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Induction of potent CD4$^+$ T cell-mediated antitumor responses by a helper HER-2/*neu* peptide linked to the Ii-Key moiety of the invariant chain

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The Ii-Key fragment from the MHC class II-associated invariant chain (or Ii protein) has been shown to facilitate direct charging of MHC class II epitopes to the peptide binding groove. The purpose of the present study was to test the potential of a series of Ii-Key/HER-2/*neu*(776–790) hybrid peptides to generate increased frequencies of peptide-specific CD4$^+$ T cells over the native peptide in mice transgenic (Tg) for a chimeric human mouse class II molecule (DR4-IE) (H-2^b) as well as their antitumor potency. Following *in vivo* priming, such hybrid peptides induced increased proliferation and frequencies of IFN-γ producing CD4$^+$ T cells in response to either syngeneic dendritic cells pulsed with native peptide, or HLA-DR4$^+$ human tumor cell lines expressing HER-2/*neu*. Hybrid peptides were more stable in an off-rate kinetics assay compared to the native peptide. In addition, antigen-specific CD4$^+$ T cells from hybrid peptide immunized DR4-IE Tg mice synergized with HER-2/*neu*(435–443)-specific CD8$^+$ T cells from HLA-A2.1 Tg HHD (H-2^b) mice in producing antitumor immunity into SCID mice xenografted with the HER-2/*neu*$^+$, HLA-A2.1$^+$ and HLA-DR4$^+$ FM3 human melanoma cell line. High proportions of these adoptively transferred HER-2/*neu* peptide-specific CD4$^+$ and CD8$^+$ T cells infiltrated FM3-induced tumors (tumor infiltrating lymphocytes; TIL) in SCID mice. CD8$^+$ TIL exhibited long-lasting antitumor activity when cotransferred with CD4$^+$ TIL, inducing regression of FM3 tumors in a group of untreated, tumor-bearing SCID mice, following adoptive transfer. Our data show that Ii-Key modified HER-2/*neu*(776–790) hybrid peptides are sufficiently potent to provide antigen-specific CD4$^+$ T$_H$ cells with therapeutic antitumor activity.

Key words: HER-2/*neu* peptides; Ii-Key hybrid peptides; cancer vaccines; CD4$^+$ T cells; MHC class II; tumor models

Peptide-based cancer vaccines mainly consist of MHC class I-restricted tumor peptides capable of eliciting antitumor cytotoxic T lymphocyte (CTL) responses.[1–3] However, there is now increasing evidence that optimal cancer vaccines require the concurrent generation of both CD4$^+$ and CD8$^+$ T cell responses.[2] In the murine system, there are several reports demonstrating that both *in vitro* and *in vivo* generation of a specific immune response to a tumor protein-epitope requires cross-priming of tumor antigens by antigen-presenting cells and is strictly T helper (T$_H$)-cell dependent, since depletion of CD4$^+$ T cells totally abrogates the generation of peptide-specific CTL responses.[4–8]

There are also several studies reporting the existence of MHC class II-restricted T-cell responses to the HER-2/*neu* proto-oncogene which is over expressed by a variety of epithelial tumors including breast, ovarian, lung, prostate and colorectal carcinomas.[2] Immunodominant regions of the HER-2/*neu* protein encompassing residues from both the intracellular domain (ICD) and extracellular domain (ECD) have been demonstrated to induce proliferation and cytokine production by patients' CD4$^+$ T$_H$ cells.[9–13] Furthermore, vaccination of cancer patients with T$_H$ epitopes containing embedded HLA-A2 binding motifs elicited enhanced HER-2/*neu* peptide specific CTL precursor frequencies and provided durable responses detectable more than 1 year after the final vaccination.[14] Our laboratory has also recently demonstrated that optimal killing of autologous tumor cells required CTL potentiation by patients CD4$^+$ T-cells.[15]

Various protocols for generating HER-2/*neu* antigen-specific immune responses have been evaluated in preclinical models,[16,17] while clinical trials have primarily focused on assessing the feasibility of antigen-specific HLA class I peptides to elicit antitumor responses.[18–20] Even though peptide-specific CTL are generated, the lack of long-term cytotoxic longevity has so far resulted in poor clinical outcomes.[18,19] Short-lived CTL responses might be attributed to the lack of CD4$^+$ T-cell support.[14] A novel method to augment potency of MHC class II epitope peptide presentation to concomitantly boost CTL activity has now become available in the form of Ii-Key/MHC class II hybrid peptides.[21,22] Such Ii-Key hybrid peptides contain an immunoregulatory segment of the Ii protein that catalyzes direct charging of MHC class II epitopes to the peptide-binding groove, circumventing the need for intracellular epitope processing.[23] A 16-amino-acid region of the Ii protein was originally found to enhance presentation of antigenic peptides by living or fixed APCs to T-cell hybridomas.[21,22] Structure–activity relationship studies using 160 homologs of that peptide, referred to as "Ii-Key," revealed a "core" structure (LRMKLPK) that had significantly greater activity than the original 16-amino-acid peptide. The shortest active sequence consisted of 4 amino acids LRMK (Ii-Key peptide).[22,24] Covalent linkage of this Ii-Key segment through a flexible polymethylene bridge -*ava*- spacer to a MHC class II epitope significantly augments antigen presentation.[25–29] The mechanistic hypothesis postulates that the Ii-Key moiety initially binds to an allosteric site of MHC class II molecules located just distal to the epitope binding groove, and induces a conformational change facilitating charging of the tethered peptide. Then, the allosteric-site ligand, with a lesser binding affinity than the antigenic peptide for the antigenic peptide-binding groove, dissociates to allow stabilization of the MHC Class II/epitope complex.

The major objective of our study was to evaluate the immunogenicity and potentiation of antitumor CTL activity mediated by a homologous series of Ii-Key/MHC class II epitope HER-2/*neu*(776–790) hybrid peptides. Members in the series differed in the length of the spacer sequence between the putative P1 site residue (Y) of the presumed epitope and LRMK (Ii-Key). The HER-2/*neu*(776–790) peptide sequence is promiscuously presented by various alleles including HLA-DR4[13,30,31] and is naturally processed and presented by numerous types of human tumor cells.[15] It is derived from the ICD of the HER-2/*neu* protein and serves as a compelling tumor rejection target antigen. T-cell immunity directed against the ICD has been shown to generate more potent HER-2/*neu*-specific immunogenic activity than the ECD.[14,32]

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However, antitumor protection mediated *via* the ICD protein required collaboration of both $CD4^+$ and $CD8^+$ T cells.[33] The intracellular location of the ICD makes it potentially more immunogenic due to its sequestering from the immune system during development.[32] Therefore, immunity directed against peptides from the ICD present in the tumor microenvironment or shed by apoptotic cells might better serve to override immunological tolerance to the HER-2/*neu* protein.

Ii-Key/HER-2/*neu* hybrid peptides are currently being evaluated for safety, tolerability and assessment of immunological responses in a phase I clinical trial with HER-2/neu^+ breast cancer patients.[34] Here, we report that Ii-Key/HER-2/*neu*(776–790) hybrid peptides prime higher frequencies of $CD4^+$ T cells compared to the native peptide following active vaccination in DR4-IE Tg mice. Furthermore, such hybrid peptide-induced $CD4^+$ T cells stimulated higher HER-2/*neu* peptide-specific CTL responses and resulted in tumor regression of SCID mice xenografted with a HER-2/neu^+ tumor cell line. Our data might be useful in the design of additional MHC class II vaccine peptides for HER-2/*neu* and other clinically significant tumor associated antigens (TAA). Ii-Key/MHC class II hybrid peptides combined either with CTL peptides or used in patients with a pre-existing CTL response might have significant therapeutic value in the treatment of cancer.

Methods

Animals

C57BL/6-DR4 ($H\text{-}2^b$)[29,35] mice were purchased from Charles River (Germany). Colonies were bred at the Hellenic Pasteur Institute animal facility in accordance with the Home Office Codes of Practice for the housing and care of animals. HLA-DR4 expression was confirmed by flow cytometry and PCR genotyping. HHD ($H\text{-}2^b$) mice (knocked out for β_{2m} and D^b and transgenic for HLA-A2.1) were provided by Prof. Francois Lemonier at the Institute Pasteur, Paris.[36] CB-17-Prkdcscid (BALB/c SCID) ($H\text{-}2^d$) mice, 8–10 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, Maine). Animals were maintained in an isolated environment with sterile food and water, which contained trimethoprim sulfamethoxazole (Biochraft, Elmwood Park, NJ).

Peptides

A homologous series of Ii-Key/HER-2/*neu*(776–790) hybrid peptides were synthesized as recently described[15] by systematically varying the structure and length of the spacer which connects the Ii-Key (LRMK) segment to the promiscuous HER-2/*neu*(776–790) MHC class II epitope through a flexible polymethylene, *-ava-* (5-aminovaleric acid = 5-aminopentanoic acid), chain (Table I). All peptides were found to be 98% pure by analytical HPLC and mass spectrometry (Commonwealth Biotechnologies, Richmond, VA). The above peptides were N-acetylated and C-amidated to inhibit exopeptidases. The control peptide Ii-Key/HIV/gag(164–181; YVDRFYKTLRAEQASQEV) which binds to several common HLA-DR alleles also including HLA-DR4[37] was linked to the Ii-Key segment in the same way to produce the Ii-Key/HIVgag(Ac-LRMK-ava-YVDRFYKTLRAEQASQEV-NH_2) hybrid peptide. The human HER-2/*neu*(435–443) and gp100(44–59) peptides were synthesized as recently described.[3,35] All peptides were found to be >95% pure by analytical HPLC and mass spectrometry (Commonwealth Biotechnologies).

Cell lines

The human colorectal adenocarcinoma cell line HT-29 (American Type Culture Collection, Marassas, VA) was cultured in McCoy's 5A medium (Life Technologies, Gaithersburg, MD). The human melanoma cell line FM3[38] was grown in RPMI-1640 (Life Technologies). T2 cells (TAP-deficient HLA-2.1 T2 cell line derived from the human T cell leukemia/B cell LCL hybrid 174) were provided by Prof. H.-G. Rammensee (Department of Immunology, University of Tuebingen). T2 cells transfected to stably express the HLA-DRB1*0401 allele (T2-DR4) were provided by Prof. T.L. Whiteside (Department of Pathology, University of Pittsburgh).[39] Both cell lines were maintained in RPMI-1640. All media were supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 50 μg/ml gentamycin.

TABLE I – II-KEY/HER-2/*neu* (776–790) MHC CLASS II EPITOPE HYBRID DESIGN

Peptide	Position	Sequence
NP	776–790	Ac-GVGSPYVSRLLGICL-NH_2
B	776–790	Ac-LRMK-ava-GVGSPYVSRLLGICL-NH_2
C	777–790	Ac-LRMK-ava-VGSPYVSRLLGICL-NH_2
E	779–790	Ac-LRMK-ava-SPYVSRLLGICL-NH_2
F	776–790	Ac-LRMK-GVGSPYVSRLLGICL-NH_2

NP is the native peptide HER-2/*neu*(776–790), whereas peptides B through F are the Ii-Key/HER-2/*neu*(776–790) hybrid peptides. The Ii-Key (LRMK) segment of the immunoregulatory Ii protein was linked through a simple polymethylene *-ava-* spacer to the promiscuous HER-2/*neu*(776–790) MHC class II epitope by systematically deleting amino acids N-terminal to the P1 (Y) site residue.

Transfection of tumor cells lines

The plasmid containing the class II transactivator (pCIITA), pEF/Bsd/CIITA, was constructed with cytomegalovirus based on constructs from Invitrogen (Carlsbad, CA) by standard molecular biology techniques. This plasmid was expanded and purified as described.[40] HT-29 cells were plated at 2×10^5 cells per well in 6-well plates. After a 24-hr incubation at 37°C in 5% CO_2 incubator, cells were washed twice in 1× phosphate-buffered NaCl solution. Then pCIITA (3–6 μg rDNA) was mixed with PolyFect transfection reagent (Qiagen, Valencia, CA), and subsequently added in duplicate wells in 1.5 ml culture medium. After 2 days of incubation, cells were removed and replaced in 24-well plates (Costar, Cambridge, MA) at 2×10^4 cells/ml with the selective antibiotic blasticidine-S-HCL (Life Technologies). Antibiotic-resistant colonies were selected and expanded further in cultures with blasticidine-S-HCL. Transfected clones were identified by expression of HLA-DR4 molecules using an anti-HLA-DR4 monoclonal antibody (mAb) (clone NFLD.D1E2, IgG1) which was obtained from Terra Nova Biotechnology (St. John's Newfoundland, Canada). As a second antibody, FITC-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark) was used. Cells were analyzed in a FACSCalibur flowcytometer (Becton Dickinson Biosciences, Mountain View, CA).

Preparation of dendritic cells

Dendritic cells (DCs) were generated from murine bone marrow (BM) cells as previously described[41] with some modifications. In brief, BM cells were flushed from the long bones of DR4-IE Tg mice and depleted of red cells with ammonium chloride. BM cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-ME and 10 mM HEPES (thereafter referred to as complete medium (CM)). About 20 ng/ml of recombinant murine (rm) GM-CSF and 20 ng/ml of rmIL-4 (R&D Systems, Mineapolis, MN) were added to CM. On day 0, 3×10^6 cells were seeded per well in 6-well plates (Costar) in 3 ml CM/well and incubated at 37°C in 5% CO_2. On days 2 and 4, floating cells were gently removed, and fresh CM supplemented with rmGM-CSF and rmIL-4 was added to the cells. On day 6, culture supernatants were aspirated and replaced with fresh CM supplemented with 100 U/ml TNFα (Sigma Chemicals, MO). Purity of $CD11c^+$ cells was >90%, as detected with PE-conjugated anti-CD11c (HL3) mAb (Pharmingen, San Diego, CA) by direct immunofluorescence.

Peptide binding on T2-DR4 cells

Peptides were labeled with phycoerythrine (PE) using to the PJ31K PhycoLink R-Phycoerythrin Conjugation kit (Prozyme, Canada) kit according to the manufacturer's instructions. PE-la-

beled peptides were incubated for 12 hr at 37°C with T2-DR4 cells or nontransfected T2 cells (as for a control). After 2 successive washings, cells were analyzed on a FACSCalibur flowcytometer (Becton Dickinson) using CellQuest software. Specific binding was defined as mean fluorescence intensity (MFI) with T2-DR4 cells-MFI with T2 control cells (ΔMFI).

MHC-peptide complex stability

The off-rate kinetics assay was performed as previously described.[3] T2-DR4 cells were incubated overnight at 37°C without peptides or with 1 μM of each PE-labeled peptide in serum-free RPMI-1640. Next, the cells were incubated with brefeldin A (10 μg/ml; Sigma-Aldrich) for 1 hr to block cell-surface expression of newly synthesized HLA molecules, washed and further incubated for 0, 2, 4, and 6 hr. At each time-point ΔMFI was calculated as MFI with PE-labeled peptides − MFI without peptides. ΔMFI measured at 0 hr was considered as 100%. ΔMFIs at all other time-points were expressed relative to ΔMFI observed in the initial labeling experiment at 0 hr, which was performed immediately after treatment with brefeldin A. The degree of stability of peptide binding on DR4 molecules was defined from the time-point at which 50% reduction of ΔMFI was measured.

ELISPOT assay

DR4-IE Tg mice were immunized once with 100 μg of peptide in 100 μl of 1× phosphate-buffered NaCl solution emulsified with an equal volume of complete Freund's adjuvant (CFA; final total volume 200 μl) subcutaneously (sc.) at the basis of the tail. On day 12, the animals were sacrificed, and inguinal, mesenteric and popliteal lymph nodes were harvested. $CD4^+$ T cells were isolated from total lymph node cells by negative selection using the $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany). Highly purified $CD4^+$ T cells (2.5×10^5 cells/well) were incubated with either peptide pulsed syngeneic DCs (2.5×10^4 cells/well), or irradiated (10,000 rad) tumor cell lines (5×10^5 cells/well) in 96-well U-bottom plates (quadruplicate wells; Costar) in 200 μl of CM. Wherever indicated, anti-HLA-DR4 mAb (clone NFLD.D1E2; Terra Nova Biotechnology) was added at 10 μg/ml final concentration. After 3 days of incubation, responder $CD4^+$ T cells were transferred to a precoated IFN-γ ELISPOT plate and incubation was extended for another day. The ELISPOT assay was performed according to the manufacturer's instructions (BD Pharmingen, Erembodegem, Belgium).

Proliferation assay

$CD4^+$ T cells from immunized DR4-IE Tg mice were cultured at 2×10^6 cells/ml with syngeneic DCs (2×10^5 cells/ml) in 200 μl of CM per well in 96-well U-bottom plates (Costar). In another series of experiments, $CD4^+$ or $CD8^+$ T cells (2×10^6 cells/ml each) from immunized DR4-IE Tg or HHD mice, respectively, were cultured with irradiated (5,000 rad) FM3 cells (5×10^6 cells/ml) as described earlier. The anti-HLA-DR4 (10 μg/ml) and anti-HLA-A2.1 (10 μg/ml) (BB7.2; provided by Prof. H.-G. Rammensee) mAbs were added at culture initiation whenever indicated. After 4 days of incubation in CO_2-incubators, 1 μCi/well tritiated thymidine ([^{3}H] TdR, 48 Ci/mmol, 1 mCi/ml; Amersham Pharmacia Biotech, Buckinghamshire, UK) was added for the last 16 hr. Cells were harvested and thymidine incorporation was measured in a liquid scintillation counter (LKB Wallac, Turku, Finland). All cultures were performed in triplicates and results are expressed as counts per minute (cpm).

Preparation of peptide-primed $CD4^+$ and $CD8^+$ T cells for adoptive transfer

DR4-IE Tg mice were immunized as described earlier, and lymph node $CD4^+$ T cells (2×10^6 cells/ml) were incubated with syngeneic DCs (2×10^5 cells/ml) pulsed with the peptide (50 μg/ml) used for immunization. After 5 days of incubation in CM, cells were harvested, washed and resuspended in 1× phosphate-buffered NaCl solution for intraperitoneal (ip.) injections. For the generation of peptide specific CTL, HLA-A2.1 HHD mice were immunized with peptide HER-2/*neu*(435–443), as recently described.[3] Highly purified $CD8^+$ T cells were isolated from total immune splenocytes by negative selection using the $CD8^+$ T cell isolation kit (Miltenyi Biotec). $CD8^+$ T cells before adoptive transfer were expanded as previously described.[3] Briefly, 1×10^5 $CD8^+$ T cells were restimulated *in vitro* with 25×10^6 HER-2/*neu*(435–443)-pulsed syngeneic irradiated naive splenocytes in the presence of low-dose interleukin-2 (IL-2). Ten days later, cells were further expanded with IL-2 and 30 ng/ml anti-CD3 mAb (Pharmigen, San Diego, CA). Before adoptive transfer, expanded $CD8^+$ T cells were tested for lysis of T2 cells pulsed with HER-2/*neu*(435–443) and FM3 human melanoma target cells, whereas both $CD4^+$ and $CD8^+$ T cells were also tested in proliferation assays cocultured with FM3 or HT-29 tumor cells. Lysis of FM3 tumor cell targets was also tested with $CD8^+$ effectors cocultured with $CD4^+$ T cells.

Tumor rejection models

SCID mice were inoculated (sc.) on the back with 5×10^5 FM3 tumor cells in 0.5 ml of 1× phosphate-buffered NaCl solution. Injections with peptide-primed $CD4^+$ and/or $CD8^+$ T-cells (1×10^6 cells from each subset per mouse in 0.5 ml of 1× phosphate-buffered NaCl solution) were administered (ip.) at a time when the tumor became palpable (about 12–16 days after inoculation). Tumor size was regularly calliper-measured and recorded as the product of the perpendicular diameters of individual's tumors every 4 days. The observation was terminated with euthanasia when the tumor mass grew to 200–250 mm^2.

Cytotoxicity assay

This was performed as recently described.[3] In brief, $CD8^+$ T cells were isolated from HHD immunized mice as described earlier, and placed at 1×10^6/ml in 100 μl CM per well in 96-well V-bottomed plates (Costar). Wherever indicated such CTL effectors were cocultured with $CD4^+$ T cells (1×10^5 cells per well) isolated from immunized DR4-IE Tg mice. T2 cells (HLA-$A2^+$) or FM3 melanoma tumor cell targets were labeled with sodium chromate (Radiochemical Centre, Amersham, The Netherlands). mAbs were present throughout the cytotoxicity assay at a final concentration (10 μg/ml) whenever indicated. For peptide recognition, T2 cells were incubated with 20 μg/ml HER-2/*neu*(435–443) overnight at 26°C, washed and labeled. Incubation was done for 6 hr in CO_2 incubators. Specific cytotoxicity was calculated as described in Ref. 3.

Tumor infiltrating lymphocytes isolation, characterization and adoptive transfer

SCID mice with regressing tumors (*i.e.*, those therapeutically treated with a combination of HER-2/*neu*(435–443)-specific $CD8^+$ T cells from HHD mice and hybrid peptide F-specific $CD4^+$ T cells from DR4-IE Tg mice) were sacrificed, their tumors were removed and digested in 1 mg/ml collagenase IV (Sigma, St Louis, MO) and 0.1 mg/ml DNase for 1 hr at 37°C. Dissociated cells were washed to remove debris and leukocytes were positively selected using CD45 MACS beads according to manufacturer's instructions (Miltenyi Biotec). Enriched leucocytes were then double stained with a PE-conjugated anti-D^b mAb (clone KH 95; BD Biosciences) and either FITC-anti-$CD4^+$ or FITC-anti $CD8^+$ mAbs (BD Biosciences) for estimating absolute numbers of the adoptively transferred $CD4^+$ and $CD8^+$ T lymphocytes which infiltrated the FM3 tumors. Pooled tumor infiltrating lymphocytes (TIL), or each of the subsets alone, were then injected intra-tumor (it.) in SCID mice with palpable FM3 tumors.

Statistical analyses

Significance of differences among all groups was assessed with the Student's *t*-test. A nonparametric Wilcoxon rank test was also

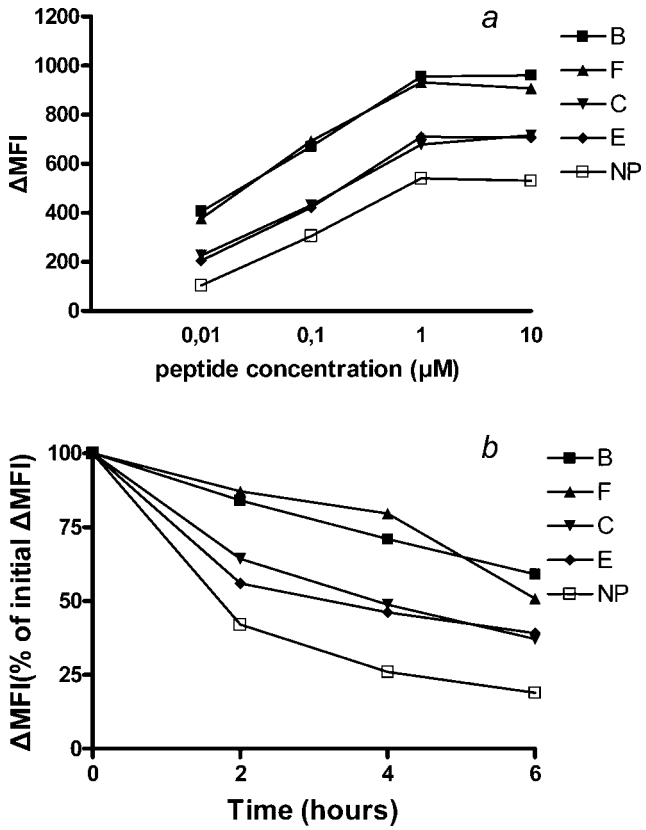

FIGURE 1 – (*a*) Dose-dependent binding of PE-labeled peptides to HLA-DR4 molecules on T2-DR4 cells. ΔMFI was defined as MFI obtained upon incubation of PE-labeled peptides with T2-DR4 cells − MFI obtained under the same experimentations with control nontransfected T2 cells. (*b*) Dissociation of PE-labeled peptides from HLA-DR4 molecules. T2-DR4 cells were incubated with peptides (1 μM) overnight, then washed and incubated for the indicated times at 37°C. ΔMFIs are expressed relative to those observed in the initial labeling experiment, which was done directly after the treatment with Brefeldin A (0 hr). One representative experiment for (*a*) and (*b*), of 3 performed, is shown.

used in the statistical analysis of the size of tumor in individual groups. The difference was considered statistically significant when $p < 0.05$.

Results

Design of Ii-Key HER-2/neu*(776–790) hybrid peptides and off-rate kinetics*

The promiscuously recognized HER-2/*neu*(776–790) epitope[13,30,31] has a tyrosine (Y) at a putative (P1) site and a leucine (L) at a putative P6 site of a predicted MHC class II epitope, both of which define a DR4-specific binding motif,[30] but also a valine (V) and a second (L) which may define a second overlapping MHC class II epitope.[15] In binding assays,[31] the HER-2/*neu*(776–790) peptide demonstrated good binding to many of the most common MHC class II alleles. Herein, a homologous series of Ii-Key/HER-2/*neu*(776–790) hybrid peptides was synthesized as recently described[15] by systematically varying the structure and length of the spacer which connects the Ii-Key (LRMK) segment to the native HER-2/*neu*(776–790) MHC class II epitope through a flexible polymethylene, -*ava*- (5-aminovaleric acid = 5-aminopentanoic acid), chain (Table I). Homologs in the series varied by 1 to 4 natural amino acid residues N-terminal to the first putative epitope and/or by addition of the polymethylene -*ava*- spacer, which has a length equivalent to 2.5 amino acids. The latter spacer was chosen

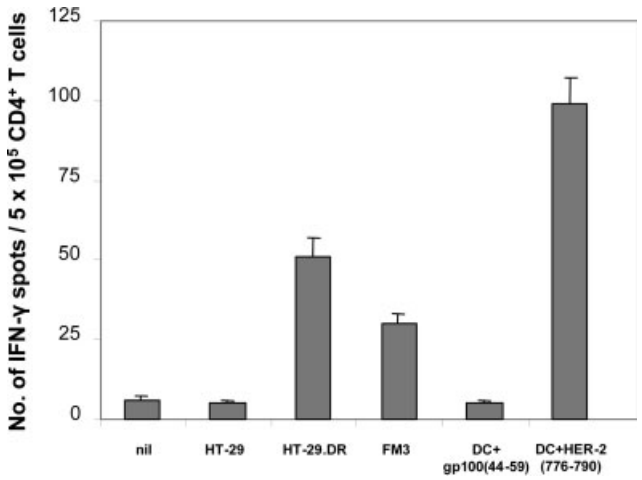

FIGURE 2 – $CD4^+$ T cells from DR4-IE Tg mice immunized with the native peptide HER-2/*neu*(776–790) recognize the HER-2/neu^+ HLA-DR^+ tumor cell lines FM3 and HT-29.DR. DR4-IE Tg mice (5 mice/group) were vaccinated with 100 μg native peptide in CFA (sc.) at the base of the tail. Twelve days later, $CD4^+$ T cells were magnetically isolated from total splenocytes and tested *ex vivo* for IFN-γ responses against either the indicated tumor cell lines and syngeneic DCs, pulsed with the native peptide (DC + HER-2/*neu*(776–790) or with control peptide gp100(44–59) (DC + gp100(44–59). Bars represent mean values (±SD) from quadruplicates.

due to its simple chemical structure and negligible interactions with side chains and the peptidyl back bone of neighboring MHC class II molecules. All peptides were N-acetylated and C-amidated to confer protection against exopeptidases, and by replacing the peptidyl backbone with the -*ava*- linker to block endopeptidases.

To compare the capacity of the native HER-2/*neu*(776–790) peptide and Ii-Key hybrids to form stable peptide/HLA-DR4 complexes, T2-DR4 cells (or non-DR4 expressing T2 cells, as for a control) were incubated with increasing doses of PE-labeled peptides. Specific binding was defined by subtracting the MFI obtained with T2 cells (always <20) from the MFI obtained with T2-DR4 cells. As shown in Figure 1*a*, there was a dose-dependent increase of binding to T2-DR4 cells, which plateaued at peptide concentrations greater than 10 μM. Overall, hybrid peptides bound DR4 molecules on T2 cells with higher affinity compared to the native peptide. Furthermore, dissociation of hybrid peptides B and F from T2-DR4 cells was significantly slower (50% reduction in ΔMFI after 6 hr) as compared to native peptide (50% reduction in ΔMFI at 2 hr) ($p < 0.001$) and to hybrid peptides C and E (50% reduction in ΔMFI at 4 hr) ($p < 0.05$) (Fig. 1*b*). The latter two peptides also showed significantly slower dissociation kinetics compared to native peptide ($p < 0.05$; Fig. 1*b*).

In vivo *priming with Ii-Key/HER-2*/neu*(776–790) hybrid peptides enhances* $CD4^+$ *T cell responses against HER-2*/neu*(776–790) peptide-pulsed syngeneic DCs and HER-2*/neu^+ $DR4^+$ *tumor lines endogenously processing the native peptide*

We next evaluated priming of DR4-IE Tg mice by Ii-Key hybrid peptides compared to the native peptide HER-2/*neu*(776–790) as measured by the ability of $CD4^+$ cells from immunized animals to specifically recognize: (*i*) syngeneic DCs pulsed with the native peptide, and (*ii*) HER-2/neu^+ tumor lines naturally presenting this epitope in the context of HLA-DR4. Lymphocytes from pooled lymph node cells were harvested from DR4-IE Tg mice 12 days after vaccination and tested in an IFN-γ based ELISPOT assay after stimulation by syngeneic DCs pulsed with the native peptide or by HER-2/neu^+ tumor cell lines expressing HLA-DR4. The tumor lines included: FM3 melanoma cells which constitutively express HLA-DR4, and HT-29 colorectal cells

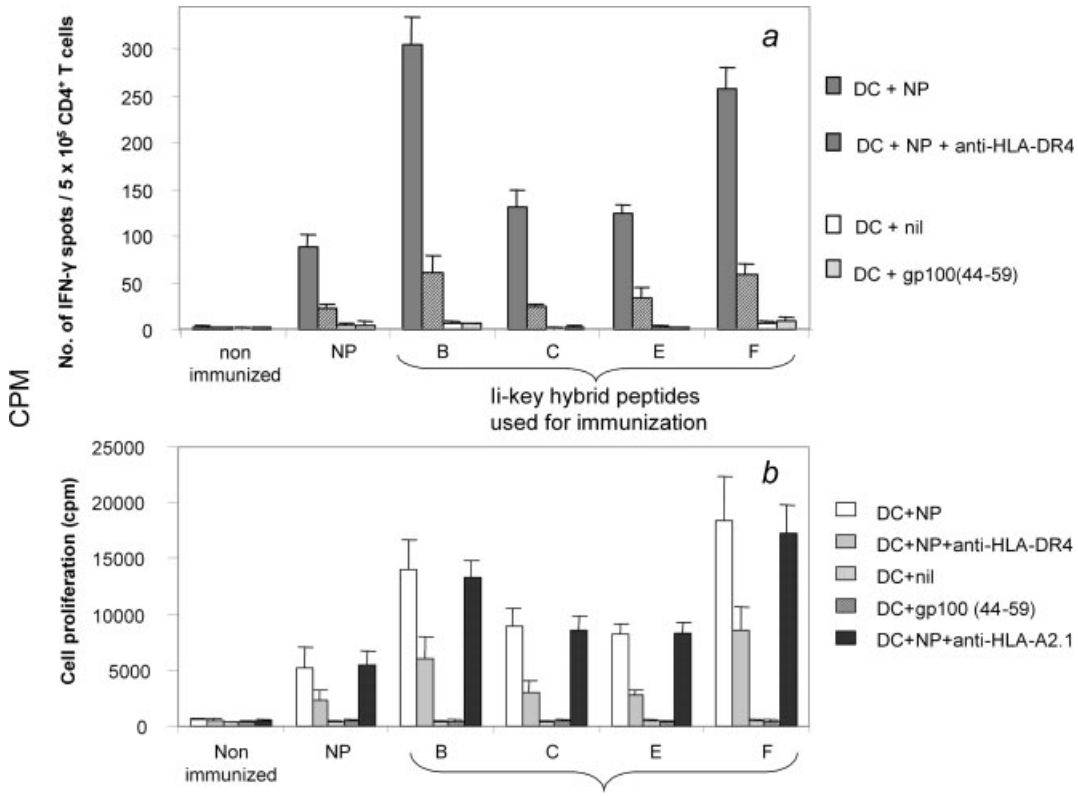

FIGURE 3 – Vaccination with Ii-Key hybrid peptides enhances potency of $CD4^+$ T-cell responses over the native peptide (NP). (*a*) DR4-IE Tg mice (5 mice/group) were immunized (sc.) at the base of the tail with 100 μg of either NP or each of the indicated hybrid peptides in CFA. $CD4^+$ T cells from these mice were then tested in the IFN-γ-based ELISPOT assay for recognition of syngeneic DC unpulsed (nil) or pulsed with the native peptide HER-2/*neu*(776–790) (NP) or with a control gp100(44–59) peptide. Anti-HLA-DR4 mAb was added at 10 μg/ml throughout the ELISPOT assay. (*b*) DR4-IE Tg mice were immunized as previously described. $CD4^+$ T cells from the immunized mice were assayed against syngeneic DC pulsed with NP, or with gp100(44–59) control peptide, or nonpulsed (nil) in a 5-day proliferation assay. Anti-HLA-DR4 was added as earlier. Bars represent mean values (±SD) from quadruplicate (*a*), or quintuplet (*b*) cultures.

which were transfected with HLA-DR4 using a DNA plasmid containing the CIITA transactivator of MHC class II gene expression (HT-29.DR; data not shown). Having initially demonstrated that HER-2/*neu*(776–790) contains a naturally processed and presented DR0401-restricted epitope using FM3 and HT-29.DR cells (Fig. 2), we then established the capacity of the Ii-Key/HER-2/*neu*(776–790) hybrid peptides to augment antigen presentation (Figs. 3*a* and 3*b*). More specifically, $CD4^+$ T cells from mice primed with the native peptide recognized HER-2/neu^+ tumor cells in an HLA-DR4 restricted fashion (*i.e.*, FM3 and HT-29.DR) significantly stronger compared to the wild-type HT-29 cell line (HER-2/neu^+ MHC class II^-) ($p < 0.001$). Recognition was markedly stronger using syngeneic DCs pulsed with HER-2/*neu*(776–790) ($p < 0.05$). No recognition was detected to syngeneic DCs pulsed with the HLA-DR4 restricted control peptide gp100(44–59) (Fig. 2) or nonpulsed (data not shown). However, $CD4^+$ T cells from mice immunized with hybrid peptides B and F reacted far more strongly to native peptide pulsed onto syngeneic DCs; almost a 3-fold increase compared to $CD4^+$ T cells from mice immunized with the native peptide; $p < 0.001$) (Fig. 3*a*). $CD4^+$ cells from the same animals did not respond to unpulsed DCs or DCs pulsed with a control HLA-DR4 restricted gp100(44–59) peptide[35] (Fig. 3*a*). Enhancement of the response towards the native peptide was also observed with $CD4^+$ T cells from mice immunized with hybrid peptides C and E, although the level of stimulation was below that observed using cells from mice inoculated with hybrid peptides B and F. Specifically, recognition of the native peptide-pulsed DR4-IE DCs was increased by almost 1.5-fold when the responding $CD4^+$ T cells were from mice immunized with hybrid peptide C or E, respectively ($p < 0.05$ compared to $CD4^+$ T cells from mice inoculated with the native peptide). All responses to DCs pulsed with native peptide HER-2/*neu*(776–790) were highly blocked in the presence of an anti-HLA-DR4 mAb (Fig. 3*a*). Immunization of DR4-IE Tg mice immunized with the control Ii-Key/HIV gag(164–176) hybrid peptide [HIVgag(164–176)], which also binds to HLA-DR4[37] led to no recognition of HER-2/*neu*(776–790) pulsed syngeneic DCs (data not shown).

The capacity of hybrid peptides B and F to prime $CD4^+$ T cells for enhanced recognition of the native peptide was also confirmed in proliferation assays. As shown in Figure 3*b*, $CD4^+$ T cells from peptide B or peptide F primed animals exhibited increased proliferation when stimulated with native peptide-pulsed syngeneic DCs (14,020 and 18,392 cpm, respectively) compared to $CD4^+$ T cells from animals primed with the native peptide (5,230 cpm; $p < 0.005$). The proliferative responses observed with hybrid peptide C or E primed $CD4^+$ T cells were also significantly higher (8,930 and 8,250 cpm, respectively) compared to those of the native peptide primed $CD4^+$ T cells (Fig. 3*b*; $p < 0.05$). However, and in agreement with our data shown in Figure 3*a*, the priming potential of these 2 latter hybrid peptides was significantly weaker ($p < 0.05$) compared to that of hybrid peptides B and F.

We then extended this analysis to test the potency of the hybrid peptides, relative to the native peptide, in stimulating $CD4^+$ T cells to recognize the HER-2/neu^+ HLA-$DR4^+$ tumor cells lines HT.29.DR and FM3 naturally expressing the HER-2/*neu*(776–790) epitope.[11] Overall, hybrid peptides B and F enhanced $CD4^+$ T cell numbers from vaccinated DR4-IE Tg mice responding to these tumor cell lines (2.1–2.7-fold enhancement of the response

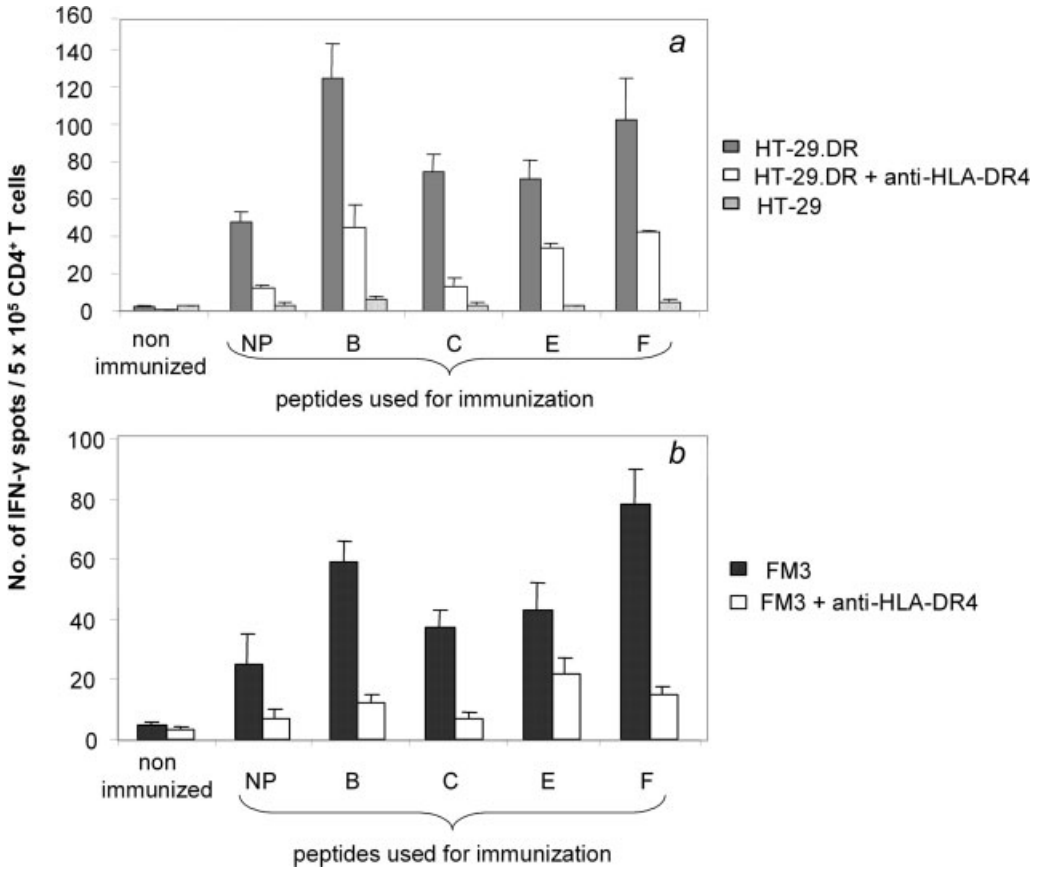

FIGURE 4 – Vaccination with Ii-Key hybrid peptides enhances the ability of $CD4^+$ T-cells to recognize the native peptide naturally processed and expressed by the human tumor cell lines FM3 and HT-29.DR. See also legend to Figure 3.

compared to native peptide; $p < 0.005$) (Figs. 4*a* and 4*b*). Hybrid peptides C and E induced a significantly higher response towards HT-29.DR and FM3 compared to native peptide ($p < 0.05$), which however, was much lower compared to the response induced by peptides B and F ($p < 0.01$) (Figs. 4*a* and 4*b*). Addition of anti-HLA-DR4 mAb in cultures abrogated to a great extent (55–89%) these responses, whereas responses towards the wild-type cell line HT-29 did not exceed background values (Fig. 4*a*).

*Adoptive transfer of $CD4^+$ T cells from Ii-Key hybrid peptide-inoculated DR4-IE Tg mice and HER-2/*neu*(435–443)-specific $CD8^+$ CTL from HLA-A2.1 Tg HHD mice induces effective antitumor responses in xenografted SCID mice*

Because $CD4^+$ T cells from DR4-IE Tg mice inoculated with hybrid peptides led to enhanced recognition of native peptide HER-2/*neu*(776–790) naturally expressed and presented by the FM3 melanoma cells, we investigated whether such hybrid peptide-stimulated $CD4^+$ T cells would more effectively synergize with $CD8^+$ CTL for the rejection of FM3-induced tumors in SCID mice. For this, we first generated HER-2/*neu* peptide-specific CTL by immunizing HLA-A2.1 Tg HHD mice with the HER-2/*neu*(435–443) CTL peptide which is naturally presented in the context of HLA-A2.1 by FM3 cells.[38] In addition, we immunized DR4-IE Tg mice with native peptide and hybrid peptides B or F to generate peptide-specific $CD4^+$ T cells *in vivo*. $CD4^+$ T cells from *in vivo* primed DR4-IE Tg mice specifically proliferated in response to FM3 cells restricted by HLA-DR4 (Fig. 5*a*; significant blocking was observed with the anti-HLA-DR4 mAb, but not with anti-HLA-A2.1 mAb). Similarly, $CD8^+$ T cells from immunized HHD mice proliferated in a similar manner (Fig. 5*b*; significant blocking was observed with the anti-HLA-A2.1 mAb, but not with anti-HLA-DR4 mAb). Furthermore, immune $CD8^+$ T cells specifically lysed either T2 cells (HLA-$A2.1^+$) pulsed with HER-2/*neu*(435–443) *in vitro* (Fig. 5*c*), or FM3 targets (Fig. 5*d*). FM3 targets were lysed in an HLA-A2.1 restricted fashion, since cytotoxicity was abrogated to a great extent in the presence of anti-HLA-A2.1 mAb (but not with the anti-HLA-DR4 mAb; Fig. 5*d*). The cytotoxic effect of $CD8^+$ CTL against the FM3 targets was highly enhanced when $CD4^+$ T cells from immunized DR4-IE Tg mice were co-added during the cytotoxicity assay. This was shown for both peptide B and peptide F primed $CD4^+$ T cells (Fig. 5*e*). Next, these $CD8^+$ CTL were tested for antitumor effects in the xenografted SCID mice. As shown in Figure 6*b*, adoptive transfer of HER-2/*neu*(435–443)-specific CTL in SCID mice with palpable FM3-induced tumors significantly delayed tumor growth over the control mice: In this group of mice, tumor size exceeded 200 mm^2 by days 68–72, whereas in untreated mice (Fig. 6*a*) this tumor growth was achieved already by days 40–44; $p < 0.05$). Transfer of HER-2/*neu*(435–443)-specific CTL along with DR4-IE $CD4^+$ T cells stimulated by the native peptide significantly improved the survival of SCID mice (Fig. 6*c*): in this group tumor size reached 215–225 mm^2 by days 88–92 ($p < 0.05$ compared to SCID mice treated with CTL alone). When the same CTL were co-transferred with DR4-IE Tg $CD4^+$ T cells stimulated with hybrid peptides the antitumor response in SCID mice was enormously increased. Thus, 4 of 9 mice treated with HER-2/*neu*(435–443)-specific $CD8^+$ CTL and hybrid peptide F-stimulated $CD4^+$ T cells (Fig. 6*d*) rejected their tumors, whereas 4 of 10 did so when treated with $CD8^+$ CTL and $CD4^+$ T cells induced by hybrid peptide B (Fig. 6*e*). In the rest of the mice from these groups tumor growth was significantly delayed (tumor size of 200 mm^2 was reached

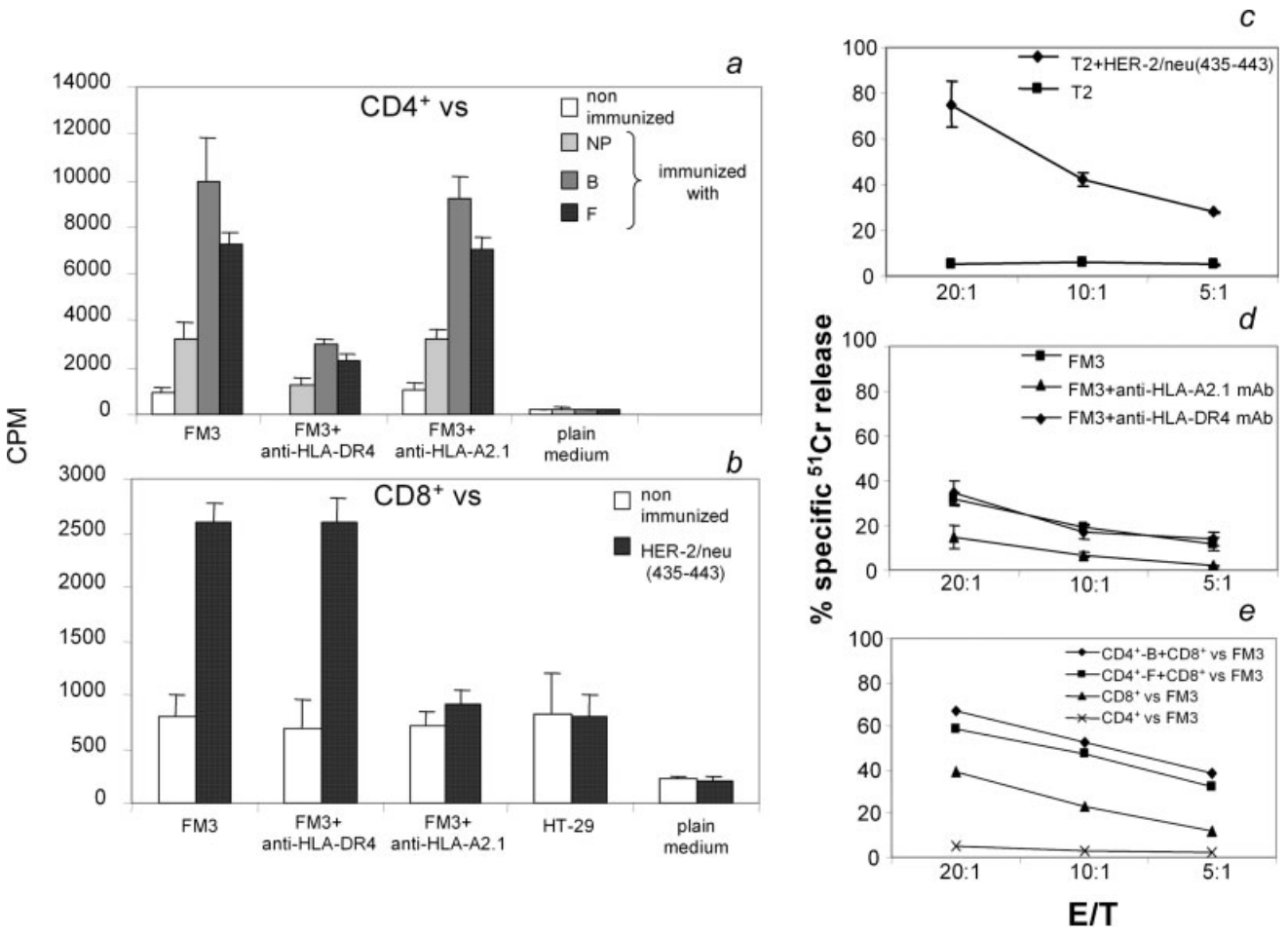

Figure 5 – HLA-restricted proliferation of immune $CD4^+$ T cells or $CD8^+$ T cells in response to irradiated FM3 cells. DR4-IE Tg mice (n = 3) were immunized with either native peptide (NP) HER-2/*neu*(776–790), or with hybrid peptides B and F. HHD mice (n = 3) were immunized with HER-2/*neu*(435–443). Splenic $CD4^+$ T cells from DR4-IE (*a*), or $CD8^+$ T cells from HHD Tg mice (*b*) were tested for proliferative responses upon stimulation with irradiated FM3 cells. Antigen-specific $CD8^+$ T cells from HHD mice (n = 3) immunized with HER-2/*neu*(435–443) lysed either T2 cells pulsed with HER-2/*neu*(435–443) CTL peptide (*c*), or FM3 melanoma cells (*d*). The cytotoxic CTL effect against the FM3 cell targets was significantly enhanced under a coculture regimen with hybrid peptides B and F primed $CD4^+$ T cells during the course of the cytotoxicity assay (*e*). Blocking with all relevant mAbs (10 μg/ml) was performed. Results are shown as mean values (±SD) from triplicate cultures. One representative experiment of 2 performed is shown.

between days 112 and 128) compared to the group of mice treated with CTL alone ($p < 0.01$) or with CTL and native peptide-induced $CD4^+$ T cells ($p < 0.05$). There was no improvement of the antitumor response induced by CTL alone when mice were treated with CTL and unprimed $CD4^+$ T cells (Fig. 6*f*). Adoptive transfer of hybrid peptide F-stimulated $CD4^+$ T cell with irrelevant CTL [primed against the HLA-A2 restricted Flu matrix peptide (58–66)] did not induce any detectable antitumor response (Fig. 6*g*). Similarly, no survival improvement was noticed upon transfer of either peptide F-stimulated $CD4^+$ cells or irrelevant CTL alone (data not shown).

We next sought to directly demonstrate the role of hybrid peptide-primed $CD4^+$ T cells in maintaining effective antitumor $CD8^+$ T cell-mediated immunity. For this, we isolated TIL from regressing tumors (day 35 post FM3 tumor inoculation) from SCID mice (n = 6) which had been treated with 1×10^6 peptide B-primed $CD4^+$ T cells and 1×10^6 HER-2/*neu*(435–443) primed $CD8^+$ T cells, as earlier. Double immunofluorescence staining with PE-conjugated anti-H-$2D^b$ (both DR4-IE Tg and HHD mice are of H-2^b haplotype) and either FITC-conjugated anti-$CD4^+$ or anti-$CD8^+$ mAbs demonstrated that regressing tumors were infiltrated by relatively high proportions of the adoptively transferred peptide-primed $CD4^+$ (range of isolated $CD4^+$ TIL: 0.9×10^5 – 1.5×10^5) or $CD8^+$ (range of isolated $CD8^+$ TIL: 0.6×10^5 – 1.4×10^5) T cells (Fig. 7*a*). Pooled TIL consisting of $\sim 1 \times 10^5$ $CD8^+$ T cells and an equal number of $CD4^+$ T cells were then injected in 100 μl PBS in nontreated SCID mice with palpable FM3 tumors. As shown in Figure 7*b*, all SCID mice treated in this way became long-term survivors. In contrast, all SCID mice receiving only $CD8^+$ TIL (Fig. 7*c*) or $CD4^+$ TIL alone developed tumors (Fig. 7*d*).

Discussion

Preclinical vaccine evaluation has been addressed though studies in transgenic mice. DR4-IE Tg mice have been used to identify several HLA-DRB1 *0401-restricted candidate autoantigen epitopes[42] and tumor epitopes from oncoproteins.[35,43,44] However, this work represents the first example of Tg mice used to identify the most potent Ii-Key/HER-2/*neu*(776–790) hybrid peptides eliciting consistently a strong response from $CD4^+$ T cells against the naturally processed and presented HLA-DR4 restricted HER-2/*neu*(776–790) epitope. Throughout our study we also sought to investigate the potential of hybrid peptide-induced DR4-IE-$CD4^+$ T cells to synergize with allogeneic $CD8^+$ T cells specific for the HLA-A2.1-restricted CTL epitope HER-2/*neu*(435–443) for the

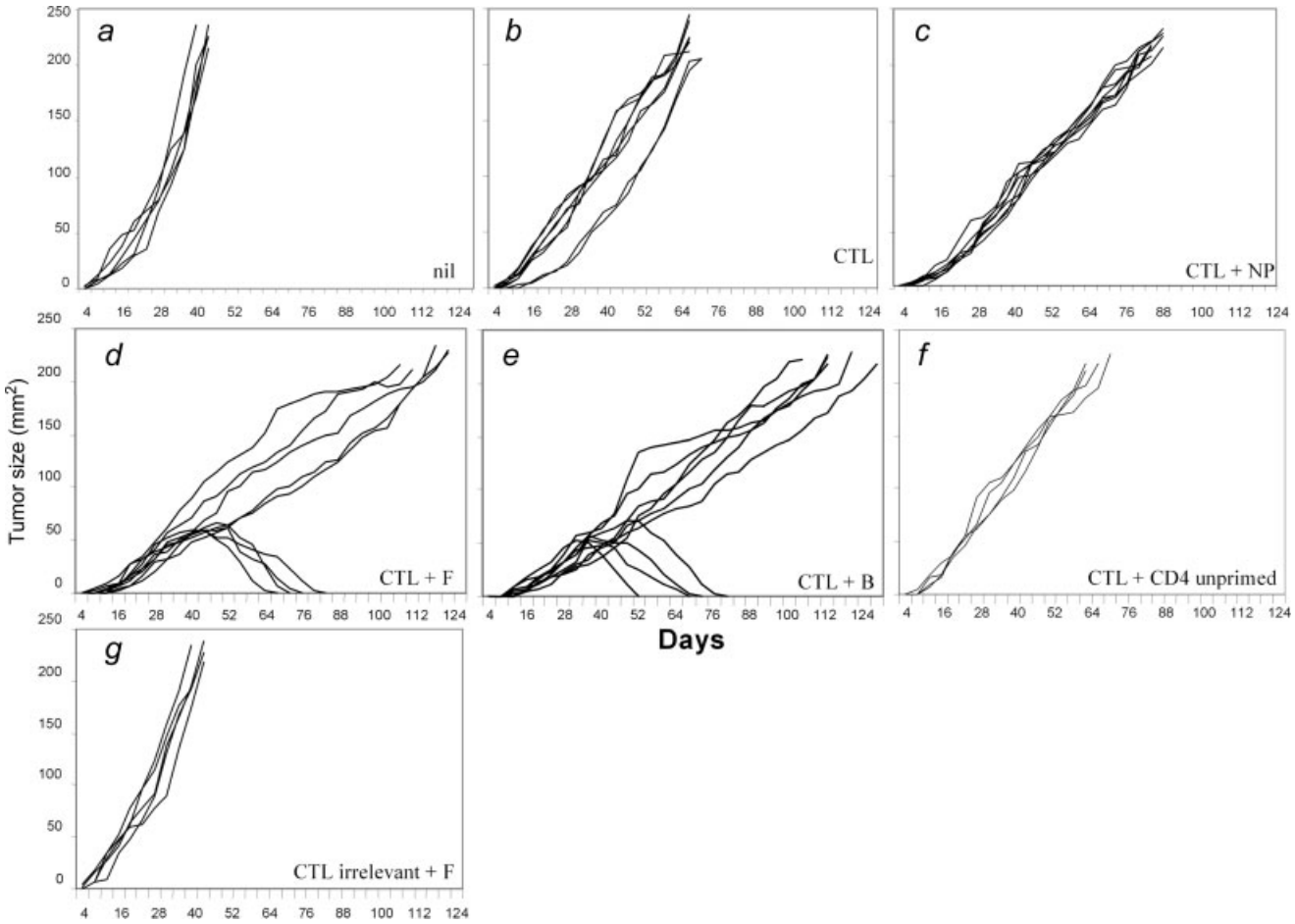

FIGURE 6 – Therapeutic efficacy of HER-2/*neu*(435–443) CTL from HLA-A2.1 (HHD) Tg mice transferred along with peptide-specific $CD4^+$ T-cells from DR4-IE Tg mice in SCID mice xenografted with the HER-2/neu^+ HLA-$A2.1^+$ $DR4^+$ human melanoma tumor cell line FM3. CTL and $CD4^+$ T cells (1×10^6 each subset) were injected into mice (ip.) in groups of 4–10 SCID mice which were previously (12–16 days) inoculated (sc.) on the back with 5×10^5 FM3. Tumor growth was caliper-measured and recorded every 4 days in all groups until tumor area had reached a tumor size >200 mm^2. Data are reported as the mean tumor area of treated mice per group. The SD was always less than 25% of the mean and thus, it was omitted. CTL primed with an irrelevant HLA-A2.1-restricted peptide [Flu matrix peptide (58–66)], injected along with $CD4^+$ T cells primed with peptide F (Group G), did not eradicate the growth of transplanted FM3 tumor cells (similar to the untreated controls). NP: native peptide; B, D, F: Ii-Key hybrid peptides. (*a*) Untreated mice. Mice treated with: (*b*) HER-2/*neu*(435–443) CTL, (*c*) HER-2/*neu*(435–443) CTL + CD4 (NP), (*d*) HER-2/*neu*(435–443) CTL + CD4 (F), (*e*) HER-2/*neu*(435–443) CTL + CD4 (B), (*f*) HER-2/*neu*(435–443) CTL + CD4 (Nil), (*g*) CTL irrelevant + CD4 (F).

generation of optimal antitumor responses in xenografted SCID mice.

Hybrid peptides were screened for binding to HLA-DR4 molecules expressed on T2-DR4 cells. Overall, both stronger binding and slower off-rate (dissociation) kinetics demonstrated by hybrids closely correlated with immunogenicity observed in functional assays. Furthermore, hybrid peptides B and F had off-rate (dissociation) kinetics greater than 6 hr, which was significantly longer compared to those of peptides C and E (4 hr) or native peptide (2 hr). A sufficiently long half-life is likely to facilitate the stimulation of many T cell receptors on a given T cell, which is thought to be important for strong T cell activation.[45] Thus, it is possible that priming with hybrid peptides B and F renders $CD4^+$ T cells capable of increased levels of proliferation and IFN-γ production upon encounter of the native peptide naturally expressed on tumor cell lines or loaded onto antigen-presenting cells.

We then set out to confirm data from our laboratory[13] as well as from others[31] that HER-2/*neu*(776–790) is a naturally processed epitope. Splenic $CD4^+$ T cells from immunized mice were tested *ex vivo* for their ability to recognize the HER-2/*neu*(776–790) peptide (*i*) presented by syngeneic DC, and (*ii*) naturally expressed by human HER-2/neu^+ and HLA-$DR4^+$ tumor cell lines. Our initial screening of the Ii-Key/HER-2/*neu*(776–790) hybrid peptides for enhanced recognition of the native HER-2/*neu*(776–790) pulsed onto DCs demonstrated a strong effect mediated by hybrid peptides B and F which contain several amino-terminal flanking residues. In contrast, hybrid peptides C and E with nested deletions N-terminal to a putative P1 site residue induced only a moderate effect in a DR4-restricted manner. When the native peptide was naturally expressed by HER-2/neu^+, HLA-$DR4^+$ tumor cell lines, its recognition was again greatly enhanced using $CD4^+$ T cells from mice immunized with hybrid peptides B and F, whereas cells derived from mice immunized with hybrid peptides C and E did not improve native peptide recognition. Overall, a much stronger response was observed toward syngeneic DCs pulsed with HER-2/*neu*(776–790), which can be explained by the fact that these DCs expressed higher levels of HLA-DR4 molecules compared to the tumor cell lines, but also due to the fact that DCs express a series of ligands for costimulatory molecules which enable optimal T cell activation.[46] In addition, the amount of cytosolic peptides generated by proteosomal cleavage of proteins and expressed in the context of MHC molecules[47] is far less compared to that usu-

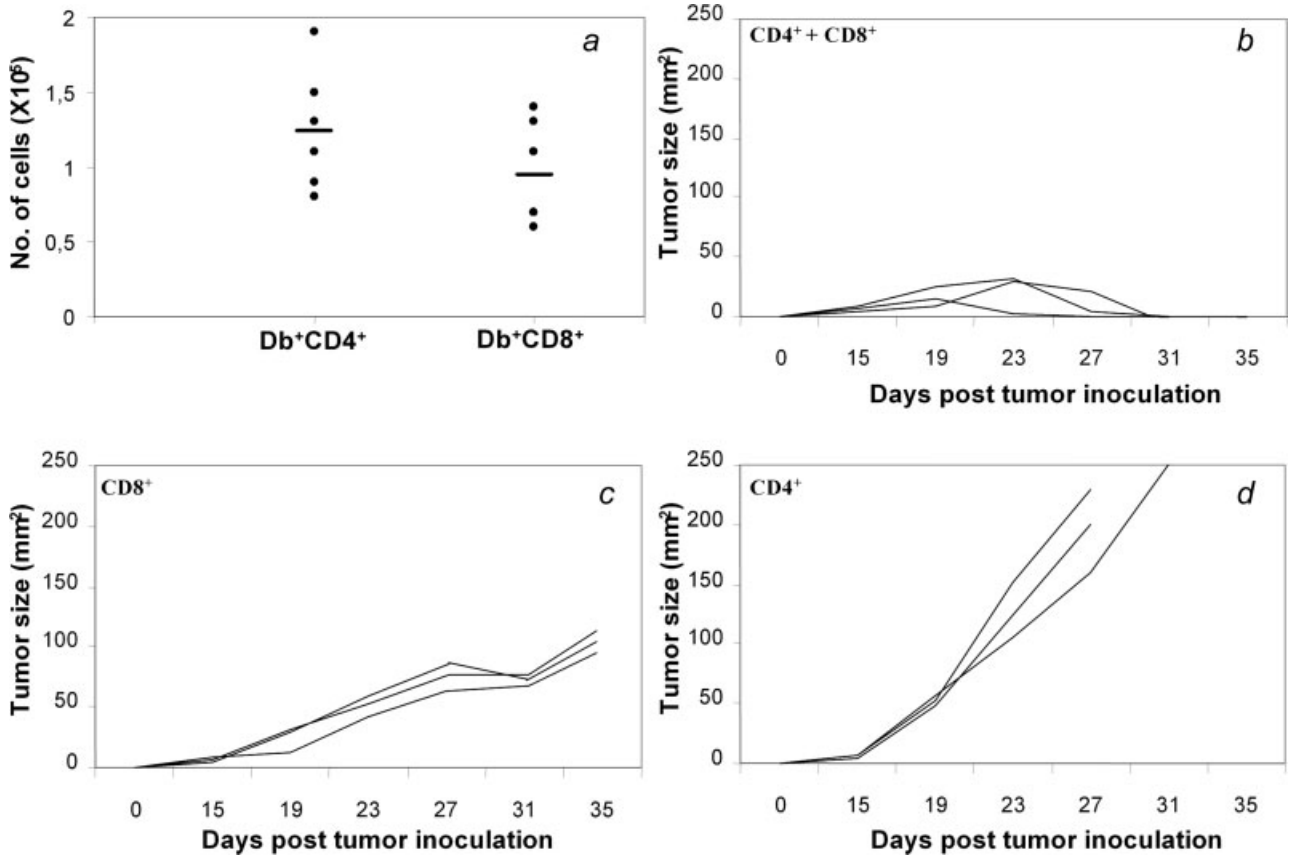

FIGURE 7 – Adoptive transfer of peptide F primed $CD4^+$ TIL along with HER-2/*neu*(435–443) primed $CD8^+$ TIL conferred antitumor immunity in SCID mice with growing FM3 tumors as previously shown in Figure 6. At the time tumors began to regress, mice were sacrificed, their tumors were removed, $CD8^+$ and $CD4^+$ TIL isolated. The absolute numbers of isolated $CD8^+$ and $CD4^+$ TIL are shown in (*a*). Pooled $CD4^+$ and $CD8^+$ TIL (*b*), or either subset alone (*c*, *d*), were then adoptively transferred to groups of untreated SCID mice with FM3 growing tumors.

ally used (up to 100 μg/ml) for pulsing antigen-presenting cells. Accordingly, the amount of the HER-2/*neu*(776–790) epitope naturally processed and expressed by the tumor lines used herein should be much less compared to the amount loaded onto the DCs (Fig. 2).

In prior studies, hybrid peptides containing longer spacer sequences were all less potent than hybrids which had shorter spacer sequences.[25,27,29] This led to a hypothesis on the mechanism whereby Ii-Key hybrid peptides facilitated direct charging of MHC class II molecules. Initially, binding of the Ii-Key moiety of the hybrid peptide to the allosteric site of the MHC class II molecule induces a conformational shift in the antigenic epitope-binding groove. This conformational change loosens the binding of any pre-bound epitope or ambient peptide and increases accessibility of the groove to interaction with another epitope, *i.e.*, the epitope portion of the Ii-Key hybrid peptide. Interaction of this epitope occurs during dissociation of the Ii-Key moiety to allow stabilization of the MHC class II complex. The observation that Ii-Key hybrid peptides containing longer spacers were less active suggested that auto-rejection may occur if the Ii-Key moiety was able to associate with the allosteric site while the tethered epitope was resident in the epitope-binding groove. However, the observation of greatest activity with the shortest Ii-Key hybrid peptide of a given series is not universal. The importance of the P1 site spacer length and activity is particularly difficult to assess in the case of promiscuous peptides that may have overlapping, slightly offset epitopes. It has been suggested that this may be the case for the HER-2/*neu*(776–790) peptide used for the Ii-Key series described here.[15] While one of the hybrid peptides with the shortest spacer (peptide F) was indeed one of the most active, peptide B, which had a longer spacer, showed comparable activity in stimulating $CD4^+$ T cells when presented either by DR4-IE DCs, or HER-2/*neu*$^+$ HLA-$DR4^+$ tumor cell lines. In addition to spacer length, we cannot rule out other variables that may influence TCR activity. In particular, the importance of flanking residues has also been shown to modulate binding activity.[48,49] It is thus possible that hybrids B and F, which contain the full length HER-2/*neu*(776–790) sequence may have benefited from amino acids near the N-terminus in priming HER-2/*neu*(776–790) antigen-specific T cells.

MHC class I-restricted antitumor CTL responses are potentiated by tumor peptide-specific $CD4^+$ T-helper cells. This potentiation of CTL activity results in higher levels of tumor cell lysis and longer duration of the cytolytic response.[50] In this report, we also tested the efficacy of the Ii-Key hybrid peptides in providing T cell help for the induction of enhanced antitumor CTL responses in xenografted SCID mice. We found that $CD4^+$ T cells from DR4-IE Tg mice stimulated with the hybrid peptides displayed a considerably higher degree of synergism (compared to $CD4^+$ T cells stimulated with the native peptide) with HER-2/*neu*(435–443)-specific syngeneic $CD8^+$ CTL for the rejection of FM3 cells in SCID mice. FM3 has a higher level of expression of the HLA class I and class II antigens and costimulatory molecules,[51,52] and therefore, it functions as an antigen-presenting cell.[53] Moreover, this particular cell line naturally expresses the CTL HER-2/*neu*(435–443) peptide in the context of HLA-A2.1[38] as well as the T_H epitope HER-2/*neu*(776–790) in the context of HLA-DR4.[13] Thus, it is reasonable to assume that synergistic interactions between peptide-specific CTL and T_H *in vivo*, resulting in FM3 tumor rejection, were dependent upon prior *in vivo* activation of the adoptively transferred peptide-specific CTL and T_H to recognize

the respective HLA-restricted peptides on FM3 tumor cells. Activated T_H cells will then produce IFN-γ (as shown herein) and possibly also IL-2,[54] both of which will further activate HER-2/*neu*(435–443)-specific CTL to lyse growing FM3 cells, thus prolonging the survival of SCID mice. We were able to demonstrate that a rather high proportion of the adoptively transferred peptide-primed $CD4^+$ and $CD8^+$ T cells infiltrate the FM3 tumors. More important, we found that isolated $CD8^+$ TIL no longer conferred effective antitumor immunity when transferred alone in SCID mice with growing FM3 tumors. To be effective for tumor regression, it was necessary for $CD8^+$ CTL to be cotransferred with $CD4^+$ TIL. These data confirmed previous reports from us[8] and others[4–6] demonstrating the requirement of $CD4^+$ T cell-help in the maintenance of $CD8^+$ T-cell responses. To date, clinical responses to either CTL or T_H tumor peptides have been below expectations.[55,56] The data presented in our study demonstrate that linking class II peptides to the Ii-Key moiety of the MHC class II-associated invariant chain augments their potency *in vivo* and *in vitro* in terms of proliferation and IFN-γ release by $CD4^+$ T cells, and that these cells are functionally more active as demonstrated by enhancement of antitumor CTL activity. Our studies provide the platform for introducing Ii-Key/HER-2/*neu* T_H epitopes in combination with CTL epitopes into the clinic or for use in patients with a pre-existing CTL activity.

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