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Modified tumour antigen-encoding mRNA facilitates the analysis of naturally occurring and vaccine-induced CD4 and CD8 T cells in cancer patients

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ORIGINAL ARTICLE

Modified tumour antigen-encoding mRNA facilitates the analysis of naturally occurring and vaccine-induced CD4 and CD8 T cells in cancer patients

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Abstract The development of effective anti-cancer vaccines requires precise assessment of vaccine-induced immunity. This is often hampered by low ex vivo frequencies of antigen-specific T cells and limited defined epitopes. This study investigates the applicability of modified, in vitro-transcribed mRNA encoding a therapeutically relevant tumour antigen to analyse T cell responses in cancer patients. In this study transfection of antigen presenting cells, by mRNA encoding the tumour antigen NY-ESO-1, was optimised and applied to address spontaneous and vaccine-induced T cell responses in cancer patients. Memory CD8+ T cells from lung cancer patients having detectable humoral immune responses directed towards NY-ESO-1 could be efficiently detected in peripheral blood. Specific T cells utilised a range of different T cell receptors, indicating a polyclonal response. Specific killing of a panel of NY-ESO-1 expressing tumour cell lines indicates recognition restricted to several HLA allelic variants, including a novel HLA-B49 epitope. Using a modified mRNA construct targeting the translated antigen to the secretory pathway, detection of NY-ESO-1-specific CD4+ T cells in patients could be enhanced, which allowed the in-depth characterisation of established T cell clones. Moreover, broad CD8+ and CD4+ T cell responses covering multiple epitopes were detected following mRNA stimulation of patients treated with a recombinant vaccinia/fowlpox NY-ESO-1 vaccine. This approach allows for a precise monitoring of responses to tumour antigens in a setting that addresses the breadth and magnitude of antigen-specific T cell responses, and that is not limited to a particular combination of known epitopes and HLA-restrictions.

Keywords T cells · Tumour immunity · Vaccination · Antigens/peptides/epitopes

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Introduction

In the decade following the definition of the first tumour antigen [1] and the advancement of antigen discovery programmes, a growing number of therapeutic tumour targets have been defined. One of the most promising categories of tumour-associated antigens (TAAs) are the cancer/testis (CT) antigens, of which over 44 families of CT antigens have been defined [2]. CT antigens are ideal immunotherapeutical targets due to their highly restricted expression pattern and broad immunogenicity [3]. A number of CT antigens have been applied in phase I/II vaccine trials, and comprehensive phase II/III trials employing CT antigens are underway or planned [4]. Although it has been difficult to definitively associate induced immune responses with objective clinical outcomes in the extensively employed peptide-based trials, lessons learned from analysis of vaccine-induced immunity have led to significant advances in the field.

NY-ESO-1, a broadly expressed and highly immunogenic CT antigen, has been employed as a prototypal

tumour antigen for cancer vaccine development [5, 6]. Recently, cancer patients were vaccinated with full length NY-ESO-1 encoded by fowlpox virus or as a recombinant protein, which resulted in the induction of a humoral response and a broad oligoclonal cellular immune response to multiple epitopes [7, 8]. Interestingly, a retrospective observation of overall survival suggests that important clinical responses may have been attained in those patients receiving full-length protein and adjuvant, in comparison to the placebo-controlled group (unpublished observation). However, this observation, although promising, needs to be confirmed by an on-going phase II study with disease-free survival as a primary endpoint.

In order to understand the mechanisms behind any observed clinical efficacy, it is critical to carefully dissect the entire vaccine-induced immune response, i.e. the sum of responses to all individual epitopes. For most tumour antigens, however, only a limited number of epitopes and restricting HLA alleles have been identified. The use of synthetic overlapping peptides [8] or viral vectors encoding the whole antigen [7] may overcome these hurdles. However, the methods have inherent pitfalls that mRNA could overcome. Issues with synthetic peptides include solubility, aggregation and that natural post-translational modifications of peptides are not represented within synthetic libraries, where modified peptides may account for a large percentage of MHC-associated peptides [9]. Moreover, long polypeptides such as those >12 amino acids require processing to allow loading on MHC class I molecules, a process involving uptake and cross presentation. This is a major drawback as cells that are often used as autologous antigen presenting cell (APC), such as EBV-transformed B-lymphoblastoid cell lines (B-LCL) or T-APC (CD4 T cell PHA blasts) are unable to uptake and cross present soluble antigen and rely on inefficient exogenous loading by serum protease degradation [10]. Another strategy employs transfection of autologous APC with gene constructs encoding the full length antigen. However, viral delivery systems are generally more difficult to generate than peptides or purified nucleic acids, many require restricted laboratory handling and, moreover, are intrinsically antigenic themselves, thus potentially influencing the immunogenicity of the TAA they encode. mRNA has been investigated as an antigen source for DC-based therapy [11–13] and also directly as a vaccine agent [14]. Recently several groups have investigated the application of full-length antigen-encoding mRNA in the context of in vitro analysis. However, many of these studies were designed as proof of principle and investigated highly immunogenic viral antigens such as influenza matrix protein [15] or CMV pp65 [16] where T cells are present in high frequencies in many healthy donors.

In this study we describe the in vitro application of modified mRNA encoding a therapeutically-relevant tumour antigen to address anti-tumour immune responses in patients. We were able to expand and detect NY-ESO-1 specific CD4+ and CD8+ T cell responses, which usually have extremely low frequencies in cancer patients. Moreover, the use of NY-ESO-1 encoding mRNA allowed the analysis of the entire NY-ESO-1 specific response without pre-existing knowledge of epitopes and/or of restriction elements. In addition, we show that this method is suited to rapidly generate functional CD8 and CD4 T cell clones to a tumour antigen in a setting independent of known epitopes or HLA-restrictions and have identified a novel NY-ESO-1 epitope restricted to HLA-B*4901.

Materials and methods

Materials

Peripheral blood samples were obtained from patients and healthy donor buffy coats (Swiss blood centre, Zurich, Switzerland) after written informed consent. Standard molecular methods were used to establish the HLA types of patients. Mononuclear cells were obtained as described [17]. Melanoma cell lines were obtained from the ESTDAB repository [18] (www.ebi.ac.uk/ipd/estdab/) and cultured as recommended, HLA data is available from the database. Patients ZH-183 and ZH-493 had resected non-small cell lung carcinoma, Stage IB and IIB, respectively. PBMC was obtained post-resection at the time points indicated in the results. Patient ZH-183 had the following HLA haplotype: HLA-A*0201/6802, B*4901/5301, C*04/07. DRB1*0102/1104, DP*0402/1701, DQ*0301/0501. Patient ZH-493: HLA-A*0201, B*2705/3501, C*01/04.

Patient NW29 and NW1672 had stage IV and stage III melanomas, respectively. Both patients were treated at Krankenhaus Nordwest, Germany as previously described [7].

In vitro stimulation of Ag-specific CD4/8 T cells with synthetic peptides

CD8 and CD4 T cells were purified serially from PBMC using positive selection with MACS technology (Miltenyi Biotech, German) according to the manufacturers instructions. T cells were stimulated in vitro with the T-cell depleted fraction of the PBMC (APC-fraction) pulsed with 1 ug/ml of the indicated synthetic peptide as previously described [17, 19]; except 800 IU/ml rhGM-CSF and 500 IU/ml rhIL-4 (Both R&D systems) were added at the time of co-culture. Cultures were maintained in 25 IU/ml rhIL-2 (R&D systems). The cytokines GM-CSF and IL-4 were added to drive differentiation of monocytes into professional APC. Forty-eight hours following the addition of

these cytokines the monocyte population had down regulated CD14 and up regulated HLA-DR, CD80, CD86 and CD83; markers for matured professional APC. In addition we have demonstrated that the addition of these cytokines to peptide stimulations significantly increases the frequency of peptide-specific CD8 T cells following in vitro stimulation (N. Nuber et al., manuscript in preparation). Sets of NY-ESO-1 overlapping 20-mer and 13-mer peptides were generously provided by E. Jaeger, Frankfurt, Germany, and W. Chen and I. Davis, Ludwig Institute for Cancer Research, Victoria, Australia (Chiron Mimotopes, Victoria, Australia), respectively.

In vitro stimulation of Ag-specific CD4/8 T cells with mRNA transduced APC

The full length NY-ESO-1 sequence was cloned in house as previously described [20] and mRNA was produced by CureVac GmBH (Tübingen, Germany) [21]. Influenza matrix protein- and green fluorescent protein (GFP)-coding mRNA was purchased commercially from CureVac GmBH. The modified Sec-NY-ESO-1 mRNA, encoding the natural NY-ESO-1 protein sequence, was produced from a synthetic codon-optimised gene, commercially produced by BlueHeron Biotechnology (Bothell, WA, USA). It was constructed as described by Kreiter et al., introducing an N-terminal signal peptide and C-terminal HLA Class I transmembrane and cytoplasmic domain [16]. mRNA was in vitro transcribed using the mMessage mMachine T7 (Ambion Inc.). Cells to be transduced ($1–10 \times 10^6$ cells), as indicated in the results, were washed once and resuspend in either 100 or 200 µl cold Optimem (Invitrogen Life Technologies) and electroporation was to be performed in a 2 or 4-mm cuvette, respectively. Cuvettes (BTX) were cooled on ice. Cells were electroporated at the indicated voltage and time, using a BTX 830 square wave electroporator (BTX) and returned on ice. Cells were then transferred to 1 ml prewarmed T cell medium and cultured for 18 h (or as indicated) in sterile 5 ml polystyrene FACS tubes (BD Falcon). Due to variation in the published protocols for electroporation of human APCs, the conditions were optimised individually for each APC type in this manuscript.

For stimulation of CD4/8 T cells, the T-cell depleted PBMC (APC fraction) was transduced with 50 µg/ml antigen encoding mRNA or at the conditions described in the results, and treated as above. Transduced APC were incubated overnight (>18 h) in T cell medium plus 800IU/ml rhGM-CSF and 500IU/ml rhIL-4 in 5 ml Polystyrene tubes (BD Falcon), followed by irradiation at 30 Gy. T cells and APC were co cultured at a 1:1 ratio (typically 4×10^5 of each) in flat-bottom 96-well plates as described above for synthetic peptides. For purification of CD45RA+ T cells, CD8 T cells were first purified by positive isolation, with DETACHaBEAD technology (Dynal, Invitrogen life sciences), and subsequently with CD45RA positive selection (Miltenyi Biotech, Germany), both according to the manufactures instructions. For in vitro stimulation of individual effector/memory T cell subsets CD8 T cells were first isolated with magnetic beads followed by staining with mouse anti-human CD45RA-FITC, CD8-PE and CCR7-APC (all BD Biosciences) and sorted as indicated with a BD FACSAria (Flow Cytometry Laboratory, University & ETH Zurich).

For generation of CD4+ T-APC, $>1 \times 10^6$ purified CD4+ T cells were cultured in T cell media with 2 µg/ml PHA (HA16, Remel Inc. USA) and 25 IU/ml rhIL-2 and maintained in culture for 7–10 days prior to use as targets.

Flow cytometry immunofluorescence analysis, multimer-guided cloning and TCR-Vβ analysis

PE-labelled HLA-A2/peptide multimers were synthesised around NY-ESO-$1_{157–165}$ (SLLMWITQC) or Flu-$M1_{58–66}$ (GILGFVFTL) and enriched CD8 T cells were stained with the appropriate PE-labelled multimers and mAbs as described previously [17]. Cells were immediately acquired on a FACSCalibur, analysis was performed with FlowJo v.7 (Tree Star Inc, USA). mAbs were from BD Biosciences. Tetramer positive cells were isolated by single-cell, multimer-guided flow cytometry using a FACSAria (BD Biosciences) and further cloned as described previously [17]. TCR-Vβ analysis of clones was performed using the IOTest Beta Mark TCRVβ repertoire kit (Beckman Coulter).

Sorting of monoclonal T cells using mRNA-transduced targets and IFN-γ secretion assay

Established CD8 and CD4 T cell lines were rechallenged with mRNA-transduced T-APC; labelled with CFSE and cultured for >18 h in T cell medium. T cell lines and targets were cultured at a 1:2 ratio for 4 h before performing the IFN-γ secretion assay (Miltenyl Biotech, Germany), according to the manufactures instructions. Single cells were sorted into 96-well format plates and expanded as described previously [17].

Intracellular IFN-γ analysis, IFN- ELISpot and chromium release assays

Recognition of target cells by T cells was tested using intracellular IFN-γ staining as described previously [22]. A specific response is deemed as greater than twofold the frequency seen with the control APC. For MHC blocking experiments, 20 µg/ml of antibody was added to the target cells for 30 min prior to co-culture. Clones LB3.1 (HLA-DR),

B7.21.3 (HLA-DP) and SPVL3 (HLA-DQ) were employed. When T-APC were used as targets, they were first labelled with 1 μM CFSE (Molecular probes) for gating out. T-APC pulsed with 13–20mer NY-ESO-1 peptide(s), was performed overnight in serum-containing media to allow for exogenous degradation and loading. Saturating concentration of 25 μg/ml for individual or peptide pools, washed and co-cultured. A mock transfection of T-APC was performed replacing mRNA with water. IFN-γ ELISpot assays were performed as described previously [23]. Lytic activity of clones was measured against peptide-pulsed T2 cells (HLA-A2+, TAP$^{-/-}$), EBV-transformed B-LCL cells, and melanoma cell lines at the indicated E:T ratios in the presence or absence of peptide in 4-h chromium release cytotoxicity assays [17].

Intracellular protein expression analysis

Staining of intracellular protein was performed as described above for IFN-γ with the following modifications. At the time points indicated, transfected cells were fixed with BD Cytofix/Cytoperm Kit (BD Biosciences). Cells were labelled with a 1:500 dilution of the mouse anti-human NY-ESO-1 mAb (Clone E978, Zymed Laboratories Inc., CA, USA) and a 1:250 dilution APC-labelled goat anti-mouse IgG (BD Biosciences) using the BD Cytofix/Cytoperm Kit.

Results

Optimisation of mRNA transfection efficiency

Two different APC fractions were used for mRNA transfection. For in vitro stimulation of effector T cells, CD4/CD8-depleted PBMC were employed. For short-term functional T cell assays, PHA-stimulated CD4 T cell blasts (T-APC) [24] were transfected with mRNA and used as targets. To establish optimal conditions for protein expression following electroporation with mRNA, the reporter molecule enhanced green fluorescent protein (eGFP) was employed. Electroporation conditions were optimised for time and voltage, with a fixed concentration of mRNA at 50 μg/ml. For T-APC, 750 V/cm and a pulse time of 10 ms resulted in a transfection efficiency of approximately 50% (35–65%, $n = 5$), observed 18 h after electroporation. Protein could be rapidly detected within 1 h following transfection and peaked around 16–18 h (data not shown). Controls for autofluorescence in the FL1 (eGFP) channel were performed on the NY-ESO-1 mRNA transfected cells (below) and was below 0.5%. Viability 24 h post transfection was greater than 70%, based upon forward scatter/side scatter FACS plots (data not shown). In CD4/CD8-depleted PBMC, detectable eGFP expression following transfection was very low, approximately 0.5–1% in both the lymphocyte and monocyte populations after 24 h (data not shown) and is consistent with that reported by Teufel et al. [15]. Intracellular staining for NY-ESO-1 was performed on T-APC and T-cell depleted PBMC following transfection with NY-ESO-1 encoding mRNA. Intracellular NY-ESO-1 protein could only be detected in approximately 10% (8–15%, $n = 5$) of T-APC after 24 h (Fig. 1a). Background staining of the eGFP mRNA-transfected control T-APC was below 0.5% (data not shown). No significant expression of NY-ESO-1 was detected in the T-cell depleted PBMC (data not shown). Transfection of the T2 cell line was used as a control, where approximately 90% of viable cells express NY-ESO-1 protein 24 h after transfection (Fig. 1b). However, based upon the widely accepted DRiP hypothesis [25]—namely that MHC peptides may be derived from mistranslated or misfolded proteins—an absence of abundant stable protein does not negate MHC peptide presentation. Thus the T-cell depleted PBMC (APC) were transduced with a viral antigen to optimise and establish the in vitro stimulation of antigen-specific T cells; hence optimisation was based on presentation and recognition of MHC-peptide. The CD4/CD8 depleted PBMC (APC-enriched fraction) was transduced with Influenza matrix protein M1 (Flu M1)-encoding mRNA (750 V/cm, 10 ms) or pulsed with the HLA-A2-restricted peptide Flu-M1$_{58–66}$. Autologous CD8 T cells were added to the APC 18 h later and maintained in culture for 10 days. Following this one in vitro stimulation with either peptide or mRNA, using HLA-peptide multimers we observed a frequency of HLA-A2.1/Flu-M1$_{58–66}$-positive cells of 21 and 18%, respectively (Fig. 1c). To determine the impact of mRNA concentration on the percentage of specific CD8 T cells, a titration experiment was performed. In the two HLA-A2 positive healthy donors shown, increasing Flu-M1 mRNA concentrations resulted in an increase in the frequency of Flu-M1$_{58–66}$ specific CD8 T cells, with a maximum frequency observed at 50 μg/ml (Fig. 1d). For all further experiments a concentration of 50 μg/ml is utilised.

Cancer/testis antigen, NY-ESO-1, encoding mRNA

Although viral antigens such as CMV pp65 are useful tools for establishing in vitro systems, often the transfer of methodologies from viral antigens to tumour antigens is problematic, in particular due to the differences in precursor frequencies between T cells specific for these two groups of antigens. Therefore to determine if the in vitro use of antigen-encoding mRNA is feasible for investigating TAA-specific T cells, NY-ESO-1 was chosen as a representative tumour antigen. Based on the observed correlation between cellular and humoral immune responses [26, 27], cancer patients with a detectable anti-NY-ESO-1 IgG titre were

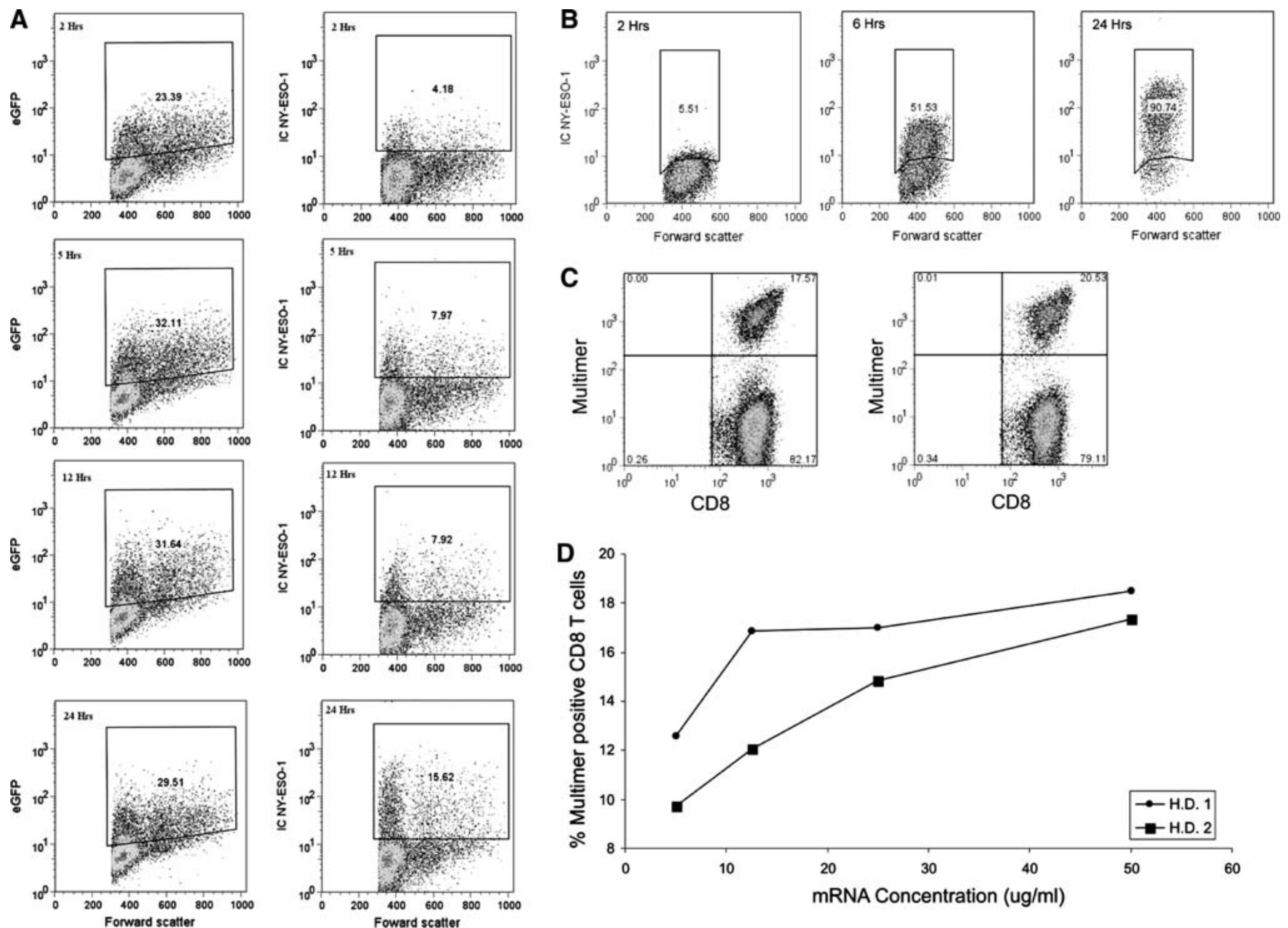

Fig. 1 Optimisation of mRNA transfection efficiency. **a** T-APC were transfected with 50 µg/ml of mRNA encoding eGFP (*left*) or NY-ESO-1 (*right*) and samples fixed at the time points indicated. Intracellular (IC) NY-ESO-1 was labelled after permeabilization, and expression of both proteins analysed in viable cells by flow cytometry. Viability 24 h post electroporation was >70% based on FACS analysis. **b** IC NY-ESO-1 expression in T2 cells was analysed at the time points indicated following transfection. **c** PBMC from an HLA-2 positive healthy donor were transduced with 25 µg/ml Flu-M1 mRNA (*left*) or pulsed with 1 ug/ml of the Flu M1 peptide p58–66 (*right*), both in the presence of rGM-CSF and rIL-4. Dot plots demonstrate the percentage of HLA-A2/Flu $M1_{58–66}$-positive CD8+ T cells labelled with multimers after 10 days of culture. **d** Quantity of Flu-M1 mRNA used to transfect PBMC from HLA-A2 positive healthy donors (H.D. 1 and 2) was titrated and the percentage of HLA-A2/Flu $M1_{58–66}$-positive CD8+ T cells after 10 days determined with fluorescent multimers

selected for in vitro stimulation (data not shown). The addition of GM-CSF and IL-4 to in vitro stimulations was included as these cytokines will drive differentiation of professional APC in PBMC, as seen by down regulation of CD14 and up-regulation of CD80, CD86 and HLA-DR. Moreover, we also observe a significant increase in the frequency and function of specific T cells stimulated in vitro with synthetic peptides using this cytokine supplementation approach (N. Nuber et al., manuscript in preparation). In patient ZH-183, one in vitro stimulation with NY-ESO-1 mRNA-transduced CD4/CD8-depleted PBMC (in the presence of GM-CSF and IL-4), a frequency of 0.4% A2/NY-ESO-$1_{157–165}$-positive cells was detected with fluorescent multimers (Fig. 2a). In contrast, in the absence of GM-CSF and IL-4, NY-ESO-$1_{157–165}$-specific CD8 T cells were not detectable (data representative of more than one experiment).

To confirm the specificity monoclonal anti-NY-ESO-$1_{157–165}$ specific CD8 T cells were obtained by flow cytometric sorting of multimer labelled cells (Fig. 2b). The avidity of the clones was assessed using T2 cells, pulsed with increasing peptide concentrations in a chromium release assay. As a representative example, clone ZP-311 1G3 exhibits an IC50 of 10^{-10} M suggesting an intermediate to high avidity (Fig. 2c). Moreover, this clone demonstrated functional killing of an allogeneic tumour cell line co expressing HLA-A2.1 and NY-ESO-1 (SK-MEL-37) whereas no killing was observed of the HLA mismatched (NW-MEL-634) or NY-ESO-1-negative control cell lines (NW-MEL-1715) (Fig. 2d).

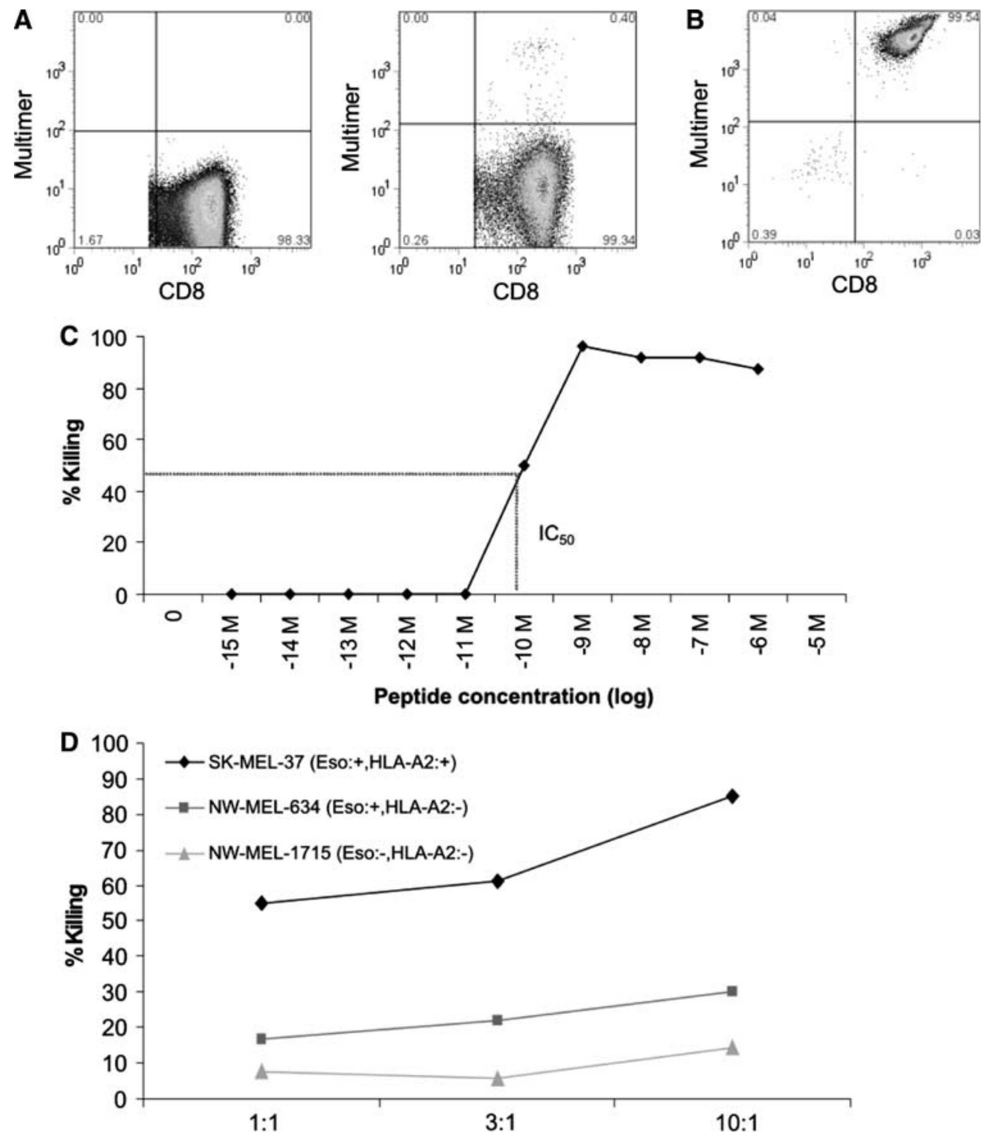

Fig. 2 Analysis of NY-EYO-$1_{157–165}$ specific CD8+ T cells in patient ZH-183 following one round of in vitro stimulation with NY-ESO-1 mRNA. **a** The CD8-depleted fraction of PBMC from HLA-A*0201-positive patient ZH-183 (Z-P-311) were transduced with NY-ESO-1 mRNA, co-cultured with autologous CD8+ T cells in the presence (*right*) or absence (*left*) of rGM-CSF and rIL-4. Cells were maintained in culture for 10 days with rIL-2 and analysed using fluorescent HLA-A2/NY-ESO-$1_{157–165}$ multimers. **b** HLA-A2/NY-$ESO_{157–165}$ positive CD8+ T cell clones were generated by FACS-assisted single-cell sorting of HLA-multimer labelled cells. **c** Functional avidity of clones was determined by peptide-titration experiments showing killing of peptide-loaded TAP-deficient T2 cells using a standard chromium release assay. **d** Specific killing of NY-ESO-1 expressing tumour cells was demonstrated in a standard chromium release assay with tumour cell lines. Eso, NY-ESO-1

Analysis of naturally occurring CD8 T cell responses to NY-ESO-1 in patients with NSCLC

To determine the breadth of the specific T cell response expanded by stimulation with NY-ESO-1 encoding mRNA, two NSCLC patients were analysed. CD8/CD4-depleted PBMC were transduced with NY-ESO-1 mRNA and used as APC for one round of in vitro stimulation with the autologous CD8 T cells. To assess the frequency of specific CD8 T cells following stimulation, T-APC were used as autologous targets in an intracellular IFN-γ assay. For this assay T-APC were either pulsed with the NY-ESO-$1_{157–165}$ peptide, mock-transduced or transduced with NY-ESO-1 mRNA (Fig. 3a). Patient ZH-183 had detectable frequencies of NY-ESO-$1_{157–165}$ specific cells at both time points. A frequency of 0.13% specific CD8 T cells was observed with NY-ESO-$1_{157–165}$ peptide-pulsed T-APC targets, whereas a threefold higher frequency of 0.41% specific CD8 T cells was observed when T-APC targets transfected with mRNA were used. HLA-A2.1 homozygous patient ZH-493 did not have any detectable NY-ESO-$1_{157–165}$ specific cells, however specific CD8 T cells were detected with mRNA transduced APC; frequency 0.26% with CD8 T cells from time point October 2005 (Fig. 3a).

To exclude that naïve cells are primed de novo, CD8 T cells from ZH-183 were separated into CD45RA positive and depleted fractions and stimulated in vitro. Thereafter, analysis with control or NY-ESO-1 peptide-pulsed APC by intracellular IFN-γ staining demonstrates that the NY-ESO-1 specific cells are exclusively found within the antigen experienced T cell repertoire of stimulated patients; where 0.42% of specific cells were seen only in the CD45RA depleted stimulation (Fig. 3b). Recognition of the control APC was below 0.05% for both the CD45RA positive and depleted fractions (result shown for the depleted fraction). Purity of the CD8/CD45RA positive fraction exhibited

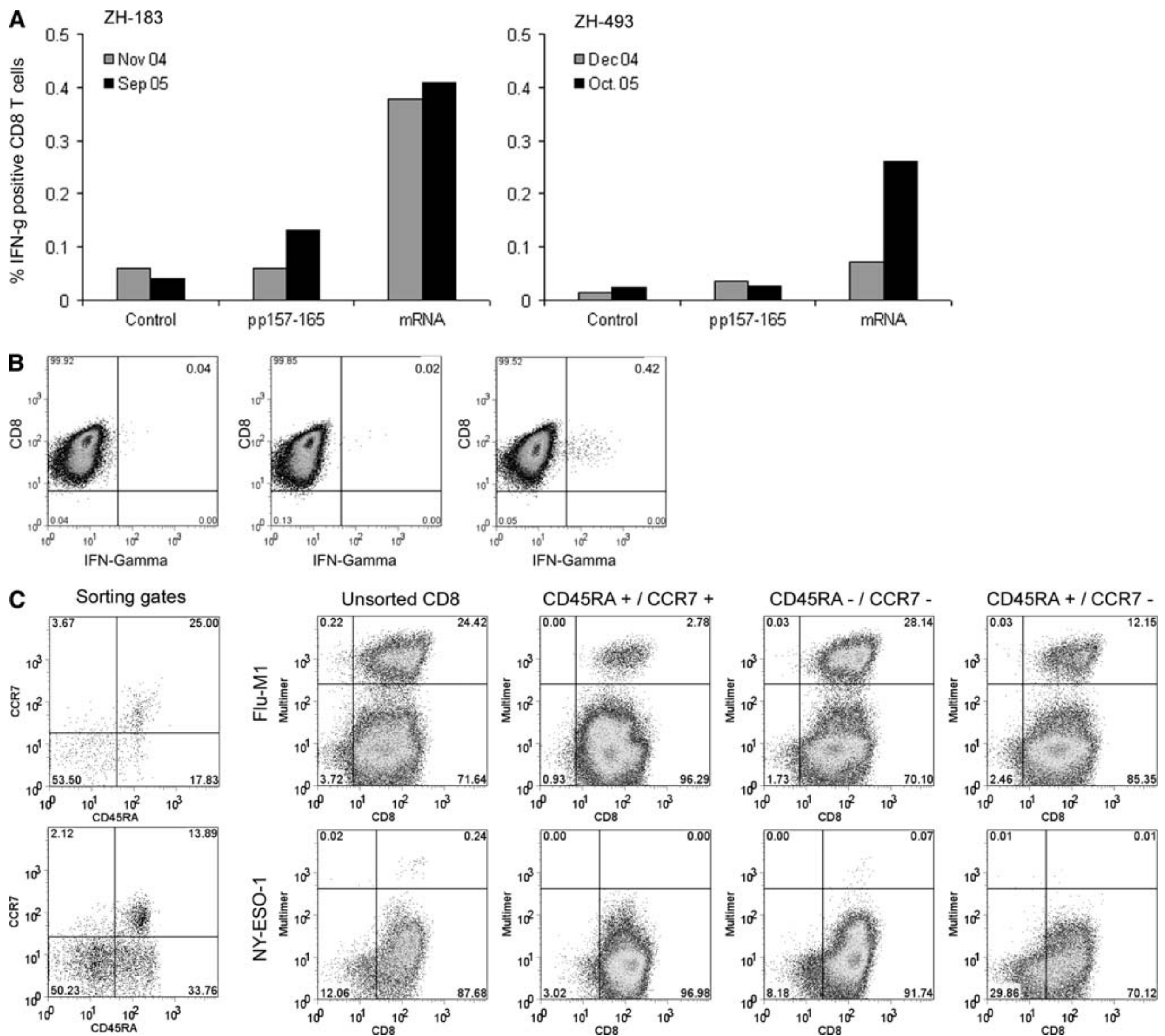

Fig. 3 Analysis of naturally occurring CD8+ responses to NY-ESO-1 in patients with NSCLC. **a** CD8 T cells from two non-small cell lung cancer (NSCLC) patients were expanded in vitro with mRNA encoding NY-ESO-1 at two time points post surgery, and whilst disease free. The frequency of NY-ESO-1 specific CD8+ T cells was determined using intracellular IFN-g analysis upon challenge with T-APC; either pulsed with the HLA-A2-restriced pp157–165 epitope or transfected with NY-ESO-1 mRNA. **b** Stimulation of naïve and antigen experienced CD8+ T cells with NY-ESO-1 mRNA. Purified CD8+ T cells from ZH-183 were further fractionated into CD45RA-enriched (*middle*) or -depleted populations (*right*) and subjected to one in vitro stimulation with NY-ESO-1 transduced autologous APC. *Dot plots* indicate intracellular IFN-γ production in APC-negative, CD3+CD8+ positive T cells following rechallenge with autologous T-APC pulsed with an NY-ESO-1 peptide or control T-APC (*left*). **c** Stimulation of sorted CD45RA/CCR7+/- CD8+ T cells with mRNA. Purified CD8+ T cells from HD-962 and ZH-107 were FACS sorted based upon CD45RA and CCR7 expression (*Left; upper and lower panels*, respectively) and each population was stimulated in vitro with autologous APC transfected with Flu-M1 or NY-ESO-1 mRNA, respectively. On day 9 specific cells were assessed with fluorescent HLA-A2.1 multimers containing the Flu-M1$_{58–66}$ (*upper panels*) and NY-ESO-1$_{157–165}$ (*lower panels*) epitopes

>95% (data not shown). Moreover, CD8 T cells isolated with magnetic beads from a healthy HLA-A2+ donor (HD-692) and a third HLA-A2+ cancer patient with detectable anti-NY-ESO-1 serology (ZH-107) were FACS sorted based upon CD45RA and CCR7 expression [28] (Fig. 3c). Unsorted CD8 T cells and the four sorted populations of CD8 T cells from HD-962 and ZH-107 were stimulated in vitro with the autologous APC-fraction transfected with mRNA encoding the Flu-M1 and NY-ESO-1 antigen, respectively. After 9 days of co-culture the cultures were labelled with anti-CD8 mAb and the HLA-A2.1 multimers containing the relevant peptides (previously described).

HLA-A2.1/Flu-M1$_{58-66}$-positive cells from HD-962 were found in the unsorted CD8 stimulation (24%) and mainly in the CD45RA−/CCR7− effector memory (28%) and the CD45RA+/CCR7−terminally differentiated effector (12%) stimulations. A smaller population was also observed in the CD45RA+/CCR7+ naïve stimulation (3%). CD45RA−/CCR7+ central memory T cells did not expand after in vitro stimulation, also observed with ZH-107. With patient ZH-107, HLA-A2.1/NY-ESO-1$_{157-165}$-positive cells were found only within the unsorted CD8 stimulation (0.24%) and the CD45RA−/CCR7− effector memory (0.07%) stimulations (Fig. 3c).

These data indicate that for NY-ESO-1 only the pool of memory cells have been expanded and naïve cells are not primed. Due to insufficient material this assay could not be performed in patient ZH-493. Moreover, NY-ESO-1 specific CD8 T cells were not detected in >5 healthy donors, which would contain only a specific naïve population, after one in vitro stimulation (data not shown). A small percentage of naïve cells specific for Flu-M1 were expanded. It is difficult to exclude that these cells expanded from CD45RA+/CCR7− CD8 T cells contaminating the naïve pool after sorting. Phenotypical analysis of the multimer positive cells indicates that the stimulated naïve pool still contained a population of specific cells with a CD45RA+/CCR7+ naïve phenotype, and thus had not been primed in vitro (data not shown).

In order to confirm antigen specificity and to assess the clonality of the response, NY-ESO-1 specific CD8 T cells from ZH-183 and ZH-493 secreting IFN-γ (following re-challenge with mRNA-transduced autologous APC) were single cell sorted. Specificity was confirmed using an IFN-γ ELISpot assay (data not shown). The TCR Vβ repertoire of specific clones was analysed. Figure 4a confirms that both patients mounted a polyclonal response as demonstrated by multiple TCR Vβ usages of the specific clones.

To ascertain the HLA-restriction of the specific clones and to demonstrate functionality, a panel of HLA-matched allogeneic melanoma cell lines that were either NY-ESO-1 expressing or non-expressing were selected from the EST-DAB cell bank [18] (data available at: www.ebi.ac.uk/ipd/estdab/). A panel of cell lines covering all the relevant MHC class I molecules for each patient were used as targets in a standard chromium release assay. Examples for three clones, two from patient ZH-183 and one from ZH-493 are shown (Fig. 4b). Clone ZH183 2D11 that is specific for the HLA-A2.1/NY-ESO-1$_{157-165}$ epitope demonstrated specific killing of ESTDAB-27, an NY-ESO-1, HLA-A2.1 positive cell line. Clone ZH183 2G5 specifically kills ESTDAB-133, an NY-ESO-1 positive line matched on HLA-B*49. Whereas, the HLA-B*49 positive, NY-ESO-1 negative cell line ESTDAB-29 was not killed. Clone ZH493 3F10 demonstrated specific killing of the NY-ESO-1 positive cell line ESTDAB-11, matched on HLA-C*0401.

To define the minimal sequence of the undefined B*49-restricted epitope, autologous APC were pulsed overnight with overlapping 20-mer peptides spanning the whole protein sequence. The HLA-B*49-restricted clone ZH183 2G5 specifically recognises peptide p119–143 in an ELISpot assay (Fig. 4c). Similar results were seen with HLA-B*49-restricted clone ZH183 3B3 (data not shown). HLA-Cw04-restricted clone ZH493 3F10 demonstrated specific recognition of peptide p91-110 (data not shown). To define the minimal HLA-B*49 restriction element, clone ZH183 2G5 was tested with overlapping 13-mers covering NY-ESO-1 positions 119–143, where specific recognition of peptide p124–136 is observed (data not shown). The use of 12-, to 8-mers covering this sequence demonstrate that the minimal epitope recognised was pp124–135 (KEFTVSGNILTI) (Fig. 4d). This peptide contains the known HLA-B*49 MHC anchor residues; glutamic acid in position 2 and a valine, leucine or isoleucine in position 9 (www.syfpeithi.de).

Analysis of naturally occurring CD4+ T cell responses to NY-ESO-1

To determine if NY-ESO-1-specific CD4+ T cell responses could be detected in patients, purified CD4+ cells were stimulated in vitro as described. For this purpose, a modified mRNA coding for an NY-ESO-1 antigen having a signal sequence to direct the translated protein to the secretory pathway (Sec-NY-ESO-1 mRNA) was used. This modification was recently described by others and shown to enhance MHC class II presentation [16]. Here, we compared the wild type (WT-NY-ESO-1) mRNA with the Sec-NY-ESO-1 to stimulate a CD4+ response in patient ZH-183. Again, one in vitro stimulation with mRNA-electroporated APC fraction was utilised and assessment of IFN-γ performed using T-APC targets pulsed with the pool of 20-mer peptides spanning the whole sequence. Peptide-pulsed T-APC were employed to exclude CD4+ cells that may have been specific for potential novel epitopes in the fusion region between the antigen and signal sequences. In this example we see a CD4+ T cell frequency of 0.59 and 1.83% for the WT- and Sec-NY-ESO-1 mRNA, respectively (Fig. 5a).

In a stimulation of CD4 T cells from ZH-183, from a second time point, read-out was performed with T-APC transduced with Sec-NY-ESO-1 mRNA, a pool of the overlapping peptides, or each of the peptides individually (Fig. 5b). In this analysis CD4 T cells specific for the peptides pp41–60 (0.27%), pp51–70 (7.17%), pp61–80 (0.77%), pp91–110 (0.14%), pp119–143 (0.35%), pp151–170 (3.35%) and pp161–180 (3.49%) were detected, where background with the control APC was <0.05%. NY-ESO-1

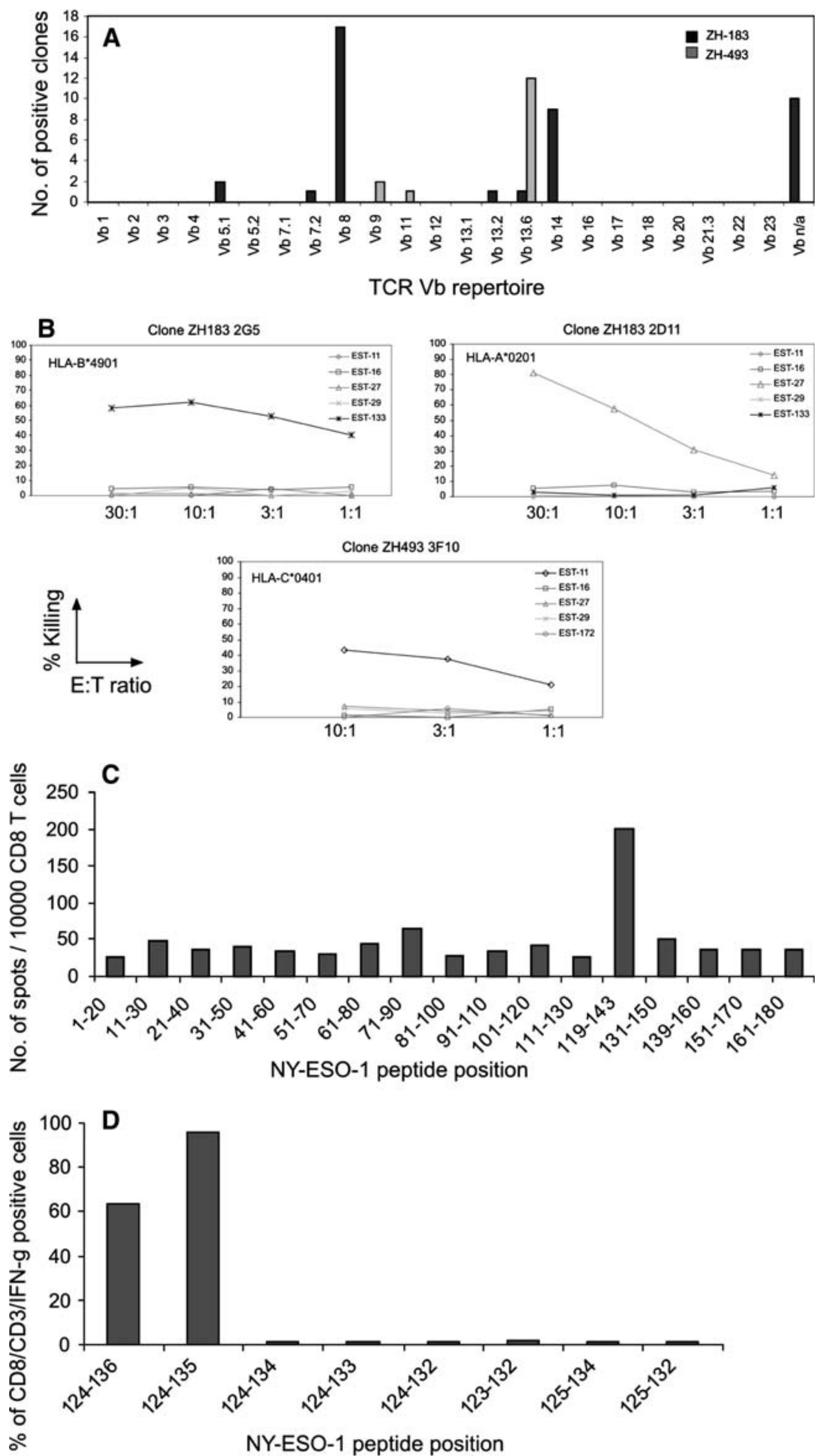

Fig. 4 Analysis of NY-ESO-1 specific CD8+ T cell clones obtained following mRNA stimulation of patients ZH-183 and ZH-493. CD8+ T cells from both patients were stimulated with autologous APC transduced with NY-ESO-1 mRNA in vitro, and after 10 days rechallenged with mRNA-transduced autologous T-APC. IFN-γ producing cells were single cell sorted, clones expanded and specificity confirmed. **a** TCR-Vβ usage of the specific clones was analysed using a commercial serological method. Vβ n/a—not available with method employed. **b** To address functionality and HLA-restriction, clones were used in a standard chromium release killing assay with allogeneic EST-DAB tumour cell lines. A panel of cell lines matched for each of the class I allelic variants relevant for each patient, expressing or non-expressing NY-ESO-1 were selected. Effector: Target ratios are indicated, in addition to the resultant HLA-restriction. **c** A set of overlapping 20-mer NY-ESO-1 peptides were used to define the region recognised by clone ZH183 2G5 in an IFN-γ ELISpot with autologous APC. **d** Subsequently 13-mers to 8-mers were employed to define the minimal epitope using autologous T-APC and an intracellular IFNγ analysis to define specificity. All polypeptides were first pulsed on APC for >18 h

specific CD4+ cells from patient ZH-183 were cloned by means of single cell sorting of IFN-γ producing cells. Specific clones were assessed by IFN-γ ELISpot with pooled or individual overlapping 20-mer peptides to determine the breadth of the response. Of 85 specific clones 70 recognised the peptide pp51–70, 1 clone pp91–110, and 14 clones recognised both pp151–170 and pp161–180 (data not shown). Recently a novel HLA-DQβ*0301 epitope contained within p51–70 was described [29]; an HLA allele expressed by patient ZH-183. Clones were tested for recognition with either an autologous B-LCL line or an allogeneic B-LCL matched or mismatched on HLA-DQβ*0301, pulsed with peptide NY-ESO-1 pp51–70, in an IFN-γ assay. Recognition of only the autologous and HLA-

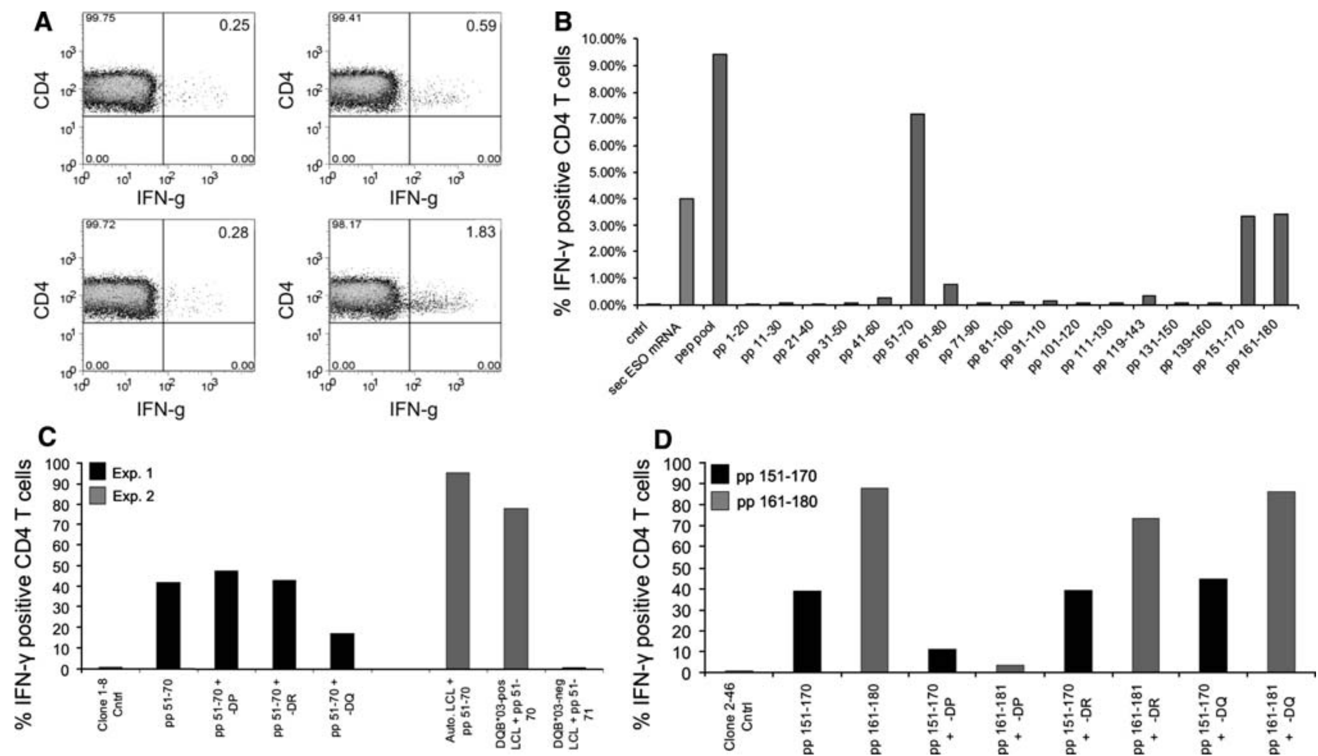

Fig. 5 Analysis of NY-ESO-1 specific CD4+ T cells obtained following mRNA stimulation of patient ZH-183. **a** CD4+ T cells were stimulated with autologous APC transduced with either the wild type (WT) (*above*), or modified (sec) NY-ESO-1 mRNA (*below*). After 20 days NY-ESO-1 specificity of gated APC-negative, CD3-positive, CD4-positive cells was assessed with an intracellular IFN-γ assay, employing autologous T-APC pulsed with a pool of 20-mer overlapping peptides spanning the full protein length (*right*), or control un-pulsed T-APC (*left*). **b** Sec-ESO-mRNA stimulated CD4+ T cells from ZH-183 were challenged with T-APC transduced with Sec-NY-ESO-1 mRNA, pulsed with the 20-mer peptide pool, or each peptide individually, and intracellular IFN-γ determined by FACS analysis. **c** HLA restriction of clone 1–8 recognising peptide p51–70 was confirmed by analysis of IFN-γ following challenge with HLA-DQ*0301 matched/mis-matched B-LCL (Exp. 2) and blocking of MHC/TCR interaction with MHC-class II mAbs (Exp. 1). **d** Clone 2–46 was challenged with autologous B-LCL pulsed with peptides p151–170 or p161–180 and blocking or recognition performed with MHC-class II mAbs

DQβ*0301 matched allogeneic B-LCL by clone 1–8 indicate restriction to this allele (Fig. 5c). Blocking experiments confirmed HLA-DQ restriction (Fig. 5c). Interestingly, CD4 T cell clone 2–46 demonstrated concurrent recognition of two NY-ESO-1 peptides: 151–170 and 161–181 (Fig. 5d), which are 20-mer peptides overlapping by ten amino acids. Blocking experiments indicate that both peptides are recognised in the context of HLA-DP (Fig. 5d).

Analysis of NY-ESO-1 vaccinina/fowlpox vaccinated patients using mRNA encoding NY-ESO-1

We extended our analysis to two patients (NW29 and NW1672) that had been enrolled in an NY-ESO-1 vaccine program [7]. CD4 and CD8 T cells were stimulated once in vitro with mRNA encoding NY-ESO-1 (WT- for CD8 and Sec-NY-ESO-1 mRNA for CD4 T cells). CD4+ T cells were also used to generate T-APC targets. PBMC were obtained at days 29, 43 and 113 of the trial for NW29, and days 260 and 365 for NW1672. Intracellular IFN-γ analysis was performed using autologous T-APC pulsed with the 20-mer peptides, mock- or WT-NY-ESO-1 mRNA-transduced.

NW29 demonstrated a high frequency of NY-ESO-1 specific CD8 T cells at all three time points after analysis with mRNA-transduced T-APC; 3.7, 5.1 and 2.6% for days 29, 43 and 113, respectively (Fig. 6a). These frequencies are approximately tenfold higher than those observed in patients with a naturally arising response. This patient was tested for recognition of peptides p81–100, p91–110 and p119–143, to which a response was reported by Jager et al. [7]. For peptide 81–100, frequencies of approximately 8% were observed at all time points. For peptide 91–110, frequencies of 2.4, 7.4 and 1.9% for days 29, 43 and 113, respectively. For peptide 119–143; 2.7, 3.3 and 0.6% for days 29, 43 and 113, respectively (Fig. 6a).

For patient NW1672 analysis of CD8 T cells at the later time points days 260 and 365 also demonstrated a strong response to autologous APC transduced with NY-ESO-1 mRNA following one in vitro stimulation; 4.6 and 3.5% of gated CD3/CD8 positive cells, respectively. To compare

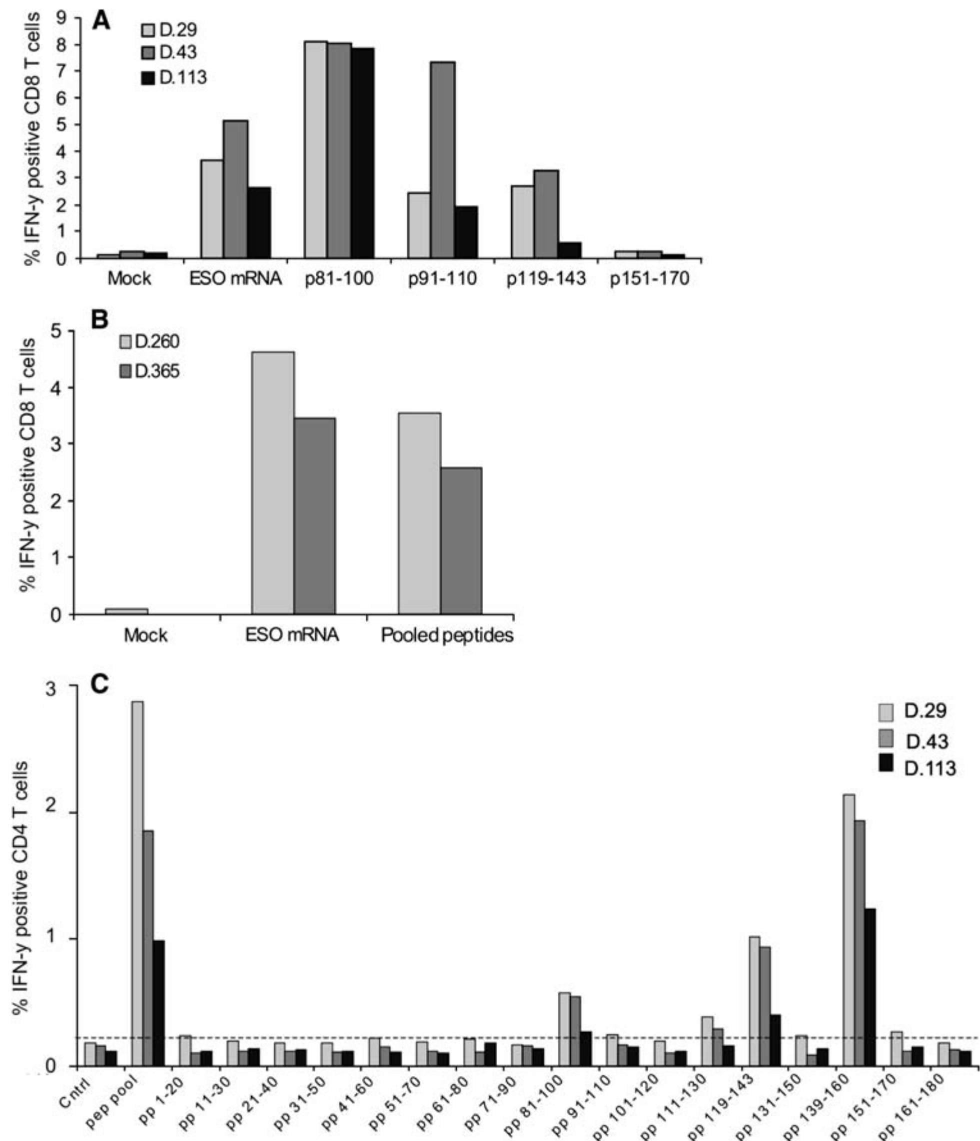

Fig. 6 Analysis of NY-ESO-1 specific CD8+ and CD4+ T cell responses in patients following vaccination with vaccinia/fowlpox NY-ESO-1. PBMC were obtained from patients NW29 and NW1672 following initiation of vaccination, at the time points indicated, and analysed for CD8+ and CD4+ T cell responses using wild type and modified (Sec) NY-ESO-1 mRNA, respectively. Following in vitro stimulation with mRNA, T cells were rechallenged with autologous T-APC and intracellular IFN-γ assessed on gated APC-negative, CD3-positive, CD4/8-positive. CD8+ from NW29 (**a**) and NW1672 (**b**) were assessed for recognition of mRNA-transduced, peptide-pool pulsed or individual 20-mer peptide pulsed T-APC, at the time points indicated. CD4+ T cells from NW29 (**c**) were assessed for recognition of peptide-pool pulsed or individual 20-mer peptide pulsed T-APC, at the time points indicated. *D* day, *PP* amino acid position. *Mock* control transfection with water

mRNA-transduced APC with APC pulsed with a pool of 20-mer peptides spanning the protein sequence, CD8 T cells from patient NW1672—following one round of in vitro stimulation—were challenged with T-APC pulsed with the peptide pool. Lower frequencies were observed with T-APC pulsed with the pool of peptides than observed for the mRNA-transfected APC; CD8 T cell frequencies from time points days 260 and 365 with the peptide-pulsed APC were; 3.6 and 2.6%, respectively (Fig. 6b).

For analysis of CD4+ responses in patient NW29, the modified Sec-NY-ESO-1 mRNA was employed for in vitro stimulation. Assessment of specific responses was made with T-APC pulsed with pooled or individual 20-mer peptides. NY-ESO-1 specific CD4+ T cells recognising the peptide pool were detectable at all three time points; 2.9, 1.9 and 1.0% for days 29, 43 and 113, respectively (Fig. 6c). Responses above the background seen with control APC (<0.18%) were observed for days 29, 43 and 113 (respectively) for peptides p81–100 (0.6, 0.6 and 0.3), p119–143 (1.0, 0.9 and 0.4%) and p139–160 (2.1, 1.9 and 1.2%). Weak but clear responses were seen towards peptide p111–130 on days 29 and 43 (0.4 and 0.3%) (Fig. 6c). For patient NW1672, low frequencies of CD4+ cells were observed for a finite number of the 20-mer peptides. Responses to peptides p119–143 (0.19%), p139–160 (0.17%) and p161–180 (0.34%) can be observed on day 265, whereas on day 365 responses to p139–160 (0.61%) and p161–180 (0.48%) were observed (background 0.1%) (data not shown).

Discussion

Precise assessment of spontaneous and vaccine-induced anti-tumour immunity in cancer patients is critical for understanding the therapeutic potential of cellular immune

responses directed against tumour antigens. Using mRNA encoding for the therapeutically relevant antigen, NY-ESO-1, we provide evidence of a highly efficient approach to address the breadth and the magnitude of CD8 and CD4 T cell responses in cancer patients. Importantly, detection is not limited to a finite set of epitopes and HLA restrictions. Moreover, we document the sensitive induction of NY-ESO-1 specific T cell responses in vitro that allowed the successful amplification of a low frequency of antigen-experienced precursors otherwise undetectable directly ex vivo.

Due to the sensitivity limits of existing immunomonitoring technologies and the low precursor frequencies of tumour antigen-specific T cells in peripheral blood—even following vaccination—in vitro stimulation still remains a necessity in most cases. Although some studies have demonstrated ex vivo detection of TAA-specific peripheral T cells [30–32] these are not frequent observations. It has been suggested that the functional capacity of specific T cells may be considered more relevant than the overall frequency of cells present [33]. Clearly in vitro stimulation ablates the capacity to determine functionality as it existed in vivo. In light of immune modulation being mediated locally within the tumour [22, 34], functional analysis of peripheral blood T cells directly ex vivo also does not provide an indication of how effective a therapy may have been at eliciting functional T cells at the tumour site [35]. Where immune monitoring does play a critical role is to address the induction and the breadth of an immune response resulting from a given therapy, especially relevant for developing vaccines comprising whole antigen as opposed to single epitopes, and to address the induction of both CD8 and CD4 T cells—including induction of memory—to enable correlations with clinical outcomes.

Recently, a number of groups have applied in vitro stimulation with mRNA to address immune responses [15, 36, 37]. Few studies have expanded their analysis beyond that of proof of principle investigations involving viral antigens [15, 16, 38], where comparatively large frequencies of high avidity T cells exist within the periphery of most healthy donors, and it remains to be addressed if their methodology would allow for the detection of TAA-specific T cells. One study did, however, presented preliminary data suggesting the expansion and detection of CD4 T cells recognising NY-ESO-1 in cancer patients [38]. Here we were able to reproducibly stimulate NY-ESO-1 specific CD8 T cells in patients with a low precursor frequency of specific cells, below ex vivo detectable limits, present as a result of naturally occurring immunity to their tumour or following NY-ESO-1 vaccination. In two HLA-A*0201 positive patients with spontaneous humoral immunity, ex vivo frequencies of cells specific for the HLA-A2.1/157-165 epitope could not be detected. We demonstrate that following one in vitro stimulation, detectable frequencies of specific T cells could be expanded from the PBMC of patient ZH-183, where further cloning enabled attainment of specific, functional clones. A novel HLA B*4901-restricted epitope from NY-ESO-1, position 124–135, was identified using these clones. No specific cells were found in the HLA-A2.1 homozygous patient ZH-493 to the formerly supposed immunodominant epitope pp157–165. Subsequent cloning and analysis suggests that some clones in patient ZH-493 are restricted to HLA*Cw4. Utilising one round of in vitro stimulation with a low quantity of PBMC enables a rapid and straightforward immune monitoring strategy. We applied the system for immunomonitoring of clinical trials by analysing PBMC from patients enrolled on an NY-ESO-1 vaccinina/fowlpox trial [7]. Here we demonstrated that we were able to detect specific CD8 T cells recognising three dominant C-terminal regions of NY-ESO-1 in patient NW29 [7], and analysis of patient NW1672 demonstrated that NY-ESO-1 specific T cells were still readily expandable in high frequencies 1 year following the first vaccination. The absence of NY-ESO-1 specific T cells following in vitro stimulation of healthy donors, as well as the absence of specific cells in the naive fraction of two patients CD8 T cells, demonstrates that we are only expanding antigen-experienced cells. An important consideration when using in vitro stimulation to monitor vaccinated patients. We were unable to detect specific T cells to the tumour antigen NY-ESO-1 ex vivo (data not shown), as shown by others for viral antigens [38], and attribute this disparity to the large difference in precursor frequencies between viral and tumour antigen-specific T cells.

To enhance MHC class II processing, modifications aimed at targeting the translated protein to the secretory pathway have be described [39–41]. Recently the inclusion of an MHC class I trafficking domain, comprised of an MHC class I signal peptide and the transmembrane/cytoplasmic region fused to the N- and C-terminus of the antigen, respectively, was described [16]. This modification was shown to specifically target the encoded antigen to diverse antigen-processing compartments and significantly enhances MHC class I and II loading [38]. Concurrently we have constructed a modified NY-ESO-1 mRNA molecule in this manner (termed Sec-NY-ESO-1 mRNA). We demonstrate that in vitro stimulation with the Sec-NY-ESO-1 mRNA resulted in an increase in the frequency of NY-ESO-1 specific CD4+ T cells, confirming that this modification enhances the stimulation of CD4+ T cells [38]. In our study peptide pulsed targets were used to ensure recognition was restricted to NY-ESO-1 epitopes and not any potential novel epitopes derived from the fusion region between the antigen and signal sequences. Specific CD4 T cell clones recognising an HLA-DQβ*0301 restricted epitope [29] were readily expandable from these in vitro

stimulated cells. A number of additional CD4+ T cell clones recognised peptides p151–170 and p161–180 concurrently, suggesting the decamer motif shared between these two overlapping peptides must be involved in binding on HLA-DP molecules and TCR recognition in this context. Analysis of CD4 T cell responses in the vaccinated patients NW1672 and NW29 demonstrated a polyclonal response to NY-ESO-1 recognising a broad array of epitopes, with large frequencies of cells similar to those achieved in the CD8 repertoire being observed for patient NW29. Unfortunately in this retrospective analysis, due to limited material, no pre-vaccination PBMC were available to assess for pre-existing T cell responses in these patients and thus one can only speculate that the higher-frequencies of NY-ESO-1 specific T cells were induced as a result of the therapy. Taken together these data indicate the applicability of in vitro stimulation with antigen-encoding mRNA to monitor CD4 and CD8 T cell responses in cancer patients.

In summary, we describe the efficient and rapid analysis of tumour antigen specific CD4 and CD8 T cells, from limited amounts of cryopreserved PBMC, by employing antigen-encoding mRNA that allows the analysis of all natural epitopes from a given antigen. By extending our analysis beyond that of reference viral antigens to a therapeutically-relevant tumour-TAA and analysing NY-ESO-1 vaccinated patients, we demonstrate this system has real potential for immune monitoring of vaccine trials. By using a single in vitro stimulation with a sensitive readout strategy one can ensure that the expanded cells are indeed in vivo amplified antigen-experienced cells as opposed to in vitro primed naïve cells, which may give false positive results and an incorrect judgement of the vaccine trial. Successful immunomonitoring, in addition to the analysis of tumour susceptibility to the immune responses (e.g. MHC/antigen loss, immune infiltration etc.) and follow-up of clinical outcomes are all requirements for the continued development of effective cancer vaccines.

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