

Three novel NY-ESO-1 epitopes bound to DRB1\*0803, DQB1\*0401 and DRB1\*0901 recognized by CD4 T cells from CHP-NY-ESO-1-vaccinated patients

Yu Mizote<sup>1</sup>, Taku Taniguchi<sup>1</sup>, Kei Tanaka<sup>1</sup>, Midori Isobe<sup>1</sup>, Hisashi Wada<sup>2</sup>, Takashi Saika<sup>3</sup>, Shoichi Kita<sup>4</sup>, Yukari Koide<sup>4</sup>, Akiko Uenaka<sup>1</sup> and Eiichi Nakayama<sup>1</sup>

*Departments of <sup>1</sup>Immunology and <sup>3</sup>Urology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, <sup>2</sup>Department of Surgery, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871 and <sup>4</sup>Medical & Biological Laboratories, Ltd., 1063-103 Terasawaoka, Ina, Nagano 396-0002, Japan*

*Runnnng Title* : Three novel NY-ESO-1 CD4 T cell epitopes

*Address correspondence to:* Eiichi Nakayama, M.D., Department of Immunology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan;  
Phone: +81-86-235-7187; Fax: +81-86-235-7193;  
E-mail: [nakayama@md.okayama-u.ac.jp](mailto:nakayama@md.okayama-u.ac.jp)

*Grant support:* Grant-in-Aid (No. 17016048) for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan

Financial Disclosure: Each author certifies that he or she has no commercial associations that might pose a conflict of interest in connection with the submitted article.

## **Abstract**

Three novel NY-ESO-1 CD4 T cell epitopes were identified using PBMC obtained from patients who were vaccinated with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1). The restriction molecules were determined by antibody blocking and using various EBV-B cells with different HLA alleles as APC for presenting peptides for CD4 T cells. The minimal epitope peptides were determined using various N- and C-termini truncated peptides deduced from 18-mer overlapping peptides originally identified for recognition. Those epitopes were DRB1\*0901-restricted NY-ESO-1 87-100, DQB1\*0401- restricted NY-ESO-1 95-107 and DRB1\*0803-restricted NY-ESO-1 124-134. CD4 T cells used for determining those epitope peptides recognized EBV-B cells or DC that were treated with recombinant NY-ESO-1 protein or NY-ESO-1-expressing tumor cell lysate, suggesting that the epitope peptides are naturally processed. Those CD4 T cells showed a cytokine profile with Th1 characteristics. Furthermore, NY-ESO-1 87-100 peptide/HLA-DRB1\*0901 tetramer staining was observed. Multiple Th1-type CD4 T cell responses are beneficial for inducing effective anti-tumor responses after NY-ESO-1 protein vaccination.

*Key words* : Tumor immunology; NY-ESO-1; CD4 epitopes

## **1. Introduction**

The NY-ESO-1 antigen was initially identified in esophageal cancer by SEREX and is in the category of cancer/testis (CT) antigens [1-3]. Its expression is restricted to germ cells in the testis in normal adult tissues, but is also observed in various types of cancer tissue at different frequencies [4]. A characteristic of the NY-ESO-1 antigen is its extremely high immunogenicity. Patients with NY-ESO-1-expressing tumors frequently show a spontaneous immune response to the NY-ESO-1 antigen. An antibody response against the NY-ESO-1 antigen was frequently observed in patients with NY-ESO-1-expressing tumors [5, 6]. Most patients who showed an antibody response also showed CD4 and CD8 T cell responses [7, 8]. Such strong immunogenicity makes the NY-ESO-1 antigen a very promising candidate target molecule for a cancer vaccine.

Several clinical trials using various preparations of NY-ESO-1 protein vaccine have been reported [3, 9]. The recombinant NY-ESO-1 protein was administered with ISCOMATRIX [10], cholesterol-bearing hydrophobized pullulan (CHP) [11], CpG in Montanide [12], imiquimod [13] or as recombinant vaccinia/fowlpox NY-ESO-1 [14]. Analyses of immune responses in patients vaccinated with the NY-ESO-1 protein showed that integrated immune responses of antibodies, CD4

and CD8 T cells were induced after vaccination.

In this study, we analyzed CD4 T cell responses against NY-ESO-1 in PBMC from 3 patients who were vaccinated with a complex of CHP and NY-ESO-1 protein (CHP-NY-ESO-1) in our clinical trial [11, 15-17] and determined 3 novel NY-ESO-1 CD4 T cell epitopes. These epitopes were NY-ESO-1 87-100 bound to DRB1\*0901, NY-ESO-1 95-107 bound to DQB1\*0401 and NY-ESO-1 124-134 bound to DRB1\*0803. CD4 T cells used for determining these epitope peptides recognized EBV-B cells or DC that were treated with recombinant NY-ESO-1 protein or NY-ESO-1-expressing tumor cell lysate, suggesting that the epitope peptides are naturally processed. These CD4 T cells showed a cytokine profile with Th1 characteristics.

## **2. Materials and Methods**

### *2.1. Patients*

Patients listed in Fig. 1 were vaccinated with CHP-NY-ESO-1 in our clinical trial reported elsewhere [11, 15-17]. Patients in the E- series were esophageal cancer patients. Patients in the P- series were prostate cancer patients. M-1

was a malignant melanoma patient.

## *2.2. Blood samples*

Peripheral blood samples were drawn from vaccinated patients after informed consent was obtained. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). CD4 T cells, CD8 T cells, CD14<sup>+</sup> cells and CD19<sup>+</sup> cells were purified from PBMC using CD4, CD8, CD14 and CD19 microbeads, respectively, using a large scale column and a magnetic device (Miltenyi Biotec, Auburn, CA). The residual cells were used as antigen-presenting cells (APC). The cells were stored in liquid N<sub>2</sub> until use. HLA typing of PBMC was performed by a sequence-specific oligo-nucleotide probe and sequence-specific priming of genomic DNA using standard procedures.

## *2.3. Antibodies*

Anti-human CD4, anti-human CD8, anti-HLA class I, anti-HLA class II, anti-DQ and anti-DR monoclonal antibodies (mAbs) were purchased from BD Bioscience (San Jose, CA). Anti-DP mAb was purchased from Santa Cruz (Santa Cruz,

CA). These monoclonal antibodies were used for an antibody blocking test in ELISA. Anti-human IFN $\gamma$  mAb (1-DIK) was obtained from Mabtech (Stockholm, Sweden). Polyclonal rabbit anti-human IFN $\gamma$  serum was obtained by immunizing a rabbit with recombinant human IFN $\gamma$  protein. Horseradish peroxidase conjugated goat anti-rabbit IgG was purchased from MBL (Nagoya, Japan).

#### *2.4. Cell lines*

Epstein-Barr virus-transformed B cells (EBV-B) were generated from CD19<sup>+</sup> peripheral blood B cells using the culture supernatant from EBV-producing B95-8 cells. OU-LC-KI and OU-LC-OK were lung adenocarcinoma cell lines established in our laboratory. These cell lines were kept in tissue culture by serial passage and in liquid N<sub>2</sub>.

#### *2.5. Peptides*

The following 28 18-mer overlapping peptides spanning the NY-ESO-1 protein were synthesized: 18.1 (1-18), 18.2 (7-24), 18.3 (13-30), 18.4 (19-36), 18.5 (25-42), 18.6 (31-48), 18.7 (37-54), 18.8 (43-60), 18.9 (49-66), 18.10 (55-72), 18.11 (61-78), 18.12 (67-84), 18.13 (73-90), 18.14 (79-96), 18.15 (85-102),

18.16 (91-108), 18.17 (97-114), 18.18 (103-120), 18.19 (109-126), 18.20 (115-132), 18.21 (121-138), 18.22 (127-144), 18.23 (133-150), 18.24 (139-156), 18.25 (145-162), 18.26 (149-166), 18.27 (153-170) and 18.28 (156-173). A 30-mer NY-ESO-1 151-180 peptide was also synthesized. These peptides were synthesized using standard solid-phase methods based on N-(9-fluorenyl)-methoxycarbonyl (Fmoc) chemistry on a Multiple Peptide Synthesizer (AMS422, ABIMED, Langenfeld, Germany) at Okayama University (Okayama, Japan).

## *2.6. Recombinant proteins*

Recombinant NY-ESO-1 [18] and RL-Akt [19] proteins were prepared as described previously. cDNAs for NY-ESO-1 and RL-Akt were cloned into Sph I/Sal I and Bam HI/Sph I sites of the pQE30 vector (Qiagen Science, Valencia, CA), respectively. N-His tagged protein was purified by nickel-ion affinity chromatography under denaturing conditions.

## *2.7. Total RNA isolation and cDNA synthesis*

Total RNA was isolated from cell pellets using an RNeasy Mini Kit (QIAGEN,



Hilden, Germany). Isolated RNA (2 µg) was reverse transcribed into single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase (Ready-To-Go You-Prime First-Strand Beads, GE Healthcare, Little Chalfont, UK) and oligo (dT)<sub>15</sub> as a primer. cDNA was tested for integrity by amplification of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene.

#### *2.8. Reverse transcription (RT)-PCR analysis*

To amplify the *NY-ESO-1* cDNA segment, primers specific for *NY-ESO-1* were designed as described previously [20]. Primers for RT-PCR were: *NY-ESO-1* 5'-AGTTCTACCTCGCCATGCCT-3' (forward), 5'-TCCTCCTCCAGCGACAAACAA-3' (reverse), *GAPDH* 5'-ACCACAGTCCATGCCATCAC-3' (forward), 5'-TCCACCACCCTGTTGCTGTA-3' (reverse). The amplification program for *NY-ESO-1* was 1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C for 35 cycles after denaturing at 94°C for 1 min. These cycles were followed by a 10 min elongation step at 72°C. The amplification program for *GAPDH* was 1 min at 94°C, 1 min at 66°C, and 1.5 min at 72°C for 30 cycles after denaturing at 94°C for 1 min. These cycles were followed by a 10 min elongation step at 72°C. The

PCR products were analyzed on a 1.3% agarose gel.

#### *2.9. Preparation of monocyte-derived dendritic cells (DC)*

CD14<sup>+</sup> cells from PBMC were cultured in RPMI medium supplemented with 5% pooled human serum, 10 ng/ml IL-4 and 10 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) for 10 days at 37°C in a 5% CO<sub>2</sub> atmosphere.

#### *2.10. Preparation of tumor cell lysate*

Tumor cells ( $1 \times 10^7$ ) were subjected to five cycles of rapid freezing in liquid N<sub>2</sub> followed by thawing in a water bath at 37°C.

#### *2.11. In vitro stimulation of CD4 T cells*

Frozen cells were thawed and re-suspended in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 5% pooled human serum (CM), and kept at 37°C in a 5% CO<sub>2</sub> atmosphere for 2 hrs. CD4 T cells ( $2 \times 10^6$ ) were stimulated with 28 18-mer overlapping peptides and a 30-mer C-terminal peptide (OLPs) (1 µg/ml for each peptide) spanning the entire NY-ESO-1 using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC ( $2 \times 10^6$ ) as antigen-presenting cells

(APC) in 2 ml of CM supplemented with 20 units/ml rIL-2 (Takeda Chemical Industries, Osaka, Japan) and 10 ng/ml rIL-7 (Peprotech, London, UK) in a 24-well culture plate at 37°C in a 5% CO<sub>2</sub> atmosphere for 12 days. For the second stimulation, 1 x 10<sup>6</sup> instead of 2 x 10<sup>6</sup> responder cells were used in the culture described above.

#### *2.12. IFN $\gamma$ secretion assay*

Responder CD4 T cells (1 x 10<sup>5</sup>) were stimulated for 4 hrs with para-formaldehyde (PFA) (Wako, Osaka, Japan) (0.2%)-treated autologous EBV-B cells (1 x 10<sup>5</sup>) pre-pulsed with the peptides for 30 min. The cells were then washed and suspended in 100  $\mu$ l of RPMI medium, and treated with bi-specific CD45 and the IFN $\gamma$  antibody (Miltenyi Biotec) (2  $\mu$ l) for 5 min on ice. The cells were then diluted in AIM-V medium (1-10 ml) and placed on a slowly rotating device (Miltenyi Biotec) to allow IFN $\gamma$  secretion at 37°C in a 5% CO<sub>2</sub> atmosphere. After incubation for 50 min, the cells were washed with cold buffer and treated with PE-conjugated anti-IFN $\gamma$ , and FITC-conjugated anti-CD4 mAb. After incubation for 10 min at 4°C, the cells were washed and analyzed by FACS Calibur (Becton Dickinson).

### *2.13. Peptide pulse and treatment with protein or tumor lysate on APC*

EBV-B cells were pulsed with peptides for 3 hrs or treated with protein for 24 hrs in 24-well culture plate at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells were then fixed by PFA (0.2%) and used for stimulating CD4 T cells in the assay. EBV-B cells or DC pretreated with tumor cell lysate (equivalent to  $1 \times 10^7$  cells) for 24 hrs were also used as APC for stimulation.

### *2.14. ELISA*

Supernatants from cultures of CD4 T cells ( $1 \times 10^4$ ) stimulated with EBV-B cells ( $1 \times 10^4$ ) pre-pulsed with peptide or pretreated with protein, or with EBV-B cells or DC pretreated with cell lysate were collected and the amounts of IFN $\gamma$ , TNF $\alpha$ , IL-4 or IL-10 were estimated by sandwich ELISA. For antibody blocking, each purified mAb (5  $\mu$ g/ml) was added to the assay culture.

### *2.15. Flow cytometry*

EBV-B cells were cultured with rNY-ESO-1 protein, CHP-NY-ESO-1 or CHP-MAGE at 10  $\mu$ g/ml of a protein concentration in a 24-well culture plate for

18 hrs at 37°C in a 5% CO<sub>2</sub> atmosphere. After incubation, the cells were permeabilized and washed twice with buffer (Fixation/Permeabilization kit, Becton Dickinson). The cells were then stained with anti-NY-ESO-1 mAb (E978) or an isotype control (5 µg/ml) and FITC-conjugated mouse anti-IgG. FcR blocking reagent (Miltenyi Biotec) was used for blocking nonspecific binding. The stained cells were detected by FACS Calibur. Flow cytometry results were analyzed with FlowJo (Tree Star, Ashland, OR).

#### *2.16. Tetramer staining*

NY-ESO-1 87-100 peptide/HLA-DRB1\*0901 tetramer was prepared. The method used was described previously [21]. CD4 T cells were incubated with tetramer for 2 hrs at 37°C in a 5% CO<sub>2</sub> atmosphere. FITC-conjugated anti-CD4 mAb was added at the end of tetramer staining and incubated for an additional 20 min at 4°C. After incubation, the cells were washed twice and analyzed by FACS Calibur.

#### *2.17. Confocal microscopy*

EBV-B cells were cultured with rNY-ESO-1 protein (10 µg/ml) in a Lab-tek

chamber slide (Nunk, Rochester, NY) at 37°C in a 5% CO<sub>2</sub> atmosphere for 18 hrs. After incubation, the cells on the slide were fixed with PFA (4%) and permeabilized with Triton X-100 (0.25%) (Katayama, Osaka, Japan). The cells were then stained with a biotin-conjugated mouse anti-NY-ESO-1 mAb (clone UTK-ESO4) and FITC-conjugated streptavidin (Biomeda Corporation, Foster, CA). For intracellular localization, rhodamine conjugated wheat germ agglutinin (WGA) (Vector Laboratories, Burlingame, CA), PE-conjugated mouse anti-CD107a (LAMP-1) mAb (EXBIO, Praha, Czech Republic) or Lysotracker Red DND-99 (Invitrogen Molecular Probes, Eugene, OR) were used. The stained cells were visualized under a confocal microscope (LSM 510, Carl Zeiss, Jena, Germany).

### **3. Results**

#### *3.1. Recognition of two dominant peptide regions in NY-ESO-1 by CD4 T cells*

In a recent clinical trial, we used CHP-NY-ESO-1 to immunize patients with an NY-ESO-1-expressing tumor. By analyzing responses of T cells in PBMC from patients using 28 18-mer overlapping peptides and a 30-mer peptide of the

C-terminus spanning the entire NY-ESO-1 protein (OLPs), we showed that CD4 and CD8 T cells recognized the two dominant regions in the NY-ESO-1 molecule [11]. These include region 73-114 consisting of 5 peptides: 73-90, 79-96, 85-102, 91-108 and 97-114 and region 115-144 consisting of 3 peptides: 115-132, 121-138 and 127-144. As shown in Fig. 1, CD4 T cells frequently recognized 18-mer peptides 79-96, 85-102 and 91-108 in region 73-114, and peptides 121-138 and 127-144 in region 115-144 in patients with different HLA types. Within 11 patients shown in Fig. 1, CD4 T cell response in 3 patients was investigated in this study. Patient E-2 showed strong CD4 T cell response even before vaccination. Patients E-8 and P-4 showed only marginal CD4 T cell response before vaccination and showed marked increase after 4 vaccinations [11, 17].

### *3.2. NY-ESO-1 124-134 peptide recognition by E-2 CD4 T cells restricted to DRB1\*0803*

We determined the restriction molecule and the minimal NY-ESO-1 epitope recognized by E-2 CD4 T cells. MACS bead-purified CD4 T cells from E-2 PBMC obtained after vaccinations were stimulated in culture for 12 days with a

mixture of OLPs using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC as APC. On the 26th day, after stimulation twice, the responding cells were harvested and stimulated with individual NY-ESO-1 OLPs using autologous CD4- and CD8-depleted PBMC as APC for 18 hrs, and assayed for IFN $\gamma$  secretion by ELISA for the culture supernatant. As shown in Fig. 2A, CD4 T cells secreted IFN $\gamma$  strongly in response to only the 18-mer peptide 121-138 (peptide 21). The cells were then expanded by stimulation with PHA and the restriction molecule for E-2 bulk CD4 T cell recognition of peptide 121-138 was determined. As shown in Fig. 2B, the addition of anti-HLA class II and anti-DR, but not anti-HLA class I, anti-DP or anti-DQ, to the assay culture blocked IFN $\gamma$  secretion. As shown in Fig. 2C, stimulation of E-2 bulk CD4 T cells with peptide 121-138 using a panel of various EBV-B cells with different DR types as APC suggested that recognition was restricted to DRB1\*0803.

The minimal epitope peptide in the peptide 121-138 recognized by E-2 bulk CD4 T cells was determined using various truncated peptides that deleted N- and C-termini amino acids. As shown in Fig. 2D, deletion of 5 amino acids from either the N- or C-terminus of peptide 121-138 greatly reduced IFN $\gamma$  secretion from CD4 T cells. Additional results with peptides truncated at both the N- and



C-termini suggested that 124-134 was the minimal epitope peptide recognized by E-2 bulk CD4 T cells.

### *3.3. NY-ESO-1 95-107 peptide recognition by E-8 CD4 T cells restricted to DQB1\*0401*

Next, we determined the restriction molecule and minimal epitope in the peptide 91-108 in another dominant region recognized by E-8 CD4 T cells in PBMC obtained after vaccinations (Fig. 1). The response of E-8 CD4 T cells to peptide 121-138 was likely due to the presence of DR4 on E-8 cells, which has been shown to present the peptide 121-130 [22] or 119-138 [23]. MACS bead-purified E-8 CD4 T cells were stimulated twice with peptide 91-110 using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC as APC. To obtain stably responding CD4 T cells, IFN $\gamma$ -producing cells were cloned by limiting dilution in 96-well plates. Strong IFN $\gamma$  production was observed in 24/96 (25%) wells. Within those, the A1 clone proliferated well and was analyzed for peptide recognition against individual OLPs. As shown in Fig. 3A, E-8 A1 CD4 T cells recognized only the peptide 91-108 (peptide 16). Antibody blocking and stimulation of E-8 A1 CD4 T cells with peptide 91-110 using a panel of various

EBV-B cells with different HLA class II alleles as APC suggested that the recognition was restricted to DQB1\*0401 (Fig. 3B and C). Analysis using a series of truncated peptides as described above suggested that the 13-mer peptide 95-107 was the minimal epitope (Fig. 3D).

*3.4. Subdominant CD4 T cell response against NY-ESO-1 87-100 restricted to DRB1\*0901 in patient P-4 who showed a dominant response against NY-ESO-1 121-138*

We then examined whether there was any subdominant CD4 T cell response in patient P-4, who showed a dominant CD4 T cell response against the peptide 121-138, using the PBMC obtained after vaccinations (Fig. 1). The dominant response of P-4 CD4 T cells was explained by the presence of DRB1\*1502 and \*0901 on P-4 cells, both of which were shown to be capable of presenting epitopes derived from the peptide 122-138 [24].

MACS bead-purified P-4 CD4 T cells were stimulated twice with a mixture of OLPs using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC as APC in 96-well plates. Cultures were then split in duplicate and stimulated with autologous EBV-B cells pre-pulsed with a mixture of OLPs or peptide 121-138

and responses were examined by IFN $\gamma$  ELISA. As shown in Fig. 4A, responses to both a mixture of OLPs and peptide 121-138 were observed in 7/40 (17.5%) wells. However, a response to a mixture of OLPs, but not peptide 121-138, was observed in 3/40 (7.5%) wells. The P-4 C3 clone reacting against a mixture of OLPs, but not peptide 121-138, could be expanded by PHA stimulation and its recognition of individual OLPs was examined. As shown in Fig. 4B and C, P-4 C3 cells recognized the peptide 85-102 (peptide 15) and the recognition was restricted to DRB1\*0901. Analysis using truncated peptides suggested that the minimal epitope peptide recognized by P-4 C3 CD4 T cells was 87-100 and the peptide was recognized at the nanomolar level (Fig. 4D). Initial attempt for producing HLA class II tetramer based on the results obtained in this study was made for NY-ESO-1 87-100 peptide/HLA-DRB1\*0901 tetramer. As shown in Fig. 4E, P-4 C3 CD4 T cells were stained. There was no similarity in the sequence between peptide 87-100 (LLEFYLAMPEATPM) defined in this study and peptide 122-138 (LLKEFTVSGNILTIRLT) defined previously, both of which presented on the same DRB1\*0901 (Fig. 5).

### *3.5. Natural processing of the epitope peptides*

To examine whether the NY-ESO-1 124-134, NY-ESO-1 95-107 and NY-ESO-1 87-100 epitopes defined in this study were naturally processed peptides, we examined E-2 bulk, E-8 A1 and P-4 C3 CD4 T cell recognition of EBV-B cells that were treated with NY-ESO-1 protein. Treatment of EBV-B cells with recombinant NY-ESO-1 protein resulted in efficient endocytosis by confocal microscopy using anti-NY-ESO-1 mAb and WGA, LAMP-1 or LysoTracker (Fig. 6A). Endocytosis was blocked by addition of cytochalasin B. Treatment of EBV-B cells with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1) and NY-ESO-1 protein alone resulted in introduction of NY-ESO-1 protein into EBV-B cells by staining with anti-NY-ESO-1 mAb after fixation (Fig. 6B). E-2 bulk, E-8 A1 and P-4 C3 CD4 T cells secreted IFN $\gamma$  in response to EBV-B cells introduced with NY-ESO-1 protein, but not control protein (Fig. 7A). E-2 bulk CD4 T cell recognition of NY-ESO-1 protein-treated EBV-B cells was blocked by anti-DR and restricted to DRB1\*0803 as the recognition of NY-ESO-1 124-134 peptide (data not shown).

We further examined CD4 T cell recognition of tumor cell-derived NY-ESO-1 antigen processed and presented by autologous EBV-B cells, or autologous or HLA class II-matched allogeneic DC using NY-ESO-1-expressing tumor lysates.

As shown in Fig 7B, E-2 bulk, E-8 A1 and P-4 C3 CD4 T cells all recognized lysate of the NY-ESO-1-expressing lung cancer cell line OU-LC-KI, but not lysate of control OU-LC-OK.

### *3.6. Cytokines secreted from E-2 bulk, E-8 A1 and P-4 C3 CD4 T cells*

Cytokines secreted from E-2 bulk, E-8 A1 and P-4 C3 CD4 T cells in stimulation with peptides 121-138, 91-110, and 85-102, respectively, were examined by ELISA. As shown in Fig. 7C, E-2 bulk, E-8 A1 and P-4 C3 CD4 T cells secreted IFN $\gamma$  and TNF $\alpha$ , but not IL-4 or IL-10. The results suggested that the CD4 T cells induced after vaccination were the Th1-type.

## **4. Discussion**

In this study, we identified 3 novel NY-ESO-1 CD4 T cell epitopes using PBMC obtained from patients who were immunized with CHP-NY-ESO-1. The restriction molecules were determined by antibody blocking and using various EBV-B cells with different HLA alleles as APC to present peptides for CD4 T cells. The minimal epitope peptides were determined using various peptides truncated at

both the N- and C-termini that were deduced from 18-mer overlapping peptides originally identified for recognition. These epitopes were NY-ESO-1 87-100 bound to DRB1\*0901, NY-ESO-1 95-107 bound to DQB1\*0401 and NY-ESO-1 124-134 bound to DRB1\*0803. The frequency of DRB1\*0901 (13.76%), DQB1\*0401 (12.98%) and DRB1\*0803 (8.29%) alleles is quite high in the Japanese population. CD4 T cells used to determine these epitope peptides recognized EBV-B cells or DC that were treated with recombinant NY-ESO-1 protein or NY-ESO-1-expressing tumor cell lysate, suggesting that the epitope peptides are naturally processed. These CD4 T cells showed a cytokine profile with Th1 characteristics. Furthermore, staining of corresponding CD4 T cells was observed with NY-ESO-1 87-100 peptide/HLA-DRB1\*0901 tetramer which was produced as an initial attempt based on the findings obtained in this study.

Recent analyses of the immune responses of patients vaccinated with NY-ESO-1 protein showed that most of the responses of either CD4 or CD8 T cells were directed against two dominant regions in the NY-ESO-1 molecule, for which restriction molecules have not been fully elucidated [11, 22-30]. In our study, the analyses were done using 18-mer NY-ESO-1 overlapping peptides and the two regions were region 73-114 consisting of 5 18-mer peptides: 73-90,

79-96, 85-102, 91-108 and 97-114 and region 115-144, consisting of 3 peptides: 115-132, 121-138 and 127-144 [11]. CD4 T cells frequently recognized 18-mer peptides 79-96, 85-102 and 91-108 in region 73-114, and peptides 121-138 and 127-144 in region 115-144 in patients with different HLA types (Fig. 1). The peptide 91-108 was a region also frequently recognized by the antibody [15]. NY-ESO-1 87-100 bound to DRB1\*0901 and NY-ESO-1 95-107 bound to DQB1\*0401 defined in this study are in region 73-114 and NY-ESO-1 124-134 bound to DRB1\*0803 is in region 115-144. Zarour et al. [31] originally identified peptide 119-143 as a promiscuous HLA class II epitope that binds to multiple DR molecules such as DRB1\*0101, DRB1\*0401, DRB1\*0701, DRB1\*1101, DRB1\*1501, DRB3\*0101, DRB4\*0101 and DRB5\*0101 expressed at a high frequency in Caucasian population. The shorter peptide 122-138 was shown to bind to DRB1\*0802, DRB1\*0901, DRB1\*1502 and DRB1\*0405/\*0410, which are common DR alleles in the Japanese population [24]. The promiscuous peptide 119-143 was also shown to contain multiple HLA class I epitopes that bound to A66, A68, Cw3 and Cw15 [32]. Recently, an immunodominant CD4 T cell response against NY-ESO-1 119-143 (core region 123-137) presented by DR52b (DRB3\*0202) was shown in patients vaccinated with the recombinant NY-ESO-1

protein with CpG in Montanide [30]. DR52b is expressed in about half of the Caucasian population and the response was observed in all DR52b-positive patients. In this study, we showed that there was a subdominant response against peptide 87-100 in patient P-4, who showed a dominant response against peptide 121-138 only. This finding further supported the two dominant antigenic regions in the NY-ESO-1 molecule and indicated occurrence of simultaneous multiple CD4 T cell responses. The finding that a rather high frequency of NY-ESO-1-specific CD4 and CD8 T cells were detected in patients analyzed in this study is likely due to the strong immunogenicity of peptide epitopes in the dominant regions. Multiple Th1-type CD4 T cell responses are very beneficial for inducing effective immune responses against the NY-ESO-1 antigen.

Several clinical trials have been reported on NY-ESO-1 protein vaccination. Davis et al. [10] and Nicholaou et al. [33] showed that recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant efficiently induced antibodies, and both CD4 and CD8 T cell responses in malignant melanoma patients. Jäger et al. [14] reported that recombinant vaccinia and fowlpox NY-ESO-1 vaccines preferentially induced a CD8 T cell response. However, Valmori et al. [12] showed that vaccination with NY-ESO-1 protein and CpG in Montanide preferentially induced



a CD4 T cell response. The CHP-NY-ESO-1 vaccine used in our study induced both CD4 and CD8 T cell responses efficiently in most patients as did the NY-ESO-1 ISCOMATRIX vaccine [11]. The findings that either CD4 or CD8 T cells responded to the two dominant regions in the NY-ESO-1 molecule suggested that protein fragments or peptides including those two dominant regions may be substituted for NY-ESO-1 whole protein vaccine and induce efficient CD4 and CD8 T cell responses. A clinical trial using NY-ESO-1 overlapping peptides including the two immunodominant regions is currently ongoing.

### **Acknowledgments**

We thank Ms. J. Mizuuchi for preparation of the manuscript.

## References

- [1] Chen YT, Scanlan MJ, Sahin U, Türeci O, Gure AO, Tsang S, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci USA* 1997; 94: 1914-1918.
- [2] Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 2005; 5: 615-625.
- [3] Gnjjatic S, Nishikawa H, Jungbluth AA, Güre AO, Ritter G, Jäger E, et al. NY-ESO-1: Review of an immunogenic tumor antigen. *Adv Cancer Res* 2006; 95: 1-30.
- [4] Jungbluth AA, Chen YT, Stockert E, Busam KJ, Kolb D, Iversen K, et al. Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. *Int J Cancer* 2001; 92: 856-860.
- [5] Stockert E, Jäger E, Chen YT, Scanlan MJ, Gout I, Karbach J, et al. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med* 1998; 187: 1349-1354.

- [6] Jäger E, Stockert E, Zidianakis Z, Chen YT, Karbach J, Jäger D, et al. Humoral immune responses of cancer patients against "Cancer-Testis" antigen NY-ESO-1: correlation with clinical events. *Int J Cancer* 1999; 84: 506-510.
- [7] Gnjatic S, Atanackovic D, Jäger E, Matsuo M, Selvakumar A, Altorki NK, et al. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. *Proc Natl Acad Sci USA* 2003; 100: 8862-8867.
- [8] Jäger E, Nagata Y, Gnjatic S, Wada H, Stockert E, Karbach J, et al. Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. *Proc Natl Acad Sci USA* 2000; 97: 4760-4765.
- [9] Nicholaou T, Ebert L, Davis ID, Robson N, Klein O, Maraskovsky E, et al. Directions in the immune targeting of cancer: lessons learned from the cancer-testis Ag NY-ESO-1. *Immunol Cell Biol* 2006; 84: 303-307.
- [10] Davis ID, Chen W, Jackson H, Parente P, Shackleton M, Hopkins W, et al. Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4 (+) and CD8(+) T cell responses in humans. *Proc Natl Acad Sci USA* 2004; 101: 10697-10702.

- [11]Uenaka A, Wada H, Isobe M, Saika T, Tsuji K, Sato E, et al. T cell immunomonitoring and tumor responses in patients immunized with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein. *Cancer Immun* 2007; 7: 9.
- [12]Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, O'Neill D, et al. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proc Natl Acad Sci USA* 2007; 104: 8947-8952.
- [13]Adams S, O'Neill DW, Nonaka D, Hardin E, Chiriboga L, Siu K, et al. Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. *J Immunol* 2008; 181: 776-784.
- [14]Jäger E, Karbach J, Gnjjatic S, Neumann A, Bender A, Valmori D, et al. Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients. *Proc Natl Acad Sci USA* 2006; 103: 14453-14458.

[15]Kawabata R, Wada H, Isobe M, Saika T, Sato S, Uenaka A, et al. Antibody response against NY-ESO-1 in CHP-NY-ESO-1 vaccinated patients. Int J Cancer 2007; 120: 2178-2184.

[16]Tsuji K, Hamada T, Uenaka A, Wada H, Sato E, Isobe M, et al. Induction of immune response against NY-ESO-1 by CHP-NY-ESO-1 vaccination and immune regulation in a melanoma patient. Cancer Immunol Immunother 2008; 57: 1429-1437.

[17]Wada H, Sato E, Uenaka A, Isobe M, Kawabata R, Nakamura Y, et al. Analysis of peripheral and local anti-tumor immune response in esophageal cancer patients after NY-ESO-1 protein vaccination. Int J Cancer 2008; 123: 2362-2369.

[18]Murphy R, Green S, Ritter G, Cohen L, Ryan D, Woods W, et al. Recombinant NY-ESO-1 cancer antigen: production and purification under cGMP conditions. Prep Biochem Biotechnol 2005; 35: 119-134.

[19]Wada H, Matsuo M, Uenaka A, Shimbara N, Shimizu K, Nakayama E. Rejection antigen peptides on BALB/c RL male 1 leukemia recognized by cytotoxic T lymphocytes: derivation from the normally untranslated 5'-region of

the c-akt proto-oncogene activated by LTR. Cancer Res 1995; 55:

4780-4783.

[20]Kurashige T, Noguchi Y, Saika T, Ono T, Nagata Y, Jungbluth A, et al.

NY-ESO-1 expression and immunogenicity associated with transitional cell

carcinoma: correlation with tumor grade. Cancer Res. 2001; 61:

4671-4674.

[21]Cecconi V, Moro M, Del Mare S, Dellabona P, Casorati G. Use of MHC class

II tetramers to investigate CD4+ T cell responses: problems and solutions.

Cytometry A. 2008; 73: 1010-1018.

[22]Zeng G, Touloukian CE, Wang X, Restifo NP, Rosenberg SA, Wang RF.

Identification of CD4+ T cell epitopes from NY-ESO-1 presented by HLA-DR

molecules. J Immunol 2000; 165: 1153-1159.

[23]Zarour HM, Storkus WJ, Brusic V, Williams E, Kirkwood JM. NY-ESO-1

encodes DRB1\*0401-restricted epitopes recognized by melanoma-reactive

CD4+ T cells. Cancer Res 2000; 60: 4946-4952.

[24]Ohkuri T, Sato M, Abe H, Tsuji K, Yamagishi Y, Ikeda H, et al. Identification

of a novel NY-ESO-1 promiscuous helper epitope presented by multiple

MHC class II molecules found frequently in the Japanese population. *Cancer Sci* 2007; 98: 1092-1098.

[25] Jäger E, Jäger D, Karbach J, Chen YT, Ritter G, Nagata Y, et al. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4\*0101-0103 and recognized by CD4(+) T lymphocytes of patients with NY-ESO-1-expressing melanoma. *J Exp Med* 2000; 191: 625-630.

[26] Gnjatich S, Nagata Y, Jager E, Stockert E, Shankara S, Roberts BL, et al. Strategy for monitoring T cell responses to NY-ESO-1 in patients with any HLA class I allele. *Proc Natl Acad Sci USA* 2000; 97: 10917-10922.

[27] Jäger E, Karbach J, Gnjatich S, Jäger D, Maeurer M, Atmaca A, et al. Identification of a naturally processed NY-ESO-1 peptide recognized by CD8+ T cells in the context of HLA-B51. *Cancer Immun* 2002; 2: 12.

[28] Benlalam H, Linard B, Guilloux Y, Moreau-Aubry A, Derré L, Diez E, et al. Identification of five new HLA-B\*3501-restricted epitopes derived from common melanoma-associated antigens, spontaneously recognized by tumor-infiltrating lymphocytes. *J Immunol* 2003; 171: 6283-6289.

- [29]Mandic M, Castelli F, Janjic B, Almunia C, Andrade P, Gillet D, et al. One NY-ESO-1-derived epitope that promiscuously binds to multiple HLA-DR and HLA-DP4 molecules and stimulates autologous CD4+ T cells from patients with NY-ESO-1-expressing melanoma. *J Immunol* 2005; 174: 1751-1759.
- [30]Bioley G, Dousset C, Yeh A, Dupont B, Bhardwaj N, Mears G, et al. Vaccination with recombinant NY-ESO-1 protein elicits immunodominant HLA-DR52b-restricted CD4+ T cell responses with a conserved T cell receptor repertoire. *Clin Cancer Res* 2009; 15: 4467-4474.
- [31]Zarour HM, Maillere B, Brusic V, Coval K, Williams E, Pouvelle-Moratille S, et al. NY-ESO-1 119-143 is a promiscuous major histocompatibility complex class II T-helper epitope recognized by Th1- and Th2-type tumor-reactive CD4+ T cells. *Cancer Res* 2002; 62: 213-218.
- [32]Matsuzaki J, Qian F, Luescher I, Lele S, Ritter G, Shrikant PA, et al. Recognition of naturally processed and ovarian cancer reactive CD8+ T cell epitopes within a promiscuous HLA class II T-helper region of NY-ESO-1. *Cancer Immunol Immunother* 2008; 57: 1185-1195.



[33]Nicholaou T, Ebert LM, Davis ID, McArthur GA, Jackson H, Dimopoulos N, et al. Regulatory T-cell-mediated attenuation of T-cell responses to the NY-ESO-1 ISCOMATRIX vaccine in patients with advanced malignant melanoma. Clin Cancer Res 2009; 15: 2166-2173.

## Figure Legends

Fig. 1. Patients' HLA class II genotypes and NY-ESO-1 peptides recognized by CD4 T cells. MACS bead-purified CD4 T cells ( $2 \times 10^6$ ) from patients' PBMC were stimulated twice with a mixture of 28 18-mer overlapping peptides and a 30-mer C-terminal peptide spanning the entire NY-ESO-1 protein (OLPs) ( $1 \mu\text{g/ml}$ ) using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC ( $2 \times 10^6$ ) as APC. CD4 T cells ( $1 \times 10^5$ ) from the culture were then assayed for IFN $\gamma$  secretion using FACS Calibur after stimulation for 4 hrs with PFA-treated autologous CD4- and CD8-depleted PBMC ( $1 \times 10^5$ ) pre-pulsed with individual OLPs ( $1 \mu\text{M}$ ). Each bar indicates an 18-mer NY-ESO-1 peptide to which a CD4 T cell response was observed.

Fig. 2. E-2 CD4 T cell recognition of the NY-ESO-1 124-134 peptide in restriction to DRB1\*0803. E-2 CD4 T cells ( $2 \times 10^6$ ) obtained after CHP-NY-ESO-1 vaccinations were stimulated twice with a mixture of OLPs ( $1 \mu\text{g/ml}$ ) using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC ( $2 \times 10^6$ ) as APC. A. E-2 bulk CD4 T cells ( $1 \times 10^4$ ) were then assayed for IFN $\gamma$  secretion after stimulation for 18 hrs with autologous EBV-B cells ( $1 \times 10^4$ ) pre-pulsed with indi-

vidual OLPs (1  $\mu$ M). E-2 CD4 T cells recognized the peptide 21 (121-138). B. Antibody blocking. E-2 bulk CD4 T cells ( $1 \times 10^4$ ) were stimulated for 18 hrs with autologous EBV-B cells ( $1 \times 10^4$ ) pre-pulsed with the peptides 121-138 (1  $\mu$ M) in the presence of anti-HLA class I, anti-HLA class II, anti-HLA-DP, anti-HLA-DQ or anti-HLA-DR mAb (5  $\mu$ g/ml) in the culture. C. E-2 bulk CD4 T cells ( $1 \times 10^4$ ) were stimulated for 18 hrs with peptide 121-138 (1  $\mu$ M) using a panel of various EBV-B cells ( $1 \times 10^4$ ) with different HLA-DR types as APC. D. E-2 bulk CD4 T cells ( $1 \times 10^4$ ) were stimulated for 18 hrs with autologous EBV-B cells ( $1 \times 10^4$ ) pre-pulsed with truncated NY-ESO-1 121-138 peptides. IFN $\gamma$  in the culture supernatants was determined by ELISA.

Fig. 3. E-8 CD4 T cell recognition of the NY-ESO-1 95-107 peptide in restriction to DQB1\*0401. E-8 CD4 T cells ( $2 \times 10^6$ ) obtained after CHP-NY-ESO-1 vaccinations were stimulated twice with the peptide 91-110 (1  $\mu$ g/ml) using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC ( $2 \times 10^6$ ) as APC. Clone A1 was then established by limiting dilution. A. E-8 A1 CD4 T cells ( $1 \times 10^4$ ) were then assayed for IFN $\gamma$  secretion after stimulation for 18 hrs with autologous EBV-B cells ( $1 \times 10^4$ ) pre-pulsed with individual OLPs (1  $\mu$ M). E-8 A1

CD4 T cells recognized the peptide 16 (91-108). B. Antibody blocking. E-8 A1 CD4 T cells ( $1 \times 10^4$ ) were stimulated for 18 hrs with autologous EBV-B cells ( $5 \times 10^3$ ) pre-pulsed with the peptides 91-108 ( $1 \mu\text{M}$ ) in the presence of anti-CD4, anti-CD8, anti-HLA class I or anti-HLA class II mAb ( $5 \mu\text{g/ml}$ ) in the culture. C. E-8 A1 CD4 T cells ( $1 \times 10^4$ ) were stimulated for 4 hrs with the peptide 91-108 ( $1 \mu\text{M}$ ) using a panel of various EBV-B cells ( $1 \times 10^4$ ) with different HLA class II types as APC. D. E-8 A1 CD4 T cells ( $1 \times 10^4$ ) were stimulated for 18 hrs with autologous EBV-B cells ( $1 \times 10^4$ ) pre-pulsed with a series of 15-mer overlapping peptides encompassing peptide 91-110 ( $5 \mu\text{M}$ ) (left). Analysis with truncated 95-107 peptides (right). IFN $\gamma$  in the culture supernatant was determined by ELISA in A, B and D. In C, IFN $\gamma$ -secreting CD4 T cells were determined by an IFN $\gamma$  secretion assay using FACS Calibur.

Fig .4. P-4 CD4 T cell recognition of NY-ESO-1 87-100 peptide in restriction to DRB1\*0901. A. P-4 CD4 T cells ( $5 \times 10^4$ ) obtained after CHP-NY-ESO-1 vaccinations were stimulated twice with a mixture of OLPs using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC ( $5 \times 10^4$ ) as APC in 96-well round-bottomed culture plates. Cultures were then split in duplicate and sti-

mulated for 18 hrs with autologous EBV-B cells ( $5 \times 10^3$ ) pre-pulsed with a mixture of OLPs or peptide 121-138 ( $1 \mu\text{M}$ ). Responses to both a mixture of OLPs and peptide 121-138 were observed in 7/40 wells (\*). Response to a mixture of OLPs, but not peptide 121-138, was observed in 3/40 wells (\*\*). B. P-4 C3 CD4 T cells ( $1 \times 10^4$ ) were stimulated for 18 hrs with autologous EBV-B cells ( $1 \times 10^4$ ) pre-pulsed with individual OLPs ( $1 \mu\text{M}$ ). Recognition of peptide 15 (85-102) was observed. C. P-4 C3 CD4 T cells ( $1 \times 10^4$ ) were stimulated for 18 hrs with autologous EBV-B cells ( $1 \times 10^4$ ) pre-pulsed with peptide 85-102 ( $1 \mu\text{M}$ ) in the presence of anti-CD4, anti-CD8, anti-HLA class I, anti-HLA class II, anti-HLA-DP, anti-HLA-DQ or anti-HLA-DR mAb ( $5 \mu\text{g/ml}$ ) in the culture in antibody blocking (left). P-4 C3 CD4 T cells ( $1 \times 10^4$ ) were stimulated for 18 hrs with peptide 85-102 ( $1 \mu\text{M}$ ) using a panel of various EBV-B cells ( $1 \times 10^4$ ) with different HLA-DR types as APC (right). D. P-4 C3 CD4 T cells ( $1 \times 10^4$ ) were stimulated for 18 hrs with autologous EBV-B cells ( $1 \times 10^4$ ) pre-pulsed with a series of 18-mer overlapping peptides encompassing peptides 79-96, 85-102 and 91-108 ( $125 \text{ nM}$ ) (left). Analysis with peptide 87-100 is shown (right). IFN $\gamma$  in the culture supernatants was determined by ELISA. E. P-4 C3 CD4 T cells were stained with NY-ESO-1 87-100 peptide / HLA-DRB1\*0901 tetramer or control

tetramer and anti-CD4 mAb. Analysis was performed using FACS Calibur.

Fig. 5. Amino acid sequence of NY-ESO-1 protein and HLA class II minimal epitope peptides identified in this study.

Fig.6. Endocytosis of NY-ESO-1 protein by EBV-B cells. A. Fluorescence microscopy. E-8 EBV-B cells were incubated with NY-ESO-1 protein (10 µg/ml) for 18 hrs and analyzed. Staining was performed with anti-NY-ESO-1 mAb (UTK-ESO4) (green), and counter staining was performed with either wheat germ agglutinin (WGA), mouse anti-CD107a mAb (LAMP-1) or LysoTracker (red) (left). Cytochalasin B (20 µM) was added to block endocytosis (right). B. E-2 EBV-B cells were cultured with CHP-NY-ESO-1 protein, NY-ESO-1 protein alone or CHP-MAGE protein (each 10 µg/ml) for 18 hrs and stained with mouse anti-NY-ESO-1 mAb (E978) or an isotype control and FITC-conjugated mouse IgG. Analysis was performed using FACS Calibur.

Fig. 7. Natural processing of epitope peptides recognized by CD4 T cells and cytokine secretion. Stimulation of E-2 bulk, E-8 A1 and P-4 C3 CD4 T cells (1 x

10<sup>4</sup>) with autologous EBV-B cells (1 x 10<sup>4</sup>) treated with NY-ESO-1 protein (10 µg/ml) (A), and with autologous EBV-B cells (1 x 10<sup>4</sup>) and autologous (E-8 A1 and P-4 C3) or HLA class II-matched allogeneic (E-2) DC treated with tumor cell lysates (B, top). In A, RL-Akt protein (10 µg/ml) was used as control protein. In B, bottom, RT-PCR analysis for expression of *NY-ESO-1* mRNA in tumor cell lines, OU-LC-KI and OU-LC-OK is shown. *GAPDH* was used for the internal control. C. E-2 bulk, E-8 A1 and P-4 C3 CD4 T cells were stimulated for 18 hrs with autologous EBV-B cells (1 x 10<sup>4</sup>) pre-pulsed with the respective cognate peptides. IFN $\gamma$ , TNF $\alpha$ , IL-4 and IL-10 in the culture supernatants were detected by ELISA.

Fig. 1



























Patients	DRB1	DQA1	DQB1	DPB1	Peptides recognized by CD4 T cells			
					1	60	120	180
E-1	*0405, *0803		*0401, *0601	*0201, *0402			115-132 	
							121-138 	
E-2	*0803, -	*0103	*0601	*0501, -			121-138 	
E-4	*1501, *0802	*01, *0401	*0602/03, *0402	*0201, *0301		79-96 	 121-138	
						85-102 	 127-144	
E-5	*1502, *1403	*0103,*05	*0601, *0301	*0501, *0901		79- 96 	 121-138	
						85-102 	 127-144	
E-6	*0405, *0803	*0103,*03	*0601, *0302	*0301, *0501			91-108 	
E-7	*0803, *0901		*0601, *0303	*0202, *1401		37-54 	121-138 	
						43-60 	127-144 	
E-8	*0405, *1405		*0401, *0503	*0201, *0501			91-108 	 121-138
P-2	*0802, -		*0402, -	*0501, -			121-138 	
P-3	*0101, *0803		*0501, *0601	*0202, *0301		85-102 	 121-138	
						91-108 		
						97-114 		
P-4	*1502, *0901		*0303, *0601	*0201, *0901			121-138 	
M-1	*1302, *1401	*01, -	*0503, *0604/05/09	*0501, -			121-138 	153-170 



Fig. 2

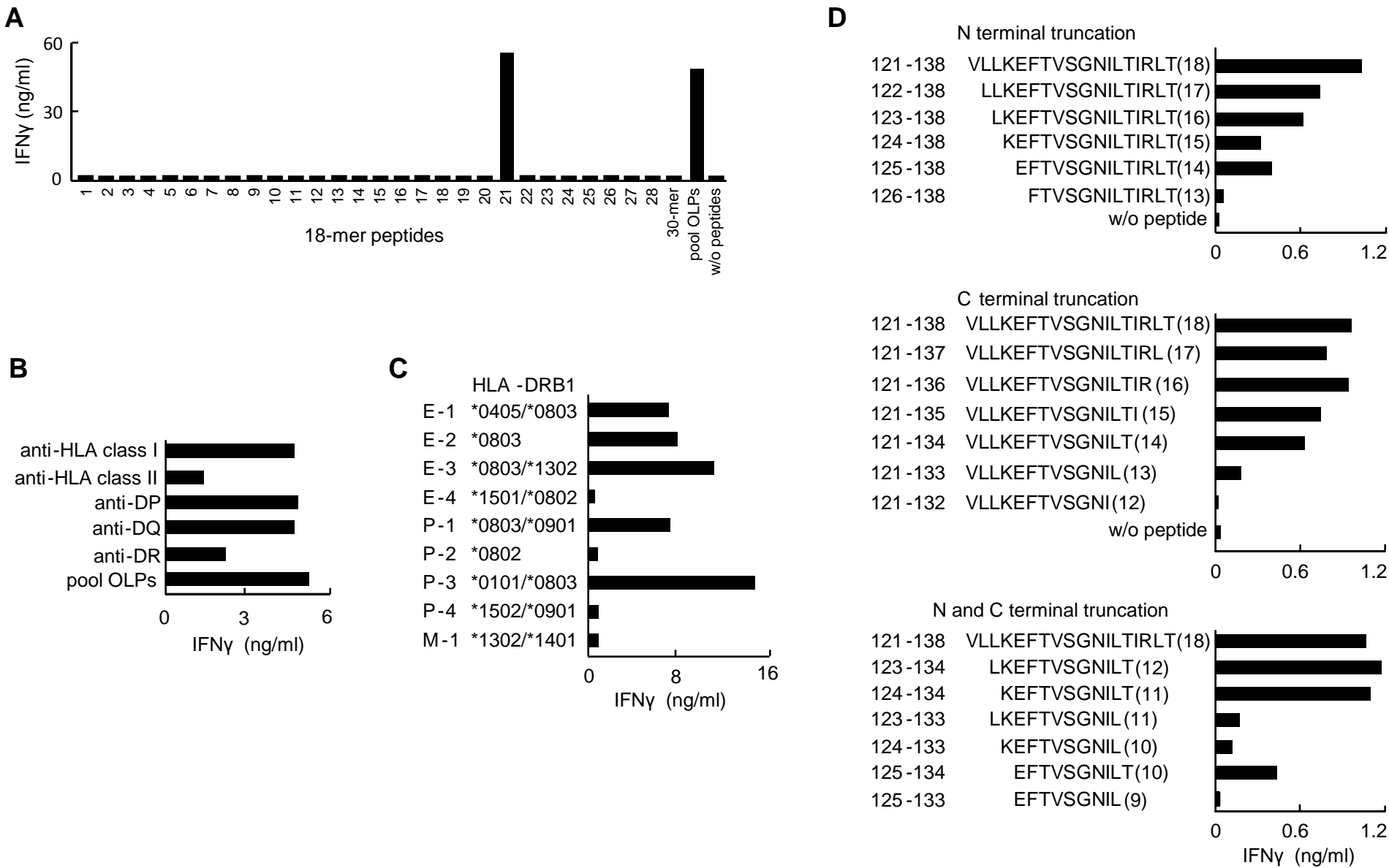


Fig. 3

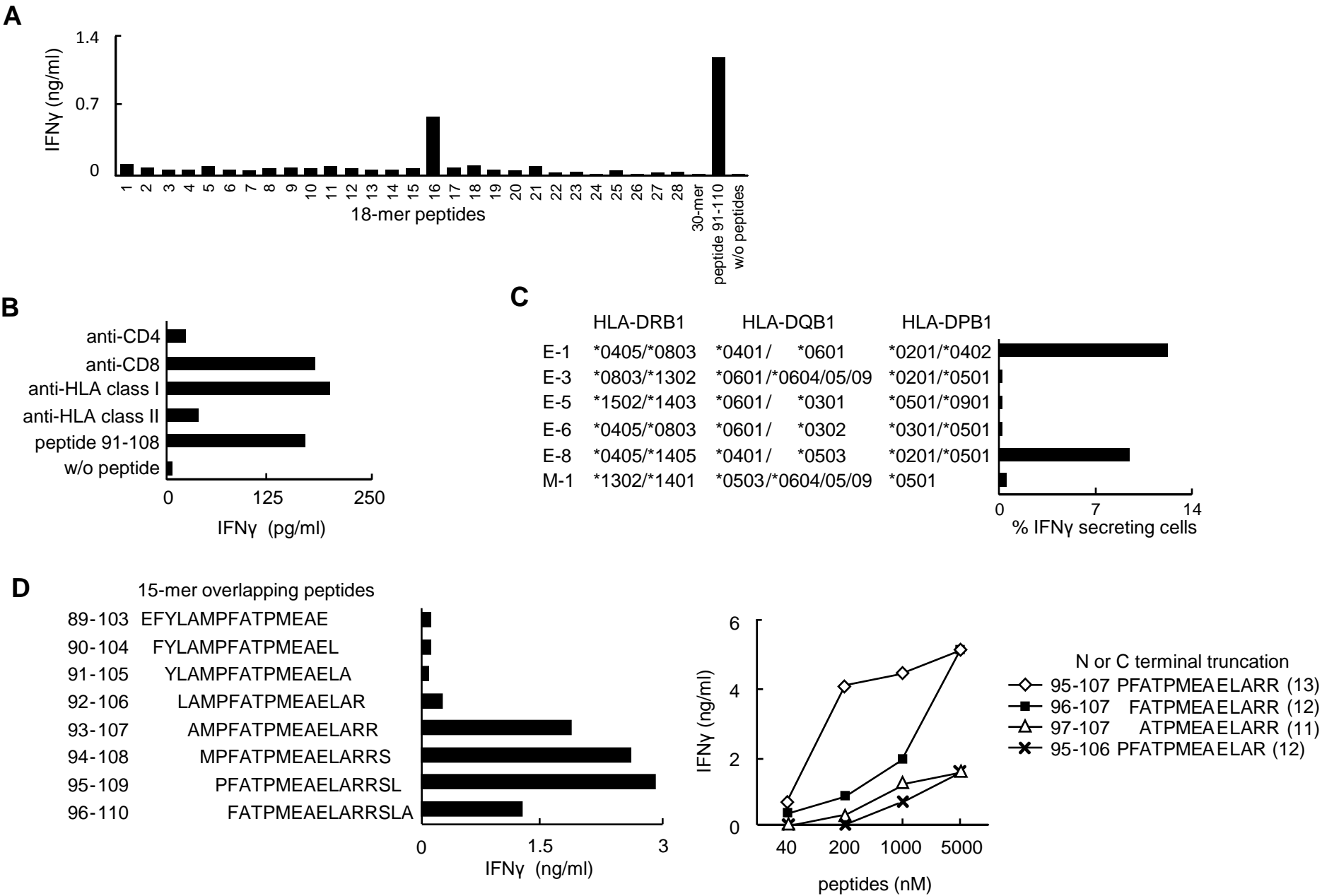


Fig. 4

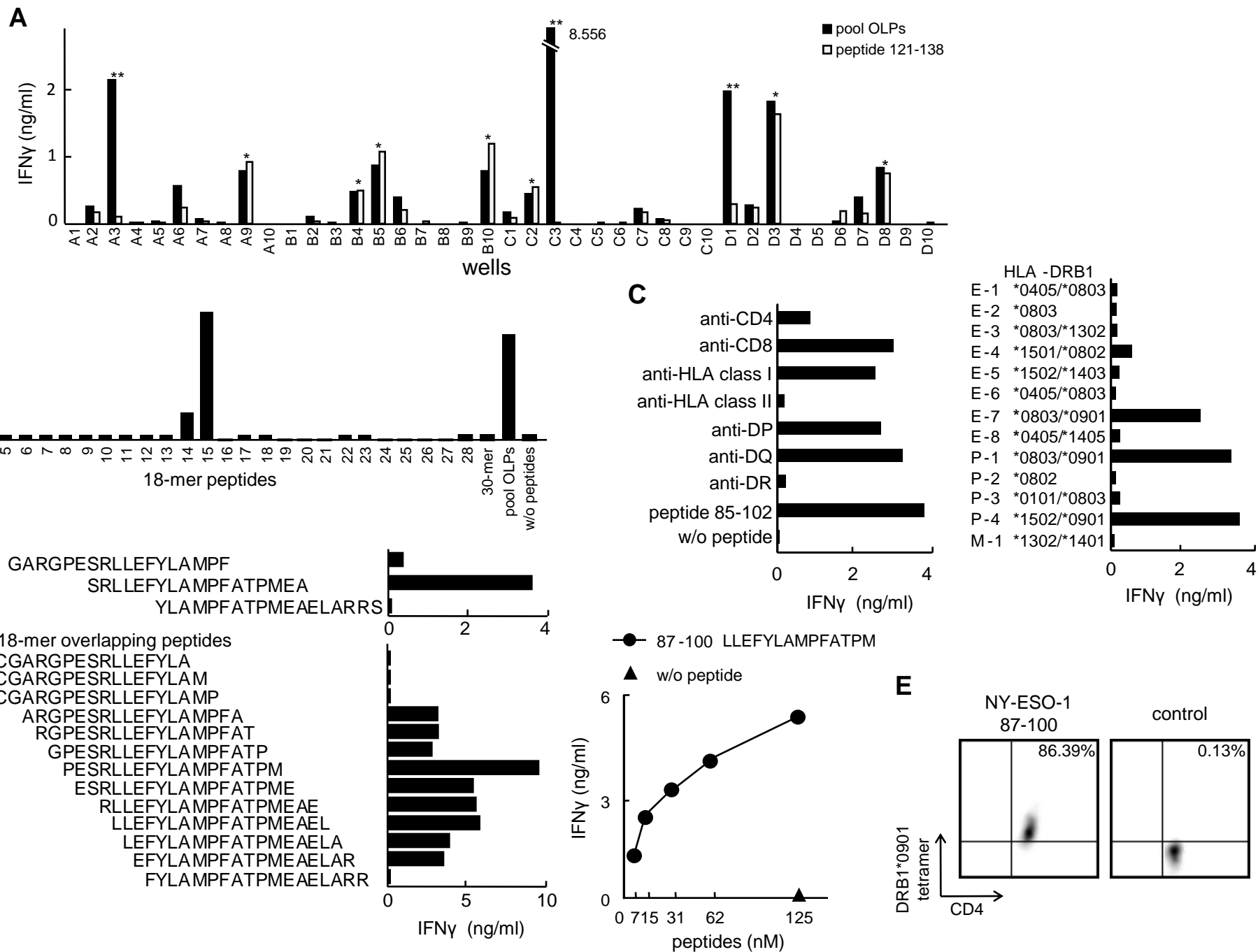


Fig. 5

1 MQAEGRGTTGGSTGDADGPGGPGIPDGPGGNAGGPGEAGATGGRGPRGAGAARASGPGGGA 60

61 PRGPHGGAASGLNGCCRCGARGPESRLLEFYLAMPFATPMEAE LARRSLAQDAPPLPVP 120

E-8 A1 DQB1\*0401 (95-107)

P-4 C3 DRB1\*0901 (87-100)

121 VLLKEFTVSGNILTIRLTAADHRQLQLSISSCLQQLSLLMWITQCF LPVFLAQPPSGQRR 180

E-2 DRB1\*0803 (124-134)

Fig. 6

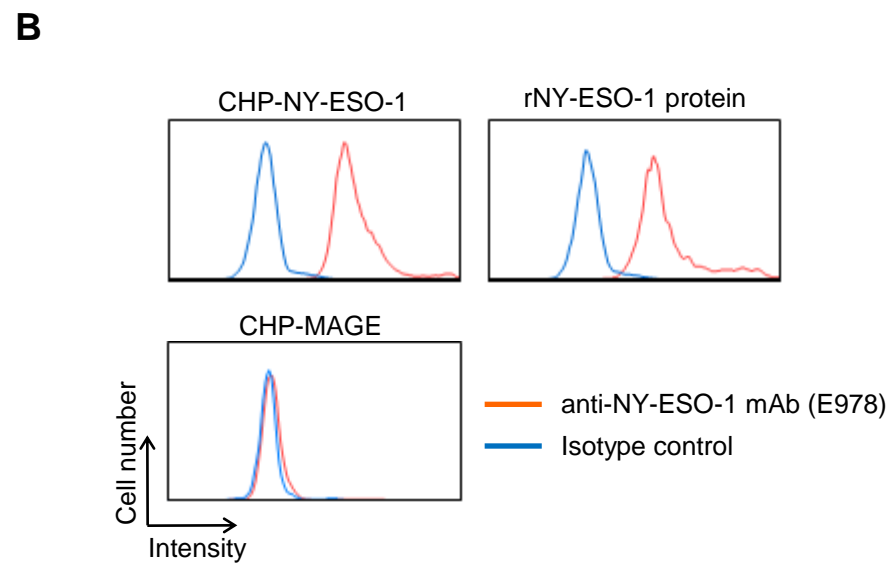
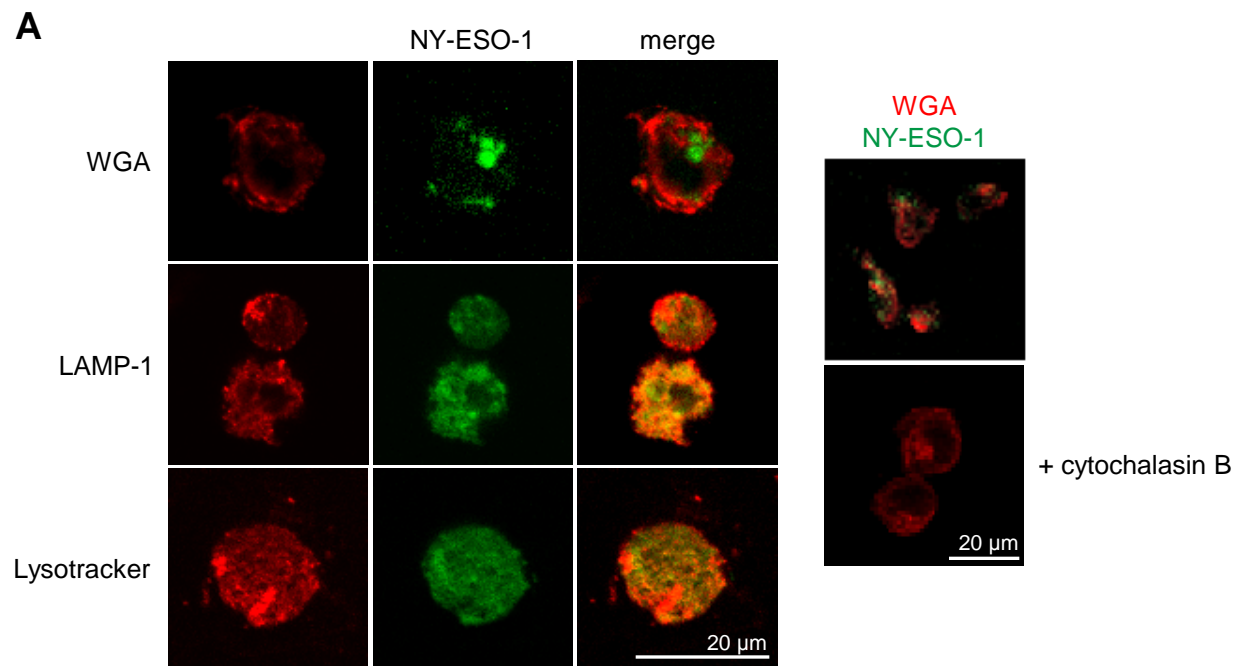
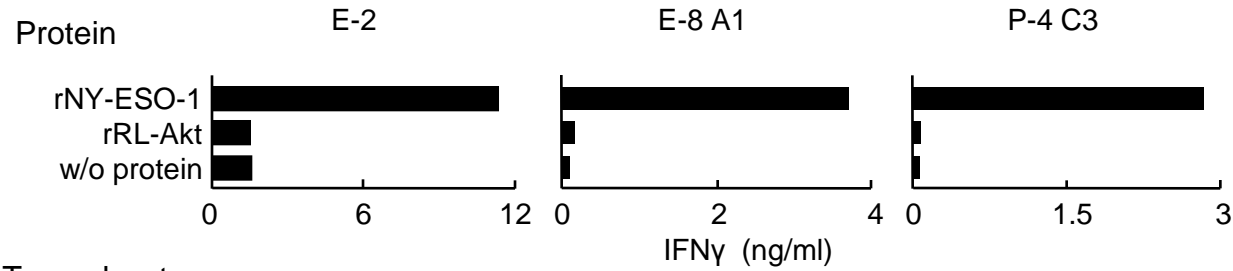
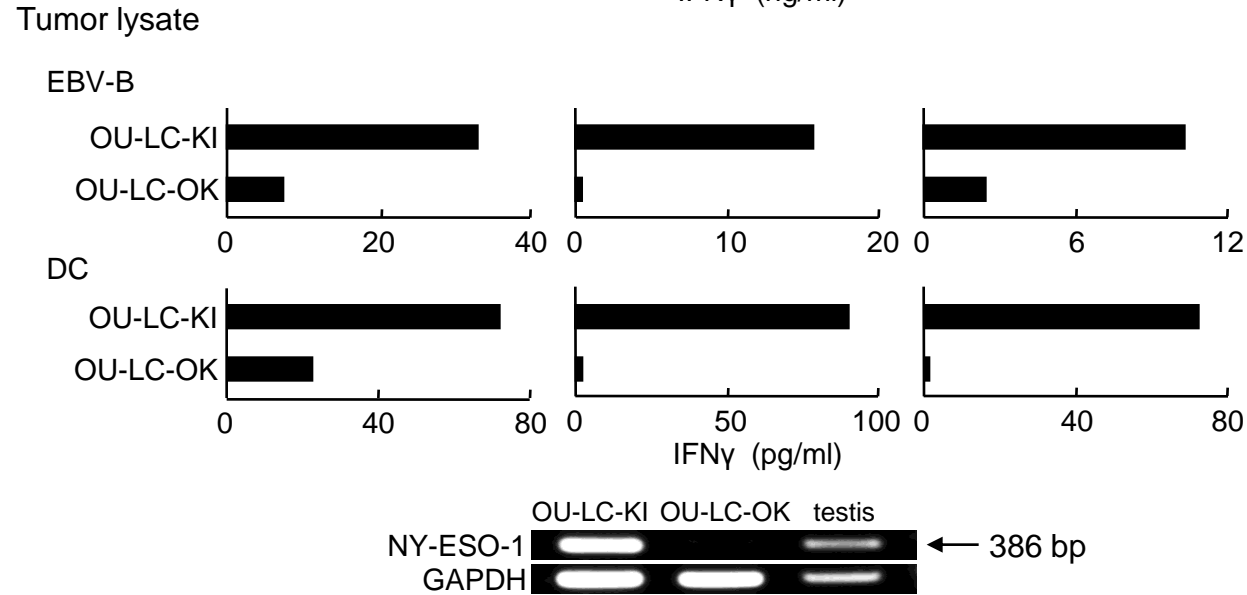


Fig. 7

A



B



C

