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ORIGINAL ARTICLE

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HER-2/neu-derived peptide 884–899 is expressed by human breast, colorectal and pancreatic adenocarcinomas and is recognized by in-vitro-induced specific CD4$^+$ T cell clones

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Abstract HER-2/neu peptides recognized in the context of HLA-DR molecules by CD4$^+$ Th lymphocytes on antigen-presenting cells have been identified. In this report, we demonstrate for the first time that HER-2/neu helper epitopes are also expressed on the surface of metastatic breast, colorectal and pancreatic carcinomas. Peripheral blood mononuclear cells from an HLA-DR4 healthy donor were used to induce HER-2/neu peptide-specific CD4$^+$ T cell clones by in vitro immunization with HER-2/neu peptide (884–899)-pulsed autologous dendritic cells (DCs). Strong proliferation and significant levels of IFN-γ were induced by the CD4$^+$ T cell clones in response to specific stimulation with autologous DCs loaded with HER-2(884–899). Furthermore, these clones also recognized HER-2/neu$^+$ tumor cell lines, and tumor cells from breast, colorectal and pancreatic adenocarcinomas induced to express HLA-DR4, but also the HLA-DR4$^+$ melanoma cell line FM3 transfected to express HER-2/neu. The recognition of tumor cells was strongly inhibited by an anti-HLA-DR mAb. Taken altogether, we provide novel information for the role of HER-2(884–899) as a naturally processed epitope expressed by breast, colorectal and pancreatic carcinomas and the capacity of HER-2/neu protein to follow the endogenous class II processing pathway. Our results suggest that HER-2(884–899) might be attractive for broadly applicable vaccines and may prove useful for adoptive immunotherapy designed for breast, colorectal and pancreatic carcinomas.

Keywords CD4$^+$ T cell clones · HER-2/neu peptide · Antigen presentation · TCRBV phenotype · Cancer patients

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Introduction

The identification of antigens expressed by human tumor cells has made possible the design of therapeutic anti-cancer vaccines eliciting immune responses capable of destroying established tumors [27]. Administration of vaccines corresponding to MHC class-I-restricted epitopes presented on the surface of tumor cells resulted in the activation of cytotoxic CD8$^+$ T lymphocytes capable of specifically recognizing tumor cells [25, 30].

Despite the emphasis on CD8$^+$ T-lymphocyte-mediated immune responses, increasing evidence from both human and animal studies has suggested that optimal cancer vaccines require the participation of both CD4$^+$ and CD8$^+$ T cells [21]. The essential role of CD4$^+$ T cells in antitumor immunity was first shown in animal models, where these cells were clearly demonstrated to provide all necessary stimuli for the induction and maintenance of antitumor CD8$^+$ T cells [8, 40]. Reports from cell-based vaccine models against MHC class-II-negative tumors [29] indicated that tumor antigens released at the tumor site are taken up by macrophages, processed, and presented to CD4$^+$ T cells, which in response, produce and secrete lymphokines that activate tumor-specific cytotoxic T lymphocytes (CTLs). More-

over, MHC class-II-knockout mice or mice depleted of $CD4^+$ T cells were no longer capable of generating CTL responses against an adenovirus E18 protein epitope, whereas wild-type mice developed helper-dependent CTLs to that particular epitope after cross-priming by antigen-presenting cells (APCs) [31].

The identification of antigens recognized by $CD4^+$ T cells on human tumors has placed strong impetus on the role of $CD4^+$ T cells in antitumor immunity. Using peptide-binding prediction algorithms, MHC class I-restricted tumor antigens, including melanoma antigens Melan-A/MART-1, gp100 and tyrosinase, tissue-specific antigen MAGE-3 and cancer-testis antigen NY-ESO-1, were demonstrated to contain MHC class-II-restricted epitopes recognized by $CD4^+$ T cells [16, 37]. Recently, a genetic approach was developed that enabled the cloning of genes coding for mutated MHC class-II-restricted antigens including CDC27, triosephosphate isomerase (TPI) and LDFP (reviewed in [36]). TPI was also identified by a biochemical approach [36].

HER-2/neu is a 185-kDa transmembrane glycoprotein with tyrosine kinase activity and extensive homology to the epidermal growth factor receptor [18]. HER-2/neu is ubiquitously expressed in many epithelial tumors and known to be over-expressed on approximately 25–30% of all ovarian and breast cancers [33], 35–45% of all pancreatic carcinomas [39] and up to 90% of colorectal carcinomas [24]. The HER-2/neu protein appears to be immunogenic because CTL responses specific for MHC class I epitopes have been observed in some cancer patients [11, 13, 41]. Furthermore, CTL responses against tumor cells from various types of cancer, including breast, ovary, pancreatic, renal, colorectal and lung adenocarcinomas, have been induced in vitro using MHC class-I-binding synthetic peptides derived from the HER-2/neu sequence [6, 22, 28], suggesting that HER-2/neu MHC class I epitopes are shared between several distinct types of epithelial tumors and can be appropriate candidates for broadly applicable vaccine therapies. There is also now evidence of the existence of MHC class-II-restricted T cell responses to HER-2/neu: $CD4^+$ T helper cells from HER-2/neu^+ breast and ovarian cancer patients can proliferate and produce lymphokines in response to stimulation with HER-2/neu recombinant protein or synthetic peptides corresponding to immunodominant regions of HER-2/neu, such as HER-2(776–788), HER-2(884–899) and HER-2(396–406) [2, 10, 14, 35]. Some of these patients indicated pre-existing immunity to these peptides in that they responded moderately after a short-term stimulation period [9, 14]. Most recently HER-2(883–899) was shown to be recognized by healthy donors' $CD4^+$ T cells in the context of four different HLA-DR alleles (i.e., DR1, DR4, DR52 and DR53) indicating a high degree of promiscuity in histocompatibility [20].

The capacity of HER-2/neu peptide-reactive $CD4^+$ T helper cells to directly recognize HER-2/neu^+ tumor cells, naturally processing and presenting the same peptide in the context of MHC class II molecules, has been considered as a prerequisite in order for the corresponding peptide to function as a therapeutically relevant T cell epitope. However, so far, there is no such evidence for HER-2/neu protein-derived peptides, in that HER-2/neu-peptide-specific $CD4^+$ T cells were defined on the basis of their capacity to respond upon recognition of peptide-pulsed APCs (being either PBMCs or DCs) [2, 14, 20, 35] or DCs pulsed with HER-2/neu recombinant protein [20].

In this report, we demonstrate for the first time that HER-2(884–899)-reactive $CD4^+$ T cell clones from an HLA-$DR4^+$ healthy donor can also specifically recognize tumor cells from patients with metastatic breast, colorectal and pancreatic cancer induced to express HLA-DR4 molecules. The same clones also recognize the HLA-$DR4^+$ melanoma cell line, FM3, transfected to express HER-2/neu (FM3/HER) and the HER-2/neu^+ colorectal cell line, HT-29, induced to express HLA-DR4. These data also demonstrate that HER-2/neu protein can follow the endogenous pathway for MHC class II presentation. The identification of HER-2(884–899) as a tumor-associated antigen in breast, colorectal and pancreatic cancer provides an opportunity for the design of novel immunotherapy and vaccine strategies.

Materials and methods

Patients

Nine HLA-$DR4^+$ patients with histologically confirmed metastatic breast ($n=3$), colorectal ($n=3$) and pancreatic ($n=3$) adenocarcinomas were studied. Pleural fluid (breast cancer) or ascites (colorectal and pancreatic cancer) were kindly provided by the Oncology Department of Hippocration State Hospital (Athens) and the Pathological Clinics of Saint Savas Cancer Hospital under the Institutional Review Board of both institutions.

Isolation of tumor cells

Pleural fluid or ascites were centrifuged at 400 *g* for 5 min to sediment cells, which were further placed on top of a 75% Ficoll-Hypaque gradient, overlaid on 100% Ficoll-Hypaque, and spun at 700 g for 25 min. Tumor cells were collected from the top of 75% Ficoll-Hypaque and cryopreserved in liquid nitrogen until used. At that time, cells were carefully thawed, slowly diluted in RPMI-1640 (Life Technologies, Gaithersburg, Md.) and washed. Tumor cells were assayed only if their viability was over 80%. Phenotype analysis showed that all tumors were negative for MHC class II gene products (but positive for MHC class I molecules; data not shown). Tumor cells were incubated in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 50μg/ml gentamycin (all purchased from Life Technologies) (complete medium) at 5×10^5 cells/ml in 24-well plates (Costar, Corning Incorporated, N.Y.) in the presence of 300 IU/ml IFN-γ (Boeringer Mannheim, Mannheim, Germany) or left untreated. After 72 h in a CO_2 incubator, tumor cells were analyzed for HLA-DR4 expression, treated with mitomycin C and used in stimulation experiments as APCs.

Cell lines

The human colorectal adenocarcinoma cell line HT-29 (American Type Culture Collection, Manassas, Va.) was cultured in

McCoy's 5A medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 50 μg/ml gentamycin. This line was induced to express HLA-DR4 upon 2-day incubation with 100 IU/ml IFN-γ in CO_2 incubator. The human melanoma cell line FM3 [32] (a kind gift of Dr. J. Zeuthen, Dept. of Tumor Cell Biology, Danish Cancer Society Research Center, Copenhagen) was grown in RPMI-1640 complete medium. The marmoset EBV transformed B cell line B95–8 (American Type Culture Collection) was grown in RPMI-1640 complete medium.

Purification of $CD4^+$ T cells

$CD4^+$ T cells were isolated from polyclonally expanded peripheral blood mononuclear cells (PBMCs). PBMCs were collected using standard procedures from a $HLA\text{-}DR4^+$ healthy volunteer and plated at 10^6 cells/well in 1 ml complete medium in 24-well plates precoated with 2 μg/ml anti-CD3 and 2 μg/ml anti-CD28 mAbs (PharMingen, San Diego, Calif.). After 7 days of culture in a CO_2 incubator, highly purified $CD4^+$ T cells (>98% purity) were isolated using Dynabeads (CD4 Positive Isolation Kit, Dynal, Oslo, Norway), as recently described [3].

Generation of dendritic cells

DCs autologous to the $CD4^+$ T cells were generated from the adherent fraction of freshly isolated PBMCs [6]. Briefly, 4×10^6 PBMCs in 2 ml X-VIVO 15 (BioWhittaker, Walkersville, Md.) were plated in 6-well plates (Costar) and incubated for 2 h in a CO_2 incubator. Non-adherent cells were gently washed out with Hanks' Balanced Salt Solution (HBSS; Life Technologies). The remaining plastic adherent cells were cultured in 2 ml X-VIVO 15 medium supplemented with 1% autologous plasma, 1000 IU/ml IL-4 (R&D Systems, Europe) and 1000 IU/ml GM-CSF (Immunex, Seattle, Wash.). Fresh medium (2 ml) with cytokines was added on days 2 and 4. For induction of maturation, TNFα (R&D Systems) was added at 10 ng/ml on day 6. Mature DCs were harvested on day 7 and used as APCs or cryopreserved for later use. The percentage of mature DCs recorded was >50%, as based on the expression of a $CD3^-$, $CD14^-$, $CD16^-$, $CD20^-$, $CD40^+$, $CD80^+$, $CD83^+$, $CD86^+$, and $HLA\text{-}DR^+$ phenotype analyzed by flow cytometry. Mature DCs used as APCs were pretreated with 100 μg/ml mitomycin C (Kyowa, Tokyo, Japan) for 45 min at 37 °C. After extensive washing with HBSS, DCs were pulsed with the appropriate concentration of peptide for 4 h at 37 °C.

EBV transformed B cells

PBMCs from another $HLA\text{-}DR4^+$ healthy volunteer were washed in PBS and resuspended in serum-free RPMI-1640 medium. EBV-containing culture supernatant from the B95–8 cell line was added for 2 h (at 37 °C, 5% CO_2). After washing, cells were cultured in complete medium in the presence of 0.5 μg/ml cyclosporin A (Sandimmun, Novartis). Generation of a long-term B-cell line was accomplished after 4 weeks. EBV-B cells were used as APCs after treatment with mitomycin C and pulsing with the appropriate peptide, as described for DCs.

Peptides

HER-2(884–899) (VPIKWMALESILRRRF) peptide and the gp100(44–59) (WNRQLYPEWTEAQRLD) were produced by the solid phase method, using an Ecosyn P peptide synthesizer (Eppendorf-Biotronik, Hamburg, Germany), employing the Fmoc strategy and a 4-carboxybenzyl alcohol resin. Purification was performed by HPLC. The purity of both peptides was >95%. Quantitative and qualitative determinations were controlled by amino acid analysis and matrix-assisted laser desorption mass spectrophotometry (Kratos Kompact Maldi II, Kratos Analytical, Manchester, UK). Peptides were lyophilized, dissolved in PBS, aliquoted at 2 mg/ml and stored frozen at –20°C until use.

Monoclonal Abs and immunophenotyping

Anti-TCR VB3.1, VB5(a), VB6.7, VB7.1, VB8(a), VB12, VB13 mAbs conjugated with FITC were obtained from Endogen (Boston, Mass.). Anti-CD83 conjugated with PE mAb was obtained from Caltag Laboratories (Burlingame, Calif.). FITC-conjugated anti-HER-2/neu mAb (clone Neu 24.7) recognizing the extracellular domain of HER-2/neu was purchased from Becton Dickinson (Mountain View, Calif.). Anti-HLA-DR4 mAb (hybridoma supernatant, clone NFLD.D1E2, IgG1, λ) was obtained from Terra Nova Biotechnology (St. John's, Newfoundland, Canada). All other mAbs were purchased from PharMingen (San Diego, Calif.). Anti-CD4, -CD8, -CD16, -CD20, -CD40, -CD80 and anti-TCR VB23 were conjugated with FITC. Anti-CD3, -CD14, -CD56, -CD86 and anti-HLA-DR mAbs were conjugated with PE. Cells to be immunostained were washed twice with ice-cold PBS/1% BSA, followed by incubation with saturating concentrations of the appropriate mAbs for 20 min at room temperature (RT). As anti-HLA-DR4 was not labeled, an additional incubation with FITC-conjugated rabbit anti-mouse Ig (DAKO A/S, Glostrup, Denmark) for 20 min at RT was performed. Thereafter, cells were washed twice in ice-cold PBS/1% BSA and fixed with 1% paraformaldehyde in PBS. Samples were analyzed using FACScan (Becton Dickinson) and CellQuest analysis software. For blocking experiments, purified azide-free anti-HLA-DR (clone L243), purified mouse anti-TNP (clone A111–3, IgG1, λ) (both from PharMingen), anti-HLA-DR4 (Terra Nova) and anti-HLA-A, B, C (clone W6/32; Serotec Ltd, Oxford, UK) were used.

Generation of a $CD4^+$ T cell line and clones specific for HER-2(884–899)

$CD4^+$ T cells were plated in 96-well U-bottom plates (Costar) at 5×10^4 cells per well with 10^4 per well autologous DCs pulsed with 50 μg/ml HER-2(884–899) peptide, in a final volume of 100 μl X-VIVO 15 medium supplemented with 1% autologous heat-inactivated plasma, 10 ng/ml IL-7 (R&D systems) and 100 pg/ml IL-12 (R&D systems). Cultures were incubated at 37 °C in a CO_2 incubator. On day 3, IL-2 was added at 20 IU/ml (Chiron Corporation, Calif.). Growing microcultures were pooled and restimulated at weekly intervals with DCs pulsed with the same peptide at either 20 μg/ml (first restimulation) or 10 μg/ml (further restimulations). After the third restimulation, microcultures were tested by ELISA for IFN-γ production and those producing high levels were pooled and further expanded with immobilized anti-CD3 and anti-CD28. Peptide-specific T cell clones were obtained from this bulk culture by limiting dilution. Cloning was accomplished in X-VIVO 15 medium supplemented with 0.75 μg/ml PHA (Sigma), 2 IU/ml IL-2, 10 ng/ml IL-7, 100 pg/ml IL-12 and 2×10^4 mitomycin-treated allogeneic PBMCs per well as feeders. Clones were expanded in 96-well U-bottom plates by using the CD3/CD28 T Cell Expander kit (Dynal).

Proliferation assay

Responder $CD4^+$ T cells (3×10^4 cells/well) were co-cultured with peptide-pulsed or unpulsed autologous DCs in 96-well U-bottom plates at a final volume of 200 μl X-VIVO 15 medium supplemented with 1% autologous plasma, 10 ng/ml IL-7 and 100 pg/ml IL-12. When EBV-B cells were used as APCs, they were added at 3×10^4 cells per well, whereas FM3 (transfected or not), HT-29 or metastatic cancer cells were used as APCs at 3×10^3 cells per well. MAbs, wherever indicated, were included at 10 μg/ml final concentration for anti-HLA-DR, anti-HLA-A, -B, -C, or isotype control, or 50% hybridoma supernatant anti-HLA-DR4 dialyzed against X-VIVO 15 throughout the culture period. On day 4, 1 μCi per well of tritiated thymidine ($[^3H]$-TdR, 48.0 Ci/mmol, 1 mCi/ml, Amersham Pharmacia Biotech, Buckinghmshire, UK) was added

for the last 16 h. Cells were then harvested and [^{3}H]-TdR incorporation was measured in a liquid scintillation counter (LKB Wallac, Turku, Finland). All cultures were performed in triplicates and results expressed as cpm.

ELISPOT assay

Responder CD4$^+$ T cells were cultured in 96-well U-bottom plates at 10^4 cells per well with autologous peptide pulsed or unpulsed DCs at 2×10^3 cells per well in 200 µl X-VIVO 15 medium supplemented with 1% autologous plasma, 2 IU/ml IL-2, 10 ng/ml IL-7 and 100 pg/ml IL-12 for 24 h, and then transferred to 96-well flat-bottom Immobilon-P PVDF plates (Multi-Screen-IP, Millipore, Bedford, Mass.) coated overnight at 4 °C with 2 µg/ml anti-IFN-γ mAb (NIB42, PharMingen). After an additional 20-h period of incubation, the nitrocellulose plates were washed and biotinylated anti-IFN-γ mAb (4S.B3; PharMingen) was added. Plates were incubated for 2 h at RT, washed and developed with alkaline phosphatase-conjugated streptavidin (Bio-Rad Laboratories, Hercules, Calif.) for 1 h at RT. After washing, BCIP/NTB substrate (Bio-Rad) was used to reveal dark-violet spots. Spots were counted under a stereomicroscope (Zeiss, Germany).

Quantitation of cytokines in culture supernatants

IFNγ, IL-4 and IL-10 secretion by HER-2(884–899)-specific CD4$^+$ T cells was estimated with commercially available ELISA Kits (Diaclone Research, Besançon, France), according to the manufacturer's instructions.

Transfection of the FM3 cell line

FM3 cells were co-transfected with a pSV2-c-erbB2 construct (kindly provided by Dr.Mien-Chie Hung M.D., Anderson Cancer Ctr., Houston, Tex.) and a pSV2neo plasmid using DNA-calcium phosphate co-precipitates. Selection with G-418 (500 µg/ml) was started 48 h later. For cloning, the cells were aliquoted into 96-well flat bottom tissue culture plates (Costar) and stable transfectants were selected. HER-2/neu-transfected FM3 lines were subcloned until all cells were found to express HER-2/neu (thereafter referred to as FM3-HER), as detected by FACS.

Results

Generation of CD4$^+$ T cells specifically recognizing peptide HER-2(884–899)

Peripheral blood-derived monocytes from an HLA-DR4$^+$ healthy donor were cultured in X-VIVO 15 serum-free medium in the presence of GM-CSF and IL-4 [6] to favor their differentiation into DCs. TNF-α was added for the last 24 h of a 7-day incubation period to induce DCs expressing high levels of CD83, CD86 and HLA-DR (data not shown). Such mature DCs were subsequently incubated for 4 h with HER-2(884–899) and utilized as APCs for sensitizing autologous CD4$^+$ T cells. The latter were isolated from PBMCs activated with immobilized anti-CD3 and anti-CD28. Cells stimulated with this combination exhibit long-term autocrine growth, do not show significant variations in their TCRBV repertoire and preferentially produce Th1-cytokines [21]. Responder CD4$^+$ T cells received 2–3 restimulations with peptide-loaded DCs at weekly intervals, or as needed. After a resting period of one week, the responder cells were tested for cytokine secretion and/or proliferation. The responder CD4$^+$ bulk culture strongly proliferated in response to stimulation with autologous DCs loaded with HER-2(884–899), which was used for in vitro sensitization [11209 cpm vs. 241 cpm with unloaded DCs, resulting in a stimulation index (SI) of 46.5] (Fig. 1). The same responders also recognized allogeneic EBV-B cells from an HLA-DR4$^+$ donor loaded with HER-2(884–899). High background values (3212 cpm) in this case could be attributed to alloreactivity, but these were still far below those achieved in the presence of the peptide (18979 cpm; SI: 5.9) (Fig. 1). The proliferating activity of CD4$^+$ T cells in the presence of either autologous DCs loaded with HER-2(884–899) or allogeneic HLA-DR4$^+$ EBV-B loaded with the same peptide was reduced to background levels by the addition in culture of anti HLA-DR4 mAb, whereas the W6/32 (anti-HLA-A,

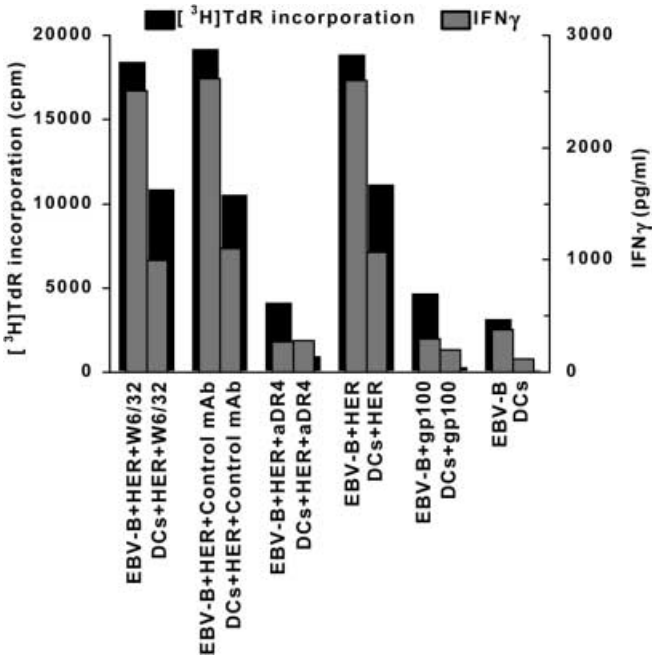


Fig. 1 Standard proliferation assay and IFN-γ production, showing recognition of autologous DCs or allogeneic HLA-DR4$^+$ EBV-B cells loaded with HER-2(884–899) by HER-2(884–899)-stimulated CD4$^+$ T cells. CD4$^+$ T cells from an HLA-DR4$^+$ healthy donor were sensitized in vitro to HER-2(884–899) as described in Materials and methods. After the third restimulation (day 29), recovered CD4$^+$ T cells were tested for proliferation by [^{3}H]-TdR incorporation and IFN-γ production by ELISA. Both types of responses were strongly blocked with an anti-HLA-DR4 mAb, but not with an isotype matched control mAb or with the W6/32 mAb (recognizing MHC class I gene products). HER-2(884–899) was used at 10 µg/ml final concentration for loading DCs or EBV-B cells. Control gp100 peptide (44–59) was also used at a final concentration of 10 µg/ml. Bars represent mean values from triplicate cultures. The SD in all cases was negligible and thus omitted. These experiments were repeated three times with similar results demonstrating the reproducibility of the experimental system

-B, -C) mAb or control mAb isotype matched to anti-HLA-DR4 had no detectable effects (Fig. 1).

Peptide-specificity of the proliferative responses observed in the previous experiments was assessed by using peptide gp100(44–59), which binds with high affinity to HLA-DR4. As also shown in Fig. 1, neither autologous DCs nor allogeneic HLA-DR4$^+$ EBV-B cells loaded with gp100 peptide could induce proliferative responses in CD4$^+$ T cells from bulk culture propagated in vitro with HER-2(884–899). In contrast, the same gp100-loaded DC could induce strong proliferative responses with a gp100-specific CD4$^+$ T cell line (data not shown).

Proliferation results were confirmed in ELISA assays (Fig. 1). CD4$^+$ T cells from the same bulk culture produced IFN-γ upon stimulation with autologous DCs or allogeneic HLA-DR4$^+$ EBV-B loaded with HER-2(884–899), but not when these cells were loaded with gp100(44–59) peptide. The recognition of peptide HER-2(884–899) was strongly inhibited by anti-HLA-DR4 mAb, but not by the control or anti-HLA class I mAb.

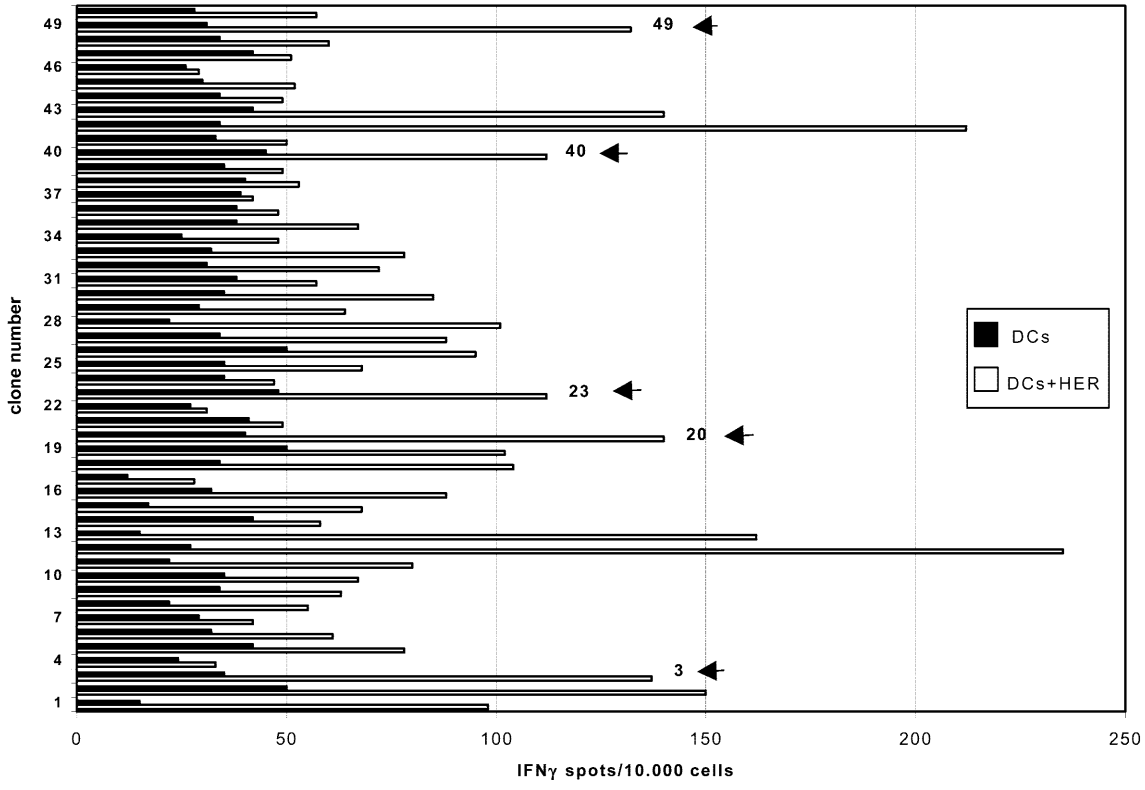
Generation and characterization of HER-2(884–899)-specific CD4$^+$ T cell clones

To examine the HER-2 peptide-specific responses in more detail, CD4$^+$ T cell clones were generated. The CD4$^+$ bulk culture was cloned by limiting dilution, using allogeneic PBMCs as feeder cells. This gave rise to a plethora of clones, which were screened for peptide recognition by using ELISPOT assays. IFN-γ spots were considered significant when the difference between the numbers counted per 1×10^4 cells in peptide-stimulated CD4$^+$ T cell cultures (ie. DCs loaded with HER-2 peptide) was higher than the mean number of spots in non-stimulated cultures (32 ± 9) plus 2 SDs (i.e., >50). In this way, we identified 18 clones (Fig. 2), from which 5 could be propagated in vitro and were further characterized. These clones (nos. 3, 20, 23, 40 and 49) produced high levels of IFN-γ (range: 1211–2594 pg/ml; mean: 1913 ± 451 pg/ml) but no detectable levels of IL-4 (range:7–12 pg/ml; mean:9.6 ± 1.8 pg/ml) and IL-10 (range: 8–21 pg/ml; mean: 14 ± 4 pg/ml) upon stimulation with autologous DCs loaded with HER-2(884–899).

Recognition of tumor cells by the HER-2(884–899) specific CD4$^+$ T cell clones

The CD4$^+$ T cell clones were tested for their ability to recognize tumor cells from HLA-DR4$^+$ patients with metastatic cancer naturally expressing the HER-2/neu protein and induced upon preincubation with IFN-γ to also express HLA-DR4. The HER-2/neu$^+$ colorectal cell line HT-29, which was induced to express HLA-DR4 upon a 2-day treatment with 100 IU/ml IFN-γ (data not shown) and the HLA-DR4$^+$ FM3

Fig. 2 ELISPOT assays showing recognition of DCs unpulsed or pulsed with HER-2(884–899) by a panel of autologous CD4$^+$ T-cell clones. All clones were generated from the CD4$^+$ T cell bulk culture that showed specificity for HER-2(884–899) in the previous experiments. Numbers of IFN-γ spots produced by 1×10^4 CD4$^+$ T cells/clone are shown from one representative experiment out of two performed. *Arrows* indicate the clones further propagated and tested in the experiments below



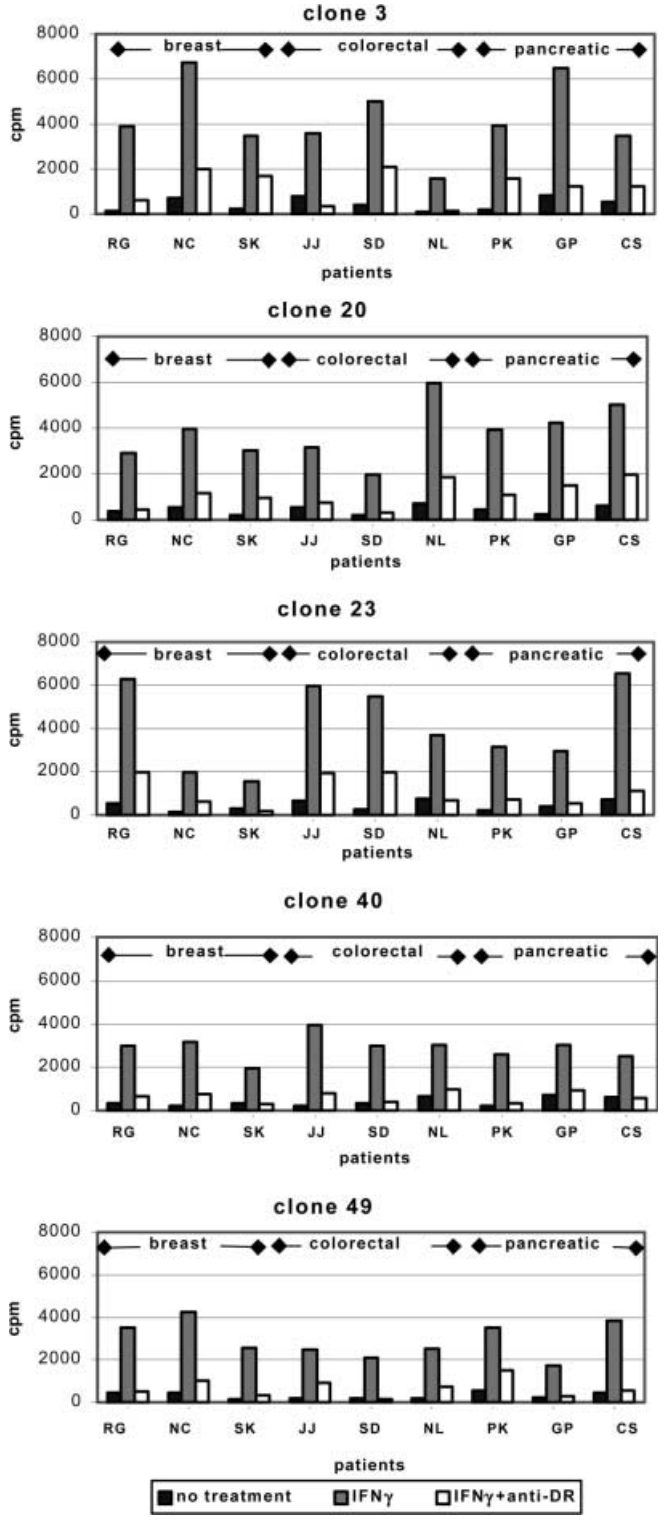


Fig. 3 HER-2(884–899)-specific T cell clones recognize HER-2/neu$^+$ tumor cells induced to express HLA-DR4. Tumor cells isolated from HLA-DR4$^+$ patients RG, NC, SK (*breast Ca*), JJ, SD, NL (*colorectal Ca*), and PK, GP, CS (*pancreatic Ca*) were used as APCs in proliferation assays, either untreated or pretreated with IFN-γ to express HLA-DR4 (see Materials and methods). Anti-HLA-DR mAb (*L243*) was present throughout the entire culture period. *Bars* represent mean values from triplicate cultures. The SD (not shown) was in all groups less than 15% of the mean value

melanoma cell line transfected to express HER-2/neu (FM3/HER), were also used as APCs.

To analyze the ability of CD4$^+$ clones 3, 20, 23, 40 and 46 to recognize tumor cells, HER-2/neu$^+$ and HLA-DR4$^+$ cancer cells from patients with metastatic breast (patients RG, NC, SK), colorectal (patients JJ, SD, NL) and pancreatic (patients PK, GP, CS) cancer were used as test APCs in a standard proliferation assay. As demonstrated in Fig. 3, all clones recognized the metastatic tumor cells to various levels (cpm range: 1550–6740, SI range: 4.5–17.0) and their proliferative responses could be blocked to a great extent with anti-HLA-DR mAb (% inhibition range: 58–93). Control APCs (i.e., HER-2/neu$^+$ tumor cells not pretreated with IFN-γ to express HLA-DR4) induced only marginal responses, which were significantly lower (cpm range: 93–750) when compared to those achieved with test APCs (i.e., the same HER-2/neu tumor cells induced to express HLA-DR4).

The reactivity of HER2(884–899)-specific CD4$^+$ T cell clones against the HER-2/neu$^+$, HLA-DR4$^+$ metastatic tumor cells was confirmed in ELISA assays (Fig. 4). The clones responded vigorously, producing high levels of IFN-γ upon stimulation with test APCs [range for DR4$^+$ pancreatic tumor cells representative of all DR4$^+$ tumors ($n=9$) tested: 1520–2550 pg/ml; mean: 2062 ± 401 pg/ml; mean: 325 ± 85 pg/ml] compared to those obtained with control APCs (range: 210–420 pg/ml). Also in this case, recognition of tumor cells by the clones was largely inhibited in the presence of anti-HLA-DR mAb (% inhibition range: 65–82) (Fig. 4). The same clones did not recognize the HER-2/

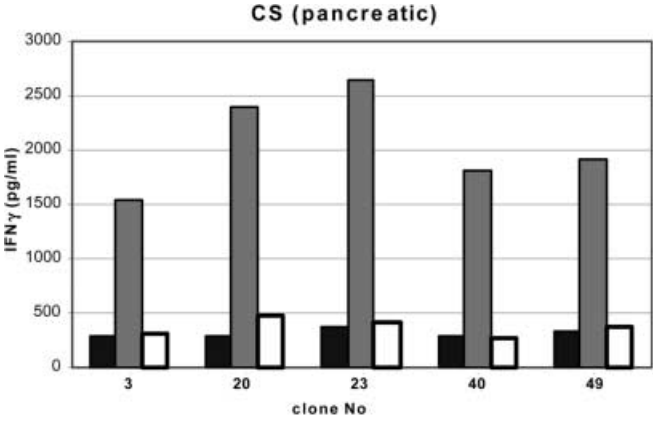
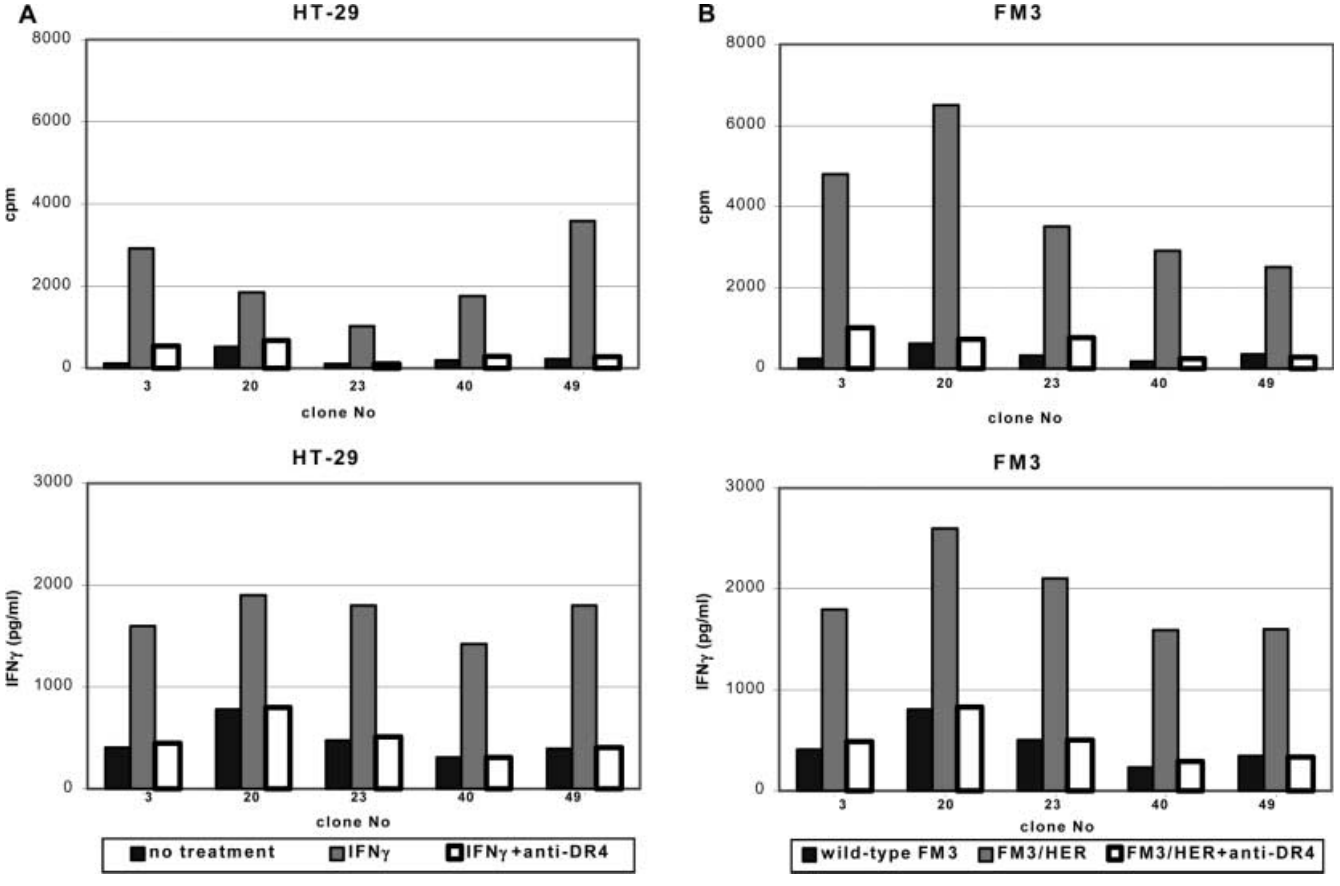


Fig. 4 IFN-γ production by HER-2(884–899)-specific T cell clones recognizing HER-2/neu$^+$ tumor cells induced to express HLA-DR4. Clones were tested in 48 h cultures for recognition of DR4$^+$ HER-2/neu$^+$ tumor cells from a patient with pancreatic Ca for IFN-γ production with the same APCs as in Fig. 3. Anti-HLA-DR mAb was present throughout the 48 h incubation period. *Bars* represent mean values from triplicate cultures (i.e. supernatants from each culture tested individually). The SD (not shown) was in all groups less than 15% of the mean value. This experiment is representative of nine conducted with tumor cells from the same patients as those listed in Fig. 3 (*filled symbols* no treatment, *hatched symbols* IFN-γ treated, *open symbols* IFN-γ treated plus anti-HLA-DR)

neu over-expressing, although HLA-DR$^-$, HT-29 colorectal cell line (cpm range: 97–530; IFN-γ range: 310–780 pg/ml), but they did so when this cell line was preincubated with IFN-γ to express HLA-DR4 (cpm range: 1025–3590 and SI range for cpm: 3.5–24.4; IFN-γ range: 1420–1900 pg/ml and SI range for IFN-γ: 2.4–4.6) (Fig. 5A). In a similar manner, the HLA-DR4$^+$ FM3/HER melanoma cells induced significantly higher proliferative responses (cpm range: 2500–4800) and IFN-γ (range: 1590–2600 pg/ml) by all clones tested, as compared to wild-type (non-transfected) FM3 cells (cpm range: 170–610 and IFN-γ range: 230–805 pg/ml) (Fig. 5B). SI for cpm ranged between 7 and 19.2, whereas that for IFN-γ ranged between 3.2 and 6.9 ($P < 0.005$ for both cpm and IFN-γ levels). As also shown in Fig. 5, addition of anti-HLA-DR mAb into the cultures greatly inhibited (up to 90%) both type of responses.

Phenotypic analysis of the CD4$^+$ T cell clones demonstrated that these were CD56$^-$ and CD16$^-$ (data not shown). We also analyzed the expression of the TCRBV regions using a panel of specific mAbs. All five CD4$^+$ T cell clones expressed TCRVB7.1 [range for mean fluorescence intensity (MFI): 1490–2250; for all other TCRBV-specific mAbs range of MFI: 30–75] indicating that T cells carrying these receptors were preferentially expanded during culture with the HER-2 peptide.

Fig. 5A, B Recognition of tumor cell lines by the HER-2(884–899)-specific T cell clones. The HER-2/neu overexpressing colorectal cell line HT-29 not treated or pretreated with IFN-γ to express HLA-DR4 **A** and the HLA-DR4$^+$ and HER-2/neu$^-$ wild-type melanoma cell line FM3 transfected to express HER-2/neu (*FM3/HER*) **B** were used as APCs in proliferation and IFN-γ production assays. Anti-HLA-DR mAb (*L243*) was present throughout the entire incubation period. Results represent one out of three similar experiments performed



Discussion

Peptide-based vaccination may become more efficient if tumor antigen-specific CD4$^+$ T cell-mediated responses are induced simultaneously by HLA-class-II tumor peptides, as suggested by several experimental tumor models [1, 5, 26, 29]. Furthermore, HLA-class-II peptides used for monitoring antigen-specific immune responses in cancer patients will provide impetus for a better understanding of the role of CD4$^+$ T cells in tumor immunology. The essential role of CD4$^+$ T cells for the generation of effective antitumor responses in humans was first shown in our recent work [4], where we were able to demonstrate that unfractionated peptide extracts from ovarian and breast tumor lysates contain antigenic peptides presented to CD4$^+$ T cells in the context of HLA-DR molecules. Peptide-specific CD4$^+$ T cells were then capable of recognizing the autologous tumor from which the peptides were derived and also

efficiently potentiated the CTL-mediated autologous tumor-specific cytotoxicity. HER-2/neu peptides predicted to bind MHC class II molecules have already previously been shown to stimulate in vitro PBMC proliferative responses in patients with breast and ovarian cancer [2, 9, 10, 14, 20, 35]. The fact that in some cases [9, 14] such responses could be obtained after one single stimulation indicated pre-existing in vivo responses to the HER-2/neu over-expressing tumors by patients. In pilot clinical settings, Disis et al [10], using HER-2/neu peptide-based vaccines, succeeded in generating immunity specific to the immunizing peptide, but also to the HER-2/neu protein, which occurred with epitope spreading. In another setting, Knutson et al. [19] immunized breast cancer patients with the 16-mer peptide epitope, HER-2 (369–384), predicted to bind to MHC class II alleles that also contained the HLA-A2-binding CTL epitope HER-2(369–377) [13]. Most of the patients demonstrated significant $CD8^+$ T cell-mediated responses against the CTL epitope concurrently with HER-2(369–384)-specific responses mediated by $CD4^+$ T cells. However, in some cases such $CD4^+$ T cell responses were also observed upon immunization with the cytotoxic CTL epitope only [i.e., HER-2(369–377)], thus making somehow less clear whether such a vaccination protocol induced a specific helper effect in vivo.

In this study, we generated $CD4^+$ T helper cell clones specific to HER-2(884–899) and demonstrated that these clones are capable of recognizing HER-2/neu^+ tumors from patients with breast, colorectal and pancreatic cancer. Such tumor cells were MHC class II^-, but were induced to express HLA-DR4 molecules. This was also the case with the HLA-$DR4^+$ melanoma cell line FM3 transfected to express HER-2/neu and with the HER-2/neu over-expressing colorectal cell line HT-29 induced to express HLA-DR4. To our knowledge this is the first report to demonstrate that HLA-$DR4^+$- and HER-2/neu-expressing tumor cells can be recognized by $CD4^+$ T helper clones specific for HER-2(884–899), indicating that this epitope is presented at the surface of tumor cells.

Besides the exogenous presentation pathway [15], MHC class II molecules also present peptides via an endogenous presentation pathway involving primarily proteins containing an endosomal targeting sequence, such as the melanocyte-specific proteins tyrosinase and gp75 [38]. There are also reports demonstrating that epitopes from MAGE-A3 protein are also recognized on tumor cells by MHC class-II-restricted cytolytic [32] or helper $CD4^+$ T cells [42]. The data presented herein suggest that HER-2/neu protein may also possess lysosomal targeting sequences that allow it to follow the endogenous MHC class II processing pathway. The identification of more antigenic HER-2/neu MHC class II epitopes presented at the surface of tumor cells will be useful to find out whether the entire HER-2/neu protein has access to endosomes. For instance, two antigenic peptides from MAGE-A3, MAGE-A3(114–127) and MAGE-A3(121–134), were demonstrated not to be expressed at the surface of MAGE-$A3^+$ tumors [7], although other epitopes from the same protein, MAGE-A3(281–295) [23] and MAGE-A3(247–258) [41], were identified to be recognized on tumor cells by $CD4^+$ T cell lines. Critical aspects in this issue are to determine where the loading of peptides from the cytosolic proteins takes place and to find out which of the peptides, and at what intensity, competes with the invariant chain for binding into the MHC class II molecules. The answer to these questions will improve our understanding of the endogenous class II presentation pathway.

To our knowledge this is also the first report on TCR usage for HER-2/neu peptide recognition by $CD4^+$ T cells. Interestingly enough, all five clones expressed TCRVB7.1. Analyzing the nucleotide sequences of the CDR3 regions will definitely provide an answer as to whether or not these are sister clones. Nevertheless, testing additional clones will provide more information on the extent of homogeneity of the TCR repertoire used for the recognition of HER-2(884–899) presented by HLA-DR4. In this context it will also be important to find out how common the use of particular TCR-gene segments (e.g. TCRVβ7.1) is for the recognition of HER-2(884–899) presented by alleles other than HLA-DR4 (e.g. HLA-DR1, -DR52, or -DR53 [20]). To this end, a $CD4^+$ T cell clone recognizing a single peptide in the context of 3 different alleles has been already reported [14]. TCR expression could provide a molecular marker for the selection of $CD4^+$ T cells recognizing HER-2/neu^+ tumors to be used along with cytotoxic effectors in adoptive immunotherapy, but also for monitoring the capacity of HER-2/neu peptide-based vaccines to induce cellular immunity in vivo.

Taken altogether, we demonstrate herein that HLA-DR4-restricted and HER-2(884–899)-specific $CD4^+$ T cell clones recognize HER-2/neu^+, HLA-$DR4^+$ breast, colorectal and pancreatic metastatic tumor cells and tumor cell lines, indicating that this helper epitope is expressed on the surface of such cells. Given the fact that HER-2/neu MHC class I epitopes recognized by CTL have already been identified in these types of cancer [6, 22, 28], this peptide represents a candidate helper epitope to be included in a vaccine preparation consisting of HER-2/neu peptides presented by MHC class I and class II molecules. With such formulations, vaccine-induced HER-2/neu-specific $CD4^+$ T cell responses may synergize with vaccine-induced CTL, resulting in the improvement of clinical results in cancer immunotherapy.

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