

Featured Article

Allogeneic Hematopoietic Cell Transplantation for Metastatic Renal Cell Carcinoma after Nonmyeloablative Conditioning: Toxicity, Clinical Response, and Immunological Response to Minor Histocompatibility Antigens

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ABSTRACT

Purpose: This phase I trial assessed the safety, efficacy, and immunologic responses to minor histocompatibility antigens following nonmyeloablative allogeneic hematopoietic cell transplantation as treatment for metastatic renal cell carcinoma.

Experimental Design: Eight patients received conditioning with fludarabine and low-dose total body irradiation followed by hematopoietic cell transplantation from an HLA-matched sibling donor. Cyclosporine and mycophenolate mofetil were administered as posttransplant immunosuppression. Patients were monitored for donor engraftment of myeloid and lymphoid cells, for clinical response by serial imaging, and for immunologic response by *in vitro* isolation of donor-derived CD8⁺ CTLs recognizing recipient minor histocompatibility (H) antigens.

Results: All patients achieved initial mixed hematopoietic chimerism with two patients rejecting their graft and recovering host hematopoiesis. Four patients developed acute, grade 2 to 3, graft-versus-host disease and four patients developed extensive chronic graft-versus-host disease. Five patients had progressive disease, two patients had stable disease, and one patient experienced a partial response after receiving donor lymphocyte infusions and IFN- α . CD8⁺ CTL clones recognizing minor H antigens were isolated from five patients studied. Clones from three patients with a partial response or stable disease recognized antigens expressed on renal cell carcinoma tumor cells.

Conclusions: Treatment of metastatic renal cell carcinoma with allogeneic hematopoietic cell transplantation after nonmyeloablative conditioning with fludarabine/total body irradiation is feasible and may induce tumor regression or stabilization in some patients. CD8⁺ CTL-recognizing minor H antigens on tumor cells can be isolated posttransplant and could contribute to the graft-versus-tumor effect. Such antigens may represent therapeutic targets for posttransplant vaccination or adoptive T-cell therapy to augment the antitumor effects of allogeneic hematopoietic cell transplantation.

INTRODUCTION

Metastatic renal cell carcinoma (1) is poorly responsive to conventional chemotherapy and results in the death of ~12,000 patients per year in the United States (1, 2). Immunotherapy with interleukin-2 (IL-2) or IFN- α may induce objective clinical responses in ~15% of patients selected for intact performance status (3). The basis for the clinical response of renal cell carcinoma to IL-2 or IFN- α is poorly understood but might involve enhancement of natural killer cell activity (4), the induction of secondary cytokines with antitumor activity (5, 6), or augmentation of a tumor-reactive cellular immune response. The contribution of T cells to the antitumor effect of cytokine therapy is suggested by studies of a murine model showing that a protective CD8⁺ CTL response can be induced against the Renca renal cell carcinoma tumor after vaccination protocols incorporating adjuvant cytokines (7, 8). In addition, CTL lines and CD8⁺ CTL clones recognizing autologous renal cell carcinoma tumor cells have been isolated from tumor-infiltrating lymphocytes or peripheral blood mononuclear cells (PBMCs) of some renal cell carcinoma patients (9–11).

The low response rate of metastatic renal cell carcinoma to IL-2 or IFN- α therapy has stimulated the search for innovative treatment modalities. One approach involves reduced intensity allogeneic hematopoietic cell transplantation, which has been used effectively to treat refractory hematologic malignancies

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(12). A subset of solid tumors including renal cell carcinoma also respond to the graft-*versus*-tumor effect of reduced intensity allogeneic hematopoietic cell transplantation. Childs *et al.* (13) reported the results of 19 patients with progressive metastatic renal cell carcinoma treated with cyclophosphamide and fludarabine followed by allogeneic hematopoietic cell transplantation and posttransplant immunosuppression with cyclosporine. Hospitalization was required for management of acute toxicity of the preparative regimen, and two patients died from transplant related causes. Ten patients (53%) achieved objective clinical responses, and 3 patients (16%) experienced complete remissions of disease. Subsequent reports from other centers confirmed that similar treatment protocols could induce objective regression of metastatic renal cell carcinoma in 8 to 57% of patients (14–18).

In recipients of MHC-matched grafts, donor-derived CD8⁺ CTL recognize minor histocompatibility (H) antigens on recipient tissues and may contribute to both posttransplant graft-*versus*-tumor effects, as well as to graft-*versus*-host disease (GVHD; ref. 19). The preparative regimens for reduced intensity allogeneic hematopoietic cell transplantation used chemotherapeutic agents that lacked activity against renal cell carcinoma. Several observations implicated a role for donor T cells in renal cell carcinoma regression. These observations included the delay of tumor regression for months after transplantation and a correlation of tumor regression with the withdrawal of immune suppression, the development of full donor chimerism of CD3⁺ cells, and the development of acute or chronic GVHD. An increased number of IFN- γ -producing CD8⁺ T cells in the posttransplant peripheral blood of allogeneic hematopoietic cell transplantation recipients has also been found to correlate with renal cell carcinoma regression (20).

The results of pilot studies of reduced intensity allogeneic hematopoietic cell transplantation for renal cell carcinoma have prompted the initiation of a multicenter phase II intergroup trial (Cancer and Leukemia Group B C90003) to additionally evaluate the response rate and toxicity of this approach, with a treatment protocol similar in design to that used in pilot studies (13, 14). However, the optimal preparative and posttransplant immunosuppressive regimens for individual malignancies have not been defined. A low-intensity regimen for nonmyeloablative allogeneic hematopoietic cell transplantation that can be administered in an outpatient setting with mild conditioning-related toxicity has been developed and used to treat >700 patients with hematologic malignancies in Seattle, Washington, and at collaborating treatment centers. The treatment regimen consists of preparative therapy with fludarabine (30 mg/m²/day for 3 days) and 2 Gy of total body irradiation followed by allogeneic peripheral blood stem cell infusion and posttransplant immunosuppression with cyclosporine and mycophenolate mofetil (12, 21). The day 100 mortality from transplant-related causes has been <10% with this regimen, and rates of GVHD are reduced compared with conventional myeloablative hematopoietic cell transplantation (22). Sustained engraftment has been achieved in >95% of hematopoietic cell transplantation recipients from HLA-identical sibling donors (23), and complete remissions have been observed in patients with refractory hematologic malignancies, suggesting the development of a potent graft-*versus*-tumor effect against hematologic tumors (24).

In this phase I trial, the toxicity and efficacy of allogeneic hematopoietic cell transplantation with the nonmyeloablative regimen of fludarabine, 2 Gy of total body irradiation, and posttransplant cyclosporine/mycophenolate mofetil were evaluated in patients with metastatic renal cell carcinoma. The close association of GVHD with regression of renal cell carcinoma in patients responding to allogeneic hematopoietic cell transplantation (13–18) suggested that donor T cells recognizing minor H antigens expressed on recipient tissues and renal cell carcinoma tumor cells could mediate graft-*versus*-tumor responses. Therefore, a major objective of this trial was to determine whether a subset of CD8⁺ minor H antigen-specific CTLs isolated from transplant recipients recognize antigens expressed by renal cell carcinoma tumor cells.

PATIENTS AND METHODS

Patient/Donor Eligibility. The eligibility criteria for this study included histologically confirmed, stage IV renal cell carcinoma, ages < 75 years, intact organ function (serum creatinine \leq 2.0 g/dL, bilirubin, aspartate aminotransferase, and alanine aminotransferase less than or equal to two times the upper limit of normal, serum ionized calcium within normal limits, cardiac ejection fraction \geq 50%, carbon monoxide diffusion capacity of the lung \geq 50% predicted), Karnofsky performance status \geq 80, absence of brain metastases, absence of ongoing bacterial, viral, or fungal infections, and sibling donors who were matched at the HLA-A, HLA-B, HLA-C, DRB1, and DQB1 loci by molecular typing methods. Donors were between 12 and 74 years of age, with no serious systemic illness, with no known allergy to granulocyte-colony stimulating factor, and not pregnant. Patients and donors gave written informed consent to participate in this study, which was approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Patient Characteristics. Between March 21, 2000, and March 13, 2002, eight consecutive patients with metastatic renal cell carcinoma underwent allogeneic hematopoietic cell transplantation from MHC-matched sibling donors (Table 1). The patients ranged from 40 to 54 years of age. All patients had undergone prior nephrectomy and had received a median of two prior systemic therapies for metastatic renal cell carcinoma. Seven patients had primarily clear cell histology, and one patient had poorly differentiated papillary histology.

Treatment Plan. The treatment protocol for nonmyeloablative hematopoietic cell transplantation has been previously described in detail (12, 21). Patients were evaluated for engraftment of donor myeloid and lymphoid cells at days 28, 56, 84, 180, and 365 after hematopoietic cell transplantation and annually thereafter as described previously.

Selection of patients for nonmobilized donor lymphocyte infusion was based on chimerism results for peripheral blood CD3⁺ cells at day 28 and day 56, in conjunction with clinical evaluation of GVHD and tumor response at day 56. Patients with full (>95%) donor CD3⁺ cell chimerism with disease progression and no GVHD were eligible for initial donor lymphocyte infusion at day 65. Patients with mixed CD3⁺ cell chimerism (>1% and <95% donor CD3⁺ cells) at day 56 and without evidence of GVHD were also eligible for donor lymphocyte infusion on day 65. However, donor lymphocyte infu-

Table 1 Patient characteristics

Patient	Age (y)/Sex	Histology/Fuhrman grade	Sites of metastases	Previous systemic therapies
1	54/M	Clear cell/3	Soft tissue, bone	IL-2, thal
2	50/F	Clear cell/3	Lung, node, bone	IL-2, T cells
3	40/M	Clear cell/2	NED *	None
4	40/M	Clear cell/4	Bone, liver	None
5	46/M	Clear cell/3	Lung, bone, liver	IL-2, T cells, thal, IFN- α
6	44/M	Papillary/NA	Lung, soft tissue, liver	IL-2, IFN- α , CRA
7	45/M	Clear cell/2	Soft tissue, node	IL-2, T cells, ABX-EGF
8	51/F	Clear cell/4	Lung, node, liver	IL-2, IFN- α

* A solitary bone metastasis was resected before transplant.

Abbreviations: IL-2, interleukin-2; thal, thalidomide; T cells, CD3/CD28-activated T cells; IFN- α , interferon-alpha; CRA, cisretinoic acid; ABX-EGF, mAb to epidermal growth factor receptor; NA, not available; NED *, no evidence of disease.

sion was deferred if patients had both stable or regressing disease and increasing donor CD3⁺ cell chimerism between day 28 and 56 with at least 50% donor CD3⁺ cells by day 56 evaluation. Donor lymphocyte infusion could be administered after an additional 2 months if a complete response and full donor chimerism were not obtained and GVHD did not develop. Patients with graft failure, defined as the absence of detectable donor cells at day 56, were not eligible for donor lymphocyte infusion.

Donors underwent leukapheresis and collection of nonmobilized PBMCs on the day of the first donor lymphocyte infusion. After determining the CD3⁺ cell content, the first dose of donor lymphocytes was infused at 1×10^7 CD3⁺ cells per kg of recipient weight. Additional cells were cryopreserved for subsequent donor lymphocyte infusion as needed. A maximum of four donor lymphocyte infusion could be administered at increasing (1/2 log) CD3⁺ cell doses. Additional donor lymphocyte infusion could be administered for disease progression in the absence of GVHD at 28 days after a prior donor lymphocyte infusion. Additional donor lymphocyte infusion could also be administered for patients with disease persistence (stable disease or partial regression) or persistent mixed chimerism in the absence of GVHD at 65 days after a prior donor lymphocyte infusion.

Response Assessment. All patients underwent baseline computed tomography scans of chest, abdomen, and pelvis, magnetic resonance imaging scans of the brain, and bone scans within 4 weeks before transplant. Tumor responses were assessed by computed tomography scans and physician examinations in comparison to baseline tests on days 56, 84, 180, 365, and yearly thereafter. Tumor responses were also assessed by magnetic resonance imaging scans of the brain and bone scans in comparison to baseline tests on days 84, 365, and yearly thereafter. Clinical responses were categorized according to the RECIST criteria (25). Data were reviewed for assignment of clinical responses as of March 31, 2003.

Cell Lines. Renal cell carcinoma lines A-498, CAKI-2, and 786-0 were obtained from American Type Culture Collection (Manassas, VA). Renal cell carcinoma lines 1.11, 1.18, and 1.24 were provided by Dr. Elizabeth Jaffee (The Johns Hopkins University, Baltimore, MD). Renal cell carcinoma lines TREP, CAJE, JAUP, GERK, LE9211, and DOBSKI were provided by Dr. Benoît van den Eynde (Ludwig Institute for Cancer Research, Brussels, Belgium). Renal cell carcinoma lines FARP

and STAR were isolated at the National Heart, Lung, and Blood Institute from primary tumor samples. Renal cell carcinoma line SST125 was isolated at the Fred Hutchinson Cancer Research Center from a metastatic tumor deposit resected from the patient's right orbit. Renal cell carcinoma line SST140 was isolated from a malignant pleural effusion sample. For 10 renal cell carcinoma tumor lines, EBV-lymphoblastoid cell lines (LCLs) derived from the same individual were also obtained. Class I MHC serotype data for the A-498 and CAKI-2 lines have been reported previously (26). The HLA-A and HLA-B genotype of the 786-0 renal cell carcinoma tumor line was determined with sequence-specific primer PCR-based typing kits for these loci (Dynal Biotech, Bromborough, United Kingdom) per the manufacturer's instructions. Class I MHC genotype for the FARP and STAR lines were determined by the HLA laboratory in the Department of Transfusion Medicine, NIH. Class I MHC genotype for the SST125 and SST140 lines were determined by the Clinical Immunogenetics Laboratory at the Seattle Cancer Care Alliance (Seattle, WA). Additional class I MHC serotype or genotype for renal cell carcinoma lines and associated EBV-LCLs were provided by the collaborating investigators. All renal cell carcinoma lines were maintained in RPMI 1640 with 25 mmol/L HEPES buffer (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Gemini Bio-Products, Woodland, CA), 2 mmol/L L-glutamine (Invitrogen), 1 mmol/L sodium pyruvate (Invitrogen), and penicillin/streptomycin (Invitrogen). Peripheral blood samples were obtained pretransplant from patients and their donors. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and used to establish EBV-LCLs as described previously (27). The T2 cell line and EBV-LCL derived from individuals of the Centre d'étude du polymorphisme humain (CEPH) reference families 1331, 1341, 1362, and 1416 were maintained as described previously (28, 29).

Mixed Lymphocyte Culture and Isolation of CD8⁺ CTL Clones. Peripheral blood samples were obtained from patients 2 to 8 at multiple time points after hematopoietic cell transplantation once donor chimerism of >80% was established in peripheral blood CD3⁺ cells. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation and used as responder T cells in mixed lymphocyte cultures as described previously (27). After two to four *in vitro* stimulations, the T-cell cultures were cloned by limiting dilution as described previously (27). Cloning wells were screened visually for growth after 11 to 13

days, and aliquots of wells with visible growth were then screened for lytic activity against chromium-51–labeled patient and donor EBV-LCL target cells. Clones that lysed patient but not donor EBV-LCL target cells were expanded in culture as described previously (30). *In vitro* studies were not initiated for patients with graft rejection (number 7) or rapid disease progression and death without complete donor CD3⁺ cell engraftment (number 8). Surface phenotype and function of expanded T-cell clones were assessed by flow cytometry 11 to 15 days after stimulation.

Flow Cytometry. After expansion in 25-cm² flasks, T-cell clones were screened by flow cytometry with monoclonal antibodies (mAbs) recognizing CD8, CD4, and TCRαβ chains (BD Biosciences, San Diego, CA).

Renal cell carcinoma tumor cell lines were analyzed by flow cytometry with class I MHC-specific mAbs recognizing HLA-A2 [clone BB7.2 (American Type Culture Collection); ref. 31], HLA-B7 (Serotec, Raleigh, NC), and HLA-B51 (One Lambda, Canoga Park, CA), as well as with the URO-2, URO-3, URO-4, and URO-8 mAbs (Signet Pathology Systems, Dedham, MA; ref. 32) defining distinct renal epithelial antigens. All 16 renal cell carcinoma lines expressed one or more of the renal epithelial antigens defined by the URO mAbs.

Cytotoxicity Assays. Aliquots of 3 to 5 × 10⁵ cells were incubated for 1 to 2 hours (renal cell carcinoma tumor cells) or overnight (EBV-LCLs) at 37°C with 50 to 100 μCi of ⁵¹Cr and used as targets in 4-hour cytotoxicity assays as described previously (27). To determine the minor antigen phenotype of EBV-LCLs from individuals in CEPH reference families 1331, 1341, 1362, and 1416, 5 × 10⁵ EBV-LCLs were infected the evening before the cytotoxicity assay with a recombinant vaccinia virus containing an HLA-A*0201 transgene (a generous gift of Dr. Jonathan Yewdell, National Institute of Allergy and Infectious Diseases, Bethesda, MD) or with wild-type vaccinia, both at a multiplicity of infection of 5:1, as described previously (28).

PCR/Restriction Fragment Length Polymorphism Assays. Genomic DNA was prepared from 3 to 5 × 10⁶ EBV-LCLs or renal cell carcinoma tumor cells with the QIAmp DNA Blood kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For determination of genotype for the HA-1 minor H antigen, PCR amplification of a 295-bp fragment of the *KIAA0223* gene was performed as described previously (33). Briefly, amplifications were performed in a 25-μL reaction volume containing genomic DNA template with 0.5 μmol/L of the forward primer 5'-GACGTCGTCGAGGACATCTCCCATC-3' and the reverse primer 5'-CTCTTGAGCCAGTGTACGCTCA-3' in 1× PCR buffer supplemented with 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxynucleoside triphosphates, and 1 unit iTaq polymerase (Bio-Rad, Hercules, CA). Cycle parameters were as follows: initial denaturation 5 minutes at 94°C; 40 cycles of denaturation at 94°C for 30 seconds; annealing at 58°C for 30 seconds; extension at 72°C for 60 seconds; and final extension at 72°C for 7 minutes. PCR products were digested with *Fnu*4HI for 2 to 3 hours at 37°C and analyzed on 2.5% agarose gels.

Linkage Analysis. Chromosomal localization of the gene encoding the minor H antigen recognized by the HLA-A*0201–restricted CTL clones 2B3 and 12B3 was performed by

genetic linkage analysis, as described previously (28). Briefly, EBV-LCLs derived from individuals in four large pedigrees—1331, 1341, 1362, and 1416—from the Utah CEPH Reference Family Collection (34) were phenotyped for minor H antigen expression in a standard 4-hour ⁵¹Cr release assay after overnight infection with a recombinant vaccinia virus carrying an HLA-A*0201 transgene. Genotypes of the CEPH EBV-LCLs for 9900 highly informative microsatellite markers were obtained from the CEPH database v9.0 for the linkage analysis.⁵ The minor H antigen phenotype was analyzed with two-point linkage analysis of the genotypes with the MLINK subroutine of the FASTLINK (v4.0) program (35–38). All markers were analyzed assuming equal allele frequencies. An autosomal dominant single gene model was assumed for the trait of minor H antigen expression. The frequency of the antigenic minor H allele recognized by the 2B3 and 12B3 CTL clones was estimated at 0.50 and the penetrance set at 0.90.

RESULTS

Engraftment and Toxicity. The content of the donor stem cell inoculum, results of the chimerism studies, and transplant toxicities are summarized in Table 2. Patients received a median of 11.2 (range, 6.8 to 18.8) × 10⁶ CD34⁺ cells per kg and 3.2 (range, 1.6 to 7.1) × 10⁸ CD3⁺ cells per kg. All patients showed initial mixed chimerism of peripheral blood CD3⁺ cells at posttransplant day 28 (range, 50 to 90% donor). Two patients subsequently lost their graft and recovered host hematopoiesis. Patient 3 underwent a second hematopoietic cell transplantation from the same donor after conditioning with cyclophosphamide and anti-thymocyte globulin and achieved sustained donor engraftment. This patient subsequently developed acute myelogenous leukemia of donor origin diagnosed 588 days after the second hematopoietic cell transplantation. Patient 7 was not retransplanted because of declining performance status related to progression of renal cell carcinoma.

All patients received the conditioning regimen in the outpatient transplant clinic. Four of the eight patients required a total of seven admissions to the hospital within the first 100 days of transplant for management of infection, GVHD, or other medical complications. The median duration of these hospitalizations was 10 days (range, 5 to 176 days). Three patients did not develop GVHD. Acute GVHD occurred in four patients, grade 2 in two patients, and grade 3 in two patients. Four patients developed extensive chronic GVHD. Infections were the most prevalent grade 3 or 4 organ toxicity (five of eight patients; Table 2) and included invasive fungal infections in patients 1 and 4. There were no deaths within the first 100 days after hematopoietic cell transplantation. Five patients died between days 120 and 854. Four deaths were attributable to progressive renal cell carcinoma, and patient 4 died at day 263 from infectious complications of steroid-refractory GVHD that developed after disease progression.

Clinical Response. The best clinical response for each patient is shown in Table 2. Six of the eight patients experienced

⁵ Internet address: <http://www.wcephb.fr/cephdb/>.

Table 2 Engraftment, toxicity, and treatment outcome

Patient	Donor sex	Cells infused		Peripheral blood CD3 ⁺ cell chimerism				Graft		GVHD		Grade 3/4 toxicity ‡						Outcome	
		CD34 ⁺ × 10 ⁶ /kg	CD3 ⁺ × 10 ⁸ /kg	28 *	56	84	180	rejection	DLI †	Acute	Chronic	GI	R	C	I	M	S		H
1	M	13.65	2.39	65	95	100	99	No	No	Grade 2	Extensive	3	3	4	3			PD, dead of RCC, day 854	
2	M	8.58	1.60	53	45	62	100	No	Yes	None	None							PR, alive day 789	
3	M	13.75	3.19	50	70	30	0	Yes	No	None	None			4				SD, alive day 708	
4	F	18.79	7.13	69	66	90	95	No	No	Grade 3	Extensive	3		3	4	3		PD, dead of GVHD/ pneumonia, day 263	
5	F	6.77	5.25	78	84	73	91	No	No	Grade 3	Extensive				3	3		PD, dead of RCC, day 245	
6	M	8.80	2.64	90	90	ND	100	No	No	None	Extensive			3	3			SD, alive day 434	
7	M	16.15	3.76	90	50	50	0	Yes	Yes	None	None							PD, dead of RCC, day 406	
8	M	8.20	3.13	72	77	73	ND	No	No	Grade 2	None	3	4	3				PD, dead of RCC, day 120	

* Days after transplant.

† Patient 2: DLI × 3 on days 70, 126, and 393; patient 7: DLI × 1 on day 111.

‡ Common Toxicity Criteria: GI, gastrointestinal; R, renal; C, cardiac; I, infection; M, metabolic; S, skin; H, hepatic.

Abbreviations: PD, progressive disease; RCC, renal cell carcinoma; PR, partial remission; SD, stable disease; ND, not determined.

progressive disease, with a median time to progression of 84 days (range, 28 to 456 days). Five of six patients with progressive disease died at a median of 254 days (range, 120 to 854 days) after hematopoietic cell transplantation. Two patients have experienced stable disease for 434+ and 708+ days. One patient, who initially experienced progression of renal cell carcinoma, achieved a partial remission after receiving three donor lymphocyte infusions and IFN- α after the third donor lymphocyte infusion. After two donor lymphocyte infusions (infused on days 70 and 126), the patient converted from mixed chimerism (50%) to full donor peripheral blood CD3⁺ cells and experienced partial regression of metastases without GVHD. Subsequently, the patient again developed disease progression despite maintaining 100% donor chimerism, and a third donor lymphocyte infusion was administered (on day 393). Thirty days after the third donor lymphocyte infusion, the patient was started on IFN- α at 3 million units per day s.c. Three months after the start of IFN- α therapy, dramatic regression of all of the patient's metastases in lung, nodes, and liver was observed. At the last follow-up, the patient had only a small radiographic abnormality in the liver that exhibited water density and did not enhance with continued i.v. contrast. The patient continued to have no evidence of GVHD 789 days after hematopoietic cell transplantation.

CTL Responses to Minor Histocompatibility Antigens.

A major objective of this study was to determine whether donor-derived cytotoxic T-cell responses specific for recipient minor H antigens expressed on renal cell carcinoma tumor cells developed in recipients of nonmyeloablative hematopoietic cell transplantation. *In vitro* studies were completed for five consecutive patients (2 to 6). A total of 39 CD8⁺ cytotoxic T-cell clones that were specific for recipient minor H antigens based on cytotoxicity for recipient but not donor EBV-LCL target cells was isolated from the five patients. The class I MHC-restricting allele was determined for 31 of the 39 CTL clones by testing the clones for cytolytic activity against panels of allogeneic EBV-LCL target cells that shared one or more class I MHC alleles with the donor-recipient pair from whom each clone was derived (data not shown). Minor H antigen-specific CTL clones

were isolated from 6 of 7 posttransplant PBMC samples evaluated *in vitro*, including samples obtained from patients receiving systemic immune suppression with cyclosporine (patients 3 and 6) or cyclosporine plus prednisone (patients 4 and 5). CTL clones recognizing two to four distinct minor H antigens were isolated from each of the five patients studied (Table 3). Flow cytometric analysis revealed that all of the CTL clones listed in Table 3 expressed both CD8 and TCR $\alpha\beta$ (data not shown).

The panel of CTL clones isolated from the five patients studied identified 14 distinct minor H antigenic specificities based on the reactivity pattern of the individual CTL clones for the allogeneic EBV-LCLs used to identify the class I MHC-restricting allele (Table 3). A total of seven of the CTL clones in Table 3 recognized minor H antigens presented by MHC alleles such as HLA-A2 (five clones), HLA-A3 (one clone), and HLA-B7 (one clone), which previously have been shown to present minor H antigens defined in our laboratory (29)⁶ or others (39–43). To determine whether these clones recognized one or more previously defined HLA-A2-, HLA-A3-, or HLA-B7-restricted minor H antigens or alternatively recognized novel specificities, the clones were tested for recognition of target cells pulsed with minor H antigen peptides and/or extended panels of target cells with previously defined minor H antigen phenotype. This analysis was sufficient to establish that all but one of the HLA-A2-, HLA-A3-, and HLA-B7-restricted clones in Table 3 recognized novel specificities. The sole exception was the HLA-A2-restricted clone 9 isolated from patient 2, which was found to recognize the HA-1 minor H antigen, VLHDDLLEA, encoded by the *KIAA0223* gene (42) and presented by HLA-A*0201 (Fig. 1A). Genotyping of patient 2 and the donor for the polymorphism that distinguishes the

⁶ E. H. Warren, N. M. Vigneron, V. Stroobant, M. A. Gavin, P. Coulie, S. M. Xuereb, J. K. Mito, S. R. Riddell, and B. J. Van den Eynde. Rearrangement and proteasome-mediated splicing of noncontiguous peptides encoded by the *SP110* gene create a human minor histocompatibility antigen recognized by CD8⁺ cytotoxic T lymphocytes, manuscript in preparation.

Table 3 CD8⁺ CTL clones recognizing unique minor histocompatibility antigens

Patient	HLA			Sample day *	Therapy †	CTL clone ‡	Class I MHC restriction	% specific lysis §		
	A	B	C					Patient	Donor	RCC reactive
2	2, 25	8, 51	w7, w14	196	None	5C10(+) ¶	HLA-B51	68	0	+
						2B3(+)	HLA-A2	63	-4	+
						Clone 9	HLA-A2	86	2	+
						Clone 17	HLA-A2	63	3	+
3	3, 24	7, 40	w4, w7	61	CSP	3A12	HLA-B7	50	1	+
						5E1	ND	48	1	ND
						2H4	HLA-A3	86	0	-
						12D10(+)	ND	66	-1	ND
4	11, 24	54, 67	w1, w7	91	CSP	23G11(+)	HLA-B54	78	2	ND
					Pred	24B4-4	HLA-A11	28	2	-
5	26, 32	7, 38	w7, w12	105	CSP	13A3	HLA-B7	78	0	ND
					Pred	13H9(+)	ND	82	1	ND
6	1, 2	8, 15	w4, w7	35	CSP	1D8	HLA-A2	82	3	+
						12B3(+)	HLA-A2	69	4	+
						24B4-6	ND	64	4	ND
						58	None	None		

* Posttransplant day peripheral blood was collected as a source for *in vitro* T-cell culture.

† Immune-modifying therapies administered at the time peripheral blood samples were obtained: CSP, cyclosporine; Pred, prednisone.

‡ CTL clones were confirmed to be of donor origin by analysis with fluorescence *in situ* hybridization with Y chromosome-specific probes in sex-mismatched donor/recipient pairs or informative microsatellite markers in sex-matched donor-recipient pairs.

§ Representative specific lysis of patient- and donor-derived EBV-LCL targets in a 4-hour chromium-release cytotoxicity assay with an E:T ratio of 10:1 or 5:1.

¶ Isolation of additional clone(s) with the same specificity for allogeneic targets indicated by (+).

|| Posttransplant day relative to patient's second transplant.

Abbreviation: ND, not determined.

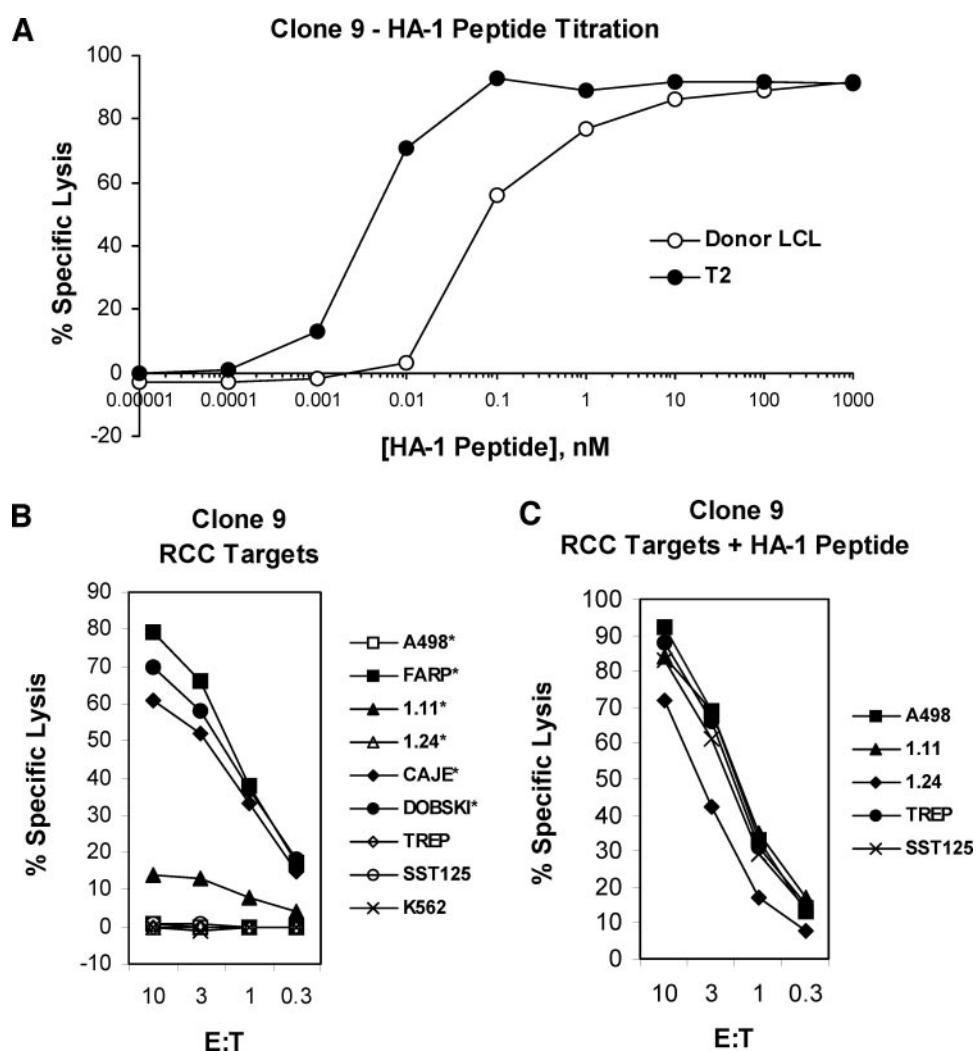
antigenic HA-1^H allele of *KIAA0223* from the nonantigenic HA-1^R allele revealed that the recipient was HA-1^{H/H}, whereas the donor was HA-1^{R/R}, thus confirming that a HA-1-specific donor anti-recipient CTL response was possible in this donor-recipient pair.

Recognition of Renal Cell Carcinoma Tumor Cells By CD8⁺ Minor H Antigen-specific CTL Clones. The association of posttransplant renal cell carcinoma regression with the development of GVHD in several studies (13–18) has suggested that minor H antigens that are expressed on renal cell carcinoma cells could potentially serve as targets for graft-versus-tumor responses. To determine whether any of the 14 distinct minor H antigens recognized by the panel of CTL clones isolated in this study (Table 3) are expressed on renal cell carcinoma tumor lines in cytotoxicity assays. Because tumor biopsy was not required for entry into this trial and therefore no autologous tumor was available from the five patients (2 to 6) from whom minor H antigen-specific clones were isolated, the clones were tested for recognition of panels of allogeneic renal cell carcinoma tumor cell lines (Table 4) that were known to express the relevant class I MHC-restricting allele for each clone. The wide variety of class I MHC alleles expressed by the renal cell carcinoma tumor lines listed in Table 4 made it possible to test the recognition of renal cell carcinoma cells by CTL clones specific for 10 of the 14 minor H antigens recognized by our panel of CTL clones, including at least one clone from each of the five patients studied. The availability of EBV-LCLs paired with 10 of the renal cell carcinoma tumor lines allowed for the definitive determination of the minor H antigen phenotype of the individuals from whom the renal cell carcinoma tumor cell lines were isolated.

CTL clones recognizing four distinct minor H antigens were isolated from patient 2, who achieved a partial response after hematopoietic cell transplantation, and all four clones demonstrated MHC-restricted cytolytic activity against renal cell carcinoma target cells; three of these clones were restricted by HLA-A2. One of the four clones was clone 9, which recognized the HLA-A*0201-restricted HA-1 minor H antigen encoded by *KIAA0223*. Surprisingly, although other groups have reported that HA-1 mRNA was detectable in a subset of renal cell carcinoma tumor cell lines propagated *in vitro* (44, 45), the cytolytic activity of HA-1-specific CTL against renal cell carcinoma tumor cells has not previously been reported. A panel of eight renal cell carcinoma tumor cell lines expressing HLA-A*0201 was collected and the HA-1 genotype for each tumor line determined by PCR-restriction fragment length polymorphism analysis of genomic DNA (Table 4). Five of these eight renal cell carcinoma tumor lines were heterozygous (A498, 1.11, 1.24, CAJE, and DOBSKI) and one line homozygous (FARP) for the antigenic HA-1^H allele of *KIAA0223* encoding the HA-1 peptide epitope. When tested in standard ⁵¹Cr release assays, clone 9 showed robust lytic activity against three of the six HA-1^H-bearing tumor lines (FARP, CAJE, and DOBSKI; Fig. 1B). The three other HLA-A*0201⁺/HA-1^H-bearing renal cell carcinoma lines that were not effectively recognized by clone 9 (A498, 1.11, and 1.24) could be sensitized for recognition with exogenous synthetic HA-1 peptide (VLHDDLLEA), as could the two HA-1^{R/R} renal cell carcinoma targets (TREP and SST125), suggesting that the levels of HLA-A*0201 on the surface of all eight renal cell carcinoma target cells were sufficient for CTL recognition (Fig. 1C).

Three additional clones isolated from patient 2, all of which recognize novel minor H antigen specificities, also showed

Fig. 1 The HA-1 minor H antigen is expressed by a subset of HLA-A*0201⁺, HA-1^H-bearing renal cell carcinoma (RCC) tumor cell lines. **A**, recognition of EBV-LCLs derived from the donor of patient 2 (genotype: HA-1^{R/R}) and of T2 cells pulsed with serial dilutions of the HA-1 peptide (VLHDDLLEA) by clone 9 CTL in a 4-hour ⁵¹Cr release assay at an E:T ratio of 10:1. **B**, A panel of eight HLA-A*0201⁺ RCC tumor cell lines was tested for HA-1 genotype by PCR-restriction fragment length polymorphism and then assessed for recognition by the CD8⁺ HA-1-specific CTL clone 9 in a ⁵¹Cr release assay at the indicated E:T ratios. Lines carrying an HA-1^H allele that encodes the antigenic HA-1 peptide are indicated by an asterisk. Class I MHC-negative K562 cells are also included as a negative control. **C**, The five RCC lines from **A** showing <20% specific lysis at E:T 10:1 were pulsed with HA-1 peptide (VLHDDLLEA) at 1 μg/mL for 1 hour at 37°C, washed twice, and then tested for recognition by CTL clone 9 in a ⁵¹Cr release assay at the indicated E:T ratios.



MHC-restricted cytolytic activity against renal cell carcinoma targets. CTL clone 5C10 was HLA-B51 restricted and recognized one of two HLA-B51⁺ EBV-LCLs (Fig. 2A) and the associated renal cell carcinoma tumor line (Fig. 2B) but did not recognize either of two additional HLA-B51⁺ renal cell carcinoma tumor lines for which paired EBV-LCLs were not available, nor did it recognize HLA-B51-negative renal cell carcinoma lines or the class I MHC-negative target cell K562 (data not shown). CTL clone 2B3 was HLA-A2-restricted and recognized one of four HLA-A2⁺ EBV-LCLs (Fig. 2C) and the associated renal cell carcinoma tumor line (Fig. 2D). This clone also recognized (35 to 55% specific lysis at E:T = 10:1) three of four additional HLA-A2⁺ renal cell carcinoma tumor lines for which no paired EBV-LCL lines were available (data not shown). CTL clone 17 was also HLA-A2 restricted and showed cytolytic activity against four of four HLA-A2⁺ EBV-LCLs (Fig. 2E) and the associated renal cell carcinoma tumor lines (Fig. 2F). This clone also lysed all four of the additional HLA-A2⁺ renal cell carcinoma tumor lines without paired EBV-LCLs (data not shown). Neither 2B3 nor clone 17 showed any recognition of HLA-A2-negative renal cell carcinoma targets or

of the class I MHC-negative K562 (data not shown). Thus, the three renal cell carcinoma-reactive HLA-A2-restricted CTL clones isolated from patient 2—clone 9, 2B3, and clone 17—recognized distinct minor H antigens that are expressed in different proportions of the HLA-A2⁺ population.

Of the eight unique CTL clones, defining eight distinct minor H antigens, that were isolated from patients 3, 4, and 5 (Table 3), MHC-restricted recognition of renal cell carcinoma tumor cells could only be conclusively demonstrated for one. For four of the clones, attempts to identify their class I MHC-restricting elements were unsuccessful despite testing the clones against extended panels of allogeneic EBV-LCL targets sharing one or more of the donor-recipient class I MHC alleles, suggesting that these CTLs may recognize minor H antigens encoded by polymorphic genes that are present at low frequency in the population. Two of the clones—2H4 and 24B4-4—showed MHC-restricted recognition of multiple allogeneic EBV-LCLs but not of their paired renal cell carcinoma tumor cell lines, suggesting that the minor H antigens recognized by these clones are expressed in EBV-LCLs but not in renal cell carcinoma tumor cells at a level sufficient to trigger the cytolytic program.

Table 4 Class I MHC typing and HA-1 genotype for 16 renal cell carcinoma tumor cell lines

Line	EBV-LCL	HLA			HA-1 genotype
		A	B	C	
A-498	No	2	8	w7	H/R *
FARP	Yes	2	51	w16	H/H
1.11	No	2, 66	18, 1	w7	H/R
1.24	Yes	1, 2	39, 63	w1, w7	H/R
TREP	No	2, 66	41, 56	w1, w17	R/R
CAJE	Yes	2, 26	44, 49	w7, w16	H/R
DOBSKI	No	1, 2	8, 51	w7, w14	H/R
SST125	Yes	1, 2	18, 37	w6, w7	R/R
786-0	No	3	7, 44	ND	ND
CAKI-2	No	1, 11	8, 51	w7, w12	ND
STAR	Yes	3	7, 15	ND	ND
1.18	Yes	1, 24	7, 8	w7	ND
JAUP	Yes	23, 26	44, 51	w1, w4	ND
GERK	Yes	3, 26	35, 40	w3	ND
LE9211	Yes	3	7, 35	w4, w7	ND
SST140	Yes	11, 26	35	w4	ND

* Antigenic HA-1^H allele encodes histidine at position 3 of the HA-1 peptide epitope (VLHDDLLEA) presented by HLA-A*0201 (42). Alternate HA-1^R allele encodes arginine at position 3.

Abbreviation: ND, not determined.

The sole renal cell carcinoma-reactive clone in this group was the HLA-B7–restricted clone 3A12, which was isolated from patient 3 who had stable disease after a second transplant. This CTL clone showed cytolytic activity against three of three HLA-B7⁺ EBV-LCLs (Fig. 3A) and the three associated renal cell carcinoma tumor lines, as well as an additional HLA-B7⁺ renal cell carcinoma tumor line (Fig. 3B).

Two of the three unique CTL clones isolated from patient 6, who had stable disease after hematopoietic cell transplantation, showed MHC-restricted recognition of renal cell carcinoma tumor cells. The HLA-A2–restricted clone 1D8 recognized three of four HLA-A2⁺ EBV-LCLs (Fig. 4A) and their associated renal cell carcinoma tumor lines (Fig. 4B). This clone also demonstrated lytic activity against three of four additional HLA-A2⁺ renal cell carcinoma tumor lines without paired EBV-LCLs (data not shown). The second renal cell carcinoma-reactive clone isolated from this patient, 12B3, which was also HLA-A2 restricted, was notable because its reactivity pattern against 11 allogeneic HLA-A2⁺ EBV-LCL lines coincided perfectly with that of CTL clone 2B3, isolated from patient 2 (data not shown), suggesting the possibility that these two clones recognize the same minor H antigen. When tested for recognition of EBV-LCL from patient 2, patient 6, and their respective donors, clones 2B3 and 12B3 both showed lytic activity against EBV-LCLs from the two patients but not the two donors. Finally, when tested against HLA-A2⁺ EBV-LCLs paired to renal cell carcinoma tumor lines, the CTL clone 12B3 recognized one of four EBV-LCLs (Fig. 4C) and its associated renal cell carcinoma tumor line (Fig. 4D)—the same EBV-LCL/renal cell carcinoma pair as recognized by clone 2B3 from patient 2—and demonstrated robust lytic activity against the same three additional HLA-A2⁺ renal cell carcinoma tumor lines without paired EBV-LCLs that were recognized by 2B3 (data not shown). These results strongly suggest that the 2B3 and 12B3 CTL clones recognize the same minor H antigen.

The Gene Encoding the Minor H Antigen Recognized by CTL Clones 2B3 and 12B3 Is Linked to Chromosome 19q. Molecular characterization of minor H antigens that are expressed on renal cell carcinoma cells and recognized by CTLs isolated from renal cell carcinoma patients whose disease progresses posttransplant will allow for further study of the contri-

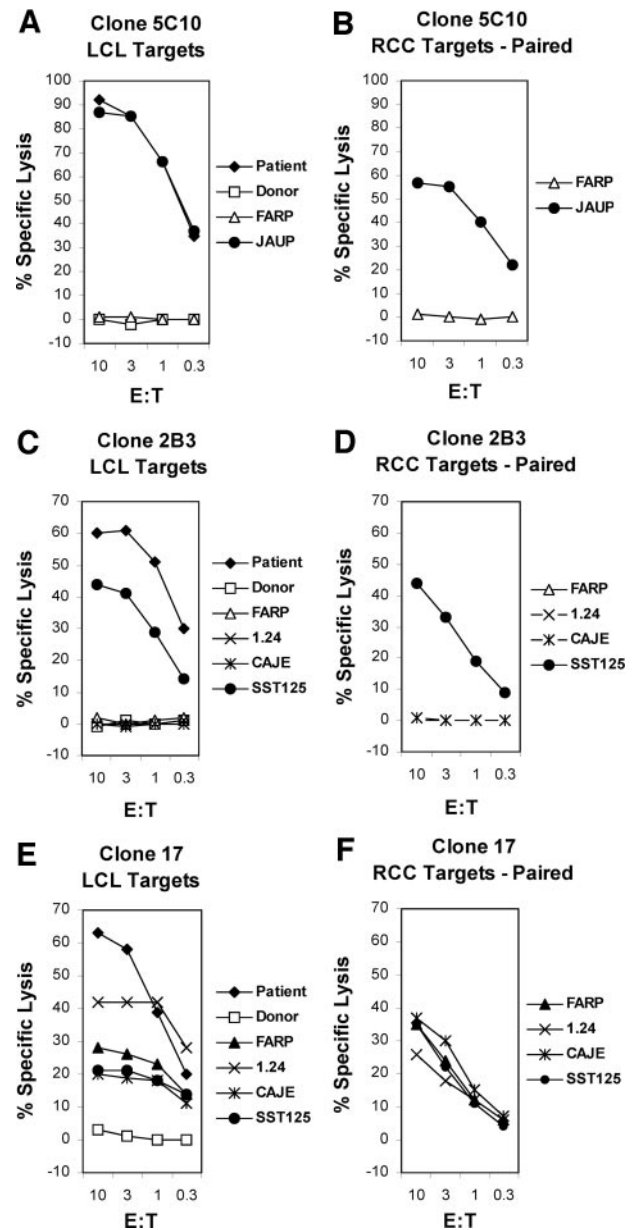


Fig. 2 Recognition of paired EBV-LCLs and renal cell carcinoma (RCC) tumor cells by CD8⁺ RCC-reactive minor H antigen-specific CTL clones isolated from patient 2. The HLA-B51–restricted clone 5C10 and the HLA-A2–restricted clones 2B3 and clone 17 were tested for cytolytic activity against EBV-LCLs derived from patient 2 and the donor, as well as allogeneic HLA-B51⁺ or HLA-A2⁺ individuals, respectively (A, C, and E), and against HLA-B51⁺ or HLA-A2⁺ RCC tumor cell lines derived from the same allogeneic individuals (B, D, and F) in a ⁵¹Cr release assay at the indicated E:T ratios.

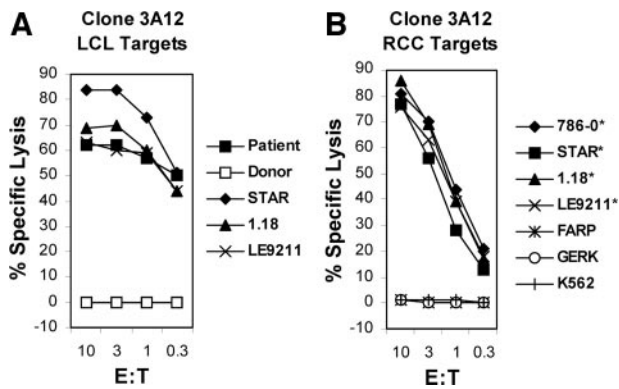


Fig. 3 Recognition of EBV-LCLs and renal cell carcinoma (RCC) tumor cells by a CD8⁺ RCC-reactive minor H antigen-specific CTL clone isolated from patient 3. The HLA-B7-restricted clone 3A12 was tested for cytolytic activity against EBV-LCLs derived from patient 3 and his donor, as well as allogeneic HLA-B7⁺ individuals (A), and against HLA-B7⁺ RCC tumor cell lines derived from the same allogeneic individuals (B) in a ⁵¹Cr release assay at the indicated E:T ratios. Cytolytic activity against an additional HLA-B7⁺ RCC tumor cell line (786-0) for which no paired EBV-LCLs was available, two HLA-B7-negative RCC tumor cell lines (FARP and GERK), and the class I MHC-negative K562 cell line are also presented in B. The HLA-B7⁺ RCC targets are indicated by an asterisk.

bution of CTL responses against these antigens to graft-versus-tumor effects. Such molecular characterization would allow, for example, the quantitation of antigen expression in malignant versus normal tissues and the enumeration of CTL responding to these antigens after allogeneic hematopoietic cell transplantation. In this study, CTL responses against the HLA-A2-restricted minor H antigen recognized by the renal cell carcinoma-reactive CTL clones 2B3 and 12B3 occurred in both patient 2, who experienced a partial response of renal cell carcinoma after hematopoietic cell transplantation, and also in patient 6, who had stable disease for >700 days posttransplant, suggesting that these responses might potentially have contributed to graft-versus-tumor activity in these two patients. Our group has previously demonstrated the feasibility of using genetic linkage analysis to identify the genes encoding minor H antigens (28). We therefore applied the same strategy to begin identification of the gene encoding the HLA-A2-restricted minor H antigen recognized by CTL clones 2B3 and 12B3.

All available EBV-LCLs derived from individuals in Utah CEPH reference families 1331, 1341, 1362, and 1416 were evaluated for minor H antigen expression by infecting them overnight with a recombinant vaccinia virus encoding HLA-A2 (*Vac/A2*) or wild-type vaccinia (*Vac/wt*) and testing them for recognition by CTL clones 2B3 and 12B3 in a standard 4-hour cytotoxicity assay. Concordant HLA-A2-dependent recognition of all 63 EBV-LCL targets by clones 2B3 and 12B3 was seen, thus confirming that these two clones recognize the same minor H antigen presented by HLA-A2. Each individual was classified as minor antigen positive or negative, as described previously (28). A genome-wide search for linkage was then conducted, with regularly spaced, highly informative microsatellite markers from the CEPH database v9.0. Two-point linkage analysis revealed that a cluster of marker loci on distal chromosome 19q13

was significantly linked (loci on distal score > 3.0) with the minor H antigen phenotype, with a maximum loci on distal score of 4.29 at $\theta = 0.001$ for marker KLK(AC) (Table 5). No other chromosomal region showed significant loci on distal scores. The candidate region, which encompasses the kallikrein gene cluster, does not contain any genes that previously have been identified as encoding any CTL-defined minor H antigens. These results therefore confirm that the minor H antigen recognized by clones 2B3 and 12B3 is indeed novel and that it is encoded by a gene located on the long arm of chromosome 19.

DISCUSSION

The initial trials of reduced intensity allogeneic hematopoietic cell transplantation regimens for metastatic renal cell carcinoma have generated considerable interest because responses were observed in patients who had experienced progressive disease after conventional immunotherapy (13–15, 17, 18), and some of these responses have been durable for >4 years (46). However, despite the use of reduced intensity conditioning regimens, the management of conditioning-related toxicity generally required inpatient care, and treatment-related complications have been responsible for deaths in 11 to 33% of patients treated in this fashion (13–17, 47). The low toxicity of

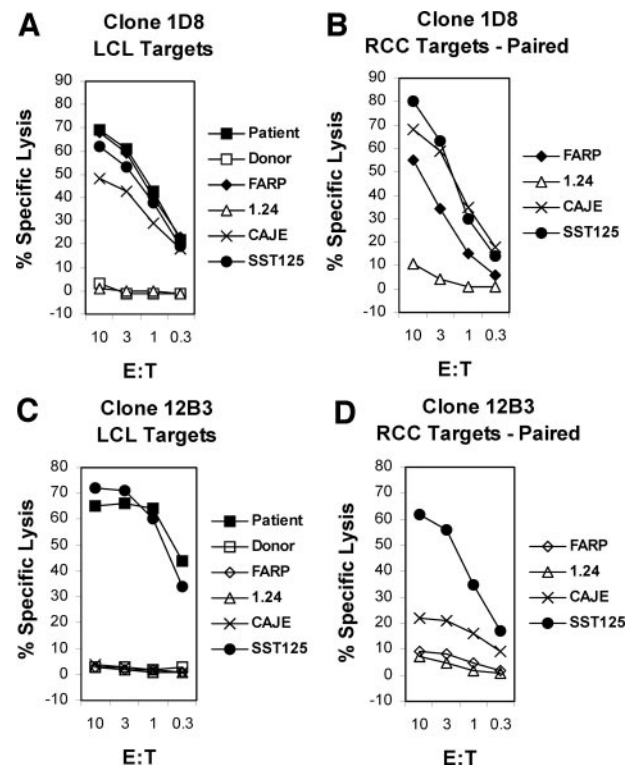


Fig. 4 Recognition of paired EBV-LCLs and renal cell carcinoma (RCC) tumor cells by CD8⁺ RCC-reactive minor H antigen-specific CTL clones isolated from patient 6. The HLA-A2-restricted clones 1D8 and 12B3 were tested for cytolytic activity against EBV-LCLs derived from patient 6 and the donor, as well as four allogeneic HLA-A2⁺ individuals (A and C), and against HLA-A2⁺ RCC tumor cell lines derived from the same allogeneic individuals (B and D) in a ⁵¹Cr release assay at the indicated E:T ratios.

Table 5 Chromosomal localization by genetic linkage analysis of the gene encoding the minor H antigen recognized by CTL clones 2B3 (from patient 2) and 12B3 (from patient 6) in four Utah CEPH genetic reference families

Marker	LOD score	Gene *	Description	Chromosome 19 position
D19S867	3.57@0.05	RPL13A	Ribosomal protein L13a	54685357
				55230307
D19S904	3.67@0.001	AL080143	Hypothetical protein	55240969
		AK131024	Hypothetical protein	55347618
				55468606
KLK(AC)	4.29@0.001	POLD1	Polymerase (DNA directed), delta 1, catalytic subunit 125 kDa	55579407
		SPIB	Spi-B transcription factor	55614027
		LOC284361	Hypothetical protein	55671593
		SCGF	Stem cell growth factor	55918452
		GPR32	G protein-coupled receptor 32	55965669
		MGC13170	Hypothetical protein	55992772
				56014215
		AY429508	Kallikrein 1 isoform 3 preproprotein	56014216
		KLK1	Kallikrein 1	56016841
		BT007253	Kallikrein 1, renal/pancreas/salivary	56014264
D19S206	3.56@0.001	LOC90353	Protein coding gene	56293587
		CD33	CD33 antigen (gp67)	56420178
		AK090470	FLJ00391 protein (Fragment)	56421033
		NKG7	Protein coding gene	56566686
		AK122619	Splice variant (Siglec8) gene	56605087
		AK091495	Hypothetical protein	56999876
		LOC147650	Hypothetical protein	56888405
		FLJ12644	Hypothetical protein	57084301
				57243159
				57259530
D19S601	2.49@0.05	AK093512	Hypothetical protein	57285645

* The genes listed represent a subset of those that are known to be encoded in the indicated intervals.

the fludarabine/total body irradiation conditioning regimen combined with posttransplant cyclosporine/mycophenolate mofetil has been established in patients with hematologic malignancies (12, 21, 23). This study confirms the limited toxicity of this conditioning regimen in patients with metastatic renal cell carcinoma. Four of the eight renal cell carcinoma patients were managed entirely in the outpatient clinic, and none of the patients died within the first 100 days after hematopoietic cell transplantation.

Mixed chimerism of peripheral blood CD3⁺ cells at 28 days after hematopoietic cell transplantation was observed in all eight renal cell carcinoma patients in this study, but two patients failed to achieve durable donor engraftment. The graft rejections were nonfatal and both patients recovered host hematopoiesis. For patients with hematologic malignancies, intensive chemotherapy received before transplant has been associated with a reduced risk for graft rejection, presumably by contributing to host immunosuppression (12, 23). Chemotherapy lacks efficacy against renal cell carcinoma and is not commonly used for these patients. In addition, the majority of renal cell carcinoma patients treated in this and similar studies had received prior IL-2 or IFN therapy, potentially augmenting host antidonor immune responses after hematopoietic cell transplantation. Increasing host immunosuppression by escalating the dose and duration of posttransplant mycophenolate mofetil to 15 mg/kg three times daily from day 0 through day 40 has been used effectively in patients with hematologic malignancies undergoing nonmyeloablative hematopoietic cell transplantation from unrelated donors (48, 49) and might decrease the rate of graft rejection while

maintaining low toxicity in renal cell carcinoma patients undergoing nonmyeloablative hematopoietic cell transplantation.

The overall incidence of acute GVHD appears to be reduced after nonmyeloablative hematopoietic cell transplantation compared with ablative hematopoietic cell transplantation (22). Nevertheless, GVHD still occurs in greater than half the patients treated by nonmyeloablative hematopoietic cell transplantation and remains a major cause of posttransplant morbidity and mortality. In this study, patient 4 died at day 263 from refractory infections related to high-dose immunosuppression for treatment of GVHD. In total, four of eight patients (six patients with sustained engraftment) developed grade 2 to 3 acute GVHD and four developed extensive chronic GVHD. Despite small patient numbers, the incidence of GVHD was similar to our experience with this regimen in patients with hematologic malignancies (22).

Striking regression of widespread metastatic lesions was seen in one patient (number 2) on this study who had earlier failed IL-2 therapy and had progressive disease at the time of transplantation, and two additional patients continue to have stable disease 434+ and 708+ days after hematopoietic cell transplantation, suggesting the development of a renal cell carcinoma-reactive graft-*versus*-tumor effect in a subset of patients. The effector mechanisms leading to renal cell carcinoma regression or stabilization after nonmyeloablative allogeneic hematopoietic cell transplantation remain largely undefined. A larger total number of donor-derived IFN- γ -secreting CD8⁺ T cells in posttransplant peripheral blood was associated with renal cell carcinoma regression in a cohort of renal cell carcinoma patients

treated by reduced intensity allogeneic hematopoietic cell transplantation at another institution (20). However, the specificity of such circulating CD8⁺ T cells for tumor-associated antigens *versus* pathogens or recipient tissue antigens that were not expressed on renal cell carcinoma was not determined. Thus, differences in the magnitude of the donor-derived tumor-reactive CTL response between responders and nonresponder renal cell carcinoma patients are unknown.

In this article, we demonstrate that CD8⁺ CTL specific for recipient minor H antigens could be detected in the peripheral blood of all five renal cell carcinoma patients studied who developed full donor chimerism of peripheral blood CD3⁺ cells after nonmyeloablative allogeneic hematopoietic cell transplantation (or after a second transplant in the case of patient 3). Furthermore, we provide direct evidence that a subset of these CD8⁺ T cells specific for minor H antigens were capable of recognizing antigen expressed on renal cell carcinoma tumor cells. Seven CD8⁺ CTL clones obtained from three patients, two with stable disease and one with a partial response, demonstrated robust MHC-restricted recognition of renal cell carcinoma tumor cell lines. These observations suggest that CD8⁺ CTL specific for minor H antigens may detect antigen on renal cell carcinoma cells *in vivo* and could contribute to tumor regression after nonmyeloablative allogeneic hematopoietic cell transplantation.

The close association of GVHD with responses of renal cell carcinoma after allogeneic hematopoietic cell transplantation observed in prior studies (13–18) suggested minor H antigen-specific CD8⁺ T cells may contribute to graft-*versus*-tumor effects in patients with renal cell carcinoma. Primary renal cell carcinoma specimens express class I HLA molecules (50), but the extent to which renal cell carcinoma tumor cells present class I MHC-restricted minor H antigens to CD8⁺ CTL had not been previously evaluated. Our group and others have previously reported that CD8⁺ CTL-recognizing minor H antigens on hematopoietic target cells can be detected in the peripheral blood of most leukemic patients after conventional myeloablative allogeneic hematopoietic cell transplantation (27, 51), and we have identified the genes encoding the epitopes recognized by several such CTL clones (29, 52, 53).⁶ The molecularly characterized minor H antigen HA-8 encoded by the *KIAA0020* gene is ubiquitously expressed in normal human tissues, and donor-recipient disparity for HA-8 has been associated with the development of acute GVHD after hematopoietic cell transplantation (54). We have found that a CTL clone specific for HA-8 lyses renal cell carcinoma targets cells *in vitro* that express both the HLA-A2-restricting allele for HA-8 and the antigenic allele of the *KIAA0020* gene.⁷ The ubiquitously expressed minor H antigen HA-3 (55) was also recognized by a HA-3-specific CTL clone in HLA-A1-expressing renal cell carcinoma target cell lines that carried the antigenic allele of *Lbc/AKAP13*.⁷ Thus, CTL responses elicited in renal cell carcinoma patients against broadly expressed minor H antigens such as HA-8 or HA-3 presented by both normal host tissues and renal cell carcinoma

tumor cells could account for the association of GVHD with tumor regression.

In this study, patient 2 achieved a partial response of renal cell carcinoma without clinical signs of GVHD. The observation of renal cell carcinoma regression without GVHD was also reported in another studies (13, 17, 56). These data suggest that posttransplant GVHD and graft-*versus*-tumor activity are not uniformly linked for all patients. Effector cells mediating graft-*versus*-tumor effects against renal cell carcinoma by recognizing antigens that are expressed in tumor cells but not broadly expressed in normal tissues could explain these clinical observations. Interestingly, a CTL clone specific for the HA-1 minor H antigen was isolated from patient 2. The expression of the *KIAA0223* mRNA that encodes HA-1 has previously been shown to be limited to hematopoietic cells and a subset of epithelial tumors (44, 45, 57). Our results extend these observations by demonstrating conclusively that the HA-1 minor antigen is expressed in a subset of renal cell carcinoma tumors that carry the antigenic HA-1^H allele. Additional novel minor H antigens recognized by other CTL clones isolated from this or other patients may also recognize minor H antigens with preferential expression in tumor cells and hematopoietic cells *versus* normal epithelia that are the target for GVHD. In this article, we have not directly searched for CTL-recognizing antigens that were expressed in renal cell carcinoma tumor but not in hematopoietic antigen presenting cells (APC). Such antigens might include kidney- or tumor-specific polymorphic minor H antigens or nonpolymorphic antigens derived from overexpressed (58) or mutated self proteins (59, 60) that could also selectively serve as targets for graft-*versus*-tumor responses but not GVHD. The use of autologous *CD80*-transfected renal cell carcinoma tumor cells as APC (61, 62) to facilitate the isolation of CD8⁺ CTL-recognizing antigens with tumor-specific expression developing posttransplant is currently under investigation in our laboratory.

The CTL clones generated in this study recognizing novel renal cell carcinoma-associated minor H antigens can be used to identify the genes that encode their peptide ligands (29, 39–43, 52, 53, 55, 63–65).⁶ The gene encoding the antigen recognized by CTL clones isolated from two different patients in this study has been linked to 19q13, a genetic locus not previously associated with a human minor H antigen. Identification of this gene and additional characterization of the minor antigen that it encodes remain an active focus of interest in our laboratory. The molecular characterization of renal cell carcinoma-associated minor histocompatibility antigens derived from patients experiencing regression of renal cell carcinoma will allow for both the assessment of antigen expression in primary renal cell carcinoma tumor samples, as well as normal host tissues, in addition to the quantitation of donor-derived CTLs responding to such antigen targets, and will thereby provide insight into the mechanisms that mediate renal cell carcinoma regression after allogeneic hematopoietic cell transplantation. The identification of additional minor H antigens such as HA-1 with preferential expression in renal cell carcinoma tumor *versus* normal epithelial tissues may form the basis for a new generation of nonmyeloablative allogeneic transplantation trials for renal cell carcinoma that could incorporate posttransplant vaccination or adoptive cellular therapy designed to augment the renal cell

⁷ S. Tykodi and E. Warren, unpublished observations.

carcinoma-reactive graft-versus-tumor response after hematopoietic cell transplantation without associated GVHD.

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REFERENCES

- Amato RJ. Chemotherapy for renal cell carcinoma. *Semin Oncol* 2000;27:177–86.
- Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. *CA - Cancer J Clin* 2002;52:23–47.
- Motzer RJ, Bander NH, Nanus DM. Renal-cell carcinoma. *N Engl J Med* 1996;335:865–75.
- Hillman GG, Droz JP, Haas GP. Experimental animal models for the study of therapeutic approaches in renal cell carcinoma. *In Vivo* 1994; 8:77–80.
- Beniers AJ, Peelen WP, Debruyne FM, Schalken JA. HLA-class-I and -class-II expression on renal tumor xenografts and the relation to sensitivity for alpha-IFN, gamma-IFN and TNF. *Int J Cancer* 1991;48: 709–16.
- Hillman GG, Puri RK, Kukuruga MA, Pontes JE, Haas GP. Growth and major histocompatibility antigen expression regulation by IL-4, interferon-gamma (IFN-gamma) and tumour necrosis factor-alpha (TNF-alpha) on human renal cell carcinoma. *Clin Exp Immunol* 1994; 96:476–83.
- Golumbek PT, Lazenby AJ, Levitsky HI, et al. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science (Wash. DC)* 1991;254:713–6.
- Seki N, Brooks AD, Carter CR, et al. Tumor-specific CTL kill murine renal cancer cells using both perforin and Fas ligand-mediated lysis in vitro, but cause tumor regression in vivo in the absence of perforin. *J Immunol* 2002;168:3484–92.
- Finke JH, Rayman P, Edinger M, et al. Characterization of a human renal cell carcinoma specific cytotoxic CD8+ T cell line. *J Immunother* 1992;11:1–11.
- Bernhard H, Karbach J, Wolfel T, et al. Cellular immune response to human renal-cell carcinomas: definition of a common antigen recognized by HLA-A2-restricted cytotoxic T-lymphocyte (CTL) clones. *Int J Cancer* 1994;59:837–42.
- Brouwenstijn N, Gaugler B, Kruse KM, et al. Renal-cell carcinoma-specific lysis by cytotoxic T-lymphocyte clones isolated from peripheral blood lymphocytes and tumor-infiltrating lymphocytes. *Int J Cancer* 1996;68:177–82.
- McSweeney PA, Niederwieser D, Shizuru JA, et al. Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. *Blood* 2001;97:3390–400.
- Childs R, Chernoff A, Contentin N, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N Engl J Med* 2000;343:750–8.
- Rini BI, Zimmerman T, Stadler WM, Gajewski TF, Vogelzang NJ. Allogeneic stem-cell transplantation of renal cell cancer after nonmyeloablative chemotherapy: feasibility, engraftment, and clinical results. *J Clin Oncol* 2002;20:2017–24.
- Bregni M, Doderio A, Peccatori J, et al. Nonmyeloablative conditioning followed by hematopoietic cell allografting and donor lymphocyte infusions for patients with metastatic renal and breast cancer. *Blood* 2002;99:4234–6.
- Hentschke P, Barkholt L, Uzunel M, et al. Low-intensity conditioning and hematopoietic stem cell transplantation in patients with renal and colon carcinoma. *Bone Marrow Transplant* 2003;31:253–61.
- Ueno NT, Cheng YC, Rondon G, et al. Rapid induction of complete donor chimerism by the use of a reduced-intensity conditioning regimen composed of fludarabine and melphalan in allogeneic stem cell transplantation for metastatic solid tumors. *Blood* 2003;102:3829–36.
- Blaise D, Bay JO, Faucher C, et al. Reduced-intensity preparative regimen and allogeneic stem cell transplantation for advanced solid tumors. *Blood* 2004;103:435–41.
- Warren EH, Gavin M, Greenberg PD, Riddell SR. Minor histocompatibility antigens as targets for T-cell therapy after bone marrow transplantation. *Curr Opin Hematol* 1998;5:429–33.
- Harlin H, Artz AS, Mahowald M, et al. Clinical responses following nonmyeloablative allogeneic stem cell transplantation for renal cell carcinoma are associated with expansion of CD8+ IFN-gamma-producing T cells. *Bone Marrow Transplant* 2004;33:491–7.
- Feinstein L, Sandmaier B, Maloney D, et al. Nonmyeloablative hematopoietic cell transplantation. Replacing high-dose cytotoxic therapy by the graft-versus-tumor effect. *Ann NY Acad Sci* 2001;938:328–37; discussion 37–9.
- Mielcarek M, Martin PJ, Leisenring W, et al. Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation. *Blood* 2003;102:756–62.
- Sandmaier BM, Maloney DG, Gooley TA, et al. Low-dose TBI conditioning for hematopoietic stem cell transplants (HSCT) from HLA-matched related donors for patients with hematologic malignancies: influence of fludarabine or cyoreductive autografts on outcome. *Blood* 2002;100(Pt. 1):145a.
- Storb R. Allogeneic hematopoietic stem cell transplantation: yesterday, today, and tomorrow. *Exp Hematol* 2003;31:1–10.
- Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst (Bethesda)* 2000;92:205–16.
- Flad T, Spengler B, Kalbacher H, et al. Direct identification of major histocompatibility complex class I-bound tumor-associated peptide antigens of a renal carcinoma cell line by a novel mass spectrometric method. *Cancer Res* 1998;58:5803–11.
- Warren EH, Greenberg PD, Riddell SR. Cytotoxic T-lymphocyte-defined human minor histocompatibility antigens with a restricted tissue distribution. *Blood* 1998;91:2197–207.
- Warren EH, Otterud BE, Linterman RW, et al. Feasibility of using genetic linkage analysis to identify the genes encoding T cell-defined minor histocompatibility antigens. *Tissue Antigens* 2002;59:293–303.
- Brickner AG, Warren EH, Caldwell JA, et al. The immunogenicity of a new human minor histocompatibility antigen results from differential antigen processing. *J Exp Med* 2001;193:195–206.
- Brodie SJ, Lewinsohn DA, Patterson BK, et al. In vivo migration and function of transferred HIV-1-specific cytotoxic T cells. *Nat Med* 1999;5:34–41.
- Parham P, Brodsky FM. Partial purification and some properties of BB7.2. A cytotoxic monoclonal antibody with specificity for HLA-A2 and a variant of HLA-A28. *Hum Immunol* 1981;3:277–99.
- Ebert T, Bander NH, Finstad CL, Ramsawak RD, Old LJ. Establishment and characterization of human renal cancer and normal kidney cell lines. *Cancer Res* 1990;50:5531–6.
- Tseng LH, Lin MT, Martin PJ, et al. Definition of the gene encoding the minor histocompatibility antigen HA-1 and typing for HA-1 from genomic DNA. *Tissue Antigens* 1998;52:305–11.

34. Dausset J, Cann H, Cohen D, et al. Centre d'étude du polymorphisme humain (CEPH): collaborative genetic mapping of the human genome. *Genomics* 1990;6:575-7.
35. Cottingham RW Jr, Idury RM, Schaffer AA. Faster sequential genetic linkage computations. *Am J Hum Genet* 1993;53:252-63.
36. Lathrop GM, Lalouel JM. Easy calculations of LOD scores and genetic risks on small computers. *Am J Hum Genet* 1984;36:460-5.
37. Lathrop GM, Lalouel JM, Julier C, Ott J. Strategies for multilocus linkage analysis in humans. *Proc Natl Acad Sci USA* 1984;81:3443-6.
38. Schaffer AA, Gupta SK, Shriram K, Cottingham RW Jr. Avoiding recomputation in linkage analysis. *Hum Hered* 1994;44:225-37.
39. Wang W, Meadows LR, den Haan JM et al. Human H-Y: a male-specific histocompatibility antigen derived from the SMCY protein. *Science (Wash. DC)* 1995;269:1588-90.
40. den Haan JM, Sherman NE, Blokland E, et al. Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science (Wash. DC)* 1995;268:1476-80.
41. Meadows L, Wang W, den Haan JM et al. The HLA-A*0201-restricted H-Y antigen contains a posttranslationally modified cysteine that significantly affects T cell recognition. *Immunity* 1997;6:273-81.
42. den Haan JM, Meadows LM, Wang W, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. *Science (Wash. DC)* 1998;279:1054-7.
43. Pierce RA, Field ED, Mutis T, et al. The HA-2 minor histocompatibility antigen is derived from a diallelic gene encoding a novel human class I myosin protein. *J Immunol* 2001;167:3223-30.
44. Fujii N, Hiraki A, Ikeda K, et al. Expression of minor histocompatibility antigen, HA-1, in solid tumor cells. *Transplantation* 2002;73:1137-41.
45. Klein CA, Wilke M, Pool J, et al. The hematopoietic system-specific minor histocompatibility antigen HA-1 shows aberrant expression in epithelial cancer cells. *J Exp Med* 2002;196:359-68.
46. Igarashi T, Mena O, Re F, Srinivasan R, Childs R. Exploring the role of allogeneic immunotherapy for non-hematologic malignancies: proof of concept and potential immune mechanisms of graft-vs-tumor effects in solid tumors. *Haematologica* 2002;87(Suppl. 1-n.6):2-5.
47. Pedrazzoli P, Da Prada GA, Giorgiani G, et al. Allogeneic blood stem cell transplantation after a reduced-intensity, preparative regimen: a pilot study in patients with refractory malignancies. *Cancer (Phila.)* 2002;94:2409-15.
48. Niederwieser D, Maris M, Shizuru JA, et al. Low-dose total body irradiation (TBI) and fludarabine followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and postgrafting immunosuppression with cyclosporine and mycophenolate mofetil (MMF) can induce durable complete chimerism and sustained remissions in patients with hematological diseases. *Blood* 2003;101:1620-9.
49. Maris MB, Niederwieser D, Sandmaier BM, et al. HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative conditioning for patients with hematologic malignancies. *Blood* 2003;102:2021-30.
50. Gastl G, Ebert T, Finstad CL, et al. Major histocompatibility complex class I and class II expression in renal cell carcinoma and modulation by interferon gamma. *J Urol* 1996;155:361-7.
51. Mutis T, Gillespie G, Schrama E, et al. Tetrameric HLA class I-minor histocompatibility antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. *Nat Med* 1999;5:839-42.
52. Warren EH, Gavin MA, Simpson E, et al. The human UTY gene encodes a novel HLA-B8-restricted H-Y antigen. *J Immunol* 2000;164:2807-14.
53. Murata M, Warren EH, Riddell SR. A human minor histocompatibility antigen resulting from differential expression due to a gene deletion. *J Exp Med* 2003;197:1279-89.
54. Akatsuka Y, Warren EH, Gooley TA, et al. Disparity for a newly identified minor histocompatibility antigen, HA-8, correlates with acute graft-versus-host disease after haematopoietic stem cell transplantation from an HLA-identical sibling. *Br J Haematol* 2003;123:671-5.
55. Spierings E, Brickner AG, Caldwell JA, et al. The minor histocompatibility antigen HA-3 arises from differential proteasome-mediated cleavage of the lymphoid blast crisis (Lbc) oncoprotein. *Blood* 2003;102:621-9.
56. Childs RW. Nonmyeloablative blood stem cell transplantation as adoptive allogeneic immunotherapy for metastatic renal cell carcinoma. *Crit Rev Immunol* 2001;21:191-203.
57. Wilke M, Dolstra H, Maas F, et al. Quantification of the HA-1 gene product at the RNA level; relevance for immunotherapy of hematological malignancies. *Hematol J* 2003;4:315-20.
58. Mollrem JJ, Lee PP, Wang C, et al. Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. *Nat Med* 2000;6:1018-23.
59. Brandle D, Brasseur F, Weynants P, Boon T, Van den Eynde B. A mutated HLA-A2 molecule recognized by autologous cytotoxic T lymphocytes on a human renal cell carcinoma. *J Exp Med* 1996;183:2501-8.
60. Gaudin C, Kremer F, Angevin E, Scott V, Triebel F. A hsp70-2 mutation recognized by CTL on a human renal cell carcinoma. *J Immunol* 1999;162:1730-8.
61. Jung D, Hilmes C, Knuth A, et al. Gene transfer of the co-stimulatory molecules B7-1 and B7-2 enhances the immunogenicity of human renal cell carcinoma to a different extent. *Scand J Immunol* 1999;50:242-9.
62. Mena O, Igarashi T, Srinivasan R, et al. Immunologic Mechanisms involved in the graft-vs-tumor effect in renal cell carcinoma (RCC) following nonmyeloablative allogeneic peripheral blood stem cell transplantation (NST). *Blood* 2001;98:856a.
63. Dolstra H, Fredrix H, Maas F, et al. A human minor histocompatibility antigen specific for B cell acute lymphoblastic leukemia. *J Exp Med* 1999;189:301-8.
64. Pierce RA, Field ED, den Haan JM, et al. Cutting edge: the HLA-A*0101-restricted HY minor histocompatibility antigen originates from DFFRY and contains a cysteinylated cysteine residue as identified by a novel mass spectrometric technique. *J Immunol* 1999;163:6360-4.
65. Akatsuka Y, Nishida T, Kondo E, et al. Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. *J Exp Med* 2003;197:1489-500.