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EGFR inhibitors augment antitumour helper T-cell responses of HER family-specific immunotherapy

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Background: Head and neck squamous cell carcinoma (HNSCC) is a major cause of cancer-related morbidity and mortality worldwide. Epidermal growth factor receptor (EGFR)-targeted therapy is an attractive strategy alternative to conventional cancer treatments for HNSCC, but its efficacy remains controversial. T-cell-based immunotherapy has been proposed as a novel therapeutic approach to improve the clinical outcome for HNSCC. In this study, we report human epidermal receptor (HER) family epitopes that induced CD4 T-cell responses to HNSCC. The results provide support for a novel strategy to treat HNSCC by combining EGFR-targeted therapy with T-cell-based immunotherapy.

Methods: We evaluated the capacity of predicted CD4 T-cell peptide epitopes from EGFR to induce antitumour immune responses *in vitro*. In addition, EGFR inhibitors were evaluated for their ability to augment tumour MHC class II expression in HNSCC cell lines and subsequently increase T-cell recognition.

Results: Among several predicted peptide epitopes, $EGFR_{875–889}$ elicited CD4 T-cell responses that were restricted by HLA-DR4, DR15, or DR53 molecules, indicating that the peptide functions as a promiscuous T-cell epitope. The peptide-reactive T cells responded to autologous dendritic cells loaded with EGFR-expressing tumour cell lysates, indicating that these epitopes are naturally processed. In addition, the CD4 T cells were capable of directly recognising and killing HNSCC cells expressing EGFR and the appropriate HLA class II molecule. T cells reactive with the $EGFR_{875–889}$ epitope could be detected in the blood of HNSCC patients. $EGFR_{875–889}$-reactive CD4 T cells were also able to recognise several peptide analogues derived from homologous regions of EGFR family members, HER-2, HER-3 and c-MET. Finally, we examined the effects of EGFR tyrosine kinase inhibition or EGFR-blocking antibodies on CD4 T-cell tumour reactivity. Treatment of tumour cells with the EGFR inhibitors enhanced tumour recognition by $EGFR_{875–889}$-reactive T cells presumably due to the upregulation of HLA-DR expression in the HNSCC cells.

Conclusion: We identified novel CD4 T-cell EGFR epitopes and amongst these, $EGFR_{875–889}$ functions as a promiscuous helper T-cell epitope that can elicit effective antitumour T-cell responses against tumours expressing HER family members and c-MET. These observations should facilitate the translation of T-cell-based immunotherapy into the clinic for the treatment of HNSCC and provide a rational basis for EGFR inhibition, immune-targeted combination therapy.

Head and neck squamous cell carcinoma (HNSCC) often presents itself in a locally advanced stage, for which a combined therapy modality is usually recommended. As surgery, radiation and chemotherapy are the main therapeutic approaches for HNSCC, significant iatrogenic effects such as functional disabilities and pancytopenia are usually inevitable. Moreover, disease recurrences

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and distant metastases are common in patients with locally advanced disease who have received conventional therapy (Michiels *et al*, 2009). Thus, there is an urgent need to broaden and improve the treatment options for HNSCC.

The epidermal growth factor receptor (EGFR) has an important role in oncogenesis in many kinds of malignancies including HNSCC by enhancing cell proliferation and inhibiting apoptosis (Del Vecchio *et al*, 2012). Moreover, EGFR overexpression has been used as an independent prognostic indicator for HNSCC patients (Wheeler *et al*, 2012). To inhibit EGFR signalling, EGFR-targeted therapies such as the use of antagonistic antibodies and tyrosine kinase inhibitors (TKIs) have been introduced to the clinical oncology practice. The addition of an anti-EGFR antibody (cetuximab) to radiation therapy (RT) significantly improved median overall survival and median duration of loco-regional control compared with RT-alone in patients with locally advanced HNSCC (Bonner *et al*, 2006). Similarly, the use of erlotinib (an EGFR TKI) prolonged disease stabilisation in a heavily pretreated HNSCC patient population (Soulieres *et al*, 2004). Despite these promising results, these therapies did not lead to durable remissions mostly due to innate and acquired resistance to the EGFR inhibitors (Diaz *et al*, 2012).

Recently, it has been reported that EGFR blockers may overcome the inhibitory effect of EGFR signalling to CIITA, resulting in increased MHC expression on HNSCC cells (Pollack *et al*, 2011). In view of this, we hypothesise that the overall efficacy of EGFR inhibitor-targeted therapy in HNSCC patients could be enhanced by the addition of T-cell-based immunotherapy, which could also be directed towards the EGFR molecule. There is little doubt that effective antitumour immunotherapy will require the coordinate participation of both CD4 (helper) and CD8 (cytotoxic) T-cell responses. Although considerable work has been devoted towards the identification of CD8 cytotoxic T lymphocyte (CTL) epitopes from EGFR (Shomura *et al*, 2004), little effort has been directed to identify EGFR peptide epitopes recognised by CD4 T helper lymphocytes (HTLs). In addition to being critical for the generation and persistence of CTLs, HTLs can also function as effector cells by exhibiting direct antitumour cytotoxic activity against MHC class II (MHC-II)-expressing tumours (Hahn *et al*, 1995). Here, we describe several novel EGFR peptide epitopes that were capable of eliciting antigen-specific and HLA-DR-restricted CD4 T-cell responses against EGFR-expressing HNSCC cells. Interestingly, one of these epitopes ($EGFR_{875–889}$) was shown to induce HTLs that crossreacted with the analogous corresponding HER-2, HER-3 and c-Met peptides and tumour cell lines expressing these antigens. Treatment of HNSCC tumours with EGFR inhibitors (erlotinib and cetuximab) resulted in enhanced antitumour recognition by the EGFR-reactive CD4 helper T cells owing to the upregulation of tumour MHC-II molecules. Overall, these results suggest that EGFR-encoded T helper epitopes, some which may be crossreactive towards the homologous HER-2, HER-3 and c-Met regions, may be utilised in vaccination strategies that could be combined with EGFR blockade for a more effective treatment of HNSCC.

MATERIALS AND METHODS

Cell lines. Epstein–Barr virus-transformed B lymphoblastoid cell lines (EBV-LCLs) were produced from peripheral blood mononuclear cells (PBMCs) of HLA-typed volunteers using culture supernatant from the EBV-producing B95-8 cell lines obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Mouse fibroblast cell lines (L-cells) transfected with and expressing individual human HLA-DR molecules were kindly provided by Dr Robert W Karr (Karr Pharma, Saint Louis, MO, USA) and by Dr Takehiko Sasazuki (Kyushu University, Fukuoka, Japan). The HNSCC cell lines Ho-1-u-1 (oropharynx SCC), HSC-2 (oral SCC, DR13/13), HSC-3 (tongue SCC, DR15/15), HSC-4 (tongue SCC, DR1/4) and Sa-3 (gingival SCC, DR9/10) were supplied by the RIKEN Bio-Resource Center (Tsukuba, Japan). The HNSCC cell lines HPC-92Y (hypopharyngeal SCC, DR4/9) and CA9-22 (gingival SCC, DR8/15) were kindly provided by Dr Syunsuke Yanoma (Yokohama Tsurugamine Hospital, Yokohama, Japan) and Dr Yasuharu Nishimura (Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan), respectively. Tumour cell lines SAS (tongue SCC, DR9/15), MCF-7 (breast cancer, DR3/15) and Jurkat (T-cell lymphoma) were purchased from ATCC. All cell lines were maintained in tissue culture as recommended by supplier.

Western blotting. One million cells were washed in phosphate-buffered saline (PBS) and lysed in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA). The cell lysate was subjected to electrophoresis in a 4–12% NuPAGE bis-Tris SDS-PAGE gel (Invitrogen) under reducing condition and transferred to Immobilon-P (Millipore, Bedford, MA, USA) membrane. The membrane was blocked in PBS containing 0.01% Tween 20 and 5% non-fat dry milk for 1 h at room temperature and incubated with polyclonal rabbit anti-human EGFR (sc-03, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1 : 500 in blocking buffer, anti-human c-Met rabbit polyclonal antibody (C-28, Santa Cruz Biotechnology) diluted 1 : 200, or anti-β-actin mAb (C4, Santa Cruz Biotechnology) diluted 1 : 1000 in blocking buffer as the control, for 18 h at 4 °C. After washing, the membrane was incubated with horseradish peroxidase-labeled sheep anti-rabbit or -mouse IgG and subjected to the enhanced chemiluminescence assay using the ECL detection system (Amersham, Buckinghamshire, UK).

Synthetic peptides. Potential HLA-DR-restricted CD4 T-cell epitopes were selected from the amino-acid sequence of EGFR using the computer-based algorithms peptide/MHC binding for three common HLA-DR alleles (DRB1*0101, DRB1*0401, and DRB1*0701) developed by Southwood *et al* (1998). The predicted peptide epitopes were synthesised by solid phase organic chemistry and purified by high-performance liquid chromatography (HPLC). The purity (>80%) and identity of peptides were assessed by HPLC and mass spectrometry, respectively. The synthetic peptides used throughout this study were $EGFR_{85–99}$ (VAGYVLIALNT VERI), $EGFR_{875–889}$ (KVPIKWMALESILHR), $EGFR_{1136–1150}$ (PEYLNTVQPTCVNST). These peptides were selected on the basis of having top 10 scores for at least two of the three HLA-DR alleles. The peptide $EGFR_{875–889}$ analogues, $HER\text{-}2_{883–897}$ (KVPIKWMALESILRR), $HER\text{-}3_{872–886}$ (KTPIKWMALESIHFG) and $c\text{-}Met_{1244–1258}$ (KLPVKWMALESLQTQ) were used in this study. The tetanus toxoid ($TT_{830\text{-}843}$) (QYIKANSKFIGITE) peptide was used as a control universal epitope peptide, as it is presented by multiple HLA-DR alleles (Panina-Bordignon *et al*, 1989). $EZH2_{95\text{-}109}$ (VIPLKTLNAVASVPI) (Hayashi *et al*, 2011) was used as irrelevant peptide.

***In vitro* induction of antigen-specific CD4 T-cell clones with synthetic peptides.** The procedure utilised for the generation of EGFR-reactive CD4 T-cell clones using peptide-stimulated lymphocytes from PBMCs of human healthy individuals has been described in detail (Kobayashi *et al*, 2000). Briefly, dendritic cells (DCs) were produced from purified CD14 monocytes (using antibody-coated magnetic microbeads from Miltenyi Biotech, Auburn, CA, USA) cultured for 7 days at 37 °C in a humidified CO_2 (5%) incubator in the presence of 50 ng ml^{-1} GM-CSF and 1000 IU ml^{-1} IL-4. Peptide-pulsed DCs (3 μg ml^{-1} for 2 h at room temperature) were irradiated (4200 rad) and co-cultured with

autologous purified CD4 T cells in 96-well flat-bottomed culture plates. One week after peptide stimulation, the CD4 T cells were restimulated in individual microcultures with peptide-pulsed irradiated autologous PBMCs and 2 days later, recombinant human IL-2 was added at a final concentration of 10 IU ml^{-1}. One week later, the T cells were tested for antigen reactivity using a cytokine-release assay as described below. Those microcultures exhibiting a significant response of cytokine-release to peptide (at least 2.5-fold over background) were cloned by limiting dilution and expanded in 24-well plates by weekly restimulation with peptides and irradiated autologous PBMCs. Complete culture medium for all procedures consisted of AIM-V medium (Invitrogen/GIBCO, Carlsbad, CA, USA) supplemented with 3% human male AB serum. All blood samples were obtained after the appropriate informed consent.

Measurement of antigen-specific responses with established CD4 T-cell clones. CD4 T cells (3×10^4 per well) were mixed with irradiated antigen-presenting cells (APCs) in the presence of various concentrations of antigen (peptides, tumour lysates) in 96-well culture plates. APCs consisted of autologous PBMCs (1×10^5 per well), HLA-DR-expressing L-cells (3×10^4 per well), autologous DCs (5×10^3 per well) or tumour cell lines (3×10^4 per well). The HNSCC cell lines were previously treated with IFN-γ at 500 U ml^{-1} for 48 h to enhance HLA-DR expression. To examine the role of EGFR inhibitor in augmenting the expression of MHC-II molecules, HNSCC cell lines were preincubated with or without 100 ng ml^{-1} DMSO, EGFR TKI, erlotinib (tyrosine kinase reversible inhibitor, 1 μM, Selleck chemicals, Houston, TX, USA), EGFR antagonistic antibody, cetuximab (2 μg ml^{-1}, Merck AG, Zug, Switzerland) or bevasizumab (2 μg ml^{-1}, Roche, Istanbul) for 2 h at 37 °C before addition of IFN-γ. The expression of HLA-DR molecules, EGFR, HER-2, HER-3 and c-MET on tumour cells were evaluated by flow cytometry using anti-HLA-DR mAb conjugated with fluorescein isothiocyanate, anti-EGFR mAb conjugated with phycoerythrin (BD Pharmingen, San Diego, CA, USA), anti-HER-2 mAb conjugated with phycoerythrin, anti-HER-3 mAb conjugated with phycoerythrin (Biolegend, San Diego, CA, USA) and anti-c-MET conjugated with phycoerythrin (R&D Systems, Minneapolis, MN, USA), respectively. Tumour cell lysates were prepared by three freeze–thaw cycles of 1×10^8 tumour cells, resuspened in 1 ml of serum-free AIM-V medium. Tumour cell lysates served as an antigen at 5×10^5 cell equivalents per ml. Culture supernatants were collected after 48 h for measuring antigen-induced lymphokine (IL-4, IFN-γ, and GM-CSF) production by the CD4 T cells using ELISA kits (BD Pharmingen). To demonstrate antigen-specificity and HLA-DR restriction, CD4 T-cells responses were blocked by adding anti-HLA-DR mAb L243 (IgG2a prepared from supernatants of the hybridoma HB-55 obtained from the ATCC) or the anti-HLA-A/B/C mAb W6/32 (IgG2a, ATCC) at 10 μg ml^{-1} throughout the 48-h incubation period.

Cell-mediated cytotoxicity assays. Cytotoxic activity of CD4 T cell clones was measured using a colorimetric CytoTox 96 assay (Promega, Madison, WI, USA). This system quantifies the release of lactate dehydrogenase (LDH) from target cells. T cells were mixed with 2×10^4 target cells at different effector to target (E:T) ratios in 96-well round-bottomed plates. After 6 h incubation at 37 °C, 50 μl supernatant samples were collected from each well to measure LDH content.

Measurement of peptide-specific responses in HNSCC patients. PBMCs were isolated from fresh heparinized blood by Ficoll–Conray centrifugation and washed twice with RPMI 1640. PBMCs were resuspended in complete medium and placed at 1.5×10^5 cells per well and cultured in triplicate in 96-well round-bottomed plates in the presence of 10 μg ml^{-1} peptide. Negative controls (in the absence of peptide) were prepared in eight replicate samples. One week later, the cultures were restimulated with peptide-pulsed (10 μg ml^{-1}) irradiated autologous PBMCs (5×10^4 per well). After 7 days of restimulation, supernatants were harvested for examining the ability of peptide-induced lymphokine production (IL-4, IFN-γ and GM-CSF) by the HNSCC patient's PBMCs. The institutional ethics committee had approved the study protocol (approval number 1066) and the appropriate written informed consent for blood donation was obtained from all patients and healthy donors before blood sampling.

RESULTS

Selection of potential HLA class II-restricted EGFR peptide epitopes. The identification of promiscuous HLA class II-binding peptide epitopes would be advantageous for the design of T-cell epitope-based vaccines for a broad cancer patient population. To predict promiscuous HLA class II-binding peptides, we used computer-based MHC-II peptide-binding algorithms for three common HLA class II molecules, HLA-DR1, DR4 and DR7 (Southwood *et al*, 1998). Using these algorithms the peptides that score high for binding to DR1, DR4 and DR7 demonstrated high capacity to bind to additional HLA class II alleles such as DR9, DR13, DR15 and DR53 (Kobayashi and Celis, 2008). Utilising this prediction system with the EGFR sequence, we selected peptides, $EGFR_{85–99}$ (VAGYVLIALNTVERI), $EGFR_{875–889}$ (KVPIKWMA-LESILHR) and $EGFR_{1136–1150}$ (PEYLNTVQPTCVNST), which exhibited the highest combined binding scores to all three HLA class II alleles (data not presented).

Induction of CD4 T-cell responses to EGFR peptide epitopes. We synthesised the three potential EGFR peptide epitopes and proceeded to study whether these peptides could induce CD4 helper T-cell responses *in vitro*. PBMCs were obtained from four healthy MHC-II typed individuals (donor 1 'S'; HLA-DR4/15, donor 2 'H'; HLA-DR4/9, donor 3 'T'; HLA-DR9/12 and donor 4 'M'; HLA-DR9/13) to select CD4 T cells and prepare autologous DCs, which were used as APCs. After three peptide restimulation cycles, peptides ($EGFR_{85–99}$, $EGFR_{875–889}$ and $EGFR_{1136–1150}$) were able to induce peptide-reactive CD4 T-cell responses in all of the four donors. As shown in Figure 1A, peptide dose titration curves showed that the affinity of the CD4 T cells from all four donors for peptide $EGFR_{875–889}$ was substantially higher as compared with the T cells reactive with peptides $EGFR_{85–99}$ and $EGFR_{1136–1150}$. These results suggest that amongst the three potential CD4 T-cell epitopes, $EGFR_{875–889}$ could be the most effective peptide for inducing antigen-specific CD4 T-cell responses capable of recognising tumour cells. In addition, the amino-acid sequence of $EGFR_{875–889}$ is similar with the homologous regions of the HER-2, HER-3 and c-Met proteins (Table 1). Thus, we decided to focus our research effort on $EGFR_{875–889}$ to further evaluate its ability to elicit tumour-reactive CD4 T cells. To carry out tumour recognition and fine MHC restriction analysis, we isolated T-cell clones from the $EGFR_{875–889}$ reactive CD4 T-cell lines of the four normal blood donors. As shown in Figure 1B, the response to $EGFR_{875–889}$ peptide observed with T-cell clones, S11 and S22 (from donor 1), H22 (donor 2), T8 (donor 3) and M8 (donor 4) was mediated via MHC-II, as this recognition was inhibited by antibody L243 (but not by the w6/32 anti-HLA class I antibody). As the L243 antibody is specific for HLA-DR and does not react with other MHC-II molecules (HLA-DP, -DQ), these results indicate that the presentation of EGFR peptide epitopes to the CD4 T-cell clones is via HLA-DR molecules. Next, to identify the HLA-DR restriction alleles for the $EGFR_{875–889}$ epitope, we assessed the T-cell responses using a panel of HLA-DR-transfected mouse L-cells as APCs. As shown in Figure 1C, T-cell clone S11 was

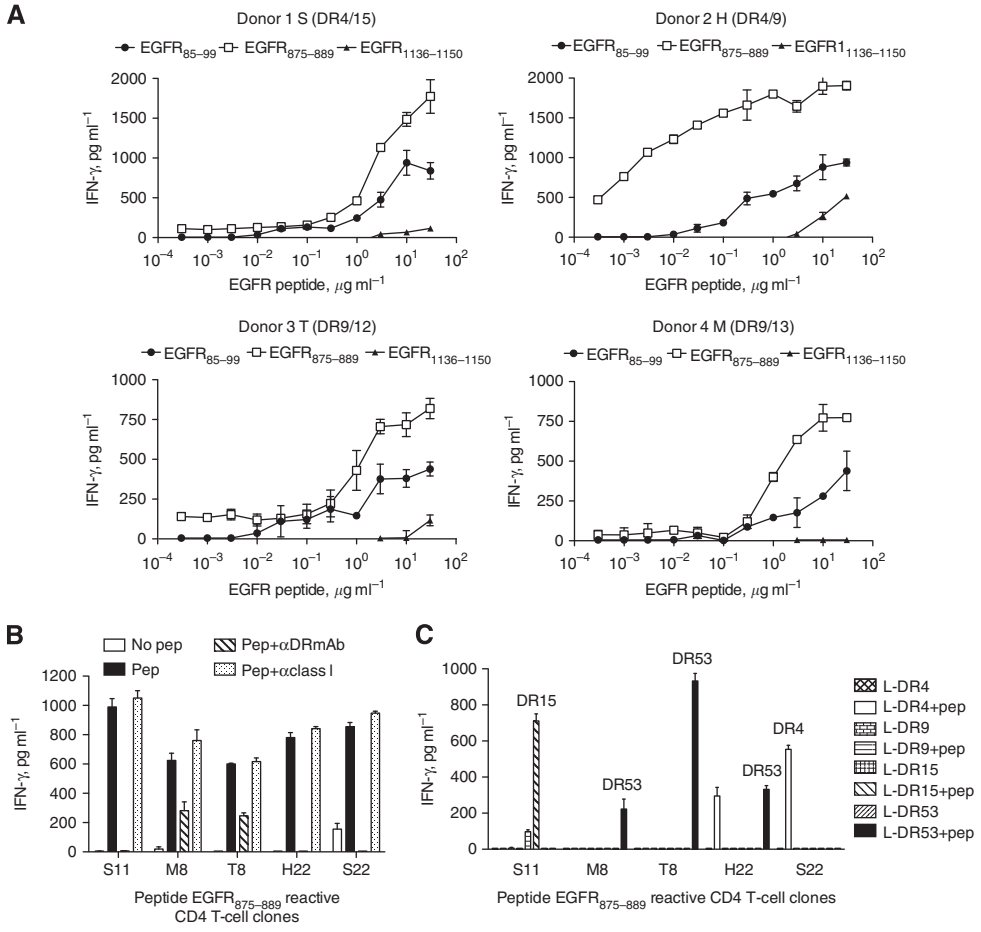

Figure 1. Induction of CD4 T-cell responses using predicted peptide epitopes derived from EGFR. (**A**) CD4 T-cell responses against candidate EGFR peptide epitopes were elicited from four healthy individuals and were subsequently tested for their capacity to recognise various concentrations of peptides presented by autologous PBMCs. Points, mean of triplicate determinations, bars s.d. Points without bars had s.d. <10% the value of the mean. Results are representative of three experiments that were performed with the same samples. (**B**) Anti-HLA-DR mAb L243 (10 $\mu g\,ml^{-1}$) inhibited IFN-γ production from $EGFR_{875–889}$ reactive CD4 T-cell clones. Anti-HLA Class I antibody was used as control. (**C**) IFN-γ productions of $EGFR_{875–889}$ reactive CD4 T-cell clones were evaluated using L-cells as APCs to define the restricting HLA-DR elements. Columns means of triplicate determinations, bars s.d. Columns without bars had s.d. <10% the values of the mean. Results are representative of at least two experiments.

Table 1. Peptide sequences of $EGFR_{875–889}$ and its homologous HER family and c-Met analogue peptide

$EGFR_{875–889}$	KVPIKWMALESILHR
HER-$2_{883–897}$	KVPIKWMALESIL**R**R
HER-$3_{872–886}$	K**T**PIKWMALESI**HFG**
c-$Met_{1244–1258}$	K**L**P**V**KWMALES**LQTQ**

Underlined and bold letters indicate amino acids that are different from peptide $EGFR_{875–889}$.

restricted by HLA-DR15, whereas clones M8, T8, and H22 were restricted by HLA-DR53 and clone S22 by HLA-DR4. These results indicate that $EGFR_{875–889}$ can be presented to CD4 T cells by multiple HLA-DR alleles, thus behaving as a classic promiscuous MHC-II epitope.

Direct recognition of EGFR-expressing HNSCC cells by $EGFR_{875–889}$-reactive CD4 T cells. It is important to assess whether peptide-reactive CD4 T cells have the ability to recognise naturally processed antigens directly on MHC-II expressing tumour cells (Kobayashi and Celis, 2008). Furthermore, in some instances CD4 T cells have the capacity to kill the tumour cells in an antigen-specific and MHC-II-restricted manner (Hayashi *et al*, 2011). In view of this, we studied whether the $EGFR_{875–889}$-reactive CD4 T-cell clones could directly recognise HLA-DR matching EGFR-positive HNSCC cells as APCs. Before performing these experiments, we assessed the expression of EGFR protein and surface MHC-II molecules in several HNSCC cell lines. As shown in Figure 2A, EGFR was expressed on all eight HNSCC cell lines. On the other hand, the Jurkat T-cell lymphoma did not express the EGFR and therefore was used for future studies as a negative control. With the exception of two HNSCC cell lines (CA9-22 and Ho-1-u-1) and Jurkat, six HNSCC cell lines expressed HLA-DR (after treatment with 500 U ml^{-1} IFN-γ for 48 h; Figure 2B). These results indicated that several of the HNSCC cell lines could be used as APCs and that MHC-II restriction studies could be performed, as MHC-II typing information was available for all the tumour lines (Materials and Methods). As shown in Figure 3A, all five $EGFR_{875–889}$ reactive CD4 T-cell clones were effective in directly reacting with EGFR-expressing tumours in an MHC-II-restricted manner. Moreover, the capacity of EGFR-expressing HNSCC cells to stimulate the CD4 T-cell clones was inhibited by the addition of anti-HLA-DR L243 mAb, confirming that the endogenously

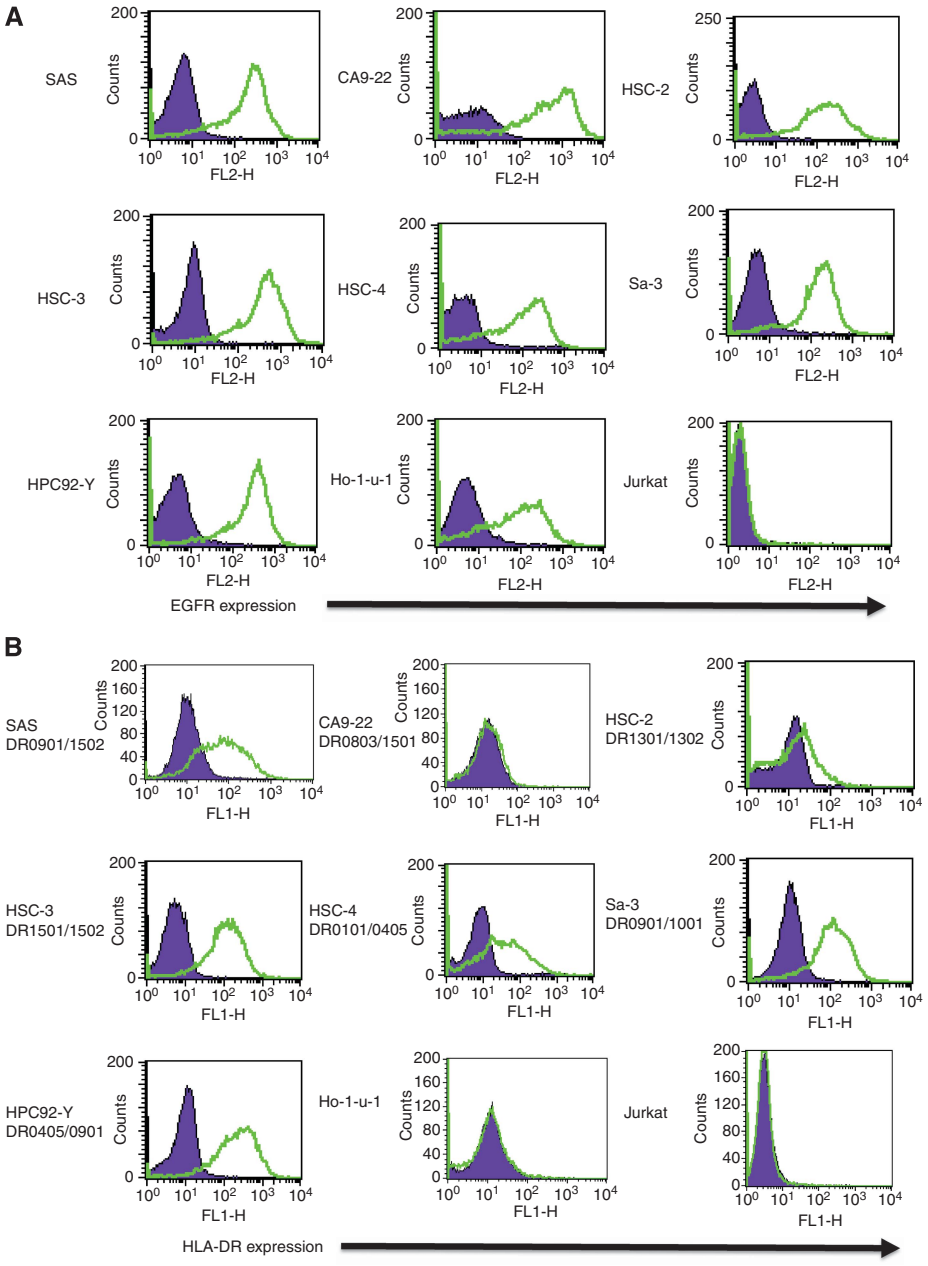

Figure 2. EGFR and HLA-DR expression in HNSCC. (**A**) Expression of EGFR in HNSCC cell lines. EGFR expression of HNSCC cell lines was examined by flow cytometry. Jurkat cells were used as negative control. (**B**) HLA-DR expression in HNSCC cell lines. HLA-DR expression in HNSCC cell lines was examined by flow cytometry 48 h after IFN-γ treatment as described in 'Materials and Methods'. Jurkat was used as negative control.

processed peptide epitope was presented via HLA-DR expressed on the tumour cells. Tumour cell lines that did not express the appropriate antigen or the corresponding matched HLA-DR molecule failed to stimulate the CD4 T cells, demonstrating that direct tumour recognition by the T-cell clones was both antigen-specific and HLA-DR-restricted.

Next, we evaluated the cytotoxic activity of the $EGFR_{875–889}$-reactive CD4 T-cell clones against the HNSCC tumour cells. As shown in Figure 3B, three of the CD4 T-cell clones, S11 (DR15-restricted), H22 (DR53-restricted) and T8 (DR53-restricted), effectively lysed EGFR-expressing HNSCC cell lines in a dose-dependent manner. On the other hand, clones M8 (DR53-restricted) and S22 (DR4 restricted) were unable to kill MHC-II-matched HNSCC cells (data not shown). Taken together, these results illustrate that EGFR peptide-reactive CD4 T-cell clones not only recognise EGFR-expressing tumours but some also have the ability to kill tumour cells directly.

Recognition of naturally processed exogenous antigen by EGFR-reactive CD4 T-cell clones. Having observed the reactivity of the CD4 T-cell clones to $EGFR_{875–889}$ peptide (Figure 1) and directly on tumour cell lines (Figure 3), we next assessed the capacity of professional APCs such as DCs to capture and process antigens derived from dead EGFR-expressing tumour cells and to present the EGFR epitope to the CD4 T-cell clones. Autologous DCs were pulsed with tumour cell freeze–thaw lysates and subsequently evaluated as APCs with the $EGFR_{875–889}$-reactive CD4 T-cell clones. To eliminate the possibility that tumour-derived MHC-II peptide complexes within the lysate would directly stimulate the T cells, lysates were prepared using EGFR-expressing MHC-II-mismatched tumours. As shown in Supplementary Figure 1, T-cell clones S11 and H22 specifically recognised naturally processed antigen presented by DCs in a HLA-DR-restricted manner, as these responses were inhibited by anti-HLA-DR mAb and were not detected using DCs loaded with

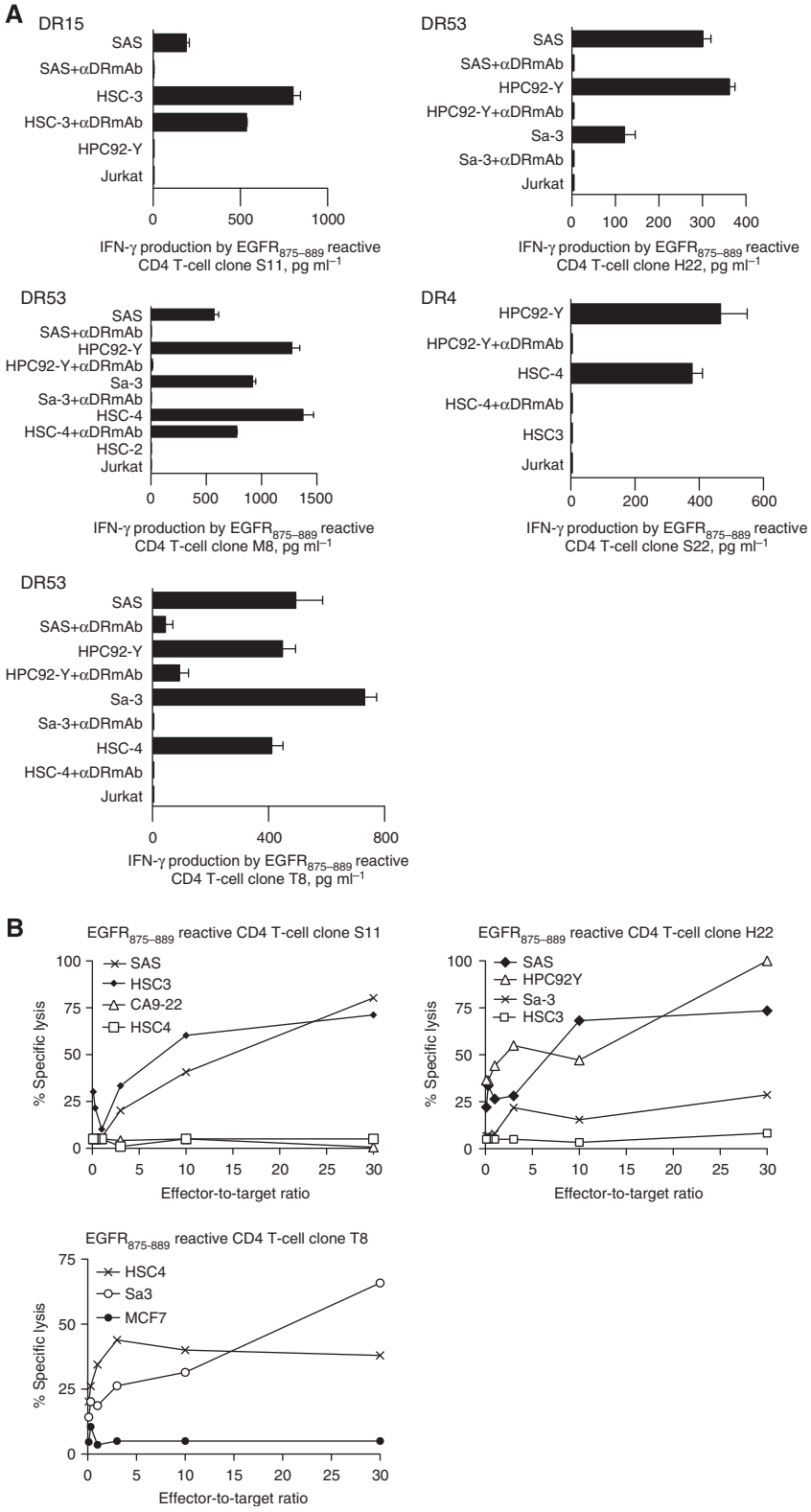

Figure 3. Direct recognition of EGFR expressing HNSCC by $EGFR_{875–889}$ reactive CD4 T-cell clones. (**A**) $EGFR_{875–889}$ reactive CD4 T-cell clones were tested for their capacity to recognise antigen directly on EGFR-positive HLA-DR matched or mismatched HNSCC cells by IFN-γ production. The HLA-DR-negative cell line Jurkat was used as negative control. HLA-DR restriction of these responses was demonstrated by blocking tumour recognition with anti-HLA-DR mAb L243 (10 μg ml^{-1}). Columns without bars had s.d. <10% the values of the mean. Results are representative of three separate experiments. (**B**) $EGFR_{875–889}$-reactive CD4 T-cell clones were evaluated for their capacity to kill EGFR-positive HLA-DR-matched HNSCC cells. HLA-DR unmatched cell lines were used as negative controls. Points, mean of triplicate determinations, bars s.d. Points without bars had s.d. <10% the value of the mean. Results are representative of three separate experiments.

PBMC lysates (negative control). However, CD4 T-cell clones M8, T8, and S22, which were efficient in directly recognising endogenous processed epitope on tumour cells, were not able to recognise tumour cell lysates presented by DCs (data not shown). One possible explanation for the lack of recognition of tumour lysate pulsed DCs by T-cell clones M8, T8, and S22 could be due to differences in the mode of antigen processing and MHC-II peptide loading between endogenously processed epitopes presented by tumours and the exogenously processed epitopes presented by DCs. Nevertheless, the results demonstrate that CD4 T cells are capable of recognising the $EGFR_{875–889}$ presented by several MHC-II molecules, either directly on tumour cells or indirectly by professional APCs loaded with tumour cell lysates.

EGFR inhibitors augment HLA-DR expression on tumour cell lines. Important factors that can facilitate tumour escape from immune surveillance can be alterations in the expression of immune-related receptor molecules and cytokines capable of inhibiting immune responses (Katayama *et al*, 2011; Chuang *et al*, 2012). Numerous reports have shown that absence or low expression of MHC molecules on tumours can decrease the therapeutic effects of antitumour vaccines (Vertuani *et al*, 2009). As reduced levels of MHC-I and MHC-II expression induced by EGFR activation have been reported in keratinocytes and HNSCC cell lines (Pollack *et al*, 2011), we assessed whether EGFR inhibitors influenced the expression of MHC-II in HNSCC cell lines, and consequently affected recognition by T cells. The use of erlotinib, an EGFR tyrosine kinase inhibitor (TKI), augmented HLA-DR expression in several HNSCC cell lines (Figure 4A). Similarly, the EGFR-blocking antibody cetuximab enhanced HLA-DR expression mediated by IFN-γ pre-treatment (Figure 4B). However, HLA-DR expression in SAS, Ho-1-u-1, and HPC-92Y cell lines was not changed with EGFR inhibitors (data not shown). These results suggest that by increasing MHC-II expression, EGFR inhibitors could be used to enhance CD4 T-cell recognition, which could potentiate the antitumour effects of immunotherapy.

EGFR inhibitors enhance tumour recognition by $EGFR_{875–889}$ reactive CD4 T cells. To assess whether the elevated expression of HLA-DR molecules on tumour cells induced by EGFR inhibitors would result in enhanced antitumor immunity, we co-incubated $EGFR_{875–889}$ reactive CD4 T-cell clones with tumour cell lines that were pretreated or not with the EGFR inhibitors. The results presented in Figure 5A and B indicate that overall, the EGFR inhibitors increased recognition of the tumour cells by the CD4 T cells. In one instance, the effects of the EGFR inhibitors was quite dramatic, where the HLA-DR15-restricted CD4 T-cell clone S11 was unable to recognise the CA9-22 (DR15 + /EGFR +) tumour unless it was pretreated with the EGFR inhibitors. Further, a potentiating effect of EGFR inhibitors was evident in the recognition of tumour cells by the HLA-DR4-restricted CD4 T-cell clone S22. In addition, the EGFR inhibitors were also shown to increase the cytotoxic activity of the EGFR-reactive S11 CD4 T-cell clone (Figure 5C). These findings suggest that EGFR inhibitors could be used to potentiate CD4 T-cell-based immunotherapy for HNSCC.

Crossreactivity of $EGFR_{875–889}$ reactive CD4 T-cell clones against homologous peptides from various EGFR family members. EGFR, also known as HER-1, is a member of a family of growth factor receptors that includes HER-2 and HER-3 (Kraus *et al*, 1989). As previously mentioned, we observed that the sequence of the $EGFR_{875–889}$ epitope was highly similar with homologous peptides derived from other members of the HER gene family and c-Met (Table 1). In view of this, we tested whether

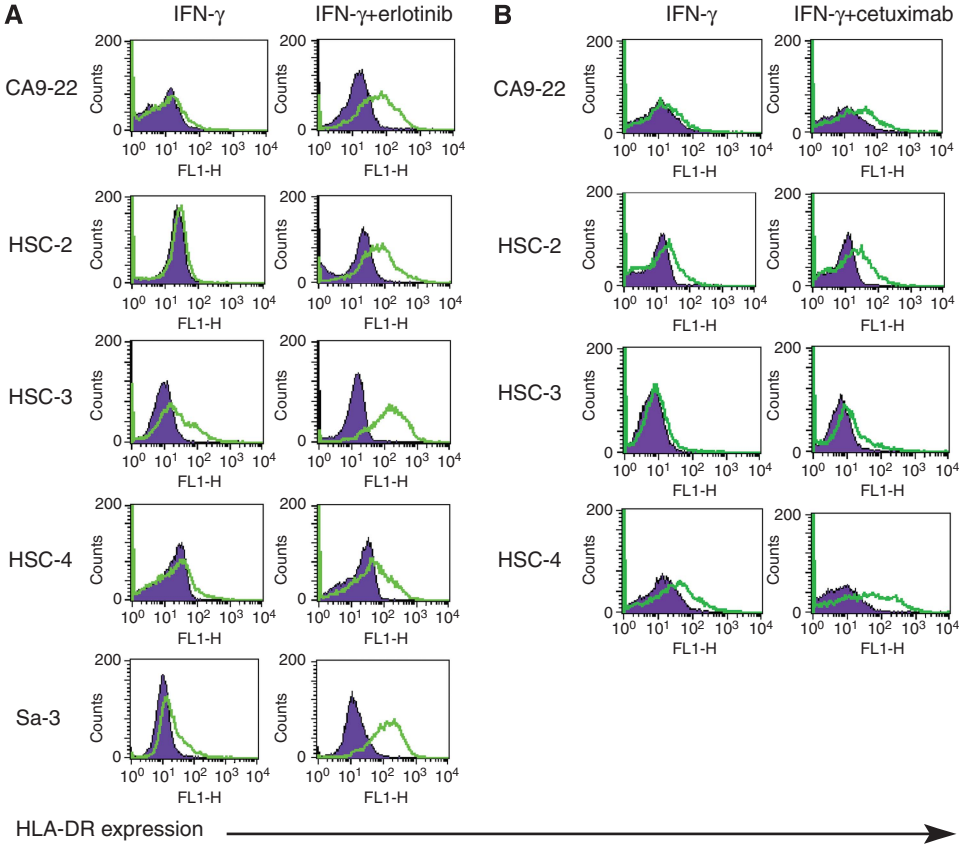

Figure 4. Upregulation of HLA-DR expression in HNSCC by EGFR inhibitors. (**A**) HLA-DR expression of HNSCC cell lines was examined by flow cytometry. HNSCC cells were treated with IFN-γ (50 U ml^{-1}) alone or with IFN-γ and elrotinib (1 mM) for 48 h. (**B**) HLA-DR expression of HNSCC cell lines was examined by flow cytometry. HNSCC cells were treated with IFN-γ (50 U/ml) alone or with IFN-γ and cetuximab (2 mg ml^{-1}) for 48 h.

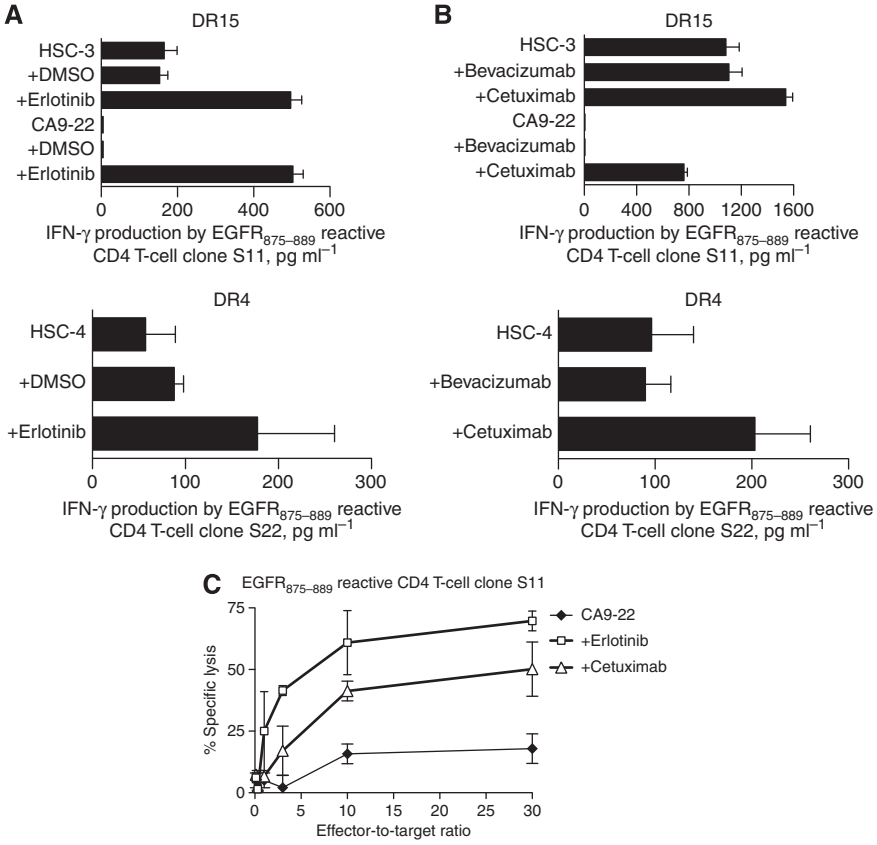

Figure 5. EGFR inhibitors augmented antitumour responses of $EGFR_{875–889}$ reactive CD4 T-cell clones. (**A**) $EGFR_{875–889}$-reactive CD4 T-cell clones were tested for their capacity to recognise antigen directly on EGFR-positive HLA-DR-matched HNSCC cells by IFN-γ production. Tumour cells were pretreated 48 h with or without erlotinib (1 mM). DMSO was used as negative control. Columns without bars had s.d. $<10\%$ the values of the mean. Results are representative of three separate experiments. (**B**) $EGFR_{875–889}$-reactive CD4 T-cell clones were tested for their capacity to recognise antigen directly on EGFR-positive HLA-DR-matched HNSCC cells by IFN-γ production. Tumor cells were pretreated 48 h with or without cetuximab (2 mg ml^{-1}). Anti VEGF antibody bevacizumab was used as negative control. Columns without bars had s.d. $<10\%$ the values of the mean. Results are representative of at least two separate experiments. (**C**) $EGFR_{875–889}$-reactive CD4 T-cell clone S11 was evaluated for its capacity to kill EGFR-positive HLA-DR-matched HNSCC cell CA19-9 by cytotoxic assay. CA19-9 was pretreated with elrotinib (1 mM, square) or cetuximab (2 mg ml^{-1}, triangle) for 48 h. Points, mean of triplicate determinations, bars s.d. Points without bars had s.d. $<10\%$ the value of the mean. Results are representative of two experiments that were done with the same samples.

synthetic peptides corresponding to the analogous $EGFR_{875–889}$ epitope would be recognised by the various CD4 T-cell clones. As shown in Figure 6A, several degrees of crossreactivity were observed with the $EGFR_{875–889}$-reactive CD4 T-cell clones in response to peptides $HER\text{-}2_{883–897}$, $HER\text{-}3_{872–886}$ and $c\text{-}Met_{1244–1258}$, whereas the irrelevant (negative control) peptide $EZH2_{95\text{-}109}$ did not stimulate any of the CD4 T-cell clones. In addition, we tested for the ability of the S11 T-cell clone (DR15-restricted, which also reacted with $HER\text{-}3_{872–886}$) to recognise the EGFR-negative but HER-3-positive breast tumour cell line MCF-7 (HLA-DR15-positive). As shown in Figure 6B, the S11 clone was effective in directly recognising MCF-7 and this recognition was completely inhibited by anti-HLA-DR mAbs. Next, the DR53-restricted M8, T8, and H22 T-cell clones were evaluated for their ability to recognise Epstein–Barr virus-transformed lymphoblastoid cell lines (EBV-LCL) that express c-Met (Figure 6C) but not EGFR, HER-2, and HER-3 (data not shown). Two MHC-II-typed EBV-LCLs were selected to study c-Met recognition in an MHC-restricted manner: LCL1 (DR4+/53+) and LCL2 (DR15+/53−). Jurkat T-cell lymphoma (DR-negative) was used as a negative control. The data presented in Figure 6D and E show that the DR53-restricted M8 and T8 clones were efficient in recognising the DR53- and c-Met-positive LCL1 cells but not the DR53-negative EBV-LCLs. On the other hand, clone H22 (also DR53-restricted) was unable to recognise the DR53 and c-Met-positive LCL1 cells (Figure 6F). These results were consistent with the previous observations that the M8 and T8 clones could crossreact with the c-Met analogue peptide but clone H22 could not (Figure 6A). The DR15-restricted CD4 T-cell clone S11, which was unable to respond to $c\text{-}Met_{1244–1258}$ (Figure 6A) as expected, did not react with the HLA-DR-matched, c-Met-positive LCL2 line (data not shown).

EGFR peptide recognition by PBMCs from HNSCC patients. It is important to determine the presence of precursor T cells specific for EGFR peptides in the peripheral blood of HNSCC patients for estimating the potential use of these peptides in cancer vaccines. To evaluate the responses of CD4 T cells from HNSCC patients to $EGFR_{875–889}$, we stimulated PBMCs from five HNSCC patients with the peptide in short-term cultures. As only small volumes of blood from these advanced HNSCC patients were available, we were unable to establish long-term T-cell lines and to perform HLA typing for patients. Tetanus toxin peptide, $TT_{830–843}$ was used as a positive control, as this antigen has faculty to induce robust CD4 T-cell responses in the majority of people regardless of their HLA-DR alleles. Seven days after the second stimulation, tissue-culture supernatants were collected and amounts of IFN-γ and GM-CSF were measured. In most instances, substantial T-cell responses to $EGFR_{875–889}$ were observed in the HNSCC

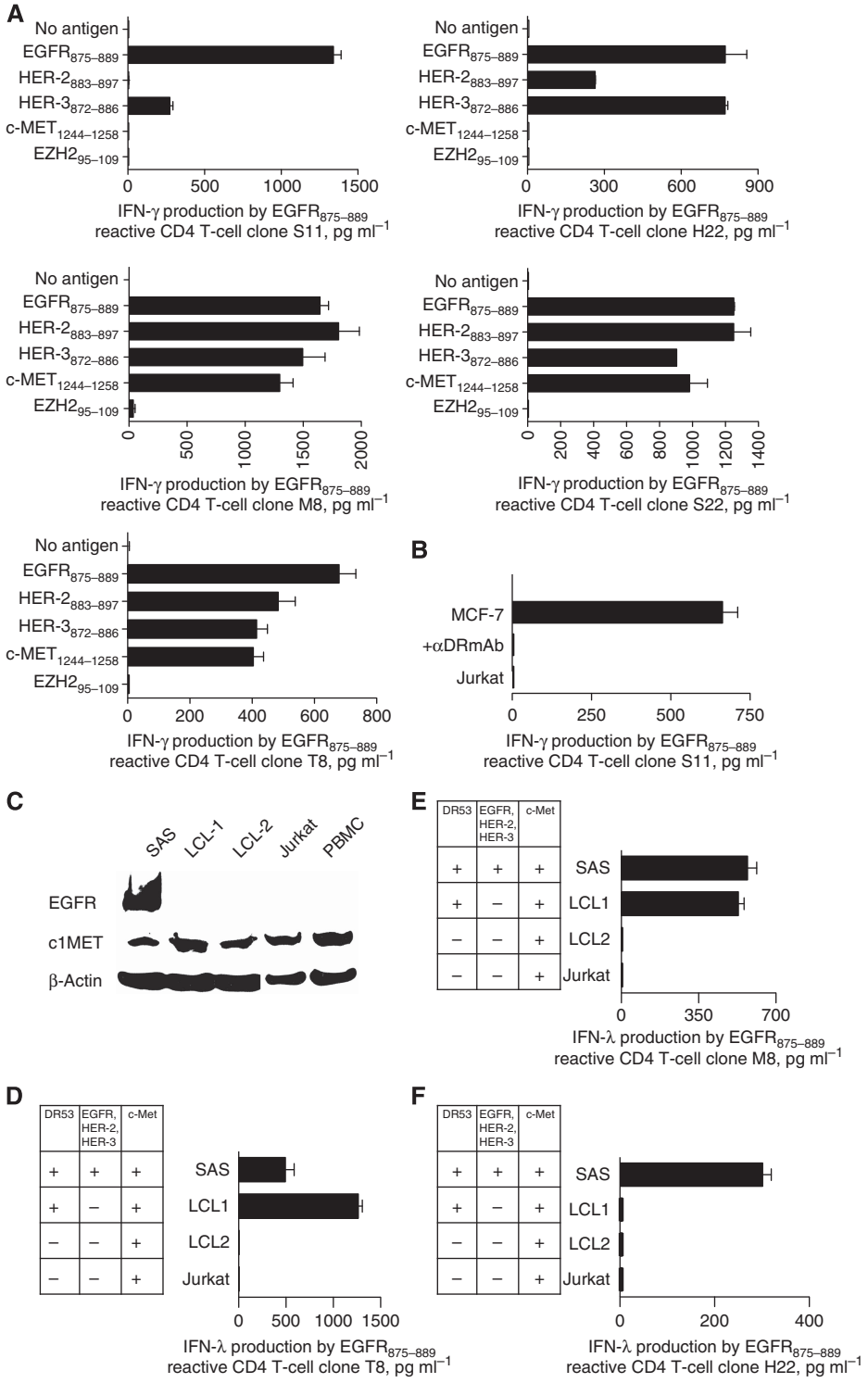

Figure 6. Responses of $EGFR_{875–889}$-reactive CD4 T-cell clones to $EGFR_{875–889}$ analogs and HER-2 or c-Met-expressing tumour cells. (**A**) $EGFR_{875–889}$-reactive CD4 T-cell clones were tested for their capacity to recognise EGFR analogue peptides (3 μg ml^{-1}) by IFN-γ production. Autologous PBMCs were used as APCs. Columns means of triplicate determinations, bars s.d. Columns without bars had s.d. <10% the values of the mean. Results are representative of at least two separate experiments. (**B**) HLA-DR15-restricted $EGFR_{875–889}$ reactive CD4 T-cell clone S11 was tested for the capacity to recognise EGFR-negative HER-2, HER-3 and c-Met positive cell line MCF-7 by IFN-γ production. Anti-HLA-DR mAb L243 (10 μg ml^{-1}) was added to demonstrate that T-cell reactivity was through TCR/HLA-DR interaction. Columns means of triplicate determinations, bars s.d. Columns without bars had s.d. <10% the values of the mean. Results are representative of at least two separate experiments. (**C**) EGFR and c-Met expression of HNSCC cell line SAS, EBV-LCLs and Jurkat T-cell lymphoma cell lines were examined by western blot. PBMC was used as negative control. (**D–F**) HLA-DR53-restricted $EGFR_{875–889}$ reactive CD4 T-cell clones were tested for the capacity to recognise EGFR-negative, c-Met-positive cell lines by IFN-γ production. Columns means of triplicate determinations, bars s.d. Columns without bars had s.d. <10% the values of the mean. Results are representative of at least two separate experiments.

Table 2. Assessment of T-cell responses to the $EGFR_{875–889}$ peptide in HNSCC patients and healthy donors

							EGFR 875	anti-HLA-DR mAb	EGFR 875	anti-HLA-DR mAb		
No.	Sex	Age (y)	Pathological stage	Primary site	No antigen (IFN-γ, pg/ml)	No antigen (GM-CSF, pg/ml)	(IFN-γ, pg ml^{-1})		(GM-CSF, pg ml^{-1})		TT 830 (IFN-γ, pg ml^{-1})	TT 830 (GM-CSF, pg ml^{-1})
1	M	69	T2N2bM0	Larynx	<	<	603 ± 199	<	195 ± 45	<	1972 ± 1155	797 ± 154
2	F	73	T2N1M0	Oropharynx	<	<	294 ± 181	<	189 ± 47	<	83 ± 11	269 ± 123
3	F	76	T2N2bM0	Larynx	<	<	703 ± 978	<	<	<	125 ± 83	<
4	M	61	T2N2cM0	Oropharynx	<	<	358 ± 200	<	151 ± 55	<	357 ± 87	89 ± 114
5	F	49	T4aN2cM0	Oropharynx	<	<	688 ± 351	<	306 ± 177	<	227 ± 81	292 ± 328
6	M	29	Normal volunteer		<	<	556 ± 87	<	177 ± 38	<	1064 ± 211	625 ± 62
7	F	42	Normal volunteer		<	<	778 ± 51	<	313 ± 130	<	1168 ± 480	576 ± 54

<: less than the lower limit of detection (mean <30 pg/ml). No. 1–5 are HNSCC patients and No. 6 and 7 are healthy donors. IL-4, Il-5, IL-17, and IL-10 are not detected in all patients and healthy donors.

patients (Table 2). These responses were inhibited by anti-HLA-DR mAb, indicating that CD4 T-cell responses were mediated via the T-cell receptor and MHC-II peptide complexes.

DISCUSSION

There are many reports documenting the important role that CD4 T cells have in the generation of effective cytotoxic CD8 T-cell responses both against tumours and infectious diseases (Giuntoli *et al*, 2002). In addition to their helper function, CD4 T cells themselves may exhibit effector cytolytic activity against tumour cells (Hayashi *et al*, 2011). We believe that the identification of CD4 T helper epitopes will be essential to obtain robust antitumour immunity, as clinical trials using solely CD8 CTLs epitopes as vaccines have not shown satisfactory results (Filipazzi *et al*, 2012).

Therapeutic EGFR inhibition using TKIs or blocking antibodies that bind to the EGFR extracellular domain have become effective therapeutic approaches for several solid tumours, but unfortunately resistance develops in many cancer patients. We believe that immunological approaches, such as vaccines that stimulate antitumour T-cell responses, could be of benefit to increase the efficacy of EGFR inhibition therapy. Here, we have provided experimental evidence that peptide $EGFR_{875–889}$ was able to induce effective antitumour CD4 T-cell responses against tumours expressing EGFR such as HNSCC. Moreover, the $EGFR_{875–889}$ epitope was presented to CD4 T cells by multiple HLA-DR alleles, suggesting that this peptide would provide broad population coverage in the clinic. Significantly, we demonstrated that the $EGFR_{875–889}$ peptide could serve as an effective crossreacting epitope inducing responses to other HER family members and to the c-Met antigen.

One major mechanism of tumour resistance to targeted EGFR blockade are EGFR mutations such as T790M and EGFR variant III (Learn *et al*, 2004; Bell *et al*, 2005). These alterations would not interfere with $EGFR_{875–889}$-mediated CD4 T-cell responses because the $EGFR_{875–889}$ epitope is preserved in these instances. Activation of alternative signalling pathways such as HER-2, HER-3 and c-Met are other causes of acquired resistance to EGFR inhibitors (Quesnelle and Grandis, 2011; Grovdal *et al*, 2012; Huang *et al*, 2013; Stabile *et al*, 2013). We have preliminary evidence indicating that HER-2 expression is increased in HNSCC cells following EGFR blockade (unpublished). As HER-2 and c-Met upregulation has been reported in some cancer patients treated with EGFR inhibitors (Suda *et al*, 2010), the T-cell crossreactivity against HER family and c-Met could expand the candidates for immunotherapy using $EGFR_{875–889}$ peptide for those EGFR inhibitor-treated patients who develop treatment resistance through HER-2, HER-3, and c-Met upregulation. From these findings, the novel identified $EGFR_{875–889}$ CD4 T helper peptide epitope might be well-suited for HER family or c-Met combinatorial targeted cancer immunotherapy regardless of EGFR status or resistance to EGFR inhibitors.

The amino-acid sequence of peptide $EGFR_{875–889}$ in this study is almost identical with peptide $HER\text{-}2_{883–897}$, which differs by only one amino acid near the C terminus end (H888 in EGFR and R898 in HER-2; Table 1). We previously reported that the $HER\text{-}2_{883–897}$ peptide epitope was promiscuously presented in the context of HLA-DR1, DR4, DR52, and DR53 alleles (Kobayashi *et al*, 2000). In the present study, $EGFR_{875–889}$-specific CD4 T cells restricted by HLA-DR4 and DR53, but not those restricted by DR15, were able to cross-react with $HER\text{-}2_{883–897}$, suggesting that $HER\text{-}2_{883–897}$ may not bind well to HLA-DR15. Alternatively, it is possible that the T-cell receptor of the $EGFR_{875–889}$-specific DR15-restricted clone relies on the histidine in position 888 for antigen recognition.

It is also clear that alterations in the expression of MHC molecules and CIITA, a transcriptional regulator of MHC molecules can influence the immune response against tumours. Downregulation of MHC-II molecules on tumour cells is associated with poor prognosis in cancer patients (Walsh *et al*, 2009), highlighting the importance of normal expression of immune-related molecules in the tumour microenvironment. A key finding of this study is that EGFR TKIs and EGFR-blocking antibodies augmented the expression of MHC-II molecules on the HNSCC cells, and consequently enhanced $EGFR_{875–889}$-specific CD4 T-cell recognition against the HNSCC cells. A recent study suggested that these features were due to the key role of EGFR in regulating CIITA expression and function, which is attenuated by EGFR activation but is increased when EGFR is inhibited by EGFR TKIs and blocking antibodies (Pollack *et al*, 2011). Furthermore, as previously reported (Anido *et al*, 2003), we also observed that EGFR expression on tumour cells remains at similar levels following EGFR blockade (data not shown). Thus, EGFR inhibitors could be used as immune modulators to enhance the expression of MHC-II molecules on tumour cells, in addition to their original intended function of suppressing proliferation and survival signals derived from EGFR.

In summary, we have identified a novel CD4 T-cell epitope from EGFR ($EGFR_{875–889}$) that elicits effective antitumour T-cell responses against HER family and c-MET-expressing tumours such as HNSCC. In addition, enhancing tumour MHC-II expression by EGFR inhibitors increased the ability of EGFR-reactive CD4 T cells to recognise and kill tumour cells. These observations should enable implementing a strategy for a combined immune-targeted approach to generate effective anti-tumour therapies and prevent tumour immune evasion.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

TK carried out and participated in all of the studies. TK, YM, KO, NA, SK, and YH have made substantial contributions to acquisition of the results. TK provided the clinical samples. EC and HK designed, supervised and coordinated the study, and prepared the manuscript. All authors read and approved the final manuscript.

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