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EphA2 as Target of Anticancer Immunotherapy: Identification of HLA-A*0201-Restricted Epitopes

Pedro M. S. Alves,[1] Olivier Faure,[1,3] Stéphanie Graff-Dubois,[1] David-Alexandre Gross,[1] Sébastien Cornet,[1] Salem Chouaib,[1] Isabelle Miconnet,[1] François A. Lemonnier,[2] and Kostas Kosmatopoulos[1]

[1]INSERM487, Institut Gustave Roussy, Villejuif; [2]Unité d'Immunité Cellulaire Antivirale, Institut Pasteur, Paris; and [3]Immuno-Designed Molecules, Paris, France

ABSTRACT

EphA2 (Eck) is a tyrosine kinase receptor that is overexpressed in several human cancers such as breast, colon, lung, prostate, gastric carcinoma, and metastatic melanoma but not in nonmalignant counterparts. To validate EphA2 as a tumor antigen recognized by CD8+ T lymphocytes, we used reverse immunology approach to identify HLA-A*0201-restricted epitopes. Peptides bearing the HLA-A*0201-specific anchor motifs were analyzed for their capacity to bind and stabilize the HLA-A*0201 molecules. Two peptides, $EphA2_{58}$ and $EphA2_{550}$, with a high affinity for HLA-A*0201 were selected. Both peptides were immunogenic in the HLA-A*0201-transgenic HHD mice. Interestingly, peptide-specific murine CTLs cell lines responded to COS-7 cells coexpressing HLA-A*0201 and EphA2 and to EphA2-positive human tumor cells of various origin (renal cell, lung, and colon carcinoma and sarcoma). This demonstrates that $EphA2_{58}$ and $EphA2_{550}$ are naturally processed from endogenous EphA2. In addition, $EphA2_{58}$ and $EphA2_{550}$ stimulated specific $CD8^+$ T cells from healthy donor peripheral blood mononuclear cells. These T cells recognized EphA2-positive human tumor cells in an HLA-A*0201-restricted manner. Interestingly, EphA2-specific CD8+ T cells were detected in the peripheral blood mononuclear cells of prostate cancer patients. These results show for the first time that EphA2 is a tumor rejection antigen and lead us to propose $EphA2_{58}$ and $EphA2_{550}$ peptides for a broad-spectrum-tumor immunotherapy.

INTRODUCTION

The clinical responses observed in some patients included in tumor immunotherapy trials encouraged carrying on efforts to identify TAgs[4] and improving their vaccine potential (1–4). Numerous TAgs have been described thus far, particularly in melanoma (5). However, the restricted pattern of expression of most of these antigens limits their clinical use. It is imperative, therefore, to evaluate TAgs that are widely expressed in cancer (universal TAgs) to develop clinical immune interventions for most cancer patients regardless of their tumor type. The recently identified hTERT and Survivin antigens fit this profile of universal TAgs (6–8).

EphA2 (Eck) belongs to the Eph receptors family. The Eph family contains at least 14 members that are type I transmembrane glycoproteins (9). Upon cell-cell contact, Eph receptors interact with their ligands called ephrins. Glycosylphosphatidyl inositol-linked ephrin-A ligands bind to EphA receptors and transmembrane ephrin-B ligands bind to EphB receptors (9, 10). Eph-ephrin signaling mediates contact-dependent cell interactions involved in the spatial organization of neurons and neural crest cells, embryogenesis, tissue patterning, and blood vessels formation (9, 10).

Strikingly, EphA2 is overexpressed in several human cancers such as breast, colon, lung, prostate, gastric carcinoma, and metastatic melanoma but not in nonmalignant breast, prostate, and skin lesions (11–15). EphA2 overexpression correlates with malignant transformation (14), poor prognosis (16, 17), metastatic progression (18), and p53-mediated apoptosis (19). Interestingly, EphA2 has been detected in tumor neovasculature and was demonstrated to play a role in tumor angiogenesis (20–22). The large number of human cancers expressing the antigen and the association with tumor vascularization and metastatization prompt EphA2 as an appealing target for tumor immunotherapy.

In this article, we show that EphA2 is target of antitumor $CD8^+$ T lymphocytes, and we describe two HLA-A*0201-restricted epitopes that induce specific $CD8^+$ T cells able to recognize tumors of various origins.

MATERIALS AND METHODS

Mice. The HLA-A*0201-transgenic HHD mice were described previously (23). They are $\beta 2m^{-/-}$ and $D^{b-/-}$ and express an HLA-A*0201 monochain composed of a chimeric heavy chain ($\alpha 1$ and $\alpha 2$ domains of HLA-A*0201 allele and the $\alpha 3$ and intracellular domains of D^b allele) linked by its NH_2 terminus to the COOH terminus of the human $\beta 2m$ by a 15-amino acid peptide arm.

Peptides and Plasmids. $EphA2_{58}$ (IMNDMPIYM) and $EphA2_{550}$ (VLAGVGFFI) peptides were synthesized by Synt:em (Nîmes, France). The EphA2 cDNA was kindly provided by Dr. Elena Pasquale (The Burnham Institute, La Jolla, CA). The HHD construct was described previously (23).

Cells and Peptides. The murine RMAS/HHD cells were described previously (23). The HLA-A*0201-expressing human tumor cells were: T2 (deficient in TAP1 and TAP2 transporters), MCF-7 (breast cancer), CACO-2 (colon cancer), 1355 (lung cancer), and M113 (melanoma) kindly provided by Dr. Francine Jotereau (Nantes, France); SAOS (sarcoma) and HIEG (renal cell carcinoma) kindly provided by Dr. Eric Angevin (Villejuif, France); and LNCaP and DU-145 (prostate cancer). Cells were grown in RPMI 1640 (Invitrogen, Paisley, United Kingdom) supplemented with 10% FCS.

Measurement of Peptide RA to HLA-A*0201. The protocol used has been described previously (24). Briefly, T2 cells were incubated with various concentrations of peptides ranging from 100 to 0.1 μM overnight and then stained with BB7.2 mAb (25) to quantify the surface expression of HLA-A*0201 allele. For each peptide concentration, the HLA-A*0201-specific staining was calculated as the percentage of staining obtained with 100 μM of the reference peptide $HIVpol_{589}$ (IVGAETFYV). The RA was determined as: RA = (concentration of each peptide that induces 20% of HLA-A*0201 expression/concentration of the reference peptide that induces 20% of HLA-A*0201 expression).

Assessment of Peptide/HLA-A*0201 Complex Stability. As previously described (24), briefly T2 cells were incubated overnight with 100 μM of each peptide. Cells were then incubated with Brefeldin A (Sigma, St. Louis, MO) at 10 μg/ml for 1 h, washed, incubated at 37°C during 0, 2, 4, or 6 h in the presence of Brefeldin A (0.5 μg/ml), and then stained with BB7.2 mAb. For each time point, peptide induced HLA-A*0201 expression was calculated as: mean fluorescence of peptide preincubated T2 cells − mean fluorescence of T2 cells treated in similar conditions in the absence of peptide. DC_{50} was

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Requests for reprints: Pedro Alves, INSERM U487, Institut Gustave Roussy, PR1, 39 rue Camille Desmoulins, 94805 Villejuif, France. Phone: 33-0-142114921; Fax: 33-0-142115288; E-mail: kostas@igr.fr.

[4] The abbreviations used are: TAg, tumor antigen; hTERT, human telomerase reverse transcriptase; $\beta 2m$, $\beta 2$ microglobulin; mAb, monoclonal antibody; IL, interleukin; RA, relative affinity; PBMC, peripheral blood mononuclear cell; TNF-α, tumor necrosis factor α.

defined as the time required for the loss of 50% of the HLA-A*0201/peptide complexes stabilized at $t = 0$.

Western Blot Analysis of EphA2 Expression by Tumor Cells. Cellular samples were lysed with lysis buffer [150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 0.1% SDS, and 0.5% NP40] supplemented with Complete Protease Inhibitor mixture (Boehringer Mannheim, Mannheim, Germany). Fifty μg of protein were loaded on 10% SDS-Polyacrilamide Gel Electrophoreses (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membrane (Amersham Pharmacia, Buckinghamshire, United Kingdom). Detection of EphA2 was done with rabbit polyclonal anti-EphA2 antibody (sc-924; Santa Cruz Biotechnology, Santa Cruz, CA) and goat-antirabbit IgG, (H+L) peroxidase conjugated (Pierce, Rockford, IL). The housekeeping protein, actin, was detected using goat polyclonal antiactin antibody (sc-1615)-horseradish peroxidase (Santa Cruz Biotechnology). Membranes were then reveled with enhanced chemiluminescence kit (Amersham Pharmacia) and exposed to photosensitive film (Amersham Pharmacia).

Generation of CTL in HHD Mice. HHD mice received s.c. injections of 100 μg of peptide emulsified in incomplete Freund's adjuvant in the presence of 140 μg of the I-A^b-restricted HBVcore$_{128}$ (TPPAYRPPNAPIL) T-helper epitope. Eleven days later, 5×10^7 spleen cells were stimulated *in vitro* with peptide (10 μM). On day 6 of culture, the bulk populations were tested for specific cytotoxicity. Upon response, CTL lines were established by weekly *in vitro* restimulations with 2×10^7-irradiated spleen cells in the presence of 1–0.1 μM peptide and 50 IU/ml IL-2 (Proleukin; Chiron Corp. Emeryville, CA).

Generation of CTL from Human PBMCs. PBMCs were collected by leukapheresis from healthy HLA-A*0201 volunteers. Dendritic cells were produced from adherent cells (2×10^6 cells/ml) cultured for 7 days in the presence of 500 IU/ml granulocyte macrophage colony-stimulating factor (Leucomax; Schering-Plough, Kenilworth, NJ) and 500 IU/ml IL-4 (R&D Systems, Minneapolis, MN) in complete medium [RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 2 μM L-glutamine (Invitrogen) and antibiotics]. On day 7, dendritic cells were collected and pulsed with 40 μg/ml peptide in the presence of 3 μg/ml β2m (Sigma) for 4 h at 20°C and then irradiated (4200 rad). CD8$^+$ T cells were isolated by positive selection with immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer instructions. A total of 0.5×10^6 CD8$^+$ T cells were cocultured with 0.25×10^5 dendritic cells in a final volume of 0.5 ml/well in a 48-well plate in the presence of 10 ng/ml IL-7 (R&D Systems). Human IL-10 (R&D Systems) at 10 ng/ml was added the next day, and 30 IU/ml human IL-2 (Proleukin; Chiron Corp.) were added on day 2. Seven and 14 days after the primary stimulation, CD8$^+$ T cells were restimulated with irradiated adherent cells pulsed with 10 μg/ml peptide in the presence of 3 μg/ml β2m. Human IL-10 (10 ng/ml) and IL-2 (50 IU/ml) were added 24 and 48 h later, respectively. Seven days after the second restimulation individual wells from the cultures were tested for peptide specific cytotoxicity on peptide loaded T2 cells in the presence of cold K562 cells (hot/cold target ratio 1:33 ratio). Cells from positive wells (>10% peptide specific lysis) were tested for intracellular IFN-γ production upon tumor cell stimulation.

Cytotoxic Assay. Targets were labeled with 100 μCi of ^{51}Cr for 60 min, plated in 96-well V-bottomed plates (3×10^3 cell/well) and pulsed with peptides (1 μM) at 37°C for 2 h. Effectors were then added and incubated at 37°C for 4 h. One hundred μl of supernatant were collected and radioactivity measured in a gamma counter. Percentage of specific lysis was determined as: (experimental release − spontaneous release)/(maximal release − Spontaneous Release) × 100.

Peptide Processing Assay on Transfected COS Cells. A total of 2.2×10^4 simian COS-7 cells were plated in flat-bottomed 96-well plates in DMEM (Invitrogen) 10% FCS. Eighteen h later, cells were transfected with 100 ng of DNA plasmid with DEAE Dextran (Sigma). After 4 h, PBS +10% DMSO were added for 2 min. Transfected COS cells were incubated in DMEM +10% FCS during 40 h and then used to stimulate murine CTL in a TNF-α secretion assay.

TNF-α Secretion Assay. Transfected COS cells at day 4 or 10^5 tumor cells were used as stimulating cells. They were plated in 50 μl of RPMI +10% FCS and, when necessary, incubated with 10 μM peptide for 2 h at 37°C. A total of 5×10^4 T cells were then added in 50 μl of RPMI +10% FCS and incubated for 6 h. Each condition was tested in triplicates. Fifty μl of the supernatant were collected for quantification of TNF-α using the WEHI-164c13 cells as described previously (26).

Intracellular Detection of IFN-γ. A total of 5×10^4 T cells were incubated with 10^5 peptide loaded T2 cells or with 10^5 tumor cells in the presence of 20 μg/ml Brefeldin A at 37°C. Six h later, they were stained with phycoerythrin-conjugated anti-CD8 mAb (Caltag Laboratories, Burlingame, CA) in PBS for 25 min at 4°C and fixed with PBS 4% Paraformaldeide (Sigma). Cells were then permeabilized with PBS +0.5% BSA +0.2% saponin (Sigma) and stained with adenomatous polyposis coli-conjugated anti-IFN-γ mAb (PharMingen, Mississauga, Ontario, Canada) for 25 min at 4°C. Cells were analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA) flow cytometer.

Cancer Patient PBMC Restimulation. PBMCs were incubated in a 96 U-bottomed plate (2×10^5 cells/well) in the presence of 10 μM peptide and 20 IU/ml IL-2 in AIM-V medium (Invitrogen). Half of the media was changed every other day with fresh one containing IL-2 at 20 IU/ml. At day 9 of culture, pools of 12 wells were tested for the presence of peptide-specific CD8$^+$ T cells by staining with the appropriate HLA-A*0201/peptide tetramer.

HLA-A*0201/Peptide Tetramer Staining of PBMCs. Phycoerythrin-conjugated HLA-A*0201/peptide tetramers were synthesized by ProImmune (Oxford, United Kingdom). Cells were stained with tetramers (5 ng/ml) in PBS +0.5% human AB serum for 1 h at room temperature, washed once in the same buffer, stained with antihuman CD3-FITC (Caltag Laboratories) and antihuman CD8-APC (PharMingen) mAbs for 30 min at 4°C followed by flow cytometry acquisition.

RESULTS

Affinity of EphA2 Peptides for HLA-A*0201. To identify HLA-A*0201-restricted EphA2 epitopes that could trigger antitumor CTL responses, we screened the EphA2 sequence for peptides with high affinity for HLA-A*0201 using the BioInformatics and Molecular Analysis Section (BIMAS) prediction software (26). We selected two peptides, EphA2$_{58}$ and EphA2$_{550}$, with high predicted binding score and evaluated their capacity to bind to HLA-A*0201 and to form stable peptide/HLA-A*0201 complexes. Both EphA2$_{58}$ and EphA2$_{550}$ exhibited a strong binding affinity (RA = 1) and stabilization capacity ($DC_{50} > 4$ h; Table 1), fulfilling the characteristics of immunogenic peptides (24).

Induction of CTL Responses against the EphA2 Peptides in HHD Mice. We then assessed the immunogenicity of the EphA2$_{58}$ and EphA2$_{550}$ in the HLA-A*0201-transgenic HHD mice. Mice vaccinated with EphA2$_{58}$ and EphA2$_{550}$ developed CTL that killed RMAS/HHD cells pulsed with the cognate peptide but not RMAS/HHD cells pulsed with an irrelevant peptide (Fig. 1*A*), demonstrating that both peptides are immunogenic *in vivo*. We established CTL lines specific for EphA2$_{58}$ (mCTL58) and EphA2$_{550}$ (mCTL550) from responding mice and tested their efficiency to recognize the cognate peptides. mCTL58 and mCTL550 lines exhibited high avidity with a half maximal lysis obtained with 10 and 20 nM of peptide, respectively (Fig. 1*B*).

EphA2$_{58}$ and EphA2$_{550}$ Are Naturally Processed Epitopes. To address whether these immunogenic peptides are naturally processed by cells expressing EphA2 endogenously, we first asked whether mCTL58 and mCTL550 recognize COS cells cotransfected with cDNAs encoding for the HHD construct (α1 and α2 domains of HLA-A*0201 allele and the α3 and intracellular domains from H-2D^b allele; Ref. 23) and for EphA2 antigen. CTL activation was measured by TNF-α secretion. mCTL58 and mCTL550 were activated by COS cells coexpressing HHD and EphA2 but not by COS cells expressing only HHD or EphA2 (Fig. 2*A*). These results show that EphA2$_{58}$ and

Table 1 *Affinity for HLA-A*0201 of EphA2-derived peptides*

Peptide	Sequence	RA[a]	DC_{50} (h)[b]
EphA2$_{58}$	IMNDMPIYM	1	4
EphA2$_{550}$	VLAGVGFFI	1	4–6

[a] The RA is determined as described in "Materials and Methods."
[b] Dissociation complex 50. DC_{50} was defined as the time required for the loss of 50% of the HLA-A*0201/peptide complexes stabilized at $t = 0$.

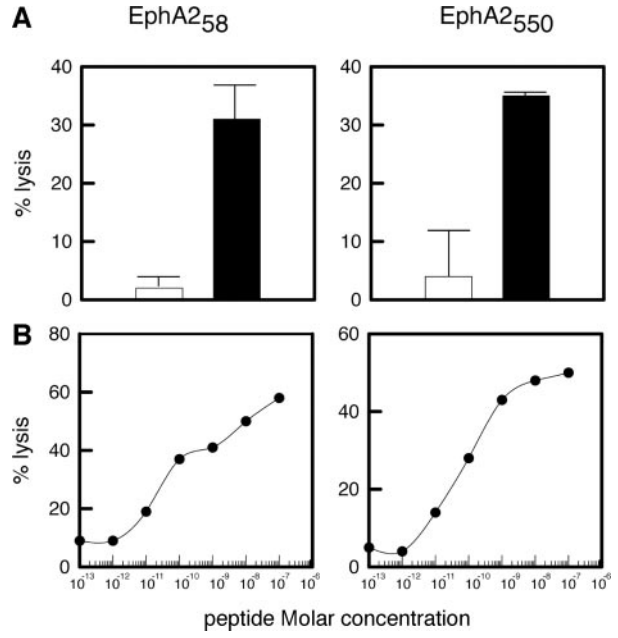

Fig. 1. Immunogenicity of EphA2$_{58}$ and EphA$_{550}$. *A*, Spleen cells from peptide vaccinated HHD mice were *in vitro* stimulated with the corresponding peptide for 7 days. CTL were tested against RMAS/HHD pulsed with 10 μM of the cognate (■) or an irrelevant (□) peptide at a E/T ratio of 60:1. Results represent the mean ± SD of triplicates and were confirmed in three independent experiments. *B*, mCTL58 and mCTL550 cell lines were established as described in "Materials and Methods." They were tested against RMAS/HHD targets in the presence of various concentrations of EphA2 peptides at a E/T ratio of 30/1.

EphA2$_{550}$ are naturally processed epitopes. However, the COS-7 model ensures a very high level of antigen expression, which probably is not reached in tumor cells. To validate that EphA2$_{58}$ and EphA2$_{550}$ are efficiently presented by human tumor cells, we stimulated mCTL58 and mCTL550 with the HLA-A*0201$^+$ EphA2$^+$ CACO-2 cell line (Ref. 13 and Figs. 3, *A* and *B*) and measured TNF-α secretion. Two negative controls (LNCaP, HLA-A*0201$^+$ EphA2$^-$ and DU-145, HLA-A*0201$^-$ EphA2$^+$; Ref. 13; Fig. 3, *A* and *B*) were also included. Both mCTL lines were stimulated by CACO-2 but not by LNCaP nor by DU-145 cells (Fig. 2*B*). Because our aim was to identify tumor epitopes expressed by a variety of human cancers, we asked whether these peptides were efficiently presented by cell lines derived from EphA2-expressing tumors of different origins. Western blot analysis revealed that the HLA-A*0201-positive HIEG (renal cell carcinoma), 1355 (lung carcinoma), and SAOS (sarcoma) cell lines expressed EphA2 (Fig. 3, *A* and *B*). We then used the HIEG, 1355, SAOS, CACO-2, and LNCaP tumor cells to stimulate mCTL58 and mCTL550. Both CTL lines responded to the EphA2$^+$ HIEG, 1355, CACO-2, and SAOS but not to the EphA2- LNCaP cells (Fig. 3*C*). For both mCTL lines, the strongest TNF-α response was obtained by HIEG and SAOS. These data, taken together, indicate that EphA2$_{58}$ and EphA2$_{550}$ are naturally processed and presented by a variety of human tumor cells of different histological origins.

Induction of Human-Specific CD8$^+$ T-Cell Responses to EphA2$_{58}$ and EphA2$_{550}$ *in Vitro*. We then assessed the capacity of EphA2$_{58}$ and EphA2$_{550}$ to induce tumor specific CD8$^+$ T-cell responses *in vitro* in humans. Four healthy donors were tested, and 48 independent cultures/peptide were performed for each donor. After priming of purified CD8$^+$ T cells with peptide-pulsed autologous dendritic cells and two rounds of restimulation with peptide pulsed adherent autologous monocytes, cultures were tested in cytotoxic assay against T2 cells pulsed with peptide in the presence of cold K562 cells. Two to three independent cultures/peptide giving up to 10% peptide specific lysis were obtained in two of four donors. We then tested these CTL from positive cultures (hCTL58 and hCTL550) for their capacity to respond to tumor cells expressing EphA2. Response was evaluated by measuring the number of IFN-γ-producing cells upon activation. Results in Fig. 4 confirm the peptide specificity of hCTL58 and hCTL550. CD8+ T cells were activated by T2 cells loaded with the corresponding EphA2 peptides but not by T2 cells loaded with an irrelevant peptide. Importantly, hCTL58 and hCTL550 responded to the HLA-A*0201$^+$ EphA2$^+$ CACO-2 and 1355 but not to the HLA-A*0201$^+$ EphA2$^-$ LNCaP, M113, and MCF-7 nor to the HLA-A*0201$^-$ EphA2$^+$ DU-145 tumor cells (Fig. 5), confirming that EphA2$_{58}$ and EphA2$_{550}$ are presented by human tumor cells associated with HLA-A*0201.

Detection of EphA2-Specific CTL in Cancer Patients' PBMCs. PBMCs from healthy donors and from patients with lung, prostate cancer, or melanoma were stained with HLA-A*0201/EphA2$_{58}$ and HLA-A*0201/EphA2$_{550}$ tetramers, as described in "Materials and Methods" section. Whenever the amount of PBMCs obtained permitted, patients' PBMCs were *in vitro* restimulated with peptide in the presence of IL-2. Tetramer-positive CD8$^+$ T cells were not detected *ex vivo* in the PBMCs of any of the 13 patients tested (Fig. 6 and data not shown). In contrast, after one *in vitro* restimulation, tetramer-positive CD8$^+$ T cells appeared in one of five patients tested. In fact, in two independent cultures of PBMCs of patient VAV (prostate cancer), 0.15% of CD8$^+$ T cells were stained with the HLA-A*0201/EphA2$_{58}$ tetramer (Fig. 6), suggesting that in this patient, EphA2$_{58}$-specific CD8$^+$ T cells were *in vivo* amplified, although to a level that can only be detectable after *in vitro* restimulation.

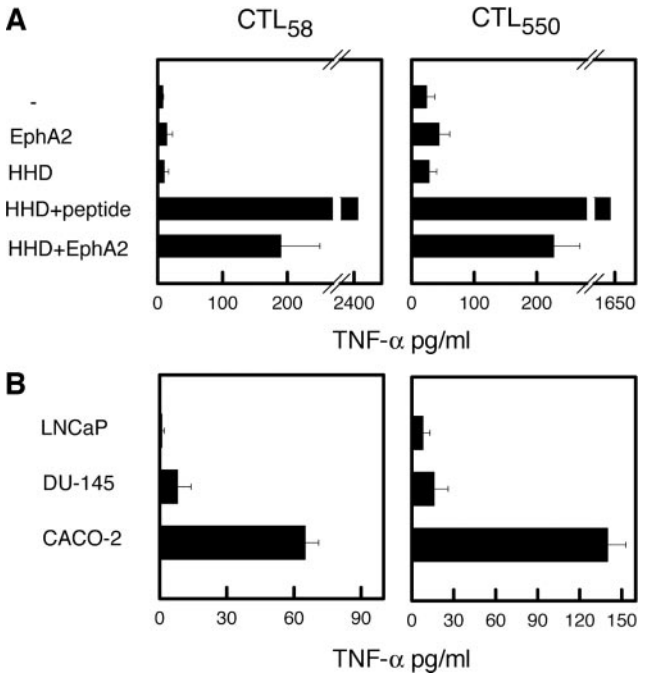

Fig. 2. Stimulation of mCTL58 and mCTL550 lines by tumor cells expressing endogenous EphA2. *A*, mCTL58 and mCTL550 cells were stimulated with COS-7 cells expressing HHD and/or EphA2 as indicated. As positive control, mCTL550 and mCTL58 cells were stimulated with HHD expressing COS-7 cells incubated with the peptide (10 μM). mCTL58 and mCTL550 response was evaluated by measuring secreted TNF-α. *B*, mCTL58 and mCTL550 cells were stimulated with the EphA2$^+$ HLA-A*0201$^+$ CACO-2, EphA2$^-$ HLA-A*0201$^+$ LNCaP, and EphA2$^+$ HLA-A*0201$^-$ DU-145 cells. mCTL58 and mCTL550 cells activation was evaluated by measuring secreted TNF-α. In both *A* and *B*, results represent the mean ± SD of triplicates and were confirmed in three independent experiments.

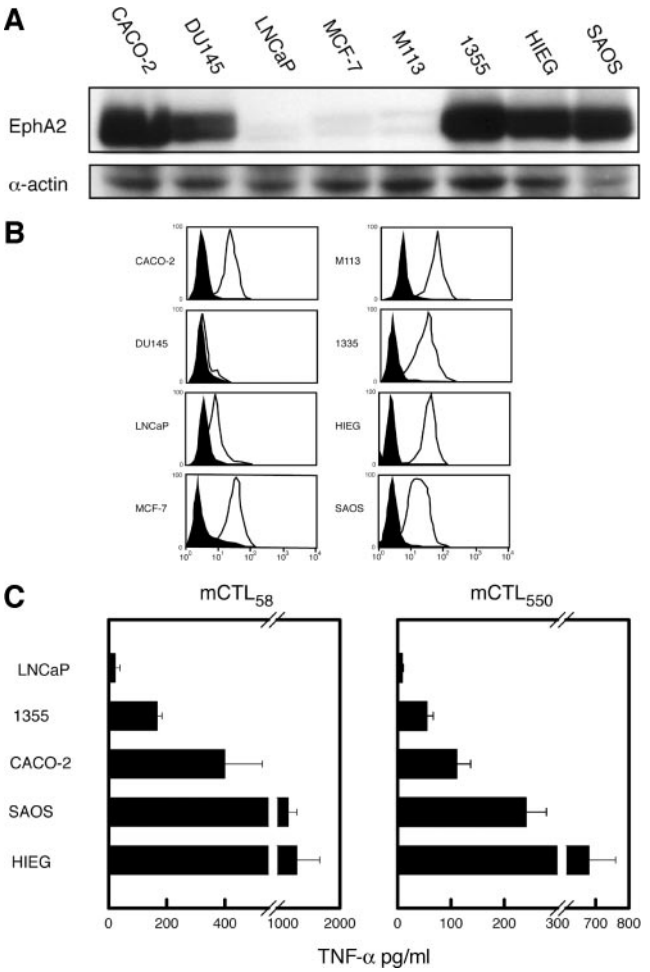

Fig. 3. Recognition of EphA2-expressing human tumor cells of different origins by mCTL58 and mCTL550. *A*, EphA2 expression analysis of human tumor cells by Western blot, as described in "Materials and Methods." *B*, HLA-A*0201 allele expression at the surface of human tumor cells analyzed by flow cytometry using BB7.2 mAb. Filled histograms represent isotypic control staining. *C*, mCTL58 and mCTL550 were stimulated with human tumor cells and activation was evaluated by measuring secreted TNF-α. Results represent the mean ± SD of triplicates and were confirmed in three independent experiments.

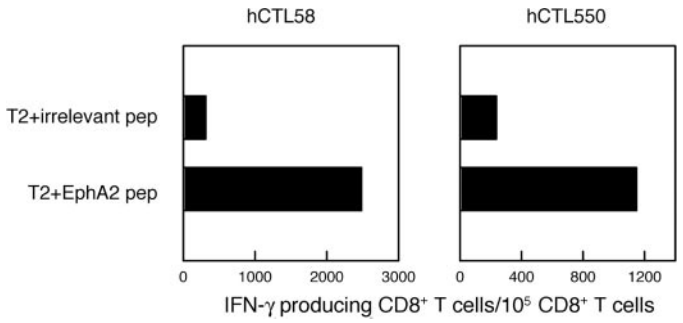

Fig. 4. Peptide specificity of hCTL58 and hCTL550. hCTL58 and hCTL550 were stimulated with T2 cells loaded with the cognate or an irrelevant peptide as indicated. CTL response was evaluated by measure of IFN-γ-producing cells upon activation as assessed by intracellular IFN-γ staining. Results from a representative donor.

DISCUSSION

The majority of TAgs identified up to now are grouped in three distinct classes: differentiation antigens of melanocytes (gp100, MELAN-A, tyrosinase, TRP-1/2) or prostate cells (PSA and PSMA); tumor-specific antigens that are either mutated (CDK4, Hsp70, β-catenin, α-actinin) or nonmutated (G250, GnTV, RAGE) with a restricted expression to some tumors; and finally tumor/testis antigens (MAGE-A, NY-ESO-1, BAGE, GAGE; for review see Ref. 27). A particular interest has been recently focused to another group of antigens that are nonmutated proteins such as hTERT (6), HER-2/neu (28), and Survivin (7, 8), which are frequently overexpressed in a large variety of tumors. These TAgs can be used for broad spectrum tumor immunotherapy. The tyrosine kinase EphA2, which belongs to the Eph family receptors, is also overexpressed in a high percentage of tumors of various origins such as breast, ovarian, lung, prostate, gastric cancer, and sarcomas (11–15). In addition, it is involved in tumor metastatization and is expressed at a higher level in metastasis than in primary tumors (11, 14). Finally, EphA2 is involved in tumor

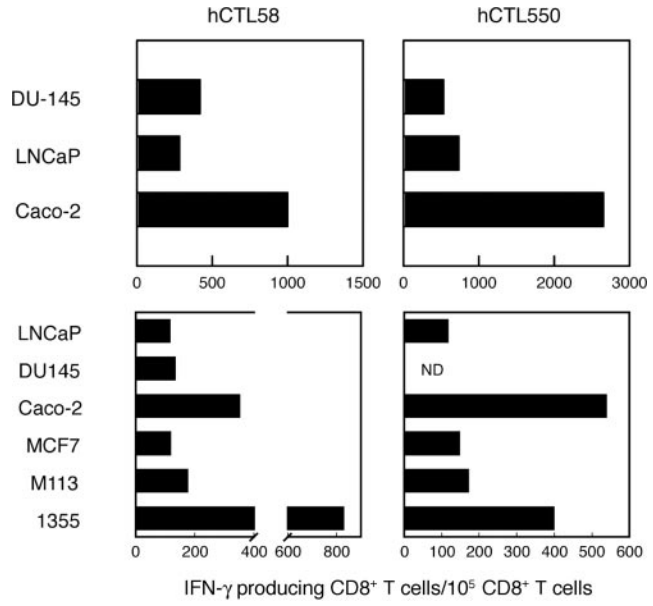

Fig. 5. Recognition of human tumor cells by hCTL58 and hCTL550. hCTL58 and hCTL550 from different healthy donors were tested for recognition of CACO-2, 1355 ($EphA2^+$ HLA-A*0201$^+$), LNCaP, M113 and MCF-7 ($EphA2^-$ HLA-A*0201$^+$), and DU-145 ($EphA2^+$ HLA-A*0201$^-$) cells. CTL response was evaluated by measure of IFN-γ-producing cells upon activation as assessed by intracellular IFN-γ staining. Results from a representative donor.

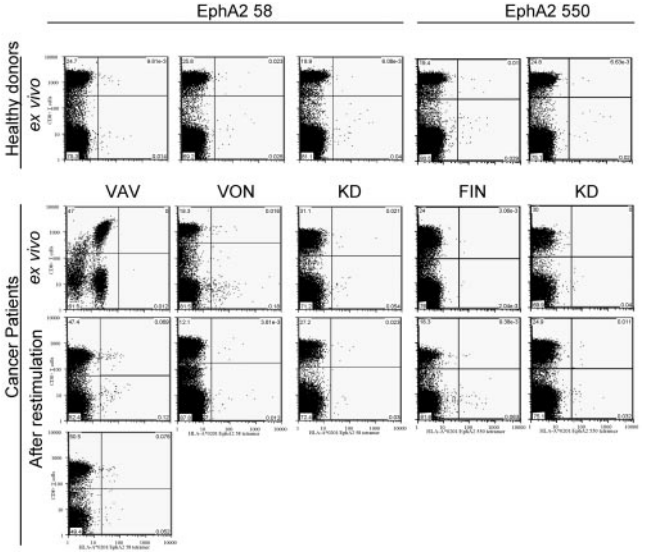

Fig. 6. Detection of CD8+ T cells specific for $EphA2_{58}$ and $EphA2_{550}$ in HLA-A*0201-positive healthy donors and cancer patients. PBMCs from healthy donors, prostate (VAV, VON, and FIN), and lung (KD) cancer patients were stained with HLA-A*0201/$EphA2_{58}$ and HLA-A*0201/$EphA2_{550}$ tetramers *ex vivo* or after *in vitro* restimulation as indicated.

angiogenesis and expressed by tumor neovasculature (20). Therefore, an EphA2-specific CTL response would particularly target tumor cells with high metastatic potential and tumor vascular endothelial cells, thus preventing tumor dissemination and growth. In this study, we describe two HLA-A*0201-restricted epitopes ($EphA2_{58}$ and $EphA2_{550}$) that derive from the EphA2 protein and that are targets of antitumor CD8+ T cells. These epitopes have a high affinity for HLA-A*0201, trigger CTL *in vivo* in the HLA-A*0201-transgenic HHD mice and *in vitro* in healthy humans.

In this study, we also present data suggesting an *in vivo* amplification of $EphA2_{58}$-specific $CD8^+$ T cells in prostate cancer patients. Although melanoma, prostate, and lung cancer patients presented frequencies of $CD8^+$ T cells specific for peptides $EphA2_{58}$ and $EphA2_{550}$ comparable with the one detected on HLA-A*0201 healthy donors when assessed by *ex vivo* staining with the appropriate tetramer, on one prostate patient (VAV), the *in vitro* restimulation of PBMCs with peptide revealed the presence of $EphA2_{58}$-specific $CD8^+$ T cells. This observation may be particularly important for peptide-based cancer immunotherapy. It provides the rational for therapeutic vaccination of cancer patients. However, a phenotypic and functional activity characterization of these *in vivo*-amplified $CD8^+$ T cells remains to be evaluated.

As with the majority of universal TAgs, EphA2 is expressed at low level by normal tissues such as lung, kidney, skin, ovaries, and even the thymus (29, 30). This raises two questions that are common for all of the ubiquitously expressed antigens: (*a*) is there tolerance to EphA2, and if there is, how can we recruit *in vivo* anti-EphA2 CTL with high avidity; and (*b*) what is the risk of autoimmunity after vaccination with EphA2 CTL epitopes? These questions are being currently addressed in the HHD mouse model by using EphA2 epitopes that, unlike $EphA2_{58}$ and $EphA2_{550}$, are shared by human and murine EphA2 sequences. However, the fact that we can induce CTL from human healthy donors after a reduced number of restimulations and, importantly, that these CTL are amplified *in vivo* in cancer patients suggest that the EphA2-specific CTL repertoire is not completely tolerized.

Several other epitopes have been described from TAgs of ubiquitous phenotype, namely HER-2/neu and hTERT (28). The inclusion of epitopes of these antigens in a polyepitopic construct would be highly favorable to the development of vaccine strategies that would trigger polyspecific responses against a large number of tumors. An additional advantage of these strategies is to diminish the risks of tumor escape by loss of the TAg (31). The EphA2-derived epitopes, here identified, could therefore be used along with hTERT and HER-2/neu epitopes in polyspecific vaccines with broad application in tumor immunotherapy.

In conclusion, in this article, we identified two HLA-A*0201-restricted EphA2-derived epitopes that induce EphA2-specific antitumor CTL response. EphA2, which is expressed by tumors of various origins, could, therefore, be considered as new universal TAg.

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