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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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 assays 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 tumor-associated epitopes have been applied clinically in cancer vaccinations (12–15), comparatively few II-restricted epitopes recognized by CD4+ T cells have been identified and clinically integrated to date (16–23). Current paradigms support the idea that CD4+ T cells that had been rendered disease-free, after surgery, 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 serve as an appropriate index of “clinically important” antitumor immunity in patients with diverse forms of cancer.

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4 The abbreviations used are: CTL, cytotoxic T lymphocyte; Th, T-helper; RCC, renal cell carcinoma; ELISPOT, enzyme-linked immunospot; PBL, peripheral blood lymphocytes; 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.
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% CO₂ 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 x 10⁶) were analyzed for EphA2 expression via Western blots using the anti-human EphA2 polyclonal antibody (clone: H-77; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) 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 H₂O and then PBS. Anti-EphA2 mAb (Ab 208; Table 1

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

<table>
<thead>
<tr>
<th>RCC patient</th>
<th>Age</th>
<th>Sex</th>
<th>Stage</th>
<th>Treatment</th>
<th>Disease status at time of evaluation (months)</th>
<th>HLA A2 (+/−)</th>
<th>DR4 (+/−)</th>
<th>Tumor expression EphA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLR30-pre</td>
<td>63</td>
<td>F</td>
<td>I</td>
<td>None</td>
<td>Local Dis.</td>
<td>+</td>
<td>−</td>
<td>NA⁻</td>
</tr>
<tr>
<td>SLR31</td>
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<td>M</td>
<td>I</td>
<td>S</td>
<td>Local Dis.</td>
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<td>−</td>
<td>NA</td>
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<td>62</td>
<td>F</td>
<td>I</td>
<td>S</td>
<td>Local Dis.</td>
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<td>−</td>
<td>NA</td>
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<tr>
<td>SLR33</td>
<td>74</td>
<td>M</td>
<td>I</td>
<td>None</td>
<td>Local Dis.</td>
<td>+</td>
<td>+</td>
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<tr>
<td>SLR34</td>
<td>75</td>
<td>F</td>
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<td>+</td>
<td>NA</td>
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<td>Local Dis.</td>
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<td>+</td>
<td>NA</td>
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<td>60</td>
<td>M</td>
<td>I</td>
<td>None</td>
<td>Local Dis.</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>NA</td>
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<td>Local Dis.</td>
<td>+</td>
<td>+</td>
<td>NA</td>
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<td>SLR39</td>
<td>65</td>
<td>M</td>
<td>I</td>
<td>S</td>
<td>NED (3)</td>
<td>+</td>
<td>−</td>
<td>3⁺</td>
</tr>
<tr>
<td>SLR30-post</td>
<td>63</td>
<td>F</td>
<td>I</td>
<td>S</td>
<td>NED (1.5)</td>
<td>+</td>
<td>−</td>
<td>NA</td>
</tr>
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<td>53</td>
<td>M</td>
<td>I</td>
<td>S</td>
<td>NED (3)</td>
<td>+</td>
<td>−</td>
<td>NA</td>
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<tr>
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<td>M</td>
<td>I</td>
<td>S</td>
<td>NED (2)</td>
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<td>+</td>
<td>NA</td>
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<td>64</td>
<td>F</td>
<td>I</td>
<td>S</td>
<td>NED (2)</td>
<td>+</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>SLR35-post</td>
<td>69</td>
<td>M</td>
<td>I</td>
<td>S</td>
<td>NED (2)</td>
<td>+</td>
<td>+</td>
<td>NA</td>
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<td>+</td>
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<td>Local Dis.</td>
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<td>3⁺</td>
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<td>IV</td>
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<td>Mets.</td>
<td>+</td>
<td>−</td>
<td>NA</td>
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<td>M</td>
<td>IV</td>
<td>S</td>
<td>Mets.</td>
<td>+</td>
<td>−</td>
<td>4⁺</td>
</tr>
<tr>
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<td>F</td>
<td>IV</td>
<td>S</td>
<td>Mets.</td>
<td>+</td>
<td>−</td>
<td>0⁺</td>
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<td>F</td>
<td>IV</td>
<td>S</td>
<td>NED (1.5)</td>
<td>+</td>
<td>−</td>
<td>NA</td>
</tr>
<tr>
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<td>M</td>
<td>IV</td>
<td>S</td>
<td>Mets. (61)</td>
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<td>−</td>
<td>NA</td>
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<tr>
<td>SLR49</td>
<td>52</td>
<td>F</td>
<td>IV</td>
<td>S, R, IFN-α, IL-2</td>
<td>Mets. (4.1)</td>
<td>+</td>
<td>−</td>
<td>2⁺</td>
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<tr>
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<td>69</td>
<td>M</td>
<td>IV</td>
<td>S</td>
<td>Mets.</td>
<td>+</td>
<td>−</td>
<td>4⁺</td>
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<tr>
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<td>IV</td>
<td>S, R, C</td>
<td>Mets (21)</td>
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<td>−</td>
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<td>SLR51</td>
<td>41</td>
<td>M</td>
<td>IV</td>
<td>S, R, IL-2</td>
<td>Mets</td>
<td>+</td>
<td>−</td>
<td>NA</td>
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<tr>
<td>SLR52</td>
<td>58</td>
<td>M</td>
<td>IV</td>
<td>S, IFN-α</td>
<td>Mets</td>
<td>+</td>
<td>−</td>
<td>NA</td>
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<td>IV</td>
<td>S</td>
<td>Mets</td>
<td>+</td>
<td>−</td>
<td>NA</td>
</tr>
<tr>
<td>SLR54</td>
<td>49</td>
<td>F</td>
<td>IV</td>
<td>C, IL-2</td>
<td>Mets</td>
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<td>−</td>
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<tr>
<td>SLR55</td>
<td>79</td>
<td>M</td>
<td>IV</td>
<td>C, IFN-α</td>
<td>Mets</td>
<td>+</td>
<td>−</td>
<td>NA</td>
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<tr>
<td>SLR56</td>
<td>56</td>
<td>M</td>
<td>IV</td>
<td>R, C, IFN-α, IL-2</td>
<td>Mets</td>
<td>+</td>
<td>−</td>
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<tr>
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<td>68</td>
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<td>IV</td>
<td>C</td>
<td>Mets</td>
<td>+</td>
<td>−</td>
<td>3⁺</td>
</tr>
<tr>
<td>SLR58</td>
<td>55</td>
<td>F</td>
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<td>Mets</td>
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<tr>
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<td>F</td>
<td>I</td>
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<td>Local Dis.</td>
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<td>−</td>
<td>NA</td>
</tr>
<tr>
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<td>58</td>
<td>M</td>
<td>I</td>
<td>None</td>
<td>Local Dis.</td>
<td>−</td>
<td>−</td>
<td>NA</td>
</tr>
<tr>
<td>SLR61</td>
<td>60</td>
<td>M</td>
<td>I</td>
<td>S</td>
<td>Local Dis.</td>
<td>−</td>
<td>+</td>
<td>2⁺</td>
</tr>
<tr>
<td>SLR62</td>
<td>64</td>
<td>M</td>
<td>I</td>
<td>S</td>
<td>NED (3)</td>
<td>−</td>
<td>−</td>
<td>NA</td>
</tr>
<tr>
<td>SLR63</td>
<td>53</td>
<td>F</td>
<td>I</td>
<td>S</td>
<td>NED (1.5)</td>
<td>−</td>
<td>−</td>
<td>NA</td>
</tr>
<tr>
<td>SLR60-post</td>
<td>58</td>
<td>M</td>
<td>I</td>
<td>S</td>
<td>NED (2)</td>
<td>−</td>
<td>−</td>
<td>NA</td>
</tr>
<tr>
<td>SLR64</td>
<td>65</td>
<td>M</td>
<td>I</td>
<td>S</td>
<td>NED (10)</td>
<td>−</td>
<td>−</td>
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<tr>
<td>SLR65</td>
<td>53</td>
<td>M</td>
<td>I</td>
<td>S</td>
<td>Local Dis.</td>
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<td>−</td>
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<tr>
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<td>M</td>
<td>IV</td>
<td>None</td>
<td>Mets.</td>
<td>−</td>
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<td>57</td>
<td>M</td>
<td>IV</td>
<td>C, R</td>
<td>Mets</td>
<td>−</td>
<td>−</td>
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<td>IV</td>
<td>S, R, C</td>
<td>Mets</td>
<td>−</td>
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<td>M</td>
<td>IV</td>
<td>S, C, R, IFN-α, IL-2</td>
<td>Mets</td>
<td>−</td>
<td>−</td>
<td>NA</td>
</tr>
</tbody>
</table>

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.

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mlgG1) 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 visualized by diaminobenzidine (DAB) staining 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-A*0201 and HLA-DRB1*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, 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 predicted using on-line algorithms as described in “Materials and Methods.”

Peptide Selection and Synthesis.

<table>
<thead>
<tr>
<th>Peptide synthesized for in vitro analysis</th>
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</thead>
<tbody>
<tr>
<td>Sequence start core AA#</td>
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<tr>
<td>666</td>
</tr>
<tr>
<td>67</td>
</tr>
<tr>
<td>55</td>
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</table>

<table>
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<th>Peptide synthesized for in vitro analysis</th>
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<tbody>
<tr>
<td>666</td>
</tr>
<tr>
<td>67</td>
</tr>
<tr>
<td>55</td>
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</tbody>
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Table 2. Selection of EphA2 peptides for analysis

A. Selected HLA-A2 presented EphA2 peptides

<table>
<thead>
<tr>
<th>Sequence start amino acid #</th>
<th>AA sequence of nonamer core</th>
<th>Binding score*</th>
<th>Peptide generated by proteasome</th>
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<tbody>
<tr>
<td>883</td>
<td>TLADFDPFRV</td>
<td>1084</td>
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<tr>
<td>546</td>
<td>VLLVLAGV</td>
<td>1006</td>
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<td>550</td>
<td>VLAGVGGFI</td>
<td>556</td>
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<td>58</td>
<td>IMNDMPYIM</td>
<td>138</td>
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<td>961</td>
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<td>162</td>
<td>KLNVEERSV</td>
<td>49</td>
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</tbody>
</table>

* 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.”

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% CO2 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 microtiter plates were coated with 10 μg/ml of antihuman IFN-γ mAb (1-D1K, Becton, Dickinson, 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 washings 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 × 10^5 cells) pulsed with 10 μg/ml synthetic peptides were seeded in triplicate in microtiter plates with antihuman IFN-γ antibody (AFHHVAREL) or Malaria CS26-345 peptide (EYLNQINSLSTEWSPCSVT), 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% CO2 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 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) 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) 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.

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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. 1A). 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. 1B; 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 546–554 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 EphA63–75 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).
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
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 EphA253–68 and EphA263–75 (but not the EphA2663–677) 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...
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 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
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 adjunctive 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 repolaring 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.

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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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