Human CD4+ T Lymphocytes Recognize a Vascular Endothelial Growth Factor Receptor-2–Derived Epitope in Association with HLA-DR

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Abstract

Purpose: Given the multiple escape mechanisms of tumor cells, immunotherapy targeting tumor-dependent stroma may be an effective cancer treatment strategy. Animal models indicate that inducing immunity to tumor endothelia engenders potent antitumor effects without significant pathology. Recently, the first human tumor endothelial antigen vascular endothelial growth factor receptor-2 (VEGFR-2) recognized by HLA class I–restricted CD8+ T cells has been characterized. In this study, we sought to investigate specific recognition of this molecule by human CD4+ T cells.

Experimental Design: To identify HLA-DR–restricted antigenic peptides on VEGFR-2 recognized by CD4+ T cells of healthy donors and cancer patients.

Results: Nine candidate VEGFR-2 peptides with high binding probability to six common HLA-DRB1 alleles were synthesized using the SYFPEITHI algorithm. One 15-mer peptide (EKRFVPDGNRISWDS), mapping to the 167-181 region of VEGFR-2, stimulated CD4+ T cells in association with several HLA-DR alleles, including DR4 and DR7. Importantly, the epitope could be naturally processed and presented both by HLA-DR–matched antigen-expressing proliferating endothelial cells and by dendritic cells loaded with the native antigen. Furthermore, circulating VEGFR-2–specific CD4+ T cells were detected in 4 of 10 healthy donors and 12 of 40 cancer patients even after single-round peptide stimulation in short-term culture. Patient’s T cells could recognize antigen-expressing proliferating endothelial cells in a HLA-DR–restricted fashion.

Conclusion: These findings indicate an important role for the 167-181 region of VEGFR-2 in the stimulation of CD4+ T cell responses to VEGFR-2 protein, and may be instrumental both for the development and monitoring of upcoming antitumor vessel vaccines against different cancers based on VEGFR-2 immunogens.

The development of vaccine therapy for cancers will likely require strategies that can both break immunologic tolerance and meanwhile enable to circumvent a variety of tumor escape mechanisms (1). One promising strategy would be to strategically direct the T cells to tumor neovessels on which tumors rely, considering that tumor endothelium is constituted by a relatively homogeneous population of genetically stable cells with low probability to lose antigen expression while being easily accessible by circulating T cells. Indeed, recent animal studies have shown that induction of immunologic responses to angiogenic proliferative tumor endothelia engenders potent antitumor effects without significant pathology (2–11). In many cases, long-lived therapeutic benefit was observed in treated animals (2–4, 6–9), thus providing the proof of principle for achievable robust efficacy by immuno-targeting of tumor blood vessels.

Given the advantages of tumor vessel–targeted vaccines, it is critical to immediately identify candidate antigens that are recognized by human HLA-restricted T cells. Until now, several CD8+ CTL epitopes have been characterized from proteins overexpressed by tumor neovessels (12–15), including endothelial-specific receptor vascular endothelial growth factor receptor-2 (VEGFR-2; refs. 12, 13). VEGFR-2 is a 1,356-amino-acid protein tyrosine kinase closely related to endothelial cell proliferation (16). It is strongly expressed in tumor but not in normal adult tissues (17–20) and can stimulate specific CTLs from cancer patient’s peripheral blood (13), making it an optimal target for immune therapy. In fact, VEGFR-2 is a validated target for the conventional antiangiogenesis cancer therapies (16, 21). Therefore, therapeutic vaccines based on VEGFR-2 CTL epitopes can be developed that specifically target tumor endothelium. For an optimal vaccine, however, both
Table 1. Peptides derived from the VEGFR-2 protein selected for this study

<table>
<thead>
<tr>
<th>Sequence (15-mer core)</th>
<th>Position of 1st core residue</th>
<th>Scores in HLA-DRB1 epitope prediction</th>
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<tbody>
<tr>
<td>VALWLCVETRAASVG</td>
<td>p9</td>
<td>32/4th 4/30th 28/1st 22/6th 23/5th 8/13th</td>
</tr>
<tr>
<td>EKRFVPDGNRISWDS</td>
<td>p167</td>
<td>21/15th 27/8th 28/1st 16/9th 18/10th 16/9th</td>
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<tr>
<td>QGLYCAASGLMLTK</td>
<td>p302</td>
<td>27/9th 9/26th 28/1st 32/1st 12/16th 8/13th</td>
</tr>
<tr>
<td>TNGYTVILNPISEK</td>
<td>p393</td>
<td>31/5th 3/31th 28/1st 32/1st 19/9th 8/13th</td>
</tr>
<tr>
<td>VRQTVLVERAPVTI</td>
<td>p656</td>
<td>26/10th 20/15th 20/4th 8/13th 29/1st 20/7th</td>
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<td>SGIVLKDGNRNLTR</td>
<td>p711</td>
<td>20/16th 38/1st 26/2nd 22/6th 7/21th 18/8th</td>
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<tr>
<td>NLEIIILGTAVIAM</td>
<td>p762</td>
<td>36/1st 12/23th 20/4th 16/9th 14/14th 18/8th</td>
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<tr>
<td>IVEFCKGLNLYLIR</td>
<td>p915</td>
<td>13/23th 16/19th 28/1st 16/9th 10/18th 32/1st</td>
</tr>
<tr>
<td>ETIFDVRVITQSDVW</td>
<td>p1075</td>
<td>30/6th 8/27th 28/1st 18/8th 22/6th 22/6th</td>
</tr>
</tbody>
</table>

NOTE: Values shown are the scores/ranking of individual peptides to each allotype used determining the SYFPEITHI epitope prediction score. Scores of 15 or above and ranking within 10th are judged as a high probability of presentation by the respective HLA-DR subtypes.

*Allele frequency in Caucasians: 6.7% for 0101, 8.8% for 0301, 5% for 0401, 13.8% for 0701, 5% for 1101, and 9.7% for 1501.

**Materials and Methods**

**Cell culture.** Human umbilical vascular endothelial cells (HUVEC, kindly provided by Dr. C. Kirkpatrick, Institute of Pathology, Johannes Gutenberg University, Mainz, Germany) were cultured in endothelial cell growth medium (PromoCell) at 37°C in 5% CO2. EBV-transformed lymphoblastoid cells produced from peripheral blood mononuclear cells (PBMC) of volunteers using culture supernatant from EBV-producing B-cell line B95.8. 293 cells were obtained from the American Type Culture Collection. Lymphoblastoid and 293 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin.

**Synthetic VEGFR-2 peptides.** Nine 15-mer peptides were selected according to HLA-DR2 peptide motifs using the SYFPEITHI algorithm6 and synthesized using Fmoc/tBu strategy. They have high algorithm scores and ranking for six common HLA-DR alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*1101, and DRB1*1501; Table 1). A 26-mer polypeptide (p161-186) covering the 167-181 region of the VEGFR-2 protein was also synthesized. The purity (>90%) and identity of peptides were determined by high-performance liquid chromatography and mass spectrometry. All peptides were dissolved in DMso.

**HLA typing.** HLA-DR alleles of cancer patients and healthy donors were determined by high-resolution molecular HLA typing methods using sequence-specific oligonucleotide probes or sequence-specific priming as previously described (28).

**Generation of dendritic cells, cell lysates, and antigen loading.** PBMCs from four HLA-DR–typed healthy donors (HD168: DRB1*0401, DRB1*0101; HD274: DRB1*0401, DRB1*1101; HD773: DRB1*0301, DRB1*0701; HD492: DRB1*1501, DRB1*0101) were used to prepare dendritic cells as described (12). Briefly, CD14+ monocytes purified by magnetic cell sorting using MiniMACs (Miltenyi Biotech) were differentiated in X-Vivo 15 medium (Cambrex) containing 10% HS, 800 units/mL of recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF), and 1,000 units/mL of rhIL-4 (R&D Systems GmbH.). On day 5, nonadherent cells were harvested and matured in fresh medium containing a six-cytokine cocktail for 24 h. Lysates of HUVEC and of 293 cells transiently transfected with full-length VEGFR-2 cDNA (12) were prepared by 10 cycles of rapid freezing and thawing. Autologous dendritic cells were loaded with lysates or peptides for 24 h during maturation and washed before their use for T cell stimulation or antigen processing.

**Generation of human CD4 T cell lines and clones.** Human CD4 T cells highly enriched from PBMC by magnetic cell sorting using MiniMACs were stimulated at 10 cells in 24-well plates with 2 × 105 autologous dendritic cells previously pulsed with 15-mer peptides (5 μmol/L for each peptide) in 2 mL of culture medium consisting of RPMI 1640, 10% human AB serum, 25 mmol/L HEPES, 0.1 mmol/L MEM nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin.

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staining assays. Descriptive analysis of data obtained from intracellular cytokine nonstimulated (background) wells. The same test was also used for stimulated wells were significantly higher ($P$ appropriate mAb for 1 h at 37°C. Antibodies were purchased from BD PharMingen. FITC, followed by fluorescence-activated cell sorting analyses. All thawed, washed, and stimulated with either Cryopreserved PBMCs from cancer patients and healthy donors were imaged before (0 h) and 20 h after the cocultivation using a ZEISS (10×). T cell recognition of endogenous p167 VEGFR-2 peptide; 1 mol/L of reverse transcriptase inhibitor AZT (Sigma) to prevent T cell transduction. To clarify that antivascular effects were achieved via specific T cell recognition of endogenous p167 VEGFR-2 peptide; 1 $\times 10^5$/well of T2.DR4 cells alone or pulsed with p167 VEGFR-2 peptide (10 μM/L) were added to T cell–HUVEC cocultures. Wells were imaged before (0 h) and 20 h after the cocultivation using a ZEISS inverted fluorescence microscope. Ex vivo analysis of T cell responses by intracellular cytokine staining. Cryopreserved PBMCs from cancer patients and healthy donors were thawed, washed, and stimulated with either (a) 10 μM/L p167 VEGFR-2 peptides or (b) no peptide, as a negative control, as described elsewhere (29), in 24-well plates (2 $\times 10^5$ cells per well) in 1 mL T cell medium (day 0). Twenty-four hours later, IL-2 (10 IU/mL) and IL-7 (10 ng/mL) were added to T cell–HUVEC cocultures. Wells were imaged before (0 h) and 20 h after the cocultivation using a ZEISS inverted fluorescence microscope. Blocking of T cell responses. APC or T cells were pretreated with the appropriate mAb for 1 h at 37°C before the test. mAbs specific for HLA class I (W6/32), HLA-DR (Ii243), DP (B7/21), DQw1 (Genox3.33), and DQw3 (IVD12) were purified from culture supernatants of hybridoma from American Type Culture Collection. All antibodies did not exhibit toxic effect on cells at indicated concentrations. In vitro antivascular assays. To assess functional activities of p167 VEGFR-2–specific CD4+ T cells in a two-dimensional environment, we used a newly developed Matrigel–GFP fluorescence coupling system, as described (12). Briefly, 1 $\times 10^5$/well of enhanced green fluorescent protein (GFP) stably expressing HUVECs were preseeded overnight on Matrigel (BD Biosciences) for tube formation, and then underwent HLA-DR induction for 48 h by 80 ng/mL rhIFN-γ. After careful removal of IFN-γ, tubular structures were cocultured with T cells for 20 h at 37°C in 5% CO2 in the presence of 10 μM/L of reverse transcriptase inhibitor AZT (Sigma) to prevent T cell transduction. To clarify that antivascular effects were achieved via specific T cell recognition of endogenous p167 VEGFR-2 peptide; 1 $\times 10^5$/well of T2.DR4 cells alone or pulsed with p167 VEGFR-2 peptide (10 μM/L) were added to T cell–HUVEC cocultures. Wells were imaged before (0 h) and 20 h after the cocultivation using a ZEISS inverted fluorescence microscope.

Results

Generation and characterization of CD4+ T cells specific for VEGFR-2 peptide p167. Recent demonstration that VEGFR-2 bears CTL epitopes (12, 13) opens the door to the possibility of developing novel antitumor vessel vaccines. To prepare efficacious vaccines incorporating both CTL and CD4+ T cell epitopes, we screened HLA-DR–restricted T cell epitope from VEGFR-2 protein sequence using the SYFPEITHI algorithm (27). Nine peptides with high binding probability to six common HLA-DRB1 alleles, *0101, *0301, *0401, *0701, *1101, and *1501, were predicted and synthesized (Table 1). To streamline the discovery of potential epitopes, we grouped
nine peptides into three subpools (pool 1: peptides p9, p167, p302; pool 2: p393, p565, p711; pool 3: p762, p915, p1075). Moreover, to bypass immunologic tolerance, which is more apparent in cancer patients than in normal individuals (31), we initiated our in vitro peptide sensitization assays using cells isolated from normal healthy donors. CD4+ T cells highly enriched from four healthy, HLA-DR–typed donors were cocultured overnight in the presence of autologous PBMC alone or pulsed with p167 peptide (5 μmol/L). Culture supernatants were assessed using R&D Systems ELISA kits for IL-2 (detectable at 7–2,000 pg/mL), IL-4 (0.1–10,000 pg/mL) and IL-10 (3.9–500 pg/mL). D, peptide titration responses of HD168.F9 ( ), HD773.B3 ( ) and HD168.D5 ( ). T cell clones. p167 peptide at various concentrations was pulsed onto autologous PBMC and used as APC to stimulate T cells in IFN-γ ELISPOT assays. Bars, SE.

To further characterize p167-reactive T cells, IFN-γ–secreting cells from respective cultures were enriched by MACS separation and cloned under limiting dilution conditions. Three clones, designated HD168.F9, HD168.D5, and HD773.B3, that specifically responded to p167 peptide in a statistically significant manner (P < 0.001) were successfully established (Fig. 2A). Fluorescence-activated cell sorting analysis revealed that cloned T cell populations were CD4+ (Fig. 2B). These T cells readily produce IL-2, in addition to IFN-γ cytokine (see Fig. 2A and below), but no IL-4 and IL-10 in response to peptide stimulation (Fig. 2C), indicative of a Th1-like subset. To evaluate the avidity of these clones for p167 ligand, we tested their reactivity in peptide titration assays (Fig. 2D). HD168.F9 and HD773.B3 T cells could significantly (P < 0.01) react with p167 at 63 and 250 nmol/L peptide concentrations, respectively, whereas efficient activation of HD168.D5 clone requires >1 μmol/L peptide concentration, suggestive of low T cell receptor avidity of these cells. Altogether, these results suggest that p167 VEGFR-2 peptide is a potential HLA-DR–restricted epitope that can stimulate Th1-like CD4+ T cells with high avidity of T cell receptor from healthy donors.

p167 VEGFR-2 peptide is recognized by CD4+ T cells in the context of HLA-DR4 in donor HD168 or HLA-DR7 in donor HD773. To identify HLA-DR allele used by p167-specific CD4+ T cells from HD168 and HD773 donors, we tested T cell reactivity with a series of allogeneic APC that share partial HLA-DR identity to HD168 (DRB1*0401, DRB1*0101) or to HD773 (DRB1*0301, DRB1*0701). In these experiments and hereafter, CD4+ T cell clones instead of cell lines were used to preclude any unspecific effect caused by reaction against peptide(s) other than p167 peptide presented by the APC. As shown in Table 2, HD168.F9 and HD168.D5 T cells efficiently recognized three donor APC that express DRB1*0401 but not HLA-DRB1*0101, whereas in cases of three donors expressing HLA-DRB1*0101 but not DRB1*0401, no recognition was observed. This shows DRB1*0401 as a presenting molecule in case of HD168.F9 and HD168.D5 clones. For HD773.B3 T cells, efficient recognition was obtained when three donors shared HLA-DRB1*0701 but not DRB1*0301, suggesting that
after processing. In contrast, no T cell reactivity was detected in clones, thus suggesting HLA-DR restriction of p167 epitope (Fig. 3C). Pretreatment of HUVECs with the addition of anti–HLA-DR antibodies inhibited recognition by HD168.F9 and HD773.B3, suggesting that HLA-DR restriction of p167 epitope after processing. In contrast, no T cell reactivity was detected with HLA-mismatched control HUVEC targets (Fig. 3B-C).

Because dendritic cells are the potent initiator of CD4+ T cell immunity and thus of high relevance for an in vivo CD4+ T cell response to VEGFR-2, we examined their capacity to present p167 epitope. Figure 3D showed that autologous dendritic cells from HD168 and HD773 efficiently presented lysates of VEGFR-2+ HUVEC or of 293 cells transiently transfected with the full-length VEGFR-2 cDNA to specific CD4+ T cells. The results presented above identify p167 VEGFR-2 peptide as a potential CD4+ T cell epitope and show that T cells with low to intermediate affinity for HLA-DR4 or HLA-DR7, as expected. Importantly, five

### Table 2. Mapping of HLA restriction element used by HD168 and HD773-derived CD4+ T-cell clones in response to p167 peptide

<table>
<thead>
<tr>
<th>T-cell clones</th>
<th>APC</th>
<th>HLA-DRB1</th>
<th>p167 peptide recognition</th>
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<tr>
<td>HD168.H9</td>
<td>HD168.D5</td>
<td>HD557</td>
<td>0401, 1401</td>
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<td>HD813</td>
<td>HD560</td>
<td>HD639</td>
<td>0101, 1302</td>
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<td>HD645</td>
<td>HD645</td>
<td>HD645</td>
<td>0101, 0901</td>
</tr>
<tr>
<td>HD530</td>
<td>HD530</td>
<td>HD530</td>
<td>0101, 0901</td>
</tr>
<tr>
<td>HD846</td>
<td>HD846</td>
<td>HD846</td>
<td>1601, 0301</td>
</tr>
<tr>
<td>HD811</td>
<td>HD811</td>
<td>HD811</td>
<td>0803, 0301</td>
</tr>
<tr>
<td>HD542</td>
<td>HD542</td>
<td>HD542</td>
<td>0701, 1301</td>
</tr>
<tr>
<td>HD632</td>
<td>HD632</td>
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<tr>
<td>HD425</td>
<td>HD425</td>
<td>HD425</td>
<td>0701, 0101</td>
</tr>
</tbody>
</table>

**DRB1*0701** is a restricting element for these T cells. Thus, p167 epitope represents a “promiscuous” epitope, as similarly described for many antigenic CD4+ T cell epitopes (32, 33).

**p167 VEGFR-2 peptide is naturally processed and presented to specific CD4+ T cells.** To assess whether the T cell epitope recognized by p167-reactive T cells was naturally presented by mitotic endothelial cells, we selected several primary HUVECs as targets for T cell recognition. Such targets were chosen because they have been widely used in the field of in vitro antangiogenesis research, positive for VEGFR-2 protein (12), and could express detectable level of HLA-DR upon IFN-γ treatment (ref. 34; Fig. 3A). Figure 3B showed that IFN-γ–treated HUVEC196 and HUVEC197 targets, which were phenotypically positive for DR4 expression following IFN-γ treatment, were significantly recognized (P < 0.001) by the HD168.F9 T cells both in IFN-γ–ELISPOT and in GM-CSF ELISA assays. T cell recognition was further extended to a certain extent when p167 peptide was added exogenously to HUVEC cultures. Similar results were obtained with clone HD773.B3 when HUVEC114-expressing HLA-DR7 were used as targets (Fig. 3C). Pretreatment of HUVECs with anti–HLA-DR antibody inhibited recognition by HD168.F9 and HD773.B3 clones, thus suggesting HLA-DR restriction of p167 epitope after processing. In contrast, no T cell reactivity was detected with HLA-mismatched control HUVEC targets (Fig. 3B-C).

To verify that the above T cell–mediated antivascular effect was indeed achieved through specific recognition of endogenously processed and presented p167 VEGFR-2 peptide, we in parallel run the T cell–tubular network cocultivation in the presence of excess numbers of p167 peptide-pulsed T2.DR4 cells. This assay condition mimics the traditional “cold” target inhibition assay and should allow to interfere with T cell recognition of endogenously presented antigens. As expected, the destruction of tubular networks by HD168.F9 T cells was markedly reduced when p167-pulsed T2.DR4 cells were present in cocultures (Fig. 3E, g, h), hence confirming that p167 peptide can be naturally processed and form a CD4+ T cell epitope on proliferating endothelial cells. However, no such inhibitory effect was observed for T2.DR4 alone (Fig. 3E, e, f).

**Detection of p167 VEGFR-2–specific CD4+ T cells in different cancer patients and healthy donors.** The results presented above identify p167 VEGFR-2 peptide as a potential CD4+ T cell epitope and show that T cells with low to intermediate affinity to this peptide and able to recognize antigen-expressing endothelial cells can be readily generated from two healthy donors who did not show any signs of autoimmunity. Nonetheless, its usefulness as an anticancer vaccine requires further investigation, because there is a concern that the anti–VEGFR-2 CD4+ T cells might have been anergized or deleted in cancer patients due to antigen-specific tolerance induced by tumors (31). Therefore, we consequently examined T cell responsiveness to p167 peptide using PBMCs of a series of 40 cancer patients, characterized by a late stage (III or IV) of the disease in most cases and not yet receiving IFN therapies (Table 3). Our initial analysis included 30 patients affected either by breast cancer, non–small cell lung carcinoma, or by colon carcinoma. This patient cohort was randomly selected, e.g., without DRB1 typing of PBMC before their analysis, because we cannot exclude that DRB1 gene products other than DR4 and DR7 may present our promiscuous epitope. After a single cycle of in vitro stimulation with p167, specific CD4+ T cells were readily detectable in 10 patients, as assessed by intracellular staining with IFN-γ and CD4-specific mAbs (Fig. 4A; Table 3). Responders included 3 non–small cell lung carcinoma, 2 colon carcinoma, and 5 breast cancer patients, with frequencies of specific CD4+ T cells in a range from 0.04% to 0.65% among patient blood CD4+ T cells. Subsequent DRB1 typing of these responders revealed them to be positive for HLA-DR4 or HLA-DR7, as expected.
responders were also DR4 or DR7 negative who shared either of DRB1*1501, DRB1*0318, or DRB1*1104, suggesting that the epitope could also be presented by at least one of these alleles to specific CD4+ T cells for recognition. To further expand our findings, we proceeded with 10 melanoma patients whose DRB1-typed PBMCs (DRB1*0101 or DRB1*0401) were available. Two additional responders, showing a specific T cell frequency of 0.18% and 0.31%, respectively, were evidenced in this cohort of patients. The overall prevalence of p167 VEGFR-2–specific T cells in patients of respective tumor types was similar, corresponding to a range of 20% to 50% of positivity in 30 nonmelanoma patients and 33% in DR4-expressing melanoma patients.

However, it is remarkable that female responders dominated, accounting to >91% (11 of 12) of the whole responding patients. A comparison of male and female patients in prior therapies that may influence VEGFR-2 expression (e.g., VEGF/VEGFR-2–targeted therapy) revealed no major difference between them. In addition, all responders, including the patient 27 who had the strongest T cell reactivity (0.65%), did not develop overt signs of autoimmune pathology.

Fig. 3. Natural processing and presentation of VEGFR-2 to p167 peptide–specific CD4+ T cells. A, HLA-DR surface expression by IFN-γ–treated HUVECs (80 ng/mL, 72 h) was assessed using HLA-DR–specific mAb. A representative result from HUVEC196 is shown. B, recognition of HUVEC cells pulsed with (●) or without (□) exogenous p167 peptide by HD168.F9 T cells was assessed in IFN-γ ELISPOT assay and GM-CSF ELISA. After IFN-γ treatment, HUVECs were extensively washed and then cocultured (3 x 10⁶/well) overnight with T cells (5,000 per well). For GM-CSF measurement, 3 x 10⁵ T cells per well were used. C, recognition was similarly assessed using HD773.B3 CD4+ T cells. T cell recognition was inhibited by anti-DR mAb (not shown) and could not be detected on HUVEC192 (DR4-, DR7-) cells. D, HD168.F9 and HD773.B3 T cells recognized autologous dendritic cells loaded with the native antigen. Autologous dendritic cells (DR4+ or DR7+) were incubated with lysates of 293 cells transiently transfected with full-length VEGFR-2 cDNA (12) or of HUVEC196 at dendritic to lysed cell ratio of 1:3 for 24 h. After washing, loaded dendritic cells were used (1 x 10⁶/well) as APCs in IFN-γ ELISPOT assays. T cell recognition was similarly assessed using a 26-mer VEGFR-2 polypeptide p161-186 at 20 μmol/L as a source of native antigen. Bars, SE. E, HLA-DR–restricted in vitro antivascular activity of HD168.F9 T cells. T cells to d, capillary tubes formed by 10⁶ enhanced GFP–expressing HUVEC197 were pretreated for 2 d with IFN for HLA-DR induction and were then incubated with 10⁴ HD168.F9 CD4+ T cells for 20 h in the absence (a, b) or presence (c, d) of 15 μg/mL L243 mAb and 10 μmol/L AZT, e to h, co-incubation was run exactly as above but in the presence of 1 x 10⁷ T2.DR4 alone (e, f) or pulsed with 10 μmol/L exogenous p167 VEGFR-2 peptide (g, h). It is of note that destruction of fluorescent tubular structures was evidenced once HD168.F9 T cells were present, and this effect was largely compromised by the excess amount of exogenous p167 VEGFR-2 peptide.
To get insight into the status of peripheral immune tolerance against the p167 VEGFR-2 peptide in healthy donors, we tried to estimate T cell reactivity using blood of 10 additional HLA-DR4–typed healthy donors. Four of 10 tested healthy donors were found to contain p167-specific CD4+ T cells (Table 3). In this donor cohort, more female than male responders were again observed, without any signs of autoimmunity.

Table 3. Assessment of p167 peptide-specific CD4+ T cells in healthy donors and cancer patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Gender</th>
<th>Age (y)</th>
<th>Tumor types</th>
<th>Stage of diseases</th>
<th>DRB1 subtype</th>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>66</td>
<td>Breast</td>
<td>IV</td>
<td>nd</td>
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<tr>
<td>2</td>
<td>F</td>
<td>40</td>
<td>Colon</td>
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<td>Colon</td>
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Healthy donors

| HD557 | F | 32 | 0401 | 0.04 | 0.20 | 0.16 |
| HD813 | F | 28 | 0401 | 0.03 | 0.30 | 0.27 |
| HD742 | F | 22 | 0401 | 0.01 | 0.02 | 0.01 |
| HD946 | F | 30 | 0401 | 0.03 | 0.12 | 0.09 |
| HD599 | F | 26 | 0401 | 0.06 | 0.07 | 0.07 |
| HD730 | M | 27 | 0401 | 0.03 | 0.03 | 0    |
| HD631 | M | 45 | 0401 | 0.06 | 0.05 | -0.01 |
| HD727 | M | 30 | 0401 | 0.02 | 0.17 | 0.15 |
| HD593 | M | 21 | 0401 | 0.03 | 0.05 | 0.07 |
| HD688 | M | 25 | 0401 | 0.04 | 0.04 | 0    |

NOTE: No patients received IFN therapies before analysis. Before analysis, patients 3 and 16 received bevacizumab (anti-VEGF) therapy, whereas 8 other patients (nos. 4, 18, 20, 22, 23, and 26-28) received anti-EGF receptor targeted therapies using either herceptin, erlotinib, or cetuximab.

Abbreviations: NSCLC, non–small-cell lung cancer. MM, malignant melanoma; nd, not determined.

*Analyzed in intracellular IFN-γ staining assay using cryopreserved PBMCs.

Values in boldface are considered as positive: percentage of IFN-γ–producing CD4+ cells was >2-fold higher compared with negative control without peptide (-Pep). These values also attained statistical significance (P < 0.05) as assessed by one-sided Student’s t test. Underlined values are actual percentages of specific CD4+ cells after single peptide stimulation in vitro.

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Cancer Research. www.aacrjournals.org Clin Cancer Res 2008;14(13) July 1, 2008 4312
isolated specific CD4+ T cells from peptide-stimulated PBMC of (i.e., able to react against proliferating endothelial cells), we specific for p167 VEGFR-2 peptide were functionally active mitotic endothelial cells.

two cancer patients, nos. 27 and 11, and tested them in IFN-

anti-VEGFR-2^ specific CD4+ T cells among p167 peptide^ stimulated PBMCs bars, SE. representaitiveactual ELISPOT resultsshown indiagram. Recognition of target assay. Before the testing, HUVECswerepretreated as inFig.3A. Bottom, representative actual ELISPOT results shown in diagram. Recognition of target cells by the patient’s T cells was blocked by anti - HLA-DR antibody. Bars, SE.

Fig. 4. Assessment of VEGFR-2–specific CD4+ T cells among circulating lymphocytes of cancer patients and healthy donors. A, the presence of anti-VEGFR-2–specific CD4+ T cells among p167 peptide–stimulated PBMCs was assessed by intracellular staining with IFN-γ and CD4-specific mAbs. Shown are representative results obtained from patients 11 and 27. Numbers in the upper right quadrants are the percentage of cytochrome-producing cells among CD4+ T cells. B, CD4+ T cells isolated from patients 11 and 27 efficiently recognize proliferating endothelial cells in HLA-DR–restricted manner. p167-specific CD4+ T cells were isolated after one-round peptide stimulation and then enriched using Miltenyi Biotec IFNγ Secretion Assay Detection Kit. At 96 h after isolation, their reactivity against HUVEC cells (HUVEC106, DR4+; HUVEC102, DR4-DR7; HUVEC114, DR7+; HUVEC192, DR4 DR7) was assessed in the presence (△) or absence (●) of L243 antibody in IFN-γ ELISPOT assay. Before the testing, HUVECs were pretreated as in Fig. 3A. Bottom, representative actual ELISPOT results shown in diagram. Recognition of target cells by the patient’s T cells was blocked by anti – HLA-DR antibody. Bars, SE.

Reactivity of patient’s VEGFR-2–specific CD4+ T cells against mitotic endothelial cells. To assess whether patient’s T cells specific for p167 VEGFR-2 peptide were functionally active (i.e., able to react against proliferating endothelial cells), we isolated specific CD4+ T cells from peptide-stimulated PBMC of two cancer patients, nos. 27 and 11, and tested them in IFN-γ ELISPOT assays. Figure 4B shows that both patient’s T cells efficiently recognized HUVEC targets. The T cell response was completely blocked by HLA-DR antibody, suggesting that these T cells are HLA-DR restricted. In contrast, no recognition was observed using HLA-mismatched targets.

Discussion

The data presented here support three new findings. First, the VEGFR-2 167-181 peptide sequence is a natural epitope for human CD4+ T cells; it displays promiscuity in the HLA restriction and can be presented by several HLA-DR alleles, including HLA-DR4 and HLA-DR7. Second, this epitope remains potentially immunogenic in cancer patients, in addition that the relevant T cells are present in healthy donors. Third, the patient’s anti-VEGFR2 167-181 –specific CD4+ T cells could still be functionally active as regards IFN-γ production upon antigen-specific recognition. To our best knowledge, this is the first study to systemically examine specific CD4+ T cell reactivity against a human tumor neovessel-associated antigen.

Until now, most studies characterizing human tumor stroma–associated antigens or antigenic peptides have concentrated on class I HLA–restricted CTL responses (12–15). Moreover, immune characterization was mainly limited to healthy volunteers, thus making it largely unclear regarding actual utility of these antigens for cancer immunotherapy. These studies showed that VEGFR-1, VEGFR-2, and fibroblast activation protein harbor antigenic peptides that can stimulate CD8+ T cell responses of healthy donors. The work by Wada et al. (13) also showed that anti–VEGFR-2 CTLs could be detected in cancer patients. However, the possibility of this antigen to significantly trigger specific CD4+ T cell responses was not examined. This might be due to the fact that antigen-specific CD8+ CTLs can recognize and kill their targets and are thus regarded as main effector arm of adaptive immunity to tumors. However, it has been increasingly clear that integrated CD8+ and CD4+ T cell responses are essential for efficient antitumor immune responses to occur in vivo (22–25, 35, 36). Therefore, because of the importance of incorporating CD4+ T cell epitopes into antitumor vaccines, the identification of MHC class II–restricted, tumor neovessel-associated antigen recognized by specific CD4+ T cells in association with frequently expressed class II alleles is presently of great interest.

In this study, we focused on T cell epitopes capable to promiscuously bind to six common HLA-DR alleles, which were shown to possess a largely overlapping peptide repertoire (26). Antigen presentation assays showing fine HLA-DR4 and HLA-DR7 restriction of VEGFR-2 167-181–specific T cells initially led us to conclude that this epitope is presented by HLA-DR4 and HLA-DR7. However, our ability to detect specific CD4+ T cells in several DR4- or DR7-negative cancer patients in subsequent experiments strongly suggests that other class II HLA alleles (i.e., DRB1*1501, DRB1*1104, or DRB1*0318) may efficiently present the 167-181 VEGFR-2 peptide as well. According to our SYFPEITHI data, DR4, DR7, and DRB1*1501 alleles displayed similar scores and ranking with respect to binding to the identified epitope, whereas binding scores for DRB1*1104 and DRB1*0318 were not covered by SYFPEITHI algorithm. Four peptide-presenting HLA-DR alleles described here together cover >40% of Caucasian population, implying that 167-181 VEGFR-2 peptide may be broadly applicable in
the development of VEGFR-2–based antitumor neovessel vaccines.

Our ex vivo T cell analysis provides first insight into natural immunogenicity status of the VEGFR-2 167-181 peptide in different cancer patients. The finding of ~33% of cancer patients containing specific CD4+ T cells was considered quite impressive, given that most patients analyzed were at the late stages (III or IV) of the diseases and that the analysis was based on 10-14 day T cell culture. Short-term in vitro expansion (not >2 weeks) usually does not generate specific T cells from naïve precursors (37). Hence, the immunogenicity discussed herein should be referred to as a memory T cell response. The present study extends previous observations on the occurrence of natural T cell immunity to conventional tumor-associated antigens in various human malignancies, including melanoma, colorectal cancer, leukemia, and breast cancer (37). However, we were unable to analyze the phenotype of these specific T cells due to their rarity in patient’s blood. In addition, the in vivo helper function of these cells in generating and/or maintenance of an optimal CTL response to VEGFR-2 remains to be determined. We were also aware that our analysis was of descriptive nature, which represents drawback of the experiment. However, our demonstration that VEGFR-2 167-181 peptide is immunogenic in cancer patients is a key prerequisite for its future clinical application in vaccine development or immune monitoring, e.g., by comparing frequencies of circulating specific T cells before and after vaccination, using validated functional (intracellular cytokine staining, ELISPOT) and/or nonfunctional (e.g., tetramer) assays.

Furthermore, we show that the occurrence of VEGFR-2 167-181–specific T cells in cancer patients is tumor type independent. This finding is consistent with overexpression of VEGFR-2 molecule in a wide range of human cancers (16, 19, 21). Strikingly surprising, however, is the broader T cell response to VEGFR-2 in females than in males. Reasons for this female preponderance are presently unknown. Prior therapies like IFN treatment or VEGF/VEGFR–targeted treatment, which may influence expressions of the MHC class II and VEGFR-2, did not seem to play a role in the observed difference. We cannot rule out that our patients may differ in general immunosuppressive status. Alternatively, it is tempting to speculate that periodic and decade-long menstruation exclusively owned by the females may contribute to this phenomenon. To clarify this issue, further studies are required, for example, using a large cohort of patients or analyzing VEGFR-2–specific CD4+ T cells that also include specific naïve precursors.

We also noticed that anti–VEGFR-2 CD4+ T cells were readily isolated from peripheral blood of healthy donors. The overall size of T cell reactivity seems to be larger in healthy individuals than in some patients. Importantly, broad T cell reactivity was not associated with apparent signs of autoimmunity. This may be due to lack of HLA class II and/or low antigen expression level on resting mature vasculatures, or low to intermediate affinity of T cells to their antigen, or both. In fact, significant activation of VEGFR-2 167-181–specific T cells was only seen when using peptide concentrations of >63 to 250 nmol/L. Our findings are, to some extent, reminiscent of a recent study on CD4+ T cell responses to the ubiquitously expressed HMW-melanoma–associated antigen (38), where strong and broad T cell reactivity to this antigen was seen more frequently in normal individuals than in melanoma patients in the absence of autoimmunity. Given the crucial role played by VEGFR-2 and its ligand in the physiopathologic angiogenic process and associated diseases, such as cancer and noncancer disorders (39, 40), we hypothesize that sizable T cells reactive to VEGFR-2 circulating in the blood of normal individuals might have physiologic functions, for instance, by overseeing proliferative endothelial cells and thus contributing to the timely and sufficient controlling of excessive angiogenesis.

Cancer immunotherapy with tumor neovessel-directed vaccines differs from conventional vaccine approaches, with the former less likely to be affected by tumor escape mechanisms, such as loss of antigen expression and poor infiltration of T cells in tumor tissues (1, 41, 42). Because tumor endothelial cells are genetically stable and have direct contact to circulating effector T cells, effective immunotherapeutic protocols directed toward tumor neovessels may deliver more sustained therapeutic benefits than approaches targeting tumor cells. On the other hand, VEGFR-2–based vaccine therapy has also several advantages over conventional targeted antiangiogenic therapy using monoclonal antibodies or inhibitors of tyrosine kinases. For example, immune recognition and CTL elimination of VEGFR-2+ tumor endothelium is not affected by the presence of other products with redundant functions, thereby being more effective at limiting resistance. In addition, inducting an effective memory response by vaccine therapy would mediate long-term therapeutic effects while omitting livelong and repeated administration of high doses of pharmacologic agents, as is required for conventional antiangiogenic therapy (16, 21, 43, 44). Some of these principles have already been proven in a variety of mouse models (2–4, 6). However, the feasibility of using VEGFR-2–based cancer vaccines can only be addressed in the clinical setting.

In conclusion, we identified a VEGFR-2 peptide that is promiscuously presented by multiple HLA-DR alleles and capable of stimulating CD4+ T cells of different cancer patients. This represents the first characterization of a tumor neovessel-associated epitope with potential immunogenicity in various cancer patients. Our data likely have significant contribution to the development of efficacious tumor neovessel-directed vaccines based on VEGFR-2 immunogens.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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References


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Human CD4+ T Lymphocytes Recognize a Vascular Endothelial Growth Factor Receptor-2–Derived Epitope in Association with HLA-DR

Yuansheng Sun, Mingxia Song, Elke Jäger, et al.

Clin Cancer Res 2008;14:4306-4315.

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