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Generation of Tumor- Reactive CTL Against the Tumor- Associated Antigen HER2 Using Retrovirally Transduced Dendritic Cells Derived from CD34+ Hemopoietic Progenitor Cells

Christian Meyer zum Büschenfelde,* Nicole Nicklisch,* Stefan Rose-John, † Christian Peschel,* and Helga Bernhard2* 

Ag-specific CD8+ CTL are crucial for effective tumor rejection. Attempts to treat human malignancies by adoptive transfer of tumor-reactive CTL have been limited due to the difficulty of generating and expanding autologous CTL with defined Ag specificity. The current study examined whether human CTL can be generated against the tumor-associated Ag HER2 using autologous dendritic cells (DC) that had been genetically engineered to express HER2. DC progenitors were expanded by culturing CD34+ hemopoietic progenitor cells in the presence of the designer cytokine HyperIL-6. Proliferating precursor cells were infected by a retroviral vector encoding the HER2 Ag and further differentiated into CD83+ DC expressing high levels of MHC, adhesion, and costimulatory molecules. Retroviral transduction of DC resulted in the expression of the HER2 molecule with a transduction efficiency of 15%. HER2-transduced DC correctly processed and presented the Ag, because HLA-A*0201-positive DC served as professional APCs for stimulating autologous T lymphocytes. Following repetitive stimulation, a HER2-specific, HLA-A*0201-restricted CTL line was generated that was capable of lysing HLA-A*0201-matched tumor cells overexpressing HER2. A CD8+ T cell clone could be generated that displayed the same specificity pattern as the parenteral CTL line. The ability to generate and expand HER2-specific, MHC class I-restricted CTL clones using HER2-transduced autologous DC in vitro facilitates the development of adoptive T cell transfer for patients with HER2-overexpressing tumors without the requirement of defining immunogenic peptides. The Journal of Immunology, 2000, 165: 4133–4140.

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R ecent studies have provided evidence that infusion of tumor-reactive T cells represents a specific modality for the treatment of cancer (for review, see Ref. 1). Adoptive transfer of T cells isolated from tumor infiltrates or peripheral blood and nonspecifically expanded in vitro first documented the therapeutic activity of tumor-reactive T cells in humans (2, 3). However, the efficacy of these polyclonal T cells was limited, most likely due to the low frequency of Ag-specific CTL present in the transferred T cell populations (4). The first time Ag-specific CTL clones were evaluated for restoration of T cell immunity was in bone marrow recipients being at risk for CMV-related disease (5). Adoptive transfer of CMV-specific CTL clones generated from the HLA-matched bone marrow donors resulted in the reconstitution of protective T cell immunity against CMV disease. In bone marrow recipients, similar results were obtained for restoration of immunity against EBV by adoptive transfer of virus-specific T cells generated from the donor (6). Ongoing clinical trials evaluate the feasibility and efficacy of autologous EBV-specific CTL for treatment of EBV-related lymphomas, such as Hodgkin’s disease (7).

The growing number of identified tumor-associated Ags serve as potential targets for adoptive therapy of Ag-specific T cells (for review, see Refs. 8–12). The human epidermal growth factor receptor 2, also known as HER2,3 neu, HER2/neu, and c-erbB2, represents a tumor-associated Ag that is an appealing immunological target for the following reasons (for review, see Ref. 13). The HER2 gene is selectively amplified, and the resulting HER2 protein is overexpressed by malignant cells, in contrast to the corresponding normal cells expressing only low levels of HER2 (14). Adenocarcinomas of different tissue origin overexpress HER2, such as adenocarcinomas of the breast, ovary, stomach, and lung (for review, see Ref. 15). In addition, HER2 overexpression is stable over time and at multiple metastatic sites (16). Some patients already have a pre-existing T cell and Ab response to HER2, indicating the immunogenicity of the molecule (17, 18). The HER2 gene encodes for a 185-kDa transmembrane protein, and portions of this protein are likely to be available to both class I and II Ag processing pathways. Naturally processed peptide epitopes recognized by autologous CD8+ cytotoxic T cells have already been identified for HLA-A*0201 and HLA-A*0301 (19–24). As

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3 Abbreviations used in this paper: HER2, neu, HER2/neu, HER2, c-erbB2, human epidermal growth factor receptor 2; DC, dendritic cells; DC /H2N, HER2-transduced DC; ECD, extracellular domain; Flt3-L, fetal liver tyrosine kinase 3 ligand; HPC, hemopoietic progenitor cells; INF-MP, influenza A matrix protein; IL-2, natural IL-2; SCF, stem cell factor; ELISPOT, enzyme-linked immunospot.
amplification of the proto-oncogene HER2 contributes to the malignant phenotype of the tumor (25). HER2-overexpressing tumors might not be able to escape from an HER2-targeted immunotherapy through immunoselection of Ag-loss variants, as observed after vaccination with peptides derived from melanoma-associated differentiation Ags (26, 27). Studies in an animal model have shown that vaccines with rat neu peptides can generate rat neu-specific T cell immunity (28). In the first clinical trial, vaccination with HER2-derived peptides elicited HER2-specific T cell immunity in women with HER2-overexpressing breast cancer (29). Beside the attempts to induce HER2-specific T cell immunity, Ab-based immunotherapies have been developed to target the HER2 Ag. In clinical phase I/II trials, infusion of a humanized anti-HER2 mAb induced impressive tumor regression in some patients with HER2-overexpressing breast cancer (30, 31).

The attempts to transfer HER2-reactive CTL have been hampered by the difficulty to generate and clone autologous CTL directed against HER2. As preliminary studies of the development of an adoptive transfer of HER2-specific T cells, the current experiments examined whether HER2-reactive CTL can be generated, cloned, and expanded in vitro. Given that one of the major functions of dendritic cells (DC) is to initiate T cell responses (for review, see Refs. 32 and 33), DC were used as professional APCs for stimulating autologous T cells in vitro. The DC were genetically engineered to express the HER2 Ag through infection with a retrovirus encoding the HER2 gene. As retroviral transduction requires dividing cells, it was necessary to develop a system in which proliferating DC precursors could be efficiently transduced and further differentiated into mature DC. These retrovirally transduced DC expressed antigenic peptides and were able to elicit HER2-specific CTL that could be cloned and expanded in vitro.

Materials and Methods

Cell lines

The following cell lines were obtained from American Type Culture Collection (Manassas, VA): ovarian cancer cell line SKOV3 (HLA-A*0201, H2N*1), breast cancer cell lines SKBR3 (HLA-A*0201, H2N*1) and MCF7 (HLA-A*0201, H2N*1), and the fibroblast cell line NIH-3T3. The EBV-transformed B cell line MZ-EBV1257 (HLA-A*0201, H2N*1) was generated as previously described (34). The HLA-A*0201 transfectant cell line SKOV3A*0201 was a gift from M. L. Dix (University of Washington, Seattle, WA). The HLA-A*0201 TAP-defective cell line, T2, was provided by P. Cresswell (Yale University, New Haven, CT). The amphotropic producer cell line GP+envAM-12 was provided by B. Gansbacher (Technical University, Munich, Germany).

Tumor cell lines, EBV-transformed B cells, and T2 cells were cultured in RPMI 1400 (Life Technologies, Paisley, Scotland) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Packed cell line GP+envAM-12 and NIH-3T3 cells were maintained in DMEM (Life Technologies) supplemented with FCS, penicillin, streptomycin, and l-glutamine at the concentrations stated above.

Synthetic peptides

Peptides were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer and purified by reverse phase HPLC (MWG AG Biotech, Ebersberg, Germany). The purity of the peptides was >90%, as indicated by analytical HPLC. Lyophilized peptides were diluted in PBS/2% DMSO (Serva Electrophoresis, Heidelberg, Germany) and stored at -20°C.

HER2-specific CTL line

Pep2-specific CTL were generated by repetitive stimulation with mature CD8+ DC as APC. DC were generated from monocytes using a protocol recently published by Jounewit et al. (35). Monocyte-derived DC were incubated with 10 μg/ml HER2p200,377 for 2 h at room temperature and then cocultured with autologous PBMC in RPMI 1640 medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine, and 5% autologous serum. The culture medium was further supplemented with 5 ng/ml rIL-7 (PharMingen International, Hamburg, Germany) on day 1 and 100 U/ml human IL-2 (nI-L-2; Biotest Pharma, Dreieich, Germany) on day 3. Responding T cells were restimulated with peptide-pulsed DC at weekly intervals in the presence of rIL-2 and rIL-7. The ratio of stimulator to responder cells was 1:20 for priming and 1:50 for restimulation. Specificity analyses of proliferating T cells were performed after three restimulations.

Retroviral vector and virus production

The retroviral vector (NAPTK) used in this study was a gift from B. Gansbacher (Technical University of Munich). NAPTK is derived from the genome of Moloney murine leukemia virus containing the bacterial neo-mycin resistance (neo) gene as a selection marker and the herpes simplex virus thymidine kinase (HSV-TK) promoter (36). To generate the plasmid pSIL-TK, containing a cDNA encoding the human HER2, was provided by Dr. G. Spies (University of Washington, Seattle, WA). The cDNA encoding the full-length HER2 or the extracellular domain (ECD) of HER2 was cloned into a unique NotI restriction site of the retroviral vector NAPTK, and the resulting retroviral vector construct was transfected into the helper-free amphotropic packaging cell line GP+envAM-12 (37) using a liposomal transfection reagent (N-[1-[2,3-dioleoyloxy]propyl]-N,N,N,N-tetramethylammonium methylsulfate, Roche, Mannheim, Germany). Colonies were isolated by neomycin selection (G418, Sigma-Aldrich, Steinheim, Germany) and expanded. Supernatants of cloned packaging cells were harvested, filtered (0.45 μm pore size), and tested for the presence of virus. Viral titration was performed on NIH-3T3 cells in the presence of neomycin. Supernatants of cell clones secreting a high virus titer (105–106 CFU/ml) were used to infect proliferating DC precursors derived from CD34+ hematopoietic progenitor cells (HPC).

Generation of DC from CD34+ HPC

DC cultures were generated from CD34+ HPC derived from peripheral blood stem cell collections from donors following mobilization with G-CSF (38). CD34+ HPC were isolated using positive selection with an immunomagnetic bead system (Milteny, Bergisch Gladbach, Germany). The purity of recovered CD34+ HPC was determined by flow cytometric analysis to be 85–95%. After purification, CD34+ HPC were cryopreserved in RPMI 1640 containing 10% DMSO (Serva Electrophoresis). CD34+ HPC were cultured at 105 cells/well in six-well plates (Greiner Labortecnique, Oberschleissheim, Germany) using 3 ml of X-VIVO 15 medium (BioWhittaker, Walkersville, MD) supplemented with 2 nM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1% human AB serum (Life Technologies, Grand Island, NY) in the presence of the following cytokines at the indicated concentrations: 50 ng/ml stem cell factor (SCF; R&D Systems, Minneapolis, MN); 75 ng/ml fetal liver tyrosine kinase 3-ligand (Flt3-L; PeproTech EC, London, U.K.); 10 ng/ml HyperIL-6 (39); 50 ng/ml IL-4, 0.5 ng/ml TGF-β1, and 2 or 100 ng/ml TNF-α (all from Strathmann-Biotech, Hannover, Germany); and 100 ng/ml GM-CSF (Novartis, Nutting, Germany).

Culture conditions for generating DC from CD34+ HPC consisted of three phases: expansion (days 0–7), differentiation (days 8–26), and maturation (days 27–28). During the expansion phase, extensive proliferation of primitive progenitor cells was induced in the presence of HyperIL-6, SCF, Flt3-L, and TGF-β1 (days 0–7). Proliferating HPC were differentiated into immature DC with IL-4, GM-CSF (days 8–26), and 2 ng/ml TNF-α (days 15–26) added to the cytokines used for expansion. Finally, maturation of DC was induced by addition of high doses of TNF-α (100 ng/ml) during the last 2 days (days 27 and 28). Following 28 days of culture, cells were harvested, phenotyped, and used for T cell stimulation.

Retroviral transduction of DC derived from CD34+ HPC

Proliferating CD34+ HPC were transduced twice with retroviral supernatant during the expansion phase. Cultured HPC were harvested on day 6, and 3 x 1010–1011 cells were resuspended in 5 ml of medium containing 1.5 ml of retroviral supernatant and 1.5 ml of X-VIVO medium supplemented with the cytokines necessary for cell expansion: HyperIL-6, SCF, Flt3-L, and TGF-β1. HPC were transduced overnight in the presence of 4 μg/ml Polybrene (Sigma, Deisenhofen, Germany). On day 7 of the expansion phase, retroviral transduction was repeated to enhance the transduction efficiency (40–42).

Polymerase chain reaction

DNA from retrovirally transduced and nontransduced DC was isolated according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Equal amounts of DNA (100 ng) from retrovirally transduced and nontransduced DC were used for each PCR. As positive control, DNA was isolated from retroviral supernatant derived from the HER2-transfected packing cell line.
The positive control contained less DNA, but equal amounts of DNA would have resulted in an overloaded gel. Primers used for amplification of HER2 were 5'-gacctggcgacagaatgca-3' and 5'-ttgacccggctcagcag-3'. PCR was performed for 33 min at 94°C, for 1.30 min at 58°C and for 1.30 min at 72°C for 35 cycles, followed by a final extension time of 8 min at 72°C. The PCR product was resolved on a 1.5% agarose gel.

Western blot analysis

Cell lysates were made from DC, retrovirally transduced DC, and SKOV3.A*0201 cells, as recently described (28). Equal amounts of protein were loaded for the retrovirally transduced DC and the nontransduced DC, but lesser amounts for the HER2-overexpressing cell line SKOV3 which served as a positive control. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose. The HER2 protein was identified using the mAb c-neu-Ab3 recognizing the intracellular domain of HER2 (Oncogene Science, Uniondale, NY) as primary Ab and rabbit anti-mouse peroxidase-conjugated Fab2 fragment (Amersham Pharmacia Biotech, Freiburg, Germany) as secondary Ab. The blots were developed using a chemiluminescent reaction (ECL, Amersham).

Flow cytometric analysis

DC or T cells were harvested, washed with PBS, and resuspended in PBS containing 0.5% BSA. Because Fc receptors are highly expressed on DC, the DC were first incubated with Fc receptor-blocking reagent (Milenyi) for 45 min at 4°C to reduce nonspecific fluorescence. Phenotypic analyses were performed by flow cytometry using saturating concentrations of the PE-conjugated mAb against following Ags: HLA-DR, CD80, CD86, CD54, CD40, CD19, and CD56 (all from Becton Dickinson, Mountain View, CA); CD83 (Coulter Immunotech, Miami, FL); and CLA, CD1a, CD14, CD34, CD3, CD4, and CD8 (all from Pharmingen). Conjugated isotype-matched mAb (all from Becton Dickinson) were used as controls. For phenotype analyses all cells were gated with the exception of dead cells, which were excluded. For detection of HER2, cells were sequentially incubated with the unconjugated mAb c-neu-Ab6 recognizing the extracellular domain for HER2 (Oncogene Science) and FITC-conjugated mAb recognizing the intracellular domain of HER2 (Oncogene Science, Uniondale, NY) as primary Ab and rabbit anti-mouse peroxidase-conjugated Fab2 fragment (Amersham Pharmacia Biotech, Freiburg, Germany) as secondary Ab. The blots were developed using a chemiluminescent reaction (ECL, Amersham).

Generation of HER2-specific CTL lines and clones using retrovirally transduced DC

DC retrovirally transduced with the HER2 gene were seeded together with autologous PBMC in RPMI 1640 medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 5% autologous serum. Cells were cocultured in a 96-well nitrocellulose filter plate coated with 7.5 × 10⁷ × 10⁴ T cells in one well of a 24-well flat-bottom plate (Greiner Laborteknik). To the DC-T cell coculture, 5 ng/ml rIL-2 was added on day 1, and 100 U/ml rIL-2 was added on day 3. Proliferating T cells were restimulated weekly using HER2-transduced DC at a 1:50 stimulator to responder ratio. Following three to five cycles of weekly stimulations, the HER2 specificity of T cells was verified by measuring Ag-specific cytotoxicity and IFN-γ production. HER2-specific polyclonal T cells were cloned by limiting dilution according to the protocol described by Greenberg et al. (43, 44). Briefly, T cells were plated at 0.3 cells/well in 96-well round-bottom plates with 30 ng/ml anti-CD3 mAb (OKT-3; Janssen, Cilag, Neuss, Germany), 5 × 10⁷/well allogeneic irradiated (30 Gy) PBMC, 10⁷/well irradiated (80 Gy) MZ-EBV1257, and 50 U/ml rIL-2 (Chiron, Emeryville, CA). Proliferating T cells were harvested, washed with PBS, and resuspended in PBS containing 0.5% BSA. Because Fc receptors are highly expressed on DC, the DC were first incubated with Fc receptor-blocking reagent (Milenyi) for 45 min at 4°C to reduce nonspecific fluorescence. Phenotypic analyses were performed by flow cytometry using saturating concentrations of the PE-conjugated mAb against following Ags: HLA-DR, CD80, CD86, CD54, CD19, and CD56 (all from Becton Dickinson, Mountain View, CA); CD83 (Coulter Immunotech, Miami, FL); and CLA, CD1a, CD14, CD34, CD3, CD4, and CD8 (all from Pharmingen). Conjugated isotype-matched mAb (all from Becton Dickinson) were used as controls. For phenotype analyses all cells were gated with the exception of dead cells, which were excluded. For detection of HER2, cells were sequentially incubated with the unconjugated mAb c-neu-Ab6 recognizing the extracellular domain for HER2 (Oncogene Science) and FITC-conjugated mAb recognizing the intracellular domain of HER2 (Oncogene Science, Uniondale, NY) as primary Ab and rabbit anti-mouse peroxidase-conjugated Fab2 fragment (Amersham Pharmacia Biotech, Freiburg, Germany) as secondary Ab. The blots were developed using a chemiluminescent reaction (ECL, Amersham).

Enzyme-linked immunospot (ELISPOT) assay

The presence of IFN-γ-producing, HER2-specific T cells was assessed in an ELISPOT assay, recently described by Herr et al. (45). Stimulator and responder cells were cultured in a 96-well nitrocellulose filter plate coated with mouse anti-human IFN-γ capture Ab Dm1.2 (Mabtech, Nacka, Sweden). Autologous DC (20,000/well), HLA-matched tumor cells (20,000/well), or peptide-pulsed T2 cells (20,000/well) were used as stimulator cells and cocultured with CTL (1,000/well) overnight (12–18 h) at 37°C with 5% CO₂. Cells were then removed by washing with PBS, and the proliferation of IFN-γ-producing cells was detected by an additional staining of biotinylated mouse anti-human IFN-γ (Mabtech) and alkaline phosphatase-conjugated streptavidin (Mabtech). The number of stained spots corresponding to the IFN-γ-producing cells was counted using a dissecting microscope coupled with a computer-assisted video image analysis (Zeiss, Göttingen, Germany). The data in the figures refer to the mean of three replicates. SDs were generally within 5–20% of the mean.

Chromium release assay

Cytolytic activity was determined as previously described (46). Briefly, 10⁶ T2 cells were labeled in 100 μl of FCS with 200 μCi/ml ⁵¹Cr (ICN Biochemicals, Irvine, CA) for 1.5 h at 37°C and then loaded with 10 μg/ml peptide for 1 h at room temperature. Tumor cell lines and DC were labeled with 100 μCi/ml ⁵¹Cr for 1 h at 37°C. ⁵¹Cr-labeled target cells and graded doses of T cells were given in 200 μl of T cell medium/well of a V-bottom 96-well tissue culture plate (Costar, Cambridge, MA). For inhibition experiments, mAb W6/32, an Ab against a common MHC class I determinant, or mAb MA2.1 recognizing HLA-A*0201 was added to the coculture, as previously described (47). Cells were incubated for 4 h at 37°C. The plates were centrifuged at 200 × g for 5 min, 100 μl of supernatant was collected, and radioactivity was measured in a gamma counter. The percentage of specific ⁵¹Cr release was calculated as follows: % specific ⁵¹Cr release = (experimental ⁵¹Cr release – spontaneous ⁵¹Cr release) × 10⁰/(maximum ⁵¹Cr release – spontaneous ⁵¹Cr release). Maximum ⁵¹Cr release was obtained by adding 100 μl of 1% Nonidet P-40 (Sigma, St. Louis, MO) to 100 μl of labeled target cells. Spontaneous ⁵¹Cr release ranged from 5 to 10% of the total counts incorporated. The data in the figures refer to the mean of two replicates. SDs were generally within 5–10% of the mean.

Results

CD34+ HPC expanded with HyperIL-6 and transduced with a retrovirus are capable of differentiating into mature CD83+ DC

Given that efficient retroviral transduction requires dividing cells, it was necessary to develop improved culture conditions that allow retroviral transduction of proliferating dendritic progenitor cells. We have previously shown that HyperIL-6 in the presence of SCF leads to an expansion of CD34+ HPC capable of differentiating into mature DC (48). Based on these findings, initial experiments asked whether the proliferative capacity of HyperIL-6 could be used for retroviral transduction of DC progenitors. Dividing CD34+ HPC were retrovirally transduced in the presence of HyperIL-6, SCF, and TGF-β1, then further differentiated into immature DC by adding IL-4, GM-CSF, and low doses of TNF-α, and finally matured under the influence of high doses of TNF-α. During the culture period of 4 wk, the total cell count increased 60- to 80-fold depending on the individual cell culture (data not shown). On day 26, 55–85% of all cultured cells displayed the typical phenotype of immature DC expressing low levels of HLA-DR, CD80, and CD86 (Fig. 1A). With TNF-α treatment, immature CD83+ DC developed into mature DC, as determined by the detection of CD83, a molecule known to be expressed by mature, myeloid-derived DC (Fig. 1B). Mature CD83+ DC highly expressed MHC class II as well as the accessory molecules CD40, CD80, CD86, and CD54 (ICAM-1). DC did not express CD1a or CLA (data not shown), molecules related to Langerhans cells. The phenotype of retrovirally transduced DC did not differ from that of nontransduced DC (data not shown). Cultured cells did not display lineage markers for B cells (CD19), T cells (CD3), NK cells (CD56), or monocytes (CD14).

DC transduced with HER2 retrovirus express HER2 protein and present HER2 peptides in context with MHC class I

DC progenitors were infected with the HER2 retrovirus on days 6 and 7 of the expansion phase (days 0–7). Following culture periods of DC differentiation (days 8–26) and maturation (days 27 and 28), DC were harvested on day 28 and assessed for successful retroviral transduction. Integration of HER2 DNA was determined by PCR using HER2-specific primers (Fig. 2A). Retrovirally transduced DC, but not native DC, were positive for HER2 DNA. DNA isolated from virus particles served as a positive control. HER2 protein synthesis was analyzed by Western blotting using an mAb
Directed against the intracellular domain of HER2 (Fig. 2B). Transduced DC expressed the HER2 protein, as documented by positive staining and correct size of 185 kDa. The ovarian cancer cell line SKOV3, which is known to overexpress HER2, served as a positive control; nontransduced DC served as a negative control. The efficacy of retroviral transduction was analyzed by FACS analysis using an mAb against the ECD of HER2 (Fig. 2C). The transduction efficiency was ~15%.

Because stimulation of Ag-specific T cells requires correct processing and presentation of antigenic epitopes, the ability of HER2-transduced DC to present immunogenic HER2 epitopes in context with MHC class I molecules was determined. For detection of MHC class I-bound peptides on the surface of HER2-transduced DC, an HLA-A*0201-restricted CTL line specific for the immunodominant peptide HER2369–377 was generated. The established peptide-specific CTL line NK1 lysed T2 cells pulsed with HER2369–377, whereas T2 cells loaded with an irrelevant peptide derived from the influenza A matrix protein (INF-MP 58–66) were not recognized (Fig. 3A). In addition, the CTL line NK1 lysed HER2-overexpressing SKOV3 cells when transfected with the relevant MHC allele HLA-A*0201 (SKOV3aA*0201), whereas SKOV3 cells naturally not expressing HLA-A*0201 were not lysed. Peptide specificity was confirmed by measuring IFN-γ secretion of CTL line NK1 upon contact with T2 loaded with HER2369–377 and SKOV3aA*0201 (Fig. 3B).

HER2-specific, HLA-A*0201-restricted lytic activity and IFN-γ secretion by the CTL line NK1 were used to analyze the correct peptide presentation of retrovirally transduced DC. HLA-A*0201-positive DC infected with HER2 retrovirus were lysed by the CTL line NK1, demonstrating that the peptide HER2369–377 had been endogenously processed and presented with HLA-A*0201 upon retroviral transduction (Fig. 3A). In addition, CTL line NK1 secreted IFN-γ upon contact with HER2-transduced HLA-A*0201-positive DC (Fig. 3B). In contrast, retrovirally transduced DC from HLA-A*0201-negative donors and nontransduced DC from HLA-A*0201-positive donors did not serve as targets for CTL line NK1.

HER2-specific, tumor-reactive CTL can be generated and cloned using HER2-transduced DC as APC

Because retrovirally transduced DC were capable of presenting HER2-derived T cell epitopes, HER2-transduced DC were used as professional APC to induce a cytotoxic T cell response in vitro. PBMC from a normal donor were stimulated with autologous HER2-transduced DC at weekly intervals. Following four stimulations, the resulting CTL line, PS, was investigated for HER2-specific cytotoxic activity in a standard chromium release assay (Fig. 4A). CTL line PS lysed HER2-overexpressing SKOV3aA*0201 cells in an HLA-A*0201-restricted manner, whereas HLA-A*0201-negative SKOV3 cells were not lysed. An HLA-A*0201-positive breast cancer cell line, MCF7, expressing low levels of HER2 was not lysed by CTL line PS. HER2 specificity was confirmed using autologous DC as target cells in an IFN-γ ELISPOT assay (Fig. 4B). The CTL line PS released IFN-γ.
upon stimulation with HER2-transduced DC, whereas nontransduced DC did not induce IFN-γ secretion. CTL lines generated from two additional HLA-A*0201-positive donors displayed a similar specificity pattern as the CTL line PS lysing HER2-overexpressing, HLA-A*0201-positive tumors (data not shown). Lysis of SKOV3tA*0201 cells (38% lysis at an E:T cell ratio of 30:1) was inhibited in the presence of mAb W6/32 (10% lysis) or mAb MA2.1 (12% lysis), confirming HLA-A*0201 as a restriction element.

The HER2-specific CTL line PS was cloned by limiting dilution and screened for HER2 specificity using autologous HER2-transduced and nontransduced DC as target cells for cytotoxicity analyses. Of four screened HER2-specific CTL clones, the CTL clone PS-D10 could be expanded and tested for cytotoxicity against a panel of cell lines (Fig. 5). CTL clone PS-D10 displayed the same specificity pattern as the parental CTL line PS. HER2-overexpressing, HLA-A*0201-positive cells (SKOV3tA*0201) were lysed, whereas HER2-overexpressing HLA-A*0201-negative cells (SKOV3 and SKBR3) were not lysed. The HLA-A*0201-positive tumor cells expressing low levels of HER2 (MCF7) and HLA-A*0201-negative cells negative for HER2 (MZ-EBV1257) were not recognized by CTL clone PS-D10. Because the CTL clone PS-D10 recognized DC transduced with a retrovirus encoding the ECD of HER2, two immunodominant peptides, HER2369–377 (20) and HER2 435–443 (24), both derived from the ECD HER2 and known to be presented with HLA-A*0201, were tested. These two tested HER2 peptides were not able to sensitize T2 cells for lysis by CTL clone PS-D10 (data not shown).

**Discussion**

The immunogenic potential of DC for cancer therapy has been widely investigated for the generation of tumor-reactive cytotoxic T cells in vitro and in vivo. Presentation of defined and/or undefined tumor-associated Ags by DC can be achieved using different methods of Ag delivery, such as tumor cell lysates, apoptotic tumor cells, heat shock proteins, recombinant antigenic proteins, synthetic or MHC class I-stripped peptides, gene transfer using
naked DNA and RNA, or infection with viral vectors recombinant for tumor Ags (for review, see Ref. 49). Optimal presentation of a defined tumor-associated Ag, such as HER2, might be achieved by the retroviral transduction of dividing dendritic progenitor cells, allowing long-term and stable expression of multiple peptide epitopes in context with different MHC class I alleles. Retroviral vectors require target cells to undergo cell division to integrate (50), in contrast to other viruses, such as vaccinia viruses (44, 51) and adenoviruses (52, 53).

In this study we have improved the culture conditions for CD34+ HPC, allowing retroviral transduction of proliferating precursors that are capable of differentiating into mature DC. The expansion phase of the cell culture was based on the proliferative signal of HyperIL-6, a fusion protein of IL-6 linked to its soluble IL-6R (39). HyperIL-6 associates with the signal transduction protein gp130 that is expressed by every cell (54) and, in contrast to IL-6R (39), HyperIL-6 associates with the signal transduction protein gp130 via HyperIL-6 together with c-bound IL-6R, including primitive HPC (39, 48, 56). Simultaneous expression gp130 that is expressed by every cell (54) and, in contrast to IL-6R (39). HyperIL-6 associates with the signal transduction protein gp130 via HyperIL-6 together with c-bound IL-6R, including primitive HPC (39, 48, 56). Simultaneous expression gp130 that is expressed by every cell (54) and, in contrast to IL-6R (39). HyperIL-6 associates with the signal transduction protein gp130 via HyperIL-6 together with c-bound IL-6R, including primitive HPC (39, 48, 56).

The ability to generate CTL against HER2-overexpressing tumors using retrovirally transduced DC allows generation of CTL without the knowledge of HLA alleles or peptide epitopes. Successful screening and cloning of CTL lysing HER2-overexpressing tumors (71). Due to the low Ag expression level of retrovirally transduced DC, stimulated T cells are confronted with low amounts of peptides that might support the generation of T cells with a sufficiently high affinity to kill tumor cells. Of note, the DC were cultured in human serum instead of FCS (40, 41), consecutively circumventing the presentation of xenogenic protein Ags.

Recognition of peptide epitopes by CTL has been shown to require the expression of the encoding gene above a certain threshold (72). Given the fact that the established HER2-specific CTL clone PS-D10 lysed tumor cells expressing high levels of HER2, but not cells with low level expression of HER2 in vitro, adoptive transfer of these T cells with intermediate affinity might lead to tumor rejection without damage in normal tissues in vivo. Similar observations have been made using a humanized anti-HER2 mAb that inhibits the growth of HER2-overexpressing tumor cells. Administration of this Ab as a single agent produced tumor responses in patients without evidence of autoimmune disease (31).

The ability to generate CTL against HER2-overexpressing tumors using retrovirally transduced DC allows generation of CTL without the knowledge of HLA alleles or peptide epitopes. Successful screening and cloning of CTL lysing HER2-overexpressing tumor cells facilitates the further development of adoptive transfer of HER2-specific T cells for patients with HER2-overexpressing tumors. Current experiments focus on the generation of HER2-specific Th cells, because long-term survival of CTL is dependent on the presence of T cell help (5).
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References


