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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
CTLs Directed against HER2 Specifically Cross-React with HER3 and HER4

Heinke Conrad, Kerstin Gebhard, Holger Krönig, Julia Neudorfer, Dirk H. Busch, Christian Peschel, and Helga Bernhard

The human epidermal growth factor receptor 2 (HER2) has been targeted as a breast cancer-associated Ag by T cell-based immunotherapeutical strategies such as cancer vaccines and adoptive T cell transfer. The prerequisite for a successful T cell-based therapy is the induction of T cells capable of recognizing the HER2-expressing tumor cells. In this study, we generated human cytotoxic T cell clones directed against the HER2_369–377 epitope known to be naturally presented with HLA-A*0201. Those HER2-reactive CTLs, which were also tumor lytic, exhibited a similar lysis pattern dividing the targets in lysable and nonlysable tumor cells. Several HER2-expressing tumor cells became susceptible to CTL-mediated lysis after IFN-γ treatment and, in parallel, up-regulated molecules of the Ag-presenting machinery, indicating that the tumor itself also contributes to the success of CTL-mediated killing. Some of the HER2_369–377-reactive T cells specifically cross-reacted with the corresponding peptides derived from the family members HER3 and/or HER4 due to a high sequence homology. The epitopes HER3_356–364 and HER4_361–369 were endogenously processed and contributed to the susceptibility of cell lysis by HER cross-reacting CTLs. The principle of “double” or “triple targeting” the HER Ags by cross-reacting T cells will impact the further development of T cell-based therapies. The Journal of Immunology, 2008, 180: 8135–8145.

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HER2 can be down-regulated or mutated following HER2-targeted immunotherapies (31–33), which may further lead to the outgrowth of HER3-overexpressing tumor cells.

In this study, we describe the generation of human CTL clones specific for HER2. Most of these CTL clones displayed a fine cross-specificity to HER2-homologous epitopes derived from HER3 and/or HER4, but not from HER1. The lysis of HER2/HER3-overexpressing tumor cells by HER2/HER3-cross-specific CTLs was dependent on the overexpression of HER2 and HER3. As HER2 and HER3 both contribute to the malignant phenotype of HER2/HER3-overexpressing breast cancer cells, the parallel targeting of HER2 and HER3 by cross-specific CTLs may inhibit the selective outgrowth of escape variants. These results will contribute to the design of T cell-based therapies for the treatment of breast cancer patients.

Materials and Methods

Cell lines

The TAP-defective HLA*0201* T2 cell line was provided by P. Creswell (Yale University School of Medicine, New Haven, CT), the HLA-A*0201-transfected cell line K562/A2 by T. Wolfèl (Klinikum der 5. hannes-Gutenberg-Universität, Mainz, Germany), and the HLA-A*0201* melanoma cell lines SK-MEL-29 and SK-MEL-37 by L. Old (Memorial Sloan-Kettering Cancer Institute, New York, NY). The HLA-A*0201-transfected ovarian cancer cell line SKOV3/A2 was supplied by M. L. Disis (University of Washington, Seattle, WA) and the HLA-A*0201 breast cancer cell lines KS24.22, GAKL, and HLA-A*0201-transfected SKBR3/A2 by B. Gückel (Eberhard-Karls-Universität, Tübingen, Germany). K562/A2/MIGR1, K562/A2/MIGR1/HER2, and K562/A2/MIGR1HER2/EC2 (where ECD is extracellular domain) were generated by retroviral transfer with MSCV-MIGR1, MSCV-MIGR1HER2, and MSCV-MIGR1HER2/ECD into the K562/A2 cell line. K562/A2, K562/A2/MIGR1, K562/A2/MIGR1HER2, K562/A2/MIGR1HER2/ECD, SKOV3/A2, and SK-MEL-29 were cultured in RPMI1640 (Invitrogen). SK-MEL-29 and SK-MEL-37, K562/A2, and GAKL were maintained in DMEM (Life Technologies). All breast cancer cell lines was documented by FACS analyses: K562/A2MIGR1-HER2 (MFI: 531), SK-MEL-29 (MFI: 501), and SK-MEL-37 (MFI: 368) (where Δ is difference and MFI is mean fluorescence intensity).

The HLA-A2 expression was confirmed by FACS analyses with a FITC-conjugated anti-HLA-A2 mAb, (BD Pharmingen). For IFN-γ production by CD8+ T cells after two or three stimulations with HER2-expressing DCs was determined in an ELISPOT reader system (KS ELISPOT; Carl Zeiss).

Cytotoxicity assay

Cytolytic activity was analyzed in a standard 4-h chromium release assay as described (19). In brief, the tumor cell lines and the transfectants (5 × 104 cells in 100 μl of FCS) were incubated with 100 μCi of 51Cr (ICN Biochemicals) for 1 h at 37°C, washed, and then used as target cells. Peptide-loaded T2 cells were first labeled with 125I for 1.5 h at 37°C and then loaded with the HER2/369–377 peptide at 10 μg/ml for an additional 1 h at room temperature. As negative controls, 51Cr-labeled T2 cells were loaded with the HLA-A*0201-transfected peptide epitope HIVpol767–848 (ILKPVEHVQG), Melan-A 26–35A27L (ELAGIGILTV), and NY-ESO-1151–165V (SLLMWITQV), respectively. The 51Cr-labeled targets were cultured with the T cells in RPMI 1640 with 10% FCS at 200 μl/well in V-bottom, 96-well tissue culture plates (Greiner). For evaluating the efficacy of CTL-mediated lysis, the T cells were serially diluted and then cocultured with a fixed amount of target cells, resulting in graded E:T ratios. For testing functional TCR avidity, the T cells were plated at a fixed E:T ratio of 30:1 while the peptide concentration was titrated. After 4 h of coculturing the effector and target cells at 37°C, 100 μl of supernatant was collected and the radioactivity was measured in a gamma counter. The killing was calculated as the percentage of specific 51Cr release using the following equation: percentage of specific lysis = [(sample release − medium release)/(maximal release − medium release)] × 100. Spontaneous release was generally <15%. The data in the figures refer to the mean of two replicates. The SD was below 5% of the mean.

IFN-γ ELISPOT assay

IFN-γ production by CD8+ T cells after two or three stimulations with HER2-expressing DCs was determined in an ELISPOT assay as described (20). The number of spots was counted by an automated ELISPOT reader system (KS ELISPOT; Carl Zeiss).

ELISA

For the detection of the IFN-γ production by the CTL clones, 2 × 104 T cells/well were cocultured with 1 × 104 target cells in 96-well round-bottom plates at 37°C. After 24 h, supernatants were collected and IFN-γ production was determined using a commercially available ELISA kit (BD Pharmingen International).

Transfection

Plasmids containing HER1, HER3, and HER4 cDNA were provided by A. Ulrich (Max-Planck-Institut für Biochemie, Martinsried, Germany). K562/A2 cells were electroporated with the vector pCDNA3.1 containing cDNA of HER1, HER2, HER3, and HER4 in a 2-mm cuvette with 200 μl of Opti-MEM using the Xcell system (BioRad).

Small interfering RNA (siRNA) treatment

The siRNA SMARTpool containing four pooled siRNA duplexes directed against ErbB2 (catalog no. M-003126-01), ErbB3 (catalog no. M-003127-02), and a nonspecific siRNA control pool (catalog no. D-001206-13) were purchased from Dharmacon. The transfection of siRNA was performed using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. The knockdown of the specified protein was determined by FACS analyses.

Generation of CTL clones

The study was in accordance with the precepts established by the Helsinki Declaration and approved by the Ethics Committee of the Technical University of Munich, Munich, Germany. HLA-A*0201-restricted, HER2/369–377-reactive CTLs were generated by restimulation with autologous dendritic cells (DCs) (34) derived from healthy donors. Three different protocols were used for the stimulation of HER2/369–377-reactive CTLs. In the first approach, PBMCs were stimulated with HER2/369–377-loaded autologous DCs in RPMI 1640 supplemented with 100 μU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% FCS. The HER2 expression of the following cell lines was documented by FACS analyses: K562/A2MIGR1-HER2 (MFI: 569), SKOV3/A2 (MFI: 326), KS24.22 (MFI: 486), GAKL (MFI: 405), SKBR3/A2 (MFI: 531), SK-MEL-29 (MFI: 240), and SK-MEL-37 (MFI: 172) (where Δ is difference and MFI is mean fluorescence intensity).

The HLA-A2 expression was confirmed by FACS analyses with a FITC-conjugated anti-HLA-A2 mAb, (BD Pharmingen). For IFN-γ treatment the medium was supplemented with 100 U/ml IFN-γ 4 h before the functional assays.
FIGURE 1. Differential lytic activity of HLA-A2-restricted HER2369–377-directed CTL clones against HLA-A2+ cell lines retrovirally transduced with HER2 or HER2-ECD. The CTL clone NvB2/12 was generated by stimulation with autologous DCs loaded with the peptide HER2369–377; the CTL clone KU1 by activation with allogeneic HLA-A2+ DCs pulsed with peptide HER2369–377, and the CTL clone KW63 by coculture with autologous DCs that had been electroporated with HER2 mRNA. A. The peptide specificity of the three HER2369–377-directed CTL clones was documented by the lysis of T2 cells pulsed with the peptide HER2369–377 (●); T2 cells loaded with peptide HIVpol476–484 (○) were used as negative control. B, CTL recognition of endogenously processed peptide HER2369–377 was evaluated by targeting HLA-A2+ K562tA2 cells that had been retrovirally transduced with MIGR1-HER2 (●) or MIGR1-HER2/ECD (●); the mock-transduced cell line K562tA2/MIGR1 (▴) was used as negative control. C, T2 cells loaded with peptide HER2369–377 and by a second unlabelled cell line K562tA2/MIGR1-HER2 (●), K562tA2/MIGR1-HER2/ECD (●), and K562tA2/MIGR1 (▴) were first treated with 100 U/ml IFN-γ for 48 h and then used as target cells for HER2369–377-directed lysis by the CTL clones NvB2/12, KW63, and KU1. Data are representative of two independent experiments.
that peptide-stimulated T cells have a deficiency in recognizing the endogenously processed peptide (29, 35, 38, 39).

Based on these findings, we were led to question whether HER2-transfected DCs are capable of inducing autologous CTLs recognizing not only target cells exogenously loaded with the peptide but also cells that present the peptide HER2369–377 following processing. Therefore, as a second approach, we stimulated CD8+ T lymphocytes with autologous DCs electroporated with mRNA coding for the full length of HER2. Following repetitive stimulations with mRNA-transfected DCs, the proliferating T cells were screened for peptide specificity, and the HER2369–377-reactive T cell populations were cloned with limiting dilution. We isolated four HER2369–377-specific T cell clones from the healthy donor KW and the reactivity pattern of the CTL clone KW63 is shown in Fig. 1 (middle panel). The peptide specificity of CTL KW63 was confirmed by lysis of T2 cells loaded with HER2369–377 and the lack of lysis of T2 cells loaded with irrelevant peptides including HIVpol476–484 (Fig. 1A, middle panel), Melan-A26–35A27L, and NY-ESO-1157–165C165V, respectively (data not shown). CTL KW63 was able to lyse IFN-γ-treated HER2- and HER2/ECD-expressing K562A2 cells that had endogenously processed HER2369–377 following transduction with HER2- and HER2/ECD, respectively (Fig. 1C, middle panel). Of note, the CTL recognition of these transfectants was dependent on their pretreatment with IFN-γ, because untreated target cells were not lysed (Fig. 1B, middle panel).

The isolation of HER2-specific T cells with a low recognition efficiency against endogenously processed HER2 may be due to the fact that HER2 is a self-Ag and T cells with high avidity TCRs against HER2 may be partly deleted in the thymus. To generate HER2-specific T cells with increased recognition efficiency, we took advantage of an HLA mismatch between the stimulating DCs and the responding T cells to isolate T cells that recognize the peptide HER2369–377 as foreign. In this way, allo-restricted T cells with peptide-dominant binding and high avidity should be selected and consecutively show high recognition efficiency. HER2−/−CD8+ T cells were stimulated with allogeneic HER2−/− DCs loaded with the peptide HER2369–377, and A2/HER2−/− multimer+ T cells were sorted and cloned. Ten HER2−/−A2-restricted T cell clones with HER2369–377-dominant binding that did not recognize irrelevant peptides such as HIVpol476–484, respectively (Fig. 1A, bottom panel), Melan-A26–35A27L, and NY-ESO-1157–165C165V, respectively (data not shown), could be expanded. As a representative, the allo-restricted CTL clone KU1 is shown in Fig. 1 (bottom row). CTL KU1 was able to lyse the HER2−/− HER2-expressing cell lines K562A2MIGR1-HER2 and K562A2MIGR1-HER2/ECD even without IFN-γ pretreatment. Of note, the lysis of the HER2−/−HER2/ECD-expressing K562A2 cells by CTL KU1 could be significantly increased following incubation with IFN-γ. The recognition patterns of the three different representative CTL clones NbVb2/12, KW63, and KU1 were confirmed by cytokine secretion (Table I).

### Tumor recognition depends on the HER2-specific T lymphocytes and the HER2-expressing tumor cells

To answer the key question of whether the HER2-specific T cells can lyse tumor cells naturally overexpressing HER2, we next investigated various HLA-A2-matched, HER2-overexpressing
FIGURE 2. Different lysability of HLA-A2* HER2-overexpressing tumor cell lines by HER2<sub>369–377</sub>-directed CTL clones. Six different HER2-overexpressing HLA-A2* tumor cell lines were used as target cells for HER2<sub>369–377</sub>-directed lysis by the CTL clones NvB2/12, KW63, and KU1. Before the cytotoxicity assay, the tumor cell lines were either treated with IFN-γ (closed symbols) or not treated (open symbols). Depending on the IFN-γ-induced lysability, three different groups of tumor cell lines were distinguished: SK-MEL-37 with or without IFN-γ (●/○) and SK-MEL-29 with or without IFN-γ (●/□) and SKOV3tA2 with or without IFN-γ (△/△) (A); KS24.22 with or without IFN-γ (●/○) and SKBR3tA2 with or without IFN-γ (●/○) and GAKL with or without IFN-γ (△/△) (B). Data are representative of three independent experiments.

The partial dependence of tumor recognition on IFN-γ suggested that deficiencies in the HLA-A2 expression may be involved in this phenomenon. The HLA-A2 surface expression was up-regulated upon treatment with IFN-γ. However, there was a threshold for HLA-A2 expression; if enough HLA-A2/peptide complexes were present on the surface for sufficient CTL recognition, the further increase of HLA-A2 expression did not result in a better CTL recognition. The HLA-A2* cell line SK-MEL-29 displaying an MFI of 254 was recognized by the HLA-A2-restricted CTL clones KU1 and KW63 without prior treatment with IFN-γ, indicating that an MFI of at least 254 was sufficient for optimal CTL recognition. The KS24.22 cells naturally displayed HLA-A2 with an MFI of 389, the SKOV3tA2 cells with an MFI of 260, and the GAKL cells with an MFI of 265, but were not recognized by the T cells. Hence, the IFN-γ-induced CTL recognition of SKOV3tA2 and KS24.22 was not due to enhanced HLA-A2 expression, because the baseline HLA-A2 expression of these tumor cell lines should have been sufficient for CTL recognition. Therefore, the reason for missing recognition by CTLs was not due to low HLA-A2 expression. Moreover, the SKBR3tA2 cells expressing low levels of HLA-A2 (MFI: 53) up-regulated their HLA-A2 expression upon IFN-γ (MFI: 332) but, nevertheless, were not recognized by the HLA-A2-restricted CTL clones. These
The data indicated that HLA-A2 expression was not responsible for lack of CTL recognition.

We next addressed the question of whether deficiencies in the HER2 processing pathway were involved in the impaired CTL recognition by investigating the components of the Ag-processing machinery (APM), including LMP2, LMP7, TAP1, TAP2, and tapasin (Table I). The tumor cell lines of group I expressed all of these five APM components in the presence or absence of IFN-γ consistent with their IFN-γ-independent perceptibility. The tumor cell lines of group II, whose lysis was dependent on the presence of IFN-γ, displayed a variable expression of the herein tested APM molecules. Upon IFN-γ treatment, single components were up-regulated in individual cell lines, e.g., tapasin in SKOV3tA2 cells, which may be responsible for IFN-γ-induced, CTL-mediated tumor lysis. However, this was not a consistent finding. For example, the KS24.22 cell line of group II expressed the five tested APM molecules. Upon IFN-γ-treatment even though the killing of KS24.22 cells was clearly dependent on IFN-γ. Moreover, SKB3tA2 and GAKL, both members of group III, were not lysed in the presence or absence of IFN-γ despite the fact that they naturally expressed the five APM molecules. In conclusion, there was no consistent correlation between the lysability and the expression of LMP2, LMP7, TAP1, TAP2, or tapasin.

The HER2 shares a high degree of homology with the other members of the HER family. Therefore, we investigated the potential reactivity of the HER2369–377-reactive CTL clones with the corresponding peptides of HER1, HER3, and HER4. The homologous HER peptides, which were identified by amino acid sequence alignment, are located in the same region of the ECD of the respective HER protein (Table II). The selected nonamers HER1364–372, HER3356–364, and HER4361–369 display the same HLA-A2 anchor motifs (iso-leucine (I) at position 2 and leucine (L) at position 9).

The HER2369–377-reactive CTL clones NvB2/12, NvB41, KW63, and KU1 were examined for their cross-reactivity with the corresponding HER peptides. In addition, the functional avidities of these CTL clones toward the peptide HER2369–377 and the other HER peptides were assessed by the recognition of serially diluted amounts of the respective peptide loaded onto T2 cells (Fig. 3). Surprisingly, the nontumor-lytic CTL clones NvB2/12 and NvB41, which had been originally generated with peptide-loaded autologous DCs, displayed a high avidity toward the synthetic peptide HER2369–377, with a half-maximum lysis of $-10^{-9}$ and $10^{-10}$ M, respectively. In contrast, the half-maximum lysis by the tumorytic CTL clones KW63 and KU1 occurred at $10^{-7}$ M, indicating low avidity TCRs against HER2369–377. The CTLs NvB41 and KW63 displayed higher avidity to HER3356–364 than to HER2369–377, even though the T cells had been originally stimulated with HER2369–377-loaded allogeneic DCs before cloning. Of note, not all CTL clones displayed an equally high avidity to the HER2- and HER3-derived peptides. In contrast, the CTL clone KU1 expressed a TCR with a higher avidity to HER2369–377 than to HER3356–364, even though the T cells had been originally stimulated with HER2369–377-loaded allogeneic DCs before cloning. The functional avidity of CTL clones was determined by the lysis of T2 cells pulsed with graded amounts of peptides at a fixed E:T ratio of 30:1. Data are representative of two independent experiments.

We next addressed the question of whether the cross-reactivity of the HER2-directed CTL clones was restricted to target cells exogenously loaded with synthetic HER3 and HER4 peptides or whether the recognition extended to the naturally processed HER3 and HER4 peptides. K562tA2 cells were transfected with cDNA coding for the full length of HER1, HER2, HER3, or HER4 and then cocultured with the CTL KU1, which is known to cross-react with synthetic HER3356–364 and HER4361–369. Indeed, CTL KU1

<table>
<thead>
<tr>
<th>Epitope</th>
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<tbody>
<tr>
<td>HER1364–372</td>
<td>SIXGLDILIL</td>
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<tr>
<td>HER2369–377</td>
<td>KIFGSLAFL</td>
</tr>
<tr>
<td>HER3356–364</td>
<td>KLIGLDIOL</td>
</tr>
<tr>
<td>HER4361–369</td>
<td>KIGHL1IFL</td>
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*a Boldfaced letters represent amino acids shared with the reference peptide HER2369–377 at the indicated positions, including the HLA-A2 anchor motifs (isoleucine (I) at position 2 and leucine (L) at position 9.*

FIGURE 3. Specificity and avidity of HER2369–377-directed CTL clones toward homologous peptide epitopes of HER3 and HER4. The CTL clones NvB2/12, NvB41, KW63, and KU1, which had been isolated for specificity to HER2369–377, were tested for their cross-reactivity toward the corresponding peptide epitopes derived from HER1, HER3, and HER4. T2 cells were loaded with HER1364–372 (A), HER2369–377 (B), HER3356–364 (C), and HER4361–369 (D). Functional avidity of CTL clones was determined by the lysis of T2 cells pulsed with graded amounts of peptides at a fixed E:T ratio of 30:1. Data are representative of two independent experiments.
clarify the role of a certain HER molecule in tumor cell recognition, the expressions of HER2 and HER3, respectively, were down-regulated by siRNA against HER2 and HER3. The successful down-regulation of the respective HER protein was assessed by flow cytometry (Fig. 4A). The transfection of SK-MEL-37 cells with siRNA against HER2 (siHER2) reduced the HER2 surface expression (ΔMFI: 125) compared with the transfection with control siRNA (siCo; ΔMFI: 183) (Fig. 4B, left histogram). The down-regulation of HER3 protein expression in SK-MEL-37 cells was almost complete (ΔMFI: 0) following transfection with siRNA against HER3 (siHER3) in contrast to siCo (ΔMFI: 196) (Fig. 4B, right histogram). The down-regulation of HER2 by siHER2 led to the decreased recognition by HER2/3-reactive CTL KU1 as determined by IFN-γ secretion (Fig. 4C). Furthermore, the silencing of HER3 protein expression was accompanied by diminished cytokine secretion by the CTL KU1.

We finally asked the question of whether additional HER2 epitopes might be shared by the other members of the HER family. T cell clones were isolated from bulk cultures that had been stimulated with autologous DCs loaded with the peptide HER2369–377 known to be naturally processed and presented with HLA-A2 (40). The established HLA-A2-restricted, HER2369–377-specific CTL clones did not cross-react with the corresponding peptides HER1765–774 and HER4771–780 (data not shown).

Discussion

The HER2 peptide sequence at position 369–377 was first identified as an immunodominant epitope for HLA-A2-restricted T lymphocytes that had been isolated from malignant ascites of patients with HER2-overexpressing ovarian cancer (37). Since this discovery, the synthetic peptide HER2369–377 has been widely investigated for the ex vivo and in vivo generation of HER2-specific CTLs following stimulation in vitro (39, 41–46) and vaccination (18, 38, 47–49), respectively. Results obtained from different research groups led to the conclusion that HER2369–377-specific T cells can potentially lyse HLA-A2-matched HER2-overexpressing tumor cells (39, 41–46, 48) and may be even able to eliminate tumor cells in vivo (18, 29, 50). However, Sette and colleagues showed for the first time that HER2369–377-specific CTLs generated by in vitro stimulations using the synthetic peptide HER2369–377 do not necessarily recognize the endogenously processed peptide and, therefore, may fail to lyse HLA-A2+ HER2-overexpressing tumor cell lines (45). Moreover, the vaccination of patients using synthetic HER2369–377 in combination with Freund’s incomplete adjuvant led to the induction of peptide-specific CTLs that failed to recognize HER2+ tumors (38).

The herein characterized HER2369–377-specific T cell clones, which had been isolated following repetitive stimulation with autologous DCs loaded with the synthetic peptide HER2369–377, were unable to lyse HLA-A2+ HER2+ tumor cells or transfectants. The inability of the HER2369–377-specific T cells to lyse tumor cells did not correlate with a low TCR avidity toward the synthetic HER2369–377 peptide, as the CTL clones NvB2/12 and NvB4 displayed a half maximum lysis of peptide-loaded T2 cells at a peptide concentration of 5 × 10−10 M (IC50 of 0.5 nM) and 10−11 M (IC50 of 0.01 nM), respectively. These TCR avidities to the HER2369–377 peptide are similar to the high avidity TCRs of virus-specific CTLs, e.g., CMV-specific T cells, that display a half-maximum lysis of peptide-loaded T2 cells at 10−10 M (IC50 of 0.1 nM) (35). Using the herein described culture condition of peptide-stimulation, we predominantly isolated HER2369–377-specific, nontumor-lytic T cell clones (five of five healthy donors). Of note, our clinical study regarding the adoptive transfer of autologous HER2369–377-specific T cells for HLA-A2+ patients

FIGURE 4. CTL recognition of endogenously processed HER2, HER3, and HER4. A, The CTL clone KU1 was cocultured with K562A2 transfected with pCDNA3.1-HER1, -HER2, -HER3, or -HER4 and the IFN-γ release was assessed as a parameter for Ag-specific CTL activation. The IFN-γ concentration of the supernatant was determined by ELISA. B, Left panel, The HER2- and HER3-overexpressing melanoma cell line SK-MEL-37 was transfected with siHER2. The down-regulation of HER2 expression was assessed with FACS using a mAb against HER2. As negative control, SK-MEL-37 was transfected with siCo. As specificity control, the HER3 expression of SK-MEL-37 was determined before and after transfection with siHER2. FACS analyses of siHER2-transfected SK-MEL-37 with anti-HER2 mAb (siHER2/anti-HER2; dotted line) demonstrated a down-regulated HER2 expression, whereas the expression of HER3 was unaffected as determined with anti-HER3 mAb (siHER2/anti-HER3; dashed line). Staining of siCo-transfected SK-MEL-37 with mAb against HER2 (siCo/anti-HER2; thick line) was used as control for the natural HER2 expression. Staining of siCo-transfected SK-MEL-37 with anti-IgG1 (siCo/anti-IgG1; thin line) was used as negative control. Right panel, SK-MEL-37 was transfected with siHER3. FACS analyses of siHER3-transfected SK-MEL-37 with mAb anti-HER3 (siHER3/anti-HER3; dotted line) showed a reduced expression of HER3, whereas the expression of HER2 was unchanged (siHER3/anti-HER2; dashed line). Unsilenced HER3 expression of siCo-transfected SK-MEL-37 was determined with a mAb against HER3 (siCo/anti-HER3; thick line). As negative control, siCo-transfected SK-MEL-37 cells were stained with anti-IgG1 mAb (siCo/anti-IgG1; thin line). C, HER2+/HER3+ SK-MEL-37 cells were transfected with siHER2 and siHER3, respectively, to determine the influence of HER2 and HER3 regarding the CTL recognition. As negative control, SK-MEL-37 was transfected with siCo. The recognition of HER2+/HER3+ SK-MEL-37 was determined by IFN-γ release with HER2/HER3-directed CTL clone KU1. Data are representative of two (A) and three (B and C) independent experiments.

recognized the naturally processed HER3 and HER4 as determined by IFN-γ secretion (Fig. 4A). Similar data were obtained for CTL KW63 (data not shown).

Because the HER2-overexpressing tumor cell lines coexpressed HER3 (data not shown), we investigated whether tumor cell lysis by HER2/3-reactive CTLs depended on HER2 and/or HER3. To
with HER2-overexpressing breast cancer was closed prematurely due to the frequent establishment of HER2369–377-specific T cell clones that failed to recognize the endogenously processed peptide HER2369–377 (29). One explanation for the preferential establishment of peptide-specific/nontumor-lytic CTL clones by peptide stimulation may be that their T cell receptors recognize a particular peptide conformation that is present when HLA-A2 molecules are exogenously loaded with peptides but different from the one conferred by endogenous presentation as suggested by Zaks and Rosenberg (38).

Another explanation of this phenomenon may be that the naturally processed HER2369–377 epitope may be glycosylated as it is derived from the ECD known to be heavily glycosylated. It has been described for the glycoprotein MUC1 that complex carbohydrates are not removed during processing (51, 52). Therefore, the majority of CTLs stimulated with the synthetic HER2369–377 peptide may not be able to recognize the naturally processed glycopeptide. A third hypothesis is based on the fact that T cells are targeted by the exogenously present pool of allogeneic T cells with peptide-dominant binding (58, 59) by stimulating T cells from an HLA-A2+ tumor cell line transfected with the cDNA encoding the HER2 ECD known to be heavily glycosylated. It has been described that the recognition by the glycopeptide is indeed “cross-reactive” due to the degeneracy of Melan-A Ag recognition (60).

The detection of the synthetic peptides HER3356–364 and/or HER4361–369 by HER2369–377-reactive CTLs does not necessarily imply their recognition of the respective epitopes, as the HER3/4 peptides may not be naturally processed or the CTLs may not recognize the processed HER3/4 peptides. Therefore, we selected the HER2/3/4-specific CTL clone KU1 with known tumor-lytic function and tested its ability to recognize the Her2+ tumor cell line transfected with the cDNA encoding the corresponding HER molecules. Indeed, the HER2/3/4-specific CTLs (e.g., CTL KU1) recognized not only the naturally processed HER2 but also HER3 and HER4 as determined by IFN-γ secretion. This is the first time that cross-reactivity of HER2-specific CTLs can be demonstrated toward newly defined epitopes derived from HER3 and HER4.

As most of the HER2+ tumor-cell lines coexpressed HER3, we sought to assess the influence of HER2 and HER3 on the tumor-recognition by the HER2/3-specific, tumor-lytic CTLs. Selective down-regulation of either HER2 or HER3 revealed that both molecules contributed to the Ag-specific recognition by HER2/3-reactive CTLs. Of note, the TCR avidities toward HER2 and HER3, as measured by the recognition of titrated synthetic peptides, did not correlate with the capacity of the HER2-directed TCR avidity, suggesting that additional factors may play a role in HER-mediated tumor lysis.

The contribution of the three HER-family members, HER2, HER3, and HER4, in the tumor susceptibility toward cross-specific CTLs opens new avenues for the development of HER-directed T cell therapies. As HER2 and HER3 both contribute to the malignant phenotype of the tumor, the “double targeting” of HER2 and HER3 may inhibit the selective outgrowth of immune escape variants (31–33). This may also be of relevance for cancer types other than breast cancer, e.g., prostate cancer, in which HER2/3 heterodimers also play a decisive role (61). The new principle of “double or triple targeting” two or three HER Ags will broaden the group of cancer patients applicable for HER-directed T cell therapy, because HER2, HER3, and HER4 are differently expressed or coexpressed by individual cancers (5, 62–64).
The members of the HER family are ubiquitously expressed in normal tissues, including the cardiovascular system and the gastrointestinal, respiratory, and urogenital tracts as well as breast and skin. Until now, HER2-directed immunotherapies, such as vaccines, mAbs, and adoptive T cell transfer have not induced autoimmune disease in patients with HER2-overexpressing tumors. However, it cannot be ruled out that the transfer of HER2-reactive T cells with cross-specificity toward HER3 and/or HER4 may lead to the development of autoimmunity. In particular, normal tissues that are dependent on the heterodimerization of HERs may be at risk, as has been reported for HER2 and HER4 in cardiomyocytes (2, 65, 66).

The efficient elimination of tumor cells by HER-specific T cells is not only dependent on the qualities of the TCR but also on the susceptibility of the tumor cell to CTL killing. For HER2-expressing tumor cells it has been shown that a decreased expression of HLA class I molecules and/or components of the APM may be responsible for the resistance toward CTL-mediated lysis (67, 68). The herein described HER2369–377-reactive, tumor-lytic T cells exhibited a similar lysis pattern by dividing the targeted HLA-A2− HER2+ cell lines in three groups as follows: 1) cell lines that were lysed independently of IFN-γ pretreatment; 2) cell lines that were only recognized after incubation with IFN-γ; and 3) cell lines that were not lysable regardless of prior IFN-γ treatment. Because HER2-overexpressing tumor cell lines vary tremendously in their expression of APM components (69), we investigated the mRNA expression of LMP2, LMP7, TAP1, TAP2, and tapasin in a panel of cell lines cultured in the presence and absence of IFN-γ. Indeed, the IFN-γ treatment resulted in the up-regulation of certain components in certain cell lines. However, we could not observe a strong correlation between the expression of APM components and the susceptibility to CTL killing. This may partly be due to the fact that, first, not all but only five APM components were examined, and second, that the increased APM component expression was determined on the mRNA level, which does not necessarily translate into a higher density of A2/HER2369–377 complexes. By using a TCR mimic mAb recognizing the A2/HER2369–377 complex, it has been recently demonstrated that the expression level of A2/HER2369–377 complexes predicts tumor cell susceptibility to HER2369–377-specific CTL killing (70). The immunohistochemical analyses of A2/HER2369–377 and other HLA/HER-peptide complexes expressed by cancer samples may have significant implications for the improvement of T cell-based immunotherapies against the HER Ags.

Based on our results, we can conclude that the success of HER2-directed T cell therapies depends on both the characteristics of the HER2-specific T cells and the HER2-expressing tumor cells. As the HER2-specific T lymphocytes vary greatly depending on their cross-specificity and tumor-lytic activity, their broad clinical application is limited due to the laborious procedure of T cell isolation and characterization if tailored for every single patient. For this reason, we will pursue an alternative methodology whereby primary human T cell populations are transduced with the HER-specific TCR of interest. The TCR gene transfer is a convenient method for producing Ag-specific T cells, further allowing individualized therapies to be available for a mass of patients (71–76).

Acknowledgments

We thank Kathrin Hofer and Kerstin Holtz for excellent technical assistance, Matthias Schiemann for expert cell sorting, Peter Cresswell, Nora Disis, Brigitte Gückel, Thomas Wölfel, and Lloyd Old for providing cell lines, Axel Ullrich for providing plasmids containing HER1, HER3, and HER4, Gert Riehmüller for helpful discussions, and Ekkehard Albert for HLA typing.

Disclosures

The authors have no financial conflict of interest.

References


