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

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

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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, $CD14^+$ cells and $CD19^+$ 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_2 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 IFNγ mAb (1-DIK) was obtained from Mabtech (Stockholm, Sweden). Polyclonal rabbit anti-human IFNγ serum was obtained by immunizing a rabbit with recombinant human IFNγ 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 $CD19^+$ 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_2.

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 $(dT)_{15}$ 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)

$CD14^+$ 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_2 atmospher.

2.10. Preparation of tumor cell lysate

Tumor cells (1×10^7) were subjected to five cycles of rapid freezing in liquid N_2 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_2 atmosphere for 2 hrs. CD4 T cells (2×10^6) 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 (2×10^6) 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_2 atmosphere for 12 days. For the second stimulation, 1 x 10^6 instead of 2 x 10^6 responder cells were used in the culture described above.

2.12. IFNγ secretion assay

Responder CD4 T cells (1 x 10^5) were stimulated for 4 hrs with para-formaldehyde (PFA) (Wako, Osaka, Japan) (0.2%)-treated autologous EBV-B cells (1 x 10^5) pre-pulsed with the peptides for 30 min. The cells were then washed and suspended in 100 µl of RPMI medium, and treated with bi-specific CD45 and the IFNγ antibody (Miltenyi Biotec) (2 µl) 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 IFNγ secretion at 37°C in a 5% CO_2 atmosphere. After incubation for 50 min, the cells were washed with cold buffer and treated with PE-conjugated anti-IFNγ, 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_2 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 x 10^7 cells) for 24 hrs were also used as APC for stimulation.

2.14. ELISA

Supernatants from cultures of CD4 T cells (1 x 10^4) stimulated with EBV-B cells (1 x 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 IFNγ, TNFα, IL-4 or IL-10 were estimated by sandwich ELISA. For antibody blocking, each purified mAb (5 μg/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 μg/ml of a protein concentration in a 24-well culture plate for

18 hrs at 37°C in a 5% CO_2 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_2 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_2 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 IFNγ secretion by ELISA for the culture supernatant. As shown in Fig. 2A, CD4 T cells secreted IFNγ 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 IFNγ 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 IFNγ 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, IFNγ-producing cells were cloned by limiting dilution in 96-well plates. Strong IFNγ 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 IFNγ 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 micrography 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 IFNγ 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 IFNγ and TNFα, 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.

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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 x 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 µg/ml) using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC (2 x 10^6) as APC. CD4 T cells (1 x 10^5) from the culture were then assayed for IFNγ secretion using FACS Calibur after stimulation for 4 hrs with PFA-treated autologous CD4- and CD8-depleted PBMC (1 x 10^5) pre-pulsed with individual OLPs (1 µM). 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 x 10^6) obtained after CHP-NY-ESO-1 vaccinations were stimulated twice with a mixture of OLPs (1 µg/ml) using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC (2 x 10^6) as APC. A. E-2 bulk CD4 T cells (1 x 10^4) were then assayed for IFNγ secretion after stimulation for 18 hrs with autologous EBV-B cells (1 x 10^4) pre-pulsed with indi-

vidual OLPs (1 µM). E-2 CD4 T cells recognized the peptide 21 (121-138). B. Antibody blocking. E-2 bulk CD4 T cells (1 x 10^4) were stimulated for 18 hrs with autologous EBV-B cells (1 x 10^4) pre-pulsed with the peptides 121-138 (1 µM) in the presence of anti-HLA class I, anti-HLA class II, anti-HLA-DP, anti-HLA-DQ or anti-HLA-DR mAb (5 µg/ml) in the culture. C. E-2 bulk CD4 T cells (1 x 10^4) were stimulated for 18 hrs with peptide 121-138 (1 µM) using a panel of various EBV-B cells (1 x 10^4) with different HLA-DR types as APC. D. E-2 bulk CD4 T cells (1 x 10^4) were stimulated for 18 hrs with autologous EBV-B cells (1 x 10^4) pre-pulsed with truncated NY-ESO-1 121-138 peptides. IFNγ 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 x 10^6) obtained after CHP-NY-ESO-1 vaccinations were stimulated twice with the peptide 91-110 (1 µg/ml) using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMC (2 x 10^6) as APC. Clone A1 was then established by limiting dilution. A. E-8 A1 CD4 T cells (1 x 10^4) were then assayed for IFNγ secretion after stimulation for 18 hrs with autologous EBV-B cells (1 x 10^4) pre-pulsed with individual OLPs (1 µM). E-8 A1

CD4 T cells recognized the peptide 16 (91-108). B. Antibody blocking. E-8 A1 CD4 T cells (1 x 10^4) were stimulated for 18 hrs with autologous EBV-B cells (5 x 10^3) pre-pulsed with the peptides 91-108 (1 μM) in the presence of anti-CD4, anti-CD8, anti-HLA class I or anti-HLA class II mAb (5 μg/ml) in the culture. C. E-8 A1 CD4 T cells (1 x 10^4) were stimulated for 4 hrs with the peptide 91-108 (1 μM) using a panel of various EBV-B cells (1 x 10^4) with different HLA class II types as APC. D. E-8 A1 CD4 T cells (1 x 10^4) were stimulated for 18 hrs with autologous EBV-B cells (1 x 10^4) pre-pulsed with a series of 15-mer overlapping peptides encompassing peptide 91-110 (5 μM) (left). Analysis with truncated 95-107 peptides (right). IFNγ in the culture supernatant was determined by ELISA in A, B and D. In C, IFNγ-secreting CD4 T cells were determined by an IFNγ 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 x 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 x 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 x 10^3) pre-pulsed with a mixture of OLPs or peptide 121-138 (1 μM). 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 x 10^4) were stimulated for 18 hrs with autologous EBV-B cells (1 x 10^4) pre-pulsed with individual OLPs (1 μM). Recognition of peptide 15 (85-102) was observed. C. P-4 C3 CD4 T cells (1 x 10^4) were stimulated for 18 hrs with autologous EBV-B cells (1 x 10^4) pre-pulsed with peptide 85-102 (1 μM) 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 μg/ml) in the culture in antibody blocking (left). P-4 C3 CD4 T cells (1 x 10^4) were stimulated for 18 hrs with peptide 85-102 (1 μM) using a panel of various EBV-B cells (1 x 10^4) with different HLA-DR types as APC (right). D. P-4 C3 CD4 T cells (1 x 10^4) were stimulated for 18 hrs with autologous EBV-B cells (1 x 10^4) pre-pulsed with a series of 18-mer overlapping peptides encompassing peptides 79-96, 85-102 and 91-108 (125 nM) (left). Analysis with peptide 87-100 is shown (right). IFNγ 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.

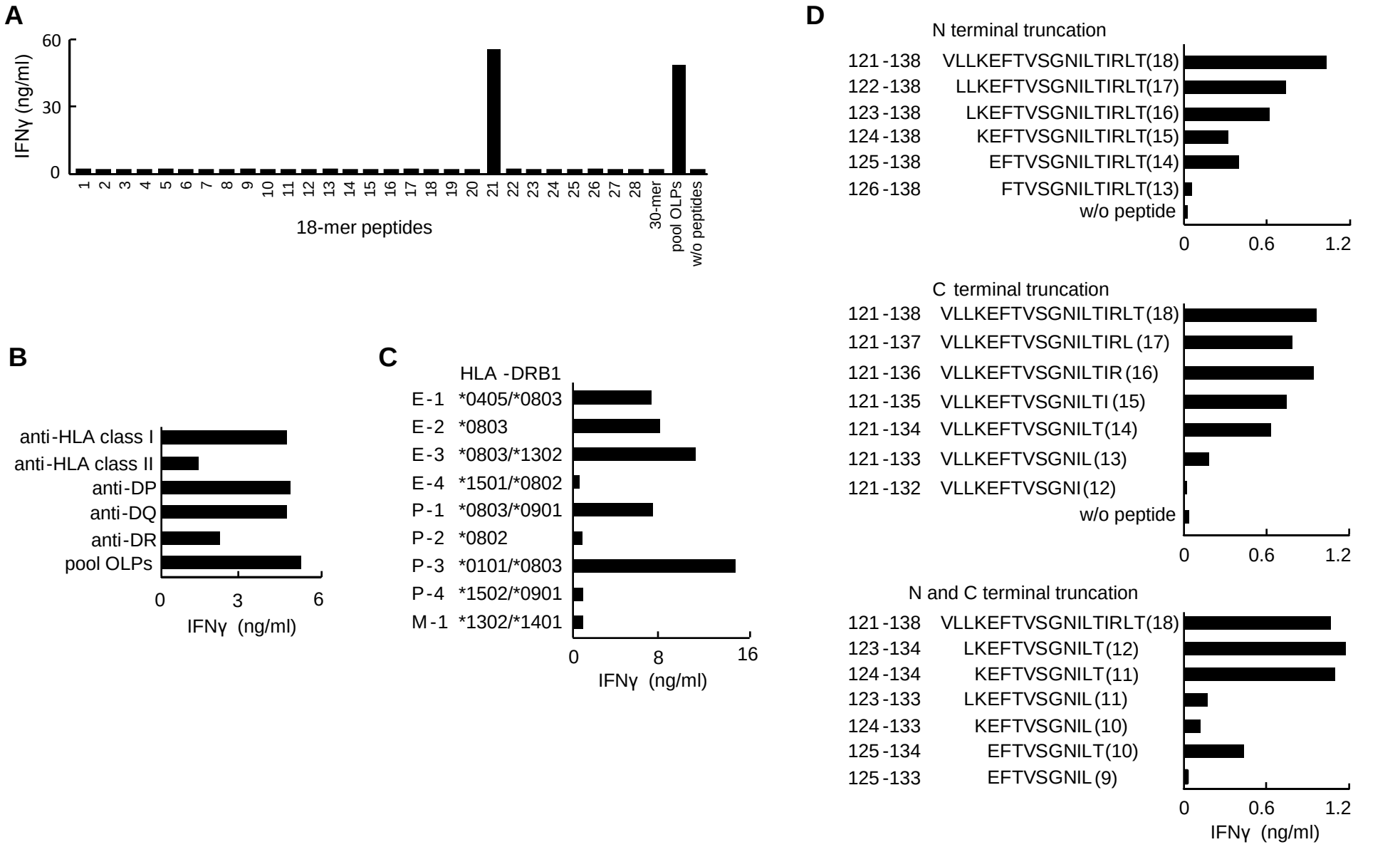
Fig. 7. Natural processing of epitope peptides recognized by CD4 T cells and cytokine secretion. Stimulation of E-2 bulk, E-8 A1 and P-4 C3 CD4 T cells (1 x

10^4) with autologous EBV-B cells (1 x 10^4) treated with NY-ESO-1 protein (10 μg/ml) (A), and with autologous EBV-B cells (1 x 10^4) 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 10^4) pre-pulsed with the respective cognate peptides. IFNγ, TNFα, 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 85-102 121-138 127-144
E-5	*1502, *1403	*0103,*05	*0601, *0301	*0501, *0901	79- 96 85-102 121-138 127-144
E-6	*0405, *0803	*0103,*03	*0601, *0302	*0301, *0501	91-108
E-7	*0803, *0901		*0601, *0303	*0202, *1401	37-54 43-60 121-138 127-144
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 91-108 97-114 121-138
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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IFNγ (ng/ml)
60
30
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 30-mer pool OLPs w/o peptides
18-mer peptides
B
anti-HLA class I
anti-HLA class II
anti-DP
anti-DQ
anti-DR
pool OLPs
0 3 6
IFNγ (ng/ml)
C
HLA -DRB1
E-1 *0405/*0803
E-2 *0803
E-3 *0803/*1302
E-4 *1501/*0802
P-1 *0803/*0901
P-2 *0802
P-3 *0101/*0803
P-4 *1502/*0901
M-1 *1302/*1401
0 8 16
IFNγ (ng/ml)
D
N terminal truncation
121-138 VLLKEFTVSGNILTIRLT(18)
122-138 LLKEFTVSGNILTIRLT(17)
123-138 LKEFTVSGNILTIRLT(16)
124-138 KEFTVSGNILTIRLT(15)
125-138 EFTVSGNILTIRLT(14)
126-138 FTVSGNILTIRLT(13)
w/o peptide
0 0.6 1.2
C terminal truncation
121-138 VLLKEFTVSGNILTIRLT(18)
121-137 VLLKEFTVSGNILTIRL(17)
121-136 VLLKEFTVSGNILTIR(16)
121-135 VLLKEFTVSGNILTI(15)
121-134 VLLKEFTVSGNILT(14)
121-133 VLLKEFTVSGNIL(13)
121-132 VLLKEFTVSGNI(12)
w/o peptide
0 0.6 1.2
N and C terminal truncation
121-138 VLLKEFTVSGNILTIRLT(18)
123-134 LKEFTVSGNILT(12)
124-134 KEFTVSGNILT(11)
123-133 LKEFTVSGNIL(11)
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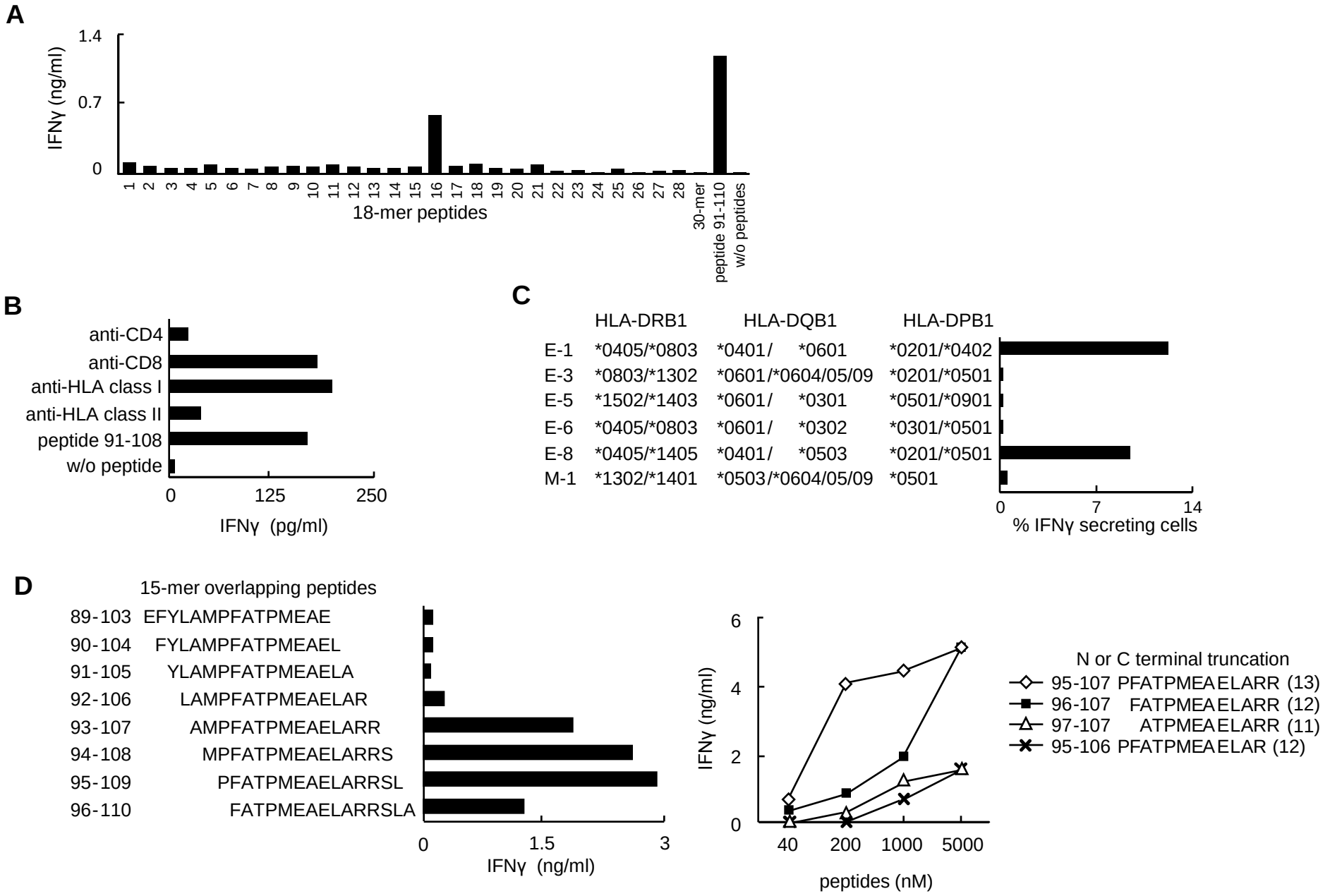
IFNγ (ng/ml)

Fig. 3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0.7
0
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30-mer peptide 91-110 w/o peptides
B
anti-CD4
anti-CD8
anti-HLA class I
anti-HLA class II
peptide 91-108
w/o peptide
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125
250
IFNγ (pg/ml)
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E-5 *1502/*1403 *0601/ *0301 *0501/*0901
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E-8 *0405/*1405 *0401/ *0503 *0201/*0501
M-1 *1302/*1401 *0503/*0604/05/09 *0501
0
7
14
% IFNγ secreting cells
D
15-mer overlapping peptides
89-103 EFYLAMPFATPMEAE
90-104 FYLAMPFATPMEAEL
91-105 YLAMPFATPMEAELA
92-106 LAMPFATPMEAELAR
93-107 AMPFATPMEAELARR
94-108 MPFATPMEAELARRS
95-109 PFATPMEAELARRSL
96-110 FATPMEAELARRSLA
0
1.5
3
IFNγ (ng/ml)
IFNγ (ng/ml)
6
4
2
0
40
200
1000
5000
peptides (nM)
N or C terminal truncation
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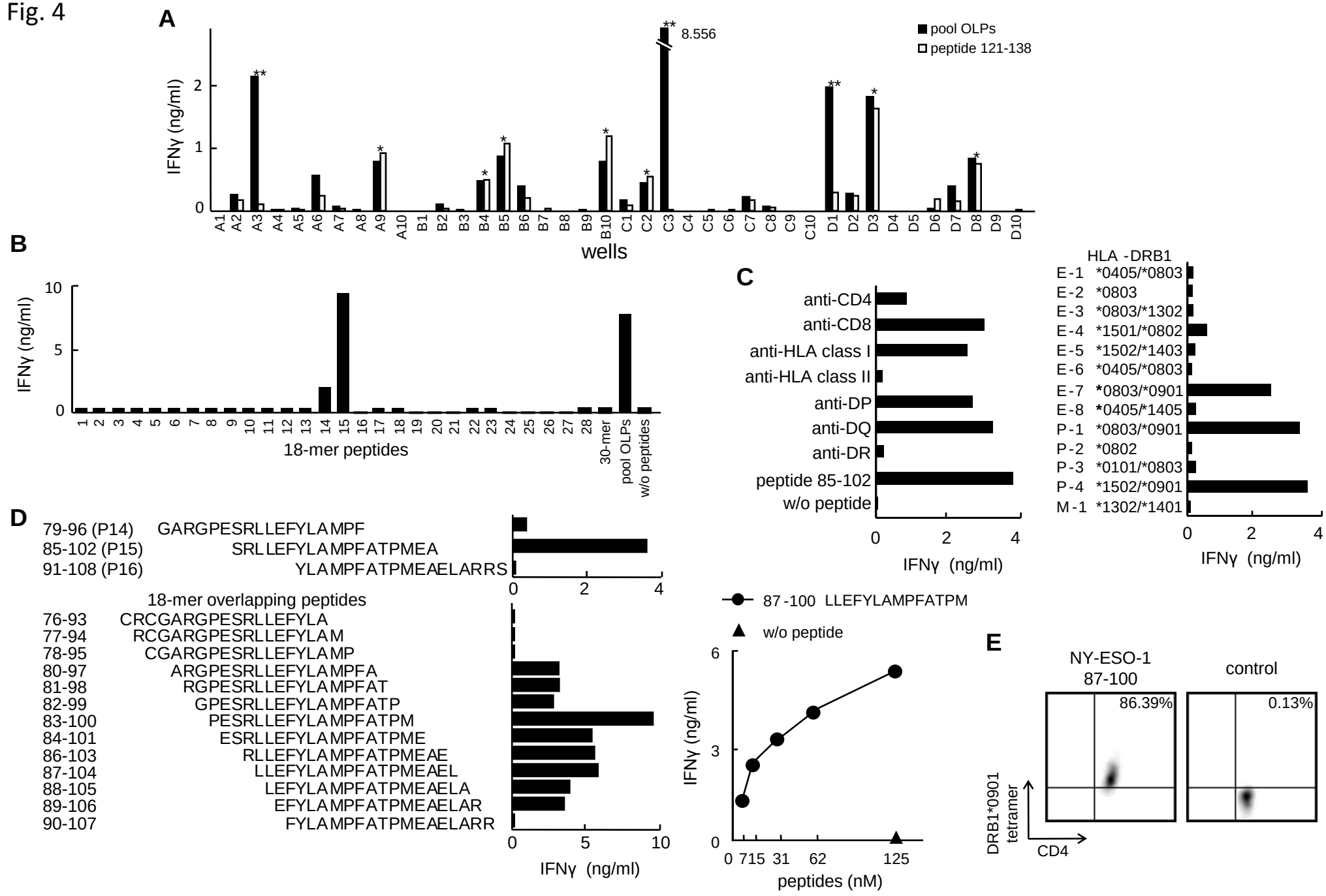
96-107 FATPMEAELARR (12)
97-107 ATPMEAELARR (11)
95-106 PFATPMEAELAR (12)

Fig. 4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IFNγ (ng/ml)
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85-102 (P15) SRLLEFYLAMPFATPMEA
91-108 (P16) YLAMPFATPMEAELARRS
18-mer overlapping peptides
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77-94 RCGARGPESRLLEFYLAM
78-95 CGARGPESRLLEFYLAMP
80-97 ARGPESRLLEFYLAMPFA
81-98 RGPESRLLEFYLAMPFAT
82-99 GPESRLLEFYLAMPFATP
83-100 PESRLLEFYLAMPFATPM
84-101 ESRLLEFYLAMPFATPME
86-103 RLLEFYLAMPFATPMEAE
87-104 LLEFYLAMPFATPMEAEL
88-105 LEFYLAMPFATPMEAELA
89-106 EFYLAMPFATPMEAELAR
90-107 FYLAMPFATPMEAELARR
IFNγ (ng/ml)
87-100 LLEFYLAMPFATPM
w/o peptide
IFNγ (ng/ml)
peptides (nM)
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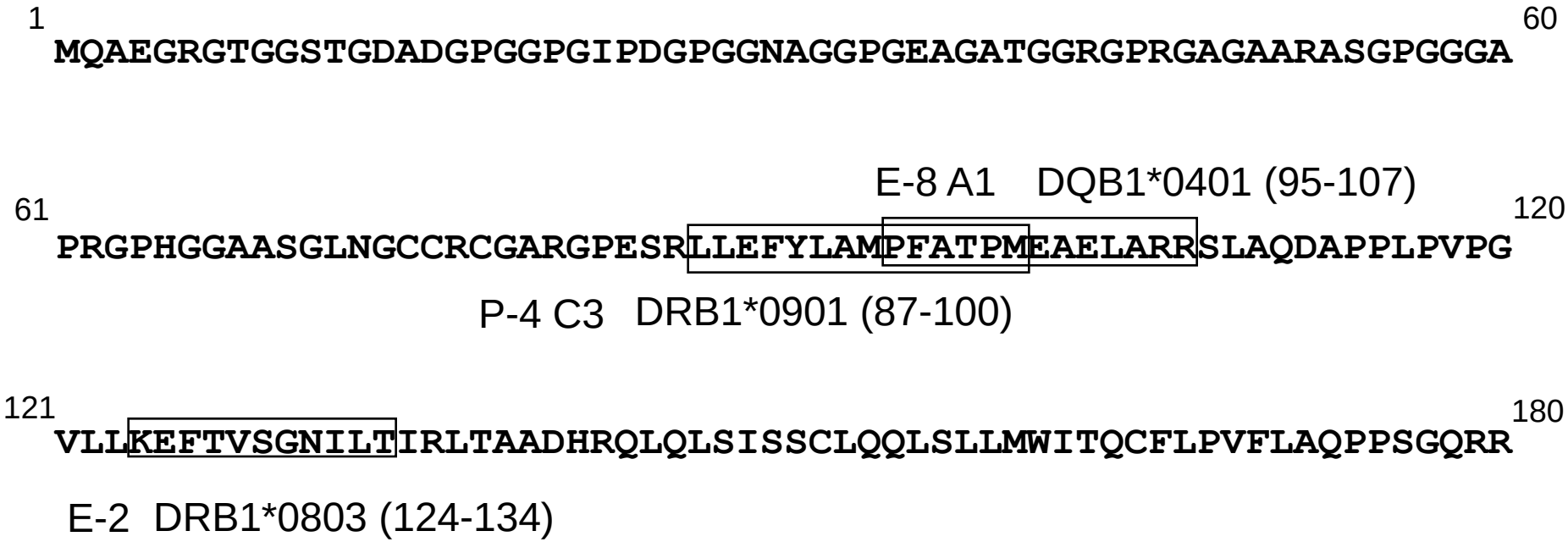
NY-ESO-1
87-100
control
86.39%
0.13%
DRB1*0901
tetramer
CD4

Fig. 5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60
E-8 A1 DQB1*0401 (95-107)
61
PRGPHGGAASGLNGCCRCGARGPESRLLEFYLAMPFATPMEAELARRSLAQDAPPLPVPG
120
P-4 C3 DRB1*0901 (87-100)
121
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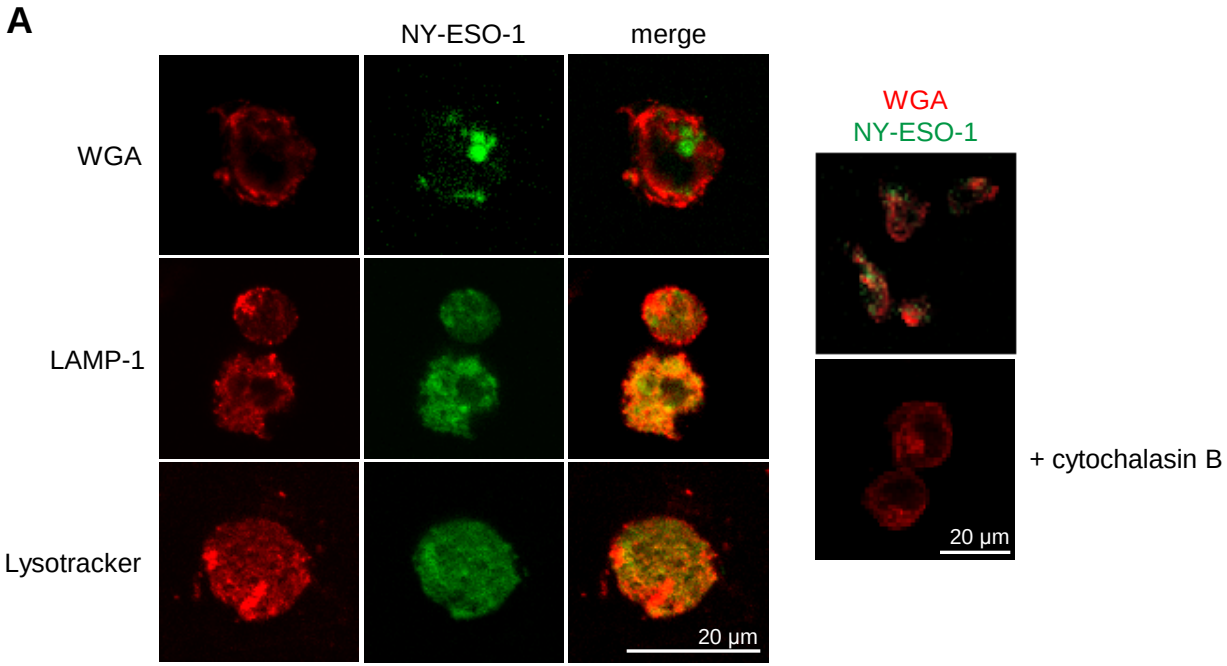
180
E-2 DRB1*0803 (124-134)

Fig. 6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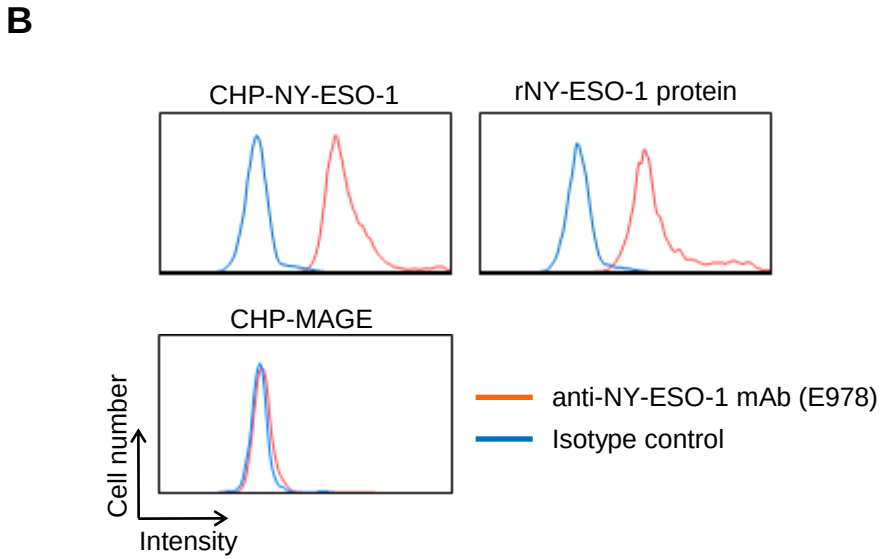
20 μm
+ cytochalasin B


B
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CHP-MAGE
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Intensity
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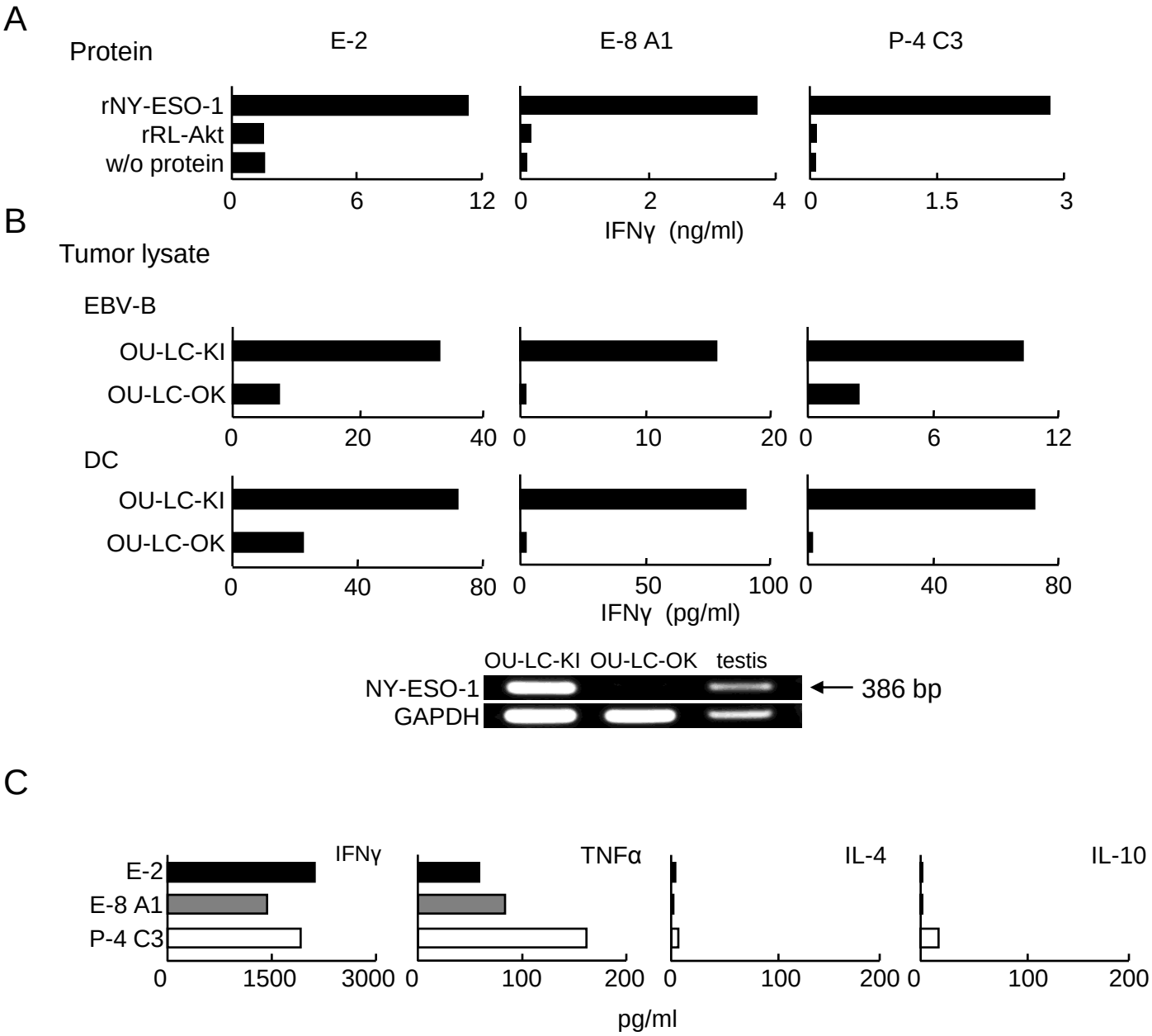
Isotype control

Fig. 7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2
4
0
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3
IFNγ (ng/ml)
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OU-LC-OK
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40
0
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IFNγ (pg/ml)
OU-LC-KI
OU-LC-OK
testis
NY-ESO-1
GAPDH
386 bp
C
IFNγ
TNFα
IL-4
IL-10
E-2
E-8 A1
P-4 C3
0
1500
3000
0
100
200
0
100
200
0
100
200
pg/ml