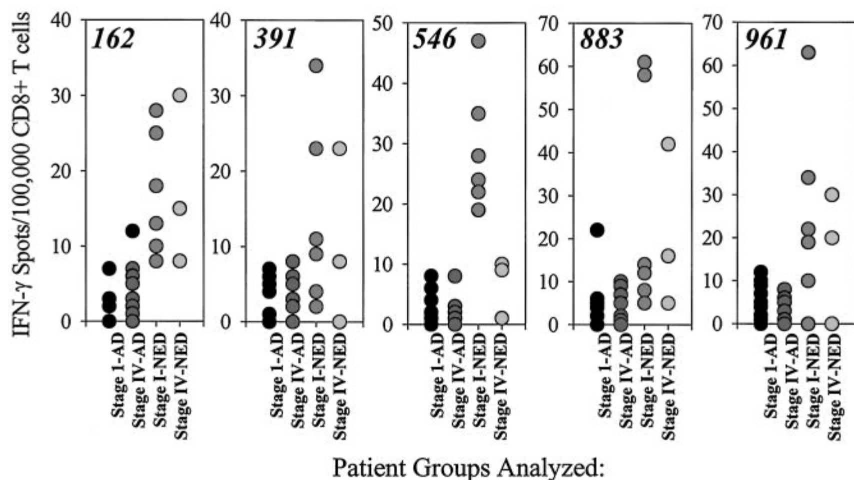

Fig. 4. IFN-γ ELISPOT analysis of RCC patient CD8+ T-cell responses to EphA2-derived epitopes *versus* disease stage. Data reported in Fig. 3 have been replotted as a function of disease-stage. *AD,* patients with active disease.

Downloaded from cancerres.aacrjournals.org on September 18, 2019. © 2003 American Association for Cancer Research.

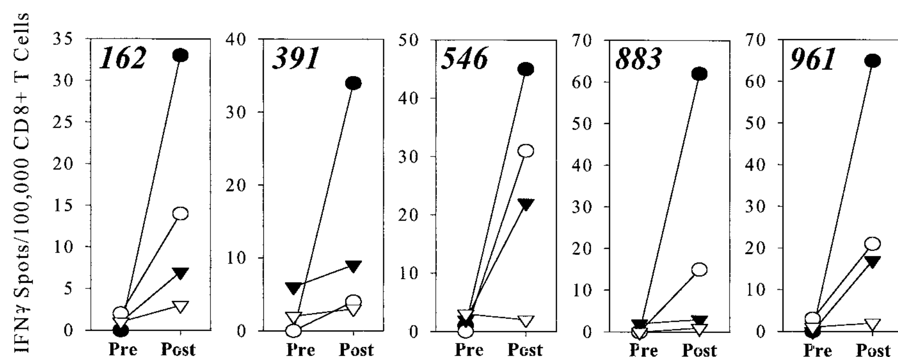

Fig. 5. Observed changes in peripheral blood CD8+ T-cell responses to EphA2 epitopes pre- *versus* postsurgery in 4 HLA-A2+ patients with RCC. Peripheral blood CD8+ T cells were isolated pre- and (6 week) postsurgery from patients with RCC, and evaluated for reactivity to EphA2 epitopes in IFN-γ ELISPOT assays, as outlined in the Fig. 3 legend. The 3 stage I RCC patients (●, ○, and ▼) were rendered free of disease as a result of surgical intervention, whereas the single stage IV RCC patient (▽) had residual disease after surgery. Each *symbol within a panel* represents the response of an individual patient.

patient, who had residual tumor burden after surgery, remained poorly responsive to EphA2 peptides (Fig. 5).

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 frequency and functional bias of patient-derived Th cells against EphA2 peptides. Peripheral blood T cells were stimulated for 1 week with peptide-pulsed immature autologous DCs (which do not appear to skew the Th1/Th2 balance; Ref. 47) before 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 1).

The functional nature of T-cell reactivity toward EphA2 related to disease progression. Patients with stage I disease displayed strongly Th1-polarized reactivity against EphA2 peptides, whereas patients with more advanced stages of the disease polarized toward strong Th2 reactivity (Fig. 6). Not every patient reacted against each peptide, but their responses were consistently polarized in accordance with the patient 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. Whereas 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 (Fig. 7). 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.,* Fig. 4, filled circles).

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

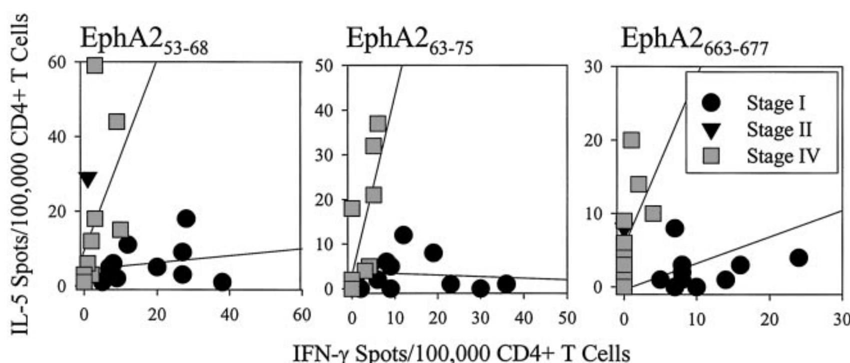

Fig. 6. Disease-stage skewing of functional CD4+ T-cell responses to EphA2 Th epitopes in HLA-DR4+ RCC patients with active disease. Peripheral blood was obtained from 19 HLA-DR4+ patients (Table 1) and CD4+ T cells isolated by positive MACS bead selection as described in "Materials and Methods." After a 1-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 to determine EphA2-specific spot numbers. Each *symbol within a panel* represents the response of an individual patient. Linear regression lines for stage I and stage IV patient data are indicated for each peptide.

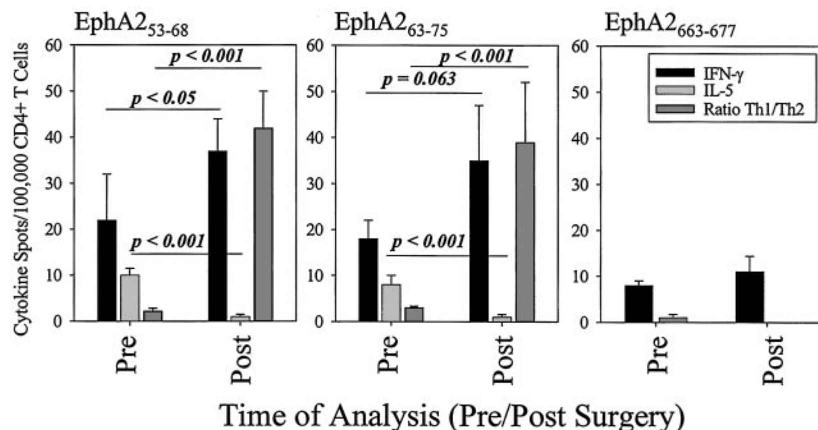

Fig. 7. Therapy-associated enhancement of Th1-type and reduction in Th2-type, CD4+ T-cell responses to EphA2 Th epitopes in an HLA-A2+/DR4+ patient with stage I RCC. Pre- and postsurgery peripheral blood was available for a single RCC patient with stage I disease. CD4+ T cells were isolated and analyzed for reactivity to EphA2 Th epitopes, as outlined in the Fig. 5 legend. A statistically significant increase in Th1-type (*IFN-γ*) and decrease in Th2-type (*IL-5*) CD4+ T-cell response postsurgery 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 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-/postsugery 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*s for significant differences are indicated; *bars,* ±SD.

Downloaded from cancerres.aacrjournals.org on September 18, 2019. © 2003 American Association for Cancer Research.

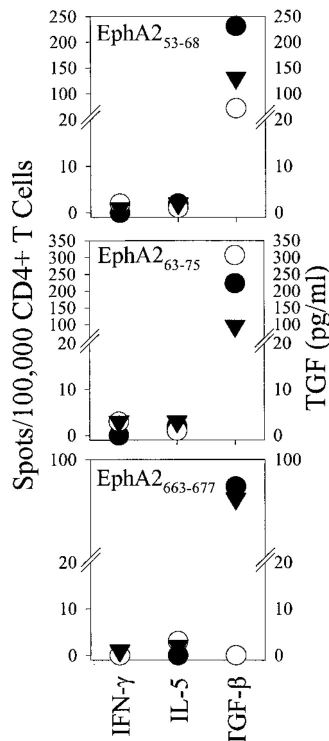

Fig. 8. Suppressor CD4+ T-cell responses to EphA2 Th epitopes in HLA-DR4+ patients with advanced stage IV RCC. Supernatants were harvested from the culture wells of IFN-γ ELISPOT assays and analyzed for levels of TGF-β1 using a commercial ELISA kit. TGF-β1 secretion in response to EphA2 peptides was 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 CD4+ T cell responses of individual patients to EphA2 peptides is also provided. *Each symbol within a panel* represents the response of an individual patient.

measured TGF-β and IL-10 production after *in vitro* peptide stimulation. TGF-β1 production by responder CD4+ T cells was only observed in a subset (*i.e.,* 3 of 8) of stage IV patients (Fig. 8), 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.

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 antitumor CD8+ and CD4+ T-cell responses in the peripheral blood of patients before, during, and after treatment (48).

The major finding of our present study is a demonstration that patients with RCC exhibit detectable CD4+ and CD8+ T-cell reactivity toward 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 after therapeutic intervention that results in disease-free status. Interestingly, 2 HLA-A2+ patients with stage IV disease were identified who were long-term survivors (>40 months) after 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 toward 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 many 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; 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 (34–38, 49, 50). 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

Table 3 *Normal donor T-cell responses to putative EphA2-derived peptide epitopes*

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+ 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 italicized.

A. HLA-A2-presented EphA2 peptides

Normal donor #	CD8+ T-cell response to peptide on T2.DR4[a] (IFN-γ spots/10^5 CD8+ T cells) 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

B. HLA-DR4-presented EphA2 peptides

Normal donor #	CD4+ T-cell response to peptide on T2.DR4[a] (IFN-γ spots/10^5 CD4+ T cells) 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

Downloaded from cancerres.aacrjournals.org on September 18, 2019. © 2003 American Association for Cancer Research.

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 natural killer cell activity, as has been reported previously (51). Notably, CD8+ T-cell reactivity against EphA2-derived epitopes significantly increased in the peripheral blood of 3 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 toward 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 antitumor CD8+ T-cell responses (52, 53). 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 (47).

Th1-type biased CD4+ T-cell responses 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 Th epitopes in a "normal" Th1-biased manner (Ref. 47; data not shown).

Whereas longitudinal data were available for only 1 HLA-DR4+ patient with stage I disease, Th1-type immunity against at least some EphA2 epitopes was strengthened and EphA2-specific, and Th2-type responses lessened after surgical resection of the patient 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 (47).

Th3/Tr CD4+ T-cell subsets may play dominant roles as antigen-specific T "suppressor" cells, in part because of secretion of immunosuppressive cytokines such as TGF-β and/or IL-10 (54). On the basis of 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 ability to productively eliminate EphA2-overexpressing tumors (55). 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 T cells that coexpress CD25, CTLA-4, or the glucocorticoid-induced tumor necrosis factor receptor (as markers of T-suppressor cells; Ref. 55).

EphA2-derived epitopes have the 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 (47), 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 repolarizing or activating conditions (56), DC-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.

ACKNOWLEDGMENTS

We thank William Knapp and Katie Olson (University of Pittsburgh), and Cynthia Combs and Christina Moon (Cleveland Clinic Foundation) for excellent technical support. We also thank Sean Alber and Dr. Simon Watkins from the University of Pittsburgh's Center for Biological Imaging for their assistance with the immunohistochemistry images. We thank Drs. John Kirkwood, William Chambers, Amy Wesa, and Herbert Zeh for careful review and suggestions used in the preparation of this manuscript.

REFERENCES

1. Boon, T., and Old, L. J. Cancer tumor antigens. Curr. Opin. Immunol., *9:* 681–683, 1997.
2. Boon, T., and van der Bruggen, P. Human tumor antigens recognized by T lymphocytes. J. Exp. Med., *183:* 725–729, 1996.
3. Boel, P., Wildmann, C., Sensi, M. L., Brasseur, R., Renauld, J. C., Coulie, P., Boon, T., and van der Bruggen, P. BAGE: a new gene encoding an antigen recognized by cytolytic T lymphocytes. Immunity, *2:* 167–175, 1995.
4. Van den Eynde, B., Peeters, O., De Backer, O., Gaugler, B., Lucas, S., and Boon, T. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on human melanoma. J. Exp. Med., *182:* 689–698, 1995.
5. Chen, Y. T., Scanlan, M. J., Sahin, U., Tureci, O., Gure, A. O., Tsang, S., Williamson, B., Stockert, E., Pfreundschuh, M., and Old, L. J. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc. Natl. Acad. Sci. USA, *94:* 1914–1918, 1997.
6. Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., De Plaen, E., Lethe, B., Coulie, P., and Boon, T. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J. Exp. Med., *178:* 489–495, 1993.
7. Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Rivoltini, L., Topalian, S. L., Miki, T., and Rosenberg, S. A. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc. Natl. Acad. Sci. USA, *91:* 3515–3519, 1994.
8. Kawakami, Y., Eliyahu, S., Jennings, C., Sakaguchi, K., Kang, X., Southwood, S., Robbins, P. F., Sette, A., Appella, E., and Rosenberg, S. A. Recognition of multiple epitopes in human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with *in vivo* tumor regression. J. Immunol., *154:* 3961–3968, 1995.
9. Cheever, M. A., Disis, M. L., Bernhard, H., Gralow, J. R., Hand, S. L., Huseby, E. S., Qin, H. L., Takahashi, M., and Chen, W. Immunity to oncogenic proteins. Immunol. Rev., *145:* 33–59, 1995.
10. Gnjatic, S., Cai, Z., Viguier, M., Chouaib, S., Guillet, J. G., and Choppin, J. Accumulation of the p53 protein allows recognition by human CTL of a wild-type p53 epitope presented by breast carcinoma and melanomas. J. Immunol., *160:* 328–333, 1998.
11. Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Buschenfelde, K. H., and Beach, D. A p16INK4a-insesitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science (Wash. DC), *269:* 1281–1284, 1995.
12. Coulie, P. G., Karanikas, V., Colau, D., Lurquin, C., Landry, C., Marchand, M., Dorval, T., Brichard, V., and Boon, T. A monoclonal cytolytic T lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene MAGE-3. Proc. Natl. Acad. Sci. USA, *98:* 10290–10295, 2001.
13. Yu, J. S., Wheeler, C. J., Zeltzer, P. M., Ying, H., Finger, D. N., Lee, P. K., Yong, W. H., Incardona, F., Thompson, R. C., Riednger, M. S., Zhang, W., Prins, R. M., and Black, K. L. Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. Cancer Res., *61:* 842–847, 2001.
14. Jager, E., Gnjatic, S., Nagata, Y., Stockert, E., Jager, D., Karbach, J., Neumann, A., Rieckenberg, J., Chen, Y. T., Ritter, G., Hoffman, E., Arand, M., Old, L. J., and Knuth, A. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. Proc. Natl. Acad. Sci. USA, *97:* 12198–12203, 2000.
15. Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat. Med., *4:* 328–332, 1998.

Downloaded from cancerres.aacrjournals.org on September 18, 2019. © 2003 American Association for Cancer Research.

16. Topalian, S. L., Rivoltini, L., Mancini, M., Markus, N. R., Robbins, P. F., Kawakami, Y., and Rosenberg, S. A. Human CD4+ T cells specially recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. Proc. Natl. Acad. Sci. USA, *91:* 9461–9465, 1994.
17. Chaux, P., Vantomme, V., Stroobant, V., Thielemans, K., Corthals, J., Luiten, R., Eggermont, A., Boon, T., and van der Bruggen, P. Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4+ T lymphocytes. J. Exp. Med., *189:* 767–777, 1999.
18. Pieper, R., Christian, R. E., Gonzales, M. I., Nishimura, M. I., Gupta, G., Settlage, R. E., Shabanowitz, J., Rosenberg, S. A., Hunt, D. F., and Topalian, S. L. Biochemical identification of a mutated human melanoma antigen recognized by CD4+ T cells. J. Exp. Med., *189:* 757–765, 1999.
19. Wang, R. F., Wang, X., Atwood, A. C., Topalian, S. L., and Rosenberg, S. A. Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science (Wash. DC), *284:* 1351–1354, 1999.
20. Topalian, S. L., Gonzales, M. I., Parkhurst, M., Li, U. F., Southwood, S., Sette, A., Rosenberg, S. A., and Robbins, P. F. Melanoma-specific CD4+ T cells recognize nonmutated HLA-DR-restricted tyrosinase epitopes. J. Exp. Med., *183:* 1965–1971, 1996.
21. Jager, E., Jager, D., Karbach, J., Chen, Y. T., Ritter, G., Nagata, Y., Gnjatic, S., Stockert, E., Arand, M., Old, L. J., and Knuth, A. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101–0103 and recognized by CD4+ T lymphocytes of patients with NY-ESO-1-expressing melanoma. J. Exp. Med., *191:* 625–630, 2000.
22. Zarour, H. M., Storkus, W. J., Brusic, V., Williams, E., and Kirkwood, J. M. NY-ESO-1 encodes DRB1*0401-restricted epitopes recognized by melanoma-reactive CD4+ T cells. Cancer Res., *60:* 4946–4952, 2000.
23. Zarour, H. M., Kirkwood, J. M., Kierstead, L. S., Herr, W., Brusic, V., Slingluff, C. L., Sidney, J., Sette, A., and Storkus, W. J. Melan-A/MART-$1_{51–73}$ represents an immunogenic HLA-DR4- restricted epitope recognized by melanoma-reactive CD4+ T cells. Proc. Natl. Acad. Sci. USA, *97:* 400–405, 2000.
24. Pardoll, D. M., and Topalian, S. L. The role of CD4+ T cell responses in antitumor immunity. Curr. Opin. Immunol., *10:* 588–594, 1998.
25. Toes, R. E., Ossendrop, F., Offringa, R., and Melief, C. J. M. CD4+ T cells and their role in antitumor immune responses. J. Exp. Med., *189:* 753–756, 1999.
26. Eph Nomenclature Committee. Unified nomenclature for Eph family receptors and their ligands. The ephrins. Cell, *90:* 403–404, 1997.
27. Mellitzer, G., Xu, Q., and Willkinson, D. G. Control of cell behavior by signaling through Eph receptors and Ephrins. Curr. Opin. Neurobiol., *10:* 400–408, 2000.
28. Willkinson, D. G. Multiple roles of Eph receptors and Ephrins in neural development. Nat. Rev. Neurosci., *2:* 155–164, 2001.
29. Knoll, B., Zarbalis, K., Wurst, W., and Drescher, U. A role for the EphA family in the topographic targeting of vomeronasal axons. Development, *128:* 895–906, 2000.
30. Oba, S., Wang, Y., Song, J., Li, Z., Kobayashi, K., Tsugane, S., Hamada, G., Tanaka, M., and Sugimura, H. Genomic structure and loss of heterozygosity of EphB2 in colorectal cancer. Cancer Lett., *164:* 97–104, 2001.
31. Dodelet, V., and Pasquale, E. Eph receptors and Ephrin ligands: embryogenesis to tumorgenesis. Oncogene, *19:* 5614–5619, 2000.
32. Miao, H., Burnette, E., Kinch, M., Simon, E., and Wang, B. Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. Nat. Cell Biol., *2:* 62–69, 2000.
33. Bittner, M., Meltzer, P., Chen, Y., Jiang, Y., Seftor, E., Hendrix, M., Radmacher, M., Simon, R., Yakhini, Z., Ben-Dor, A., Sampas, N., Dougherty, E., Wang, E., Marincola, F., Gooden, C., Lueders, J., Glatfelter, A., Leja, D., Dietrich, K., Beaudry, C., Berens, M., Alberts, D., and Sondak, V. Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature (Lond.), *406:* 536–540, 2000.
34. Easty, D., Gurthrie, B., Maung, K., Farr, C., Lindberg, R., Toso, R., Herlyn, M., Benette, D. Protein B61 as a new growth factor: expression of B61 and up-regulation of its receptor epithelial cell kinase during melanoma progression. Cancer Res., *55:* 2528–2532, 1995.
35. Easty, D., Hill, S., Hsu, M., Fallowfield, M., Florenes, V., Herlyn, M., and Bennett, D. Up-regulation of Ephrin-A1 during melanoma progression. Int. J. Cancer, *84:* 494–501, 1999.
36. Walker-Daniels, J., Coffman, K., Azimi, M., Rhim, J., Bostwick, D., Snyder, P., Kerns, B., Waters, D., and Kinch, M. Overexpression of the EphA2 tyrosinase kinase in prostate cancer. Prostate, *41:* 275–280, 1999.
37. Zantek, N. D., Walker-Daniels, J., Stewart, J., Hansen, R. K., Robinson, D., Miao, H., Wang, B., Kung, H. J., Bissell, M. J., and Kinch, M. S. MCF-10A-NeoST: a new cell system for studying cell-ECM and cell-cell interaction in breast cancer. Clin. Cancer Res., *7:* 3640–3648, 2001.
38. D'Amico, T. A., Aloia, T. A., Moore, M. B., Conlon, D. H., Herndon, J. E. 2nd, Kinch, M. S., and Harpole, G. H., Jr. Predicting the sites of metastases from lung cancer using molecular biologic markers. Ann. Thorac. Surg., *72:* 1144–1148, 2001.
39. Van den Hove, L. E., Van Gool, S. W., Van Poppel, H., Baert, L., Coorrevitis, L., Van Damme, B., and Ceupens, J. L. Phenotype, cytokine, production and cytolytic capacity of fresh (uncultured) tumor-infiltrating T lymphocytes in human renal cell carcinoma. Clin. Exp. Immunol., *109:* 501–509, 1997.
40. Finke, J. H., Rayman, P., Hart, L., Alexander, J. P., Edinger, M. G., Tubbs, R. R., Klein, E., Tuason, L., and Bukowski, R. M. Characterization of tumor-infiltrating lymphocytes subsets from human renal cell carcinoma: specific reactivity defined by cytotoxicity, interferon-γ secretion, and proliferation. J. Immunother. Emphas. Tumor Immunol., *15:* 91–104, 1994.
41. Kolbeck, P. C., Kaveggia, F. F., Johansson, S. L., Grune, M. T., and Taylor. R, J. The relationships among tumor-infiltrating lymphocytes, histopathologic findings and long-term clinical follow-up in renal cell carcinoma. Mod. Pathol., *5:* 420–425, 1992.
42. Maeurer, M. J., Martin, D. M., Castelli, C., Elder, E., Leder, G., Storkus, W. J., and Lotze, M. T. Host immune response in renal cell cancer: interleukin-4 (IL-4) and IL-10 mRNA are frequently detected in freshed collected tumor-infiltrating lymphocytes. Cancer Immunol. Immunother., *41:* 111–121, 1995.
43. Elsasser-Beile, U., Kolble, N., Grussenmeyer, T., Schultze-Seemann, W., Wetterauer, U., Gallati, H., Schultz Monting, J., and von Kleist, S. Th1 and Th2 cytokine response patterns in leukocyte culture of patients with urinary bladder, renal call and prostate carcinoma. Tumor Biol., *19:* 470–476, 1998.
44. Ryan, M. J., Johnson, G., Kirk, J., Fuerstenberg, S. M., Zager, R. A., and Torok-Storb, B. HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. Kidney Int., *45:* 48–57, 1994.
45. Honeyman, M. C., Brusic, V., Stone, N. L., and Harrison, L. C. Neural network-based prediction of candidate T-cell epitope. Nat. Biotechnol., *16:* 966–969, 1998.
46. Southwood, S., Sidney, J., Kondo, A., Del Guercio, M-F., Appella, E., Hoffman, S., Kubo, R. T., Chesnut, R. W., Grey, H. M., and Sette, A. Several common HLA-DR types share largely overlapping peptide binding repertoires. J. Immunol., *160:* 3363–3383, 1998.
47. Tatsumi, T., Kierstead, L. S., Ranieri, E., Gesualdo, L., Schena, F. P., Finke, J. H., Bukowski, R. M., Mueller-Berghaus, J., Kirkwood, J. M., Kwok, W. W., and Storkus, W. J. Disease-associated bias in T helper type 1 (Th1)/Th2 CD4+ T cells responses against MAGE-6 in HLA-DRβ1*0401+ patients with renal cell carcinoma or melanoma. J. Exp. Med., *196:* 619–628, 2002.
48. Keilholz, U., Weber, J., Finke, J., Gabrilovich, D., Kast, M., Disis, N., Kirkwood, J., Scheibenbogen, C., Schlom, J., Maino, V., Lyerly, K., Lee, P., Storkus, W. J., Marincola, F., Worobecand, A., and Atkins, M. B. Immunologic monitoring of cancer vaccine therapy. J. Immunother., *25:* 97–138, 2002.
49. Miyazaki, T., Kato, H., Fukuchi, M., Nakajima, M., and Kuwano, H. EphA2 overexpression correlates with poor prognosis in esophageal squamous cell carcinoma. Int. J. Cancer, *103:* 657–663, 2003.
50. Ogawa, K., Pasqualini, R., Lindberg, R. A., Kain, R., Freeman, A. L., and Pasquale, E. B. The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. Oncogene, *19:* 6043–6052, 2003.
51. Kiessling, R., Wasserman, K., Horiguchi, S., Kono, K., Sjoberg, J., Pisa, P., and Petersson, M. Tumor-induced immune dysfunction. Cancer Immunol. Immunother., *48:* 353–362, 1999.
52. Liu, H., Andreansky, S., Diaz, G., Hogg, T., and Doherty, P. C. Reduced functional capacity of CD8+ T cells expanded by post-exposure vaccination of γ-herpesvirus-infected CD4-deficient mice. J. Immunol., *168:* 3477–3483, 2002.
53. Moser, J. M., and Lukacher, A. E. Immunity to polyoma virus infection and tumorigenesis. Viral Immunol., *14:* 199–216, 2001.
54. Krause, I., Blank, M., and Shoenfeld, Y. Immunomodulation of experimental autoimmune disease via oral tolerance. Crit. Rev. Immunol., *20:* 1–16, 2000.
55. Levings, M. K., Sangregorio, R., Sartirana, C., Moschin, A. L., Battaglia, M., Orban, P. C., and Roncarolo, M. G. Human CD25+CD4+ T suppressor cell clones produce transforming growth factor β, but not interleukin 10, and are distinct from type 1 T regulatory cells. J. Exp. Med., *196:* 1335–1346, 2002.
56. Vieira, P. L., de Jong, E. C., Wierenga, E. A., Kapsenberg, M. L., and Kalinski, P. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. J. Immunol., *164:* 4507–4512, 2000.

Downloaded from cancerres.aacrjournals.org on September 18, 2019. © 2003 American Association for Cancer Research.

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

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

Tomohide Tatsumi, Christopher J. Herrem, Walter C. Olson, et al.

Cancer Res 2003;63:4481-4489.

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/63/15/4481

Cited articles	This article cites 56 articles, 27 of which you can access for free at: http://cancerres.aacrjournals.org/content/63/15/4481.full#ref-list-1
Citing articles	This article has been cited by 25 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/63/15/4481.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/63/15/4481. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.

Downloaded from cancerres.aacrjournals.org on September 18, 2019. © 2003 American Association for Cancer Research.