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Production of NY-ESO-1 peptide/DRB1*08:03 tetramers and ex vivo detection of CD4 T-cell responses in vaccinated cancer patients

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ABSTRACT

We established CD4 T-cell clones, Mz-1B7, and Ue-21, which recognized the NY-ESO-1 121–138 peptide from peripheral blood mononuclear cells (PBMCs) of an esophageal cancer patient, E-2, immunized with an NY-ESO-1 protein and determined the NY-ESO-1 minimal epitopes. Minimal peptides recognized by Mz-1B7 and Ue-21 were NY-ESO-1 125–134 and 124–134, respectively, both in restriction to DRB1*08:03. Using a longer peptide, 122–135, and five other related peptides, including either of the minimal epitopes recognized by the CD4 T-cell clones, we investigated the free peptide/DR recognition on autologous EBV-B cells as APC and peptide/DR tetramer binding. The results showed a discrepancy between them. The tetramers with several peptides recognized by either Mz-1B7 or the Ue-21 CD4 T-cell clone did not bind to the respective clone. On the other hand, unexpected binding of the tetramer with the peptide not recognized by CD4 T-cells was observed. The clone Mz-1B7 did not recognize the free peptide 122–135 on APC, but the peptide 122–135/DRB1*08:03 tetramer bound to the TCR on those cells. The failure of tetramer production and the unexpected tetramer binding could be due to a subtly modified structure of the peptide/DR tetramer from the structure of the free peptide/DR molecule. We also demonstrated that the NY-ESO-1 123–135/DRB1*08:03 tetramer detected ex vivo CD4 T-cell responses in PBMCs from patients after NY-ESO-1 vaccination in immunomonitoring.

1. Introduction

To analyze T-cell immunomonitoring after vaccination, peptide/MHC tetramers have become widely used [1]. Peptide/MHC tetramers identified and visualized antigen specific T-cells. MHC class I tetramers were originally developed by Altman and Davis [2], and used for various antigens including those of viral or tumor origin [3,4]. However, MHC class II tetramers have been used in only a few studies because of the difficulty in preparation [5]. The soluble form of MHC class II molecules is necessary to produce tetramers. However, production of such molecules using extracellular domains of MHC class II α and β chains is generally difficult because of a lack of assembly or aggregation [6]. These findings indicate the necessity of transmembrane regions for the proper assembly of the molecules. Kalandadze et al. [7] found that replacement of the hydrophobic transmembrane regions by the Fos and Jun leucine zipper dimerization motifs resulted in the assembly and secretion of DRαβ heterodimers in yeast. Novak et al. [8] developed MHC class II tetramers using DR molecules incorporating leucin zipper motifs to stabilize the DRα and β heterodimer. The procedure has been widely used, but successful production of MHC class II tetramers is still limited [9–13].

We recently analyzed CD4 T-cell responses against NY-ESO-1 in PBMCs from patients who were vaccinated with a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 protein (CHP-NY-ESO-1) in our clinical trial and determined three novel NY-ESO-1 CD4 T-cell epitopes: NY-ESO-1 87–100 bound to DRB1*09:01, NY-ESO-1 95–107 bound to DQB1*04:01, and NY-ESO-1 124–134 bound to DRB1*08:03 [14]. CD4 T-cells that

Abbreviations: APC, antigen-presenting cell; CHP-NY-ESO-1, complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 whole protein; Fmoc, *N*-(9-fluorenyl)-methoxycarbonyl; HD, healthy donor; MFI, mean fluorescence intensity; OLP, overlapping peptide; PBMC, peripheral blood mononuclear cell.

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recognized these epitope peptides also recognized EBV-B cells or DC that were treated with recombinant NY-ESO-1 protein or an NY-ESO-1-expressing tumor cell lysate, suggesting that the epitope peptides are naturally processed. These CD4 T-cells had a cytokine profile with Th1 characteristics.

In this study, we showed that tetramers with several peptides recognized by the CD4 T-cell clones did not bind to the same clones. On the other hand, unexpected binding of the tetramer with a peptide not recognized by CD4 T-cells was observed. The failure of tetramer production and the unexpected tetramer binding could be due to a subtly modified structure of the peptide/DR tetramer from the structure of the free peptide/DR molecule. We also demonstrated that the NY-ESO-1 123–135/DRB1*08:03 tetramer detected ex vivo CD4 T-cell responses in PBMCs from patients after NY-ESO-1 vaccination in immunomonitoring.

2. Materials and methods

2.1. Patients and blood samples

Peripheral blood samples were drawn from esophageal cancer patients E-1 and E-2, and a prostate cancer patient P-3, who were vaccinated with CHP-NY-ESO-1, and a lung cancer patient TK-OLP-01, who was vaccinated with NY-ESO-1 OLP in our clinical trials [15,16] after obtaining written informed consent. PBMCs were isolated by density gradient centrifugation using Histopaque 1077 (Sigma–Aldrich, St. Louis, MO). CD4 T-cells and $CD19^+$ cells were purified from PBMCs using CD4 and CD19 microbeads, respectively, using a large scale column and a magnetic device (Miltenyi Biotec, Auburn, CA). The cells were stored in liquid N_2 until use. HLA typing was done using PBMCs with a sequence-specific oligo-nucleotide probe and sequence-specific priming of genomic DNA using standard procedures. Patient E-2 was found to possess homozygous alleles.

2.2. Peptides

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).

2.3. Cell lines

E-2 bulk CD4 T-cells were stimulated in vitro twice as described previously [14]. Clones were then established by limiting dilution. EBV-B cells were generated from $CD19^+$ peripheral blood B cells using the culture supernatant from EBV-producing B95-8 cells.

2.4. Generation of HLA-DRB1*08:03 tetramers

HLA-DR tetramers were prepared as described previously [5]. The cDNA coding for the extracellular domains of the HLA-DRα chain was inserted by fusion PCR in a basic leucine zipper and His tag. The HLA-DRβ chain was fused with an acidic leucine zipper and the BirA substrate peptide for BirA enzyme-dependent biotinylation. The HLA-DRα and HLA-DRβ chimeric cDNA were cloned into the pcDNA3.1 vector, respectively. The expression vectors containing the HLA-DRα and HLA-DRβ chains were co-transfected into CHO cells.

2.5. ELISA

Supernatants (100 μl) from cultures of CD4 T-cells (5×10^3) stimulated for 18 h with autologous EBV-B cells (5×10^3) pre-pulsed for 30 min with peptide in a 96-well round bottomed culture plate, or with solid-phase peptide/HLA-DRB1*08:03 tetramers in a 96-well flat bottomed culture plate, were collected and the amounts of IFNγ were estimated by sandwich ELISA [14]. TNFα, IL-4, IL-10 and IL-17A in the culture supernatants were estimated by DuoSet Sandwich ELISAs (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

2.6. Flow cytometry

FITC-conjugated anti-human TCRαβ mAb (BD), PerCP Cy5.5-conjugated anti-human CD3 mAb and APC-conjugated anti-human CD4 mAb (eBioscience, San Diego, CA) were used for T-cell surface staining. The stained cells were detected by FACS Canto II (BD). Flow cytometry results were analyzed with FlowJo (Tree Star, Ashland, OR).

2.7. Tetramer staining

CD4 T-cells were incubated with tetramers for 1 h at 37 °C in a 5% CO_2 atmosphere. FITC-conjugated anti-human CD4 mAb (Miltenyi Biotec) was added at the end of tetramer staining and incubated for an additional 20 min at 4 °C.

2.8. IFNγ capture assay

The method has been described previously [14].

2.9. TCR Vβ and CDR3 sequence analysis

For TCR Vβ analysis, the IOTest Beta Mark kit (Beckman Coulter, Brea, CA) was used. The CDR3 sequence was determined by PCR as described previously [17].

3. Results

3.1. Determination of NY-ESO-1 minimal epitopes recognized by CD4 T-cell clones Mz-1B7 and Ue-21 established from PBMCs of an esophageal cancer patient E-2 immunized with CHP-NY-ESO-1

We established CD4 T-cell clones from PBMCs of an esophageal cancer patient E-2 immunized with CHP-NY-ESO-1 which recognized the 18-mer NY-ESO-1 121–138 peptide. The CD4 T-cell clones Mz-1B7 and Ue-21 produced IFNγ, TNFα, but not IL-4, IL-10 or IL-17A (Supplementary Fig. 1), indicating that they have Th1 characteristics. We determined restriction molecules by antibody blocking and minimal epitopes using various N- and C-termini truncated peptides. Assays were done by ELISA examining IFNγ in the culture supernatant from responding T-cells using autologous EBV-B cells as antigen-presenting cells (APC). As shown in Fig. 1A, recognition of the 18-mer NY-ESO-1 121–138 by CD4 T-cell clones Mz-1B7 and Ue-21 was inhibited by addition of anti-HLA-DR mAb, but not anti-HLA-DQ mAb. Since patient E-2 possessed homozygous haplotypes (DRB1*08:03, DQA1*01:03, DQB1*06:01, DPB1*05:01) according to genetic analysis (see Section 2), the two clones Mz-1B7 and Ue-21 recognized the NY-ESO-1 peptide 121–138 in restriction to DRB1*08:03.

We then investigated recognition of various N- and C-termini truncated peptides and found that a core peptide region recognized by either clone Mz-1B7 or clone Ue-21 was made up of amino acids 125–134 (Fig. 1B). Further analysis revealed that a minimal peptide recognized by clone Mz-1B7 was peptide 125–134 (10-mer) and that recognized by clone Ue-21 was peptide 124–134 (11-mer) (Fig. 1C). Thus, clones Mz-1B7 and Ue-21 recognized