In vitro Stimulation of CD8 and CD4 T Cells by Dendritic Cells Loaded with a Complex of Cholesterol-Bearing Hydrophobized Pullulan and NY-ESO-1 Protein: Identification of a New HLA-DR15–Binding CD4 T-Cell Epitope

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Abstract Purpose: NY-ESO-1 belongs to a class of cancer/testis antigens and has been shown to be immunogenic in cancer patients. We synthesized a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 protein (CHP/ESO) and investigated the in vitro stimulation of CD8 and CD4 T cells from peripheral blood mononuclear cells in healthy donors with autologous CHP/ESO-loaded dendritic cells as antigen-presenting cells.

Experimental Design: In vitro stimulation of CD8 or CD4 T cells was determined by IFN-γ ELISPOT assays against autologous EBV-B cells infected with vaccinia/NY-ESO-1 recombinant virus or wild-type vaccinia virus as targets and by ELISA measuring secreted IFN-γ.

Results: NY-ESO-1–specific CD8 and CD4 T cells were induced. In a donor expressing HLA-A2, CD8 T cells stimulated with CHP/ESO-loaded dendritic cells recognized naturally processed NY-ESO-1157–165, an HLA-A2–binding CD8 T cell epitope. NY-ESO-1 CD4 T cells were Th1-type. We identified a new HLA-DR15–binding CD4 T-cell epitope, NY-ESO-1137–50.

Conclusions: These findings indicate that CHP/ESO is a promising polyvalent cancer vaccine targeting NY-ESO-1.

NY-ESO-1 was originally identified by serologic expression cloning (SEREX) using serum from a patient with esophageal cancer, and exhibited characteristics of cancer/testis antigens whose expression is observed in various types of cancer and germ tissues such as testis, ovary, and placenta, but not in normal somatic tissues (1). The antibody response to NY-ESO-1 is much more frequent than other human tumor antigens in patients with cancer (2), and CD8 T cell response to NY-ESO-1 is mostly associated with the humoral immune response to NY-ESO-1 (3, 4), suggesting that NY-ESO-1 is a highly immunogenic human tumor antigen.

CD8 T cell epitopes from NY-ESO-1 have been identified for several MHC class I alleles such as HLA-A*0201 (5), A*2402 (6), and A*3101 (7). The CD8 T cell response was found to be polyclonal in patients with NY-ESO-1 antibody (8). Simultaneous HLA-A2- and HLA-Cw3–restricted CD8 T cell responses were elicited in a malignant melanoma patient with NY-ESO-1 antibody (8). CD4 T cell responses to NY-ESO-1 have been described and many epitopes binding to several HLA-DR alleles have been identified, including a promiscuous one (9–12). HLA-DP4–restricted CD4 T cell response was correlated with antibody response to NY-ESO-1 in patients with cancer (13, 14). Those studies showed that NY-ESO-1 contains multiple T cell epitopes and that polyvalent vaccine containing full-length protein, rather than monovalent vaccine using peptides, is likely to evoke strong immunity against NY-ESO-1, and to induce anticancer effects on tumors expressing NY-ESO-1. However, exogenous protein antigens hardly sensitize CD8 T cells because they are internalized into endosomes and processed mainly by the class II pathway. Immature dendritic cells (DC) were reported to differentiate into mature DCs after phagocytosis of NY-ESO-1/IgG immune complex, and was shown to present an HLA-A2–binding CD8 T cell epitope (15). NY-ESO-1 formulated with ISCOMATRIX, a 40 nm cage-like structure composed of saponin, phospholipids, and cholesterol was taken up by DCs, which cross-presented a CD8 T cell epitope (16). Polyclonal CD8- and CD4-T cell responses were observed in...
patients immunized with NY-ESO-1/ISCOMATRIX (17). These findings indicated that exogenous protein antigens could be cross-presented and sensitize CD8 T cells when appropriate delivery systems were used.

Cholesterol-bearing hydrophobized pullulan (CHP) is composed of a pullulan backbone and cholesterol branches, and forms colloidal stable nanoparticles in water by self-association because of the formation of cross-linking domains through the interaction of the cholesterol branches. CHP nanoparticles form complexes with soluble protein (18). Immunization of mice with HER2 protein complexed with CHP (CHP/HER2) induced antibody and CD8 T cell responses to HER2 (19). DCs treated with CHP/HER2 cross-presented the antigenic peptide, HER2<sub>A-E</sub>, on HLA-A24 (20). In the present study, we synthesized a complex of CHP and NY-ESO-1 protein (CHP/ESO) and successfully induced NY-ESO-1-specific CD8 and CD4 T cells by autologous CHP/ESO-loaded DCs. In addition, we identified a new HLA-DR15-binding CD4 T cell epitope from NY-ESO-1.

**Materials and Methods**

**Cell lines.** SK-MEL-37 is a melanoma cell line expressing NY-ESO-1 mRNA and HLA-A*0201. SK-MEL-23 is a melanoma cell line expressing HLA-A*0201, but not NY-ESO-1 mRNA.

**Antibodies.** Anti-human CD4, anti-human CD8, anti-HLA class I, anti-HLA class II, anti-DP, anti-DQ, and anti-DR monoclonal antibodies (mAb) were purchased from BD Bioscience (San Jose, CA). Anti-human IFN<sub>γ</sub> mAb (1-DIK) was obtained from Mabtech (Stockholm, Sweden). Rabbit anti-human IFN<sub>γ</sub> serum was obtained by immunizing a rabbit with recombinant human IFN<sub>γ</sub> protein. Goat anti-rabbit IgG serum conjugated with alkaline-phosphatase and goat anti-rabbit IgG serum conjugated with horseradish peroxidase were purchased from Southern Biotechnology (Birmingham, AL) and MBL (Nagoya, Japan), respectively.

**Cytokines.** Human recombinant granulocyte macrophage colony-stimulating factor and interleukin (IL)-2 were kindly provided by Kirin (Tokyo, Japan) and Takeda (Osaka, Japan) pharmaceutical companies, respectively. Human recombinant IL-4 was purchased from Peprotech (London, England). Human recombinant IFN<sub>γ</sub> was obtained from R&F (London, England).

**Peptides.** All peptides were synthesized by standard solid-phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry and a peptide synthesizer (AMS422; ABIMED, Langenfeld, Germany).

**Recombinant proteins and CHP/ESO complex.** Recombinant NY-ESO-1 and SSX2 proteins were prepared as described previously (1, 2). The CHP/ESO complex was synthesized as described previously (18).

**Preparation of monocyte-derived DCs.** Blood was drawn from healthy donors under informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation with Ficoll-Paque (Sigma-Aldrich, St. Louis, MO) and CD14<sup>+</sup> cells were purified using antibody-coated magnetic beads (Miltenyi Biotec, Auburn, CA). CD14<sup>+</sup> cells (5 × 10<sup>5</sup>) were cultured in six-well plates in the medium supplemented with 10 ng/mL IL-4 and 10 ng/mL granulocyte macrophage colony-stimulating factor for 7 days at 37°C in a 5% CO<sub>2</sub> atmosphere. The medium used was RPMI 1640 with 5% human AB sera, 2 mmol/L l-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1% nonessential amino acids. Flow cytometric analysis showed that these DCs displayed an immature phenotype (CD80<sub>low</sub>, CD83<sup>+</sup>, and CD86<sub>low</sub>). We designated these cells DCs. For the maturation of DCs, 100 ng/mL of lipopolysaccharide (Sigma-Aldrich) was added with NY-ESO-1 (1 μg/mL) or SSX2 protein (1 μg/mL) to the culture on day 6. The treated DCs exhibited a mature phenotype (CD80<sub>high</sub>, CD83<sub>low</sub>, and CD86<sub>high</sub>). We designated these cells mature DCs.

**Incubation of monocyte-derived DCs with CHP/ESO complex.** DCs (5 × 10<sup>5</sup>) were cultured with CHP/ESO (20 μg/mL CHP and 1 μg/mL NY-ESO-1 protein) for 3 hours at 37°C in a 5% CO<sub>2</sub> atmosphere. After incubation, DCs were washed with RPMI 1640 and cultured in the medium supplemented with IL-4 and granulocyte macrophage colony-stimulating factor for 18 hours at 37°C in a 5% CO<sub>2</sub> atmosphere and then used as stimulators.

**In vitro stimulation of CD8 and CD4 T cells with DCs.** CD8 and CD4 T cells were purified from PBMC using antibody-coated magnetic beads (Miltenyi Biotec, Auburn, CA). Two million purified T cells were cocultured with 5 × 10<sup>4</sup> DCs loaded with CHP/ESO, CHP, or recombinant NY-ESO-1 protein for 7 days. The responding cells were stimulated twice or thrice at weekly intervals and cultured in medium supplemented with 10 units/mL IL-2.

**Infection of EBV-B cells with recombinant vaccinia virus.** For target cells, EBV-B cells (1 × 10<sup>5</sup>) were infected with vaccinia/NY-ESO-1 recombinant virus (v.v. ESO) or wild-type vaccinia virus (v.v. WT) at 30 plaque-forming units/cell for 20 hours at 37°C in 300 μL X-VIVO 15 (Cambrex, Walkersville, MD) and then used as target cells.

**Peptide pulsing.** One million EBV-B cells (EBV-transformed B cells) or 1 × 10<sup>5</sup> DCs were pulsed with various concentrations of peptides in 1 mL of X-VIVO 15 for 1 hour at 37°C in a 5% CO<sub>2</sub> atmosphere. Peptide-pulsed EBV-B cells were used as target cells after irradiation.

**IFN<sub>γ</sub> ELISPOT assay.** This method has been described previously (4). IFN<sub>γ</sub> ELISA<sup>ss</sup>. Responding CD8<sup>+</sup> T cells (2 × 10<sup>5</sup>/well) and target cells were cultured in 96-well round-bottomed plates (Falcon, Franklin Lakes, NJ) for 20 hours at 37°C in X-VIVO 15 medium. For antibody blocking, 2.5 μg/mL mAbs were present throughout the assays. Culture supernatants were transferred to ELISA plates (Nunc, Rochester, NY) precoated with 2 μg/mL anti-human IFN<sub>γ</sub> mAb and incubated for 2 hours at 37°C. The plates were then washed and rabbit anti-human IFN<sub>γ</sub> protein diluted at 1:800 with PBS was added.

**Table 1. In vitro stimulation of CD8-T cells with autologous CHP/ESO-loaded DCs**

<table>
<thead>
<tr>
<th>HLA class I</th>
<th>Number of IFN&lt;sub&gt;γ&lt;/sub&gt; ELISPOTs</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>v.v. ESO</td>
</tr>
<tr>
<td>HD1</td>
<td>*0201/*2402</td>
</tr>
<tr>
<td>HD2</td>
<td>*0203/*1101</td>
</tr>
<tr>
<td>HD3</td>
<td>*1101/*2402</td>
</tr>
<tr>
<td>HD4</td>
<td>*0206/*2402</td>
</tr>
</tbody>
</table>

NOTE: Production of IFN<sub>γ</sub> ELISPOTs by CD8 T cells (2 × 10<sup>5</sup>) stimulated twice (HD1) or thrice (HD2, HD3, and HD4) with autologous CHP/ESO-loaded DCs against autologous EBV-B cells (2 × 10<sup>5</sup>) infected with v.v. ESO or v.v. WT. Assays were done in duplicate. Values are the means ± SD. The data are representative data from one of three experiments.
and incubated for 2 hours at 37°C. After the plates were washed extensively, goat anti-rabbit IgG serum conjugated with horseradish peroxidase diluted 1:2,000 was added and incubated for 1 hour at 37°C. After washing, the substrate solution [50 mmol/L citric acid, 100 mmol/L Na2HPO4, 0.03% orthophenylenediamine, 0.1% H2O2 in distilled water (pH 5.0)] was added to each well and incubated for 15 minutes at room temperature. After adding 6 N H2SO4, the plates were read using a U-2001 spectrophotometer (Tosoh, Tokyo, Japan).

**Statistical analysis.** The values were expressed as means ± SD. Unpaired Student’s t-test was used.

**Results**

*In vitro stimulation of CD8 T cells with CHP/ESO-loaded DCs.* We investigated the *in vitro* stimulation of CD8 T cells after purification of those cells from PBMC of a healthy donor carrying HLA-A*0201 (HD1, see Table 1 for HLA type) with autologous CHP/ESO-loaded immature DCs without treatment for maturation thrice at weekly intervals. Loading immature DCs with CHP/ESO resulted in no up-regulation of CD80 and CD86 as costimulatory molecules or of CD40 on their surface, and no IL-12 secretion (data not shown). These results indicated that DCs retained an immature phenotype after CHP/ESO loading. On day 7 after each stimulation, IFNγ ELISPOTs produced by HD1 CD8 T cells (2 × 104) were determined against autologous EBV-B cells (2 × 104) infected with v.v. ESO. As shown in Fig. 1A, production of IFNγ ELISPOTs was observed after the second stimulation. DCs loaded with NY-ESO-1 protein alone failed to stimulate CD8 T cells (Fig. 1B). As shown in Fig. 2A, production of IFNγ ELISPOTs by HD1 CD8 T cells stimulated with CHP/ESO-loaded DCs was observed against DC targets loaded with CHP/ESO, but not CHP or NY-ESO-1 protein alone. Furthermore, the response was observed against HLA-A2–positive and NY-ESO-1 mRNA–positive SK-MEL-37, and autologous EBV-B cells pulsed with NY-ESO-1–binding CD8 T cell epitope, but not HLA-A2–positive and NY-ESO-1 mRNA–negative SK-MEL-23 or autologous EBV-B cells pulsed with NY-ESO-1–binding CD8 T cell epitope.

The production of IFNγ by those HD1 CD8-T cells against autologous EBV-B cells pulsed with NY-ESO-1–binding CD8 T cell epitope was blocked by anti-CD8 mAb and anti-HLA class I mAb, but not anti-CD4 mAb or anti-HLA class II mAb (Fig. 2B).

**Fig. 1.** *In vitro* stimulation of CD8 T cells with CHP/ESO-loaded DCs. A, CD8 T cells purified from PBMC of HD1 were stimulated *in vitro* with autologous CHP/ESO-loaded DCs thrice at weekly intervals. On day 7 after each stimulation, the production of IFNγ ELISPOTs by HD1 CD8 T cells (2 × 104) was determined against autologous EBV-B cells (2 × 104) infected with v.v. ESO (filled columns) or v.v. WT (open columns) as targets. B, production of IFNγ ELISPOTs by HD1 CD8 T cells stimulated with autologous DCs loaded with CHP (20 μg/mL), NY-ESO-1 protein (1 μg/mL) alone, or CHP/ESO (20 μg/mL CHP and 1 μg/mL NY-ESO-1 protein) thrice at weekly intervals was determined as above. Assays were done in duplicate. Columns, means ± SD; *, P < 0.05.

**Fig. 2.** HD1 CD8 T cells stimulated with autologous CHP/ESO-loaded DCs recognized CHP/ESO-loaded DC targets and an HLA-A2–binding CD8 T cell epitope. A, production of IFNγ ELISPOTs by HD1 CD8 T cells (2 × 104) stimulated twice with autologous CHP/ESO-loaded DCs was determined against various target cells. Target cells were autologous EBV-B cells (2 × 104) infected with v.v. ESO or v.v. WT, autologous DCs (2 × 104) loaded with CHP (20 μg/mL), recombinant NY-ESO-1 protein (1 μg/mL) alone, or CHP/ESO (20 μg/mL CHP and 1 μg/mL NY-ESO-1 protein), irradiated melanoma cell lines (SK-MEL-23 and SK-MEL-37; 1 × 104), and irradiated autologous EBV-B cells (2 × 104) pulsed with 10−6 mol/L NY-ESO-1–binding CD8 T cell epitope (filled columns) or NY-ESO-1–negative CD8 T cell epitope (open columns) as targets. B, antibody blocking. IFNγ production by HD1 CD8 T cells (2 × 104) stimulated twice with autologous CHP/ESO-loaded DCs was determined by ELISA against irradiated autologous EBV-B cells (2 × 104) pulsed with 10−6 mol/L NY-ESO-1–binding CD8 T cell epitope in the presence of purified mAbs (2.5 μg/mL). Open columns, background IFNγ against autologous EBV-B cells alone. Assays were done in duplicate. Columns, means ± SD; *, P < 0.05.
shown in Fig. 3A, the production of IFNγ ELISPOTs by HD1 CD4 T cells (2 × 10^5) was observed against autologous EBV-B cells infected with v.v. ESO, but not v.v. WT. DCs loaded with NY-ESO-1 protein alone failed to stimulate CD4 T cells. DCs loaded with NY-ESO-1 protein alone treated with lipopolysaccharide to induce in vitro maturation (ESO/mDC) stimulated CD4 T cells. Production of IFNγ ELISPOTs by HD1 CD4 T cells stimulated with autologous CHP/ESO-loaded DCs was observed against autologous CHP/ESO-loaded DC targets without lipopolysaccharide treatment (CHP/ESO/DC) and against NY-ESO-1 protein–loaded mature DCs with lipopolysaccharide treatment (ESO/mDC; Fig. 3B).

As shown in Fig. 3C, production of IFNγ ELISPOTs by those HD1 CD4 T cells against EBV-B cells infected with v.v. ESO was blocked by anti-CD4 mAb, anti-HLA class II mAb and anti-HLA-DR mAb, but not anti-CD8 mAb, anti-HLA class I mAb, anti-DP mAb, or anti-DQ mAb.

We evaluated the in vitro stimulation of CD4 T cells by CHP/ESO-loaded DCs in two more healthy donors. As shown in Table 2, NY-ESO-1–specific CD4 T cells were induced in all three healthy donors including HD1. No IL-4 was detected in the culture, indicating that those CD4 T cells were Th1 type (data not shown).

Identification of an HLA-DRB1*15–binding CD4 T cell epitope from NY-ESO-1. Twenty-eight 18-mer peptides of NY-ESO-1 with 12 overlapping residues were synthesized to identify the epitope recognized by HD1 CD4 T cells. The production of IFNγ ELISPOTs by HD1 CD4 T cells (2 × 10^5) stimulated with autologous CHP/ESO-loaded DCs was determined against autologous EBV-B cells (2 × 10^5) pulsed with each peptide at a concentration of 10^−5 mol/L. As shown in Fig. 4A and B, production of IFNγ ELISPOTs was observed against autologous EBV-B cells pulsed with peptide no. 7 (NY-ESO-1-137-144, AGATGGRPRGGAARAS) at concentrations as low as 1 × 10^−7 mol/L. The production of IFNγ ELISPOTs by HD1 CD4 T cells against NY-ESO-1-137-144–pulsed autologous EBV-B cells was significantly blocked by anti-HLA-DR mAb (data not shown), indicating that the peptide was recognized in association with HLA-DR. To investigate the restriction molecule, autologous and allogeneic EBV-B cells were used as target cells. As shown in Table 3, NY-ESO-1-137-144 peptide-pulsed EBV-B cells derived from HLA-DR*1502–positive individuals were recognized.

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Table 2. In vitro stimulation of CD4 T cells with autologous CHP/ESO-loaded DCs

<table>
<thead>
<tr>
<th>HLA-DRB1</th>
<th>Number of IFNγ ELISPOTs</th>
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<tbody>
<tr>
<td></td>
<td>v.v. ESO</td>
</tr>
<tr>
<td>HD1 *1101/*1502</td>
<td>145 ± 11.3</td>
</tr>
<tr>
<td>HD2 *1405/*1502</td>
<td>114 ± 2.1</td>
</tr>
<tr>
<td>HD4 *0901/*1401</td>
<td>105 ± 8.5</td>
</tr>
</tbody>
</table>

NOTE: Production of IFNγ ELISPOTs by CD4 T cells (2 × 10^5) stimulated twice (HD1) or thrice (HD2 and HD4) with autologous CHP/ESO-loaded DCs against autologous EBV-B cells (2 × 10^5) infected with v.v. ESO or v.v. WT. Assays were done in duplicate. Values are the means ± SD. The data are representative data from one of three experiments.
Furthermore, CD4 T cells from an HLA-DR*1502–positive individual were stimulated in vitro with the peptide (Fig. 4C). To define the minimal sequence, we synthesized peptides truncated at either the NH2 or COOH terminus of NY-ESO-137-54 and analyzed HD1 CD4 T cell recognition of these peptides. As shown in Fig. 5, truncation at the COOH terminus of the first four amino acids did not affect recognition. Further truncation of the peptide at the COOH terminus resulted in decreased peptide recognition. Truncation of the first amino acid at the NH2 terminus caused loss of recognition. Thus, it was determined that NY-ESO-137-50 was the minimal epitope recognized by CD4 T cells binding to HLA-DR15.

### Discussion

NY-ESO-1 is one of a few tumor antigens which elicit simultaneous humoral and cellular immune responses spontaneously (3, 4) and after vaccination (17, 21) in patients with cancer. In the present study, we showed in vitro stimulation of CD8 T cells with immature DCs loaded with a complex of CHP and NY-ESO-1 protein. CD8 T cells induced in a healthy donor carrying HLA-A*0201 (HD1) recognized HLA-A2–binding CD8 T cell epitope peptide (NY-ESO-1157-165). NY-ESO-1–specific CD8 T cells were similarly induced in three more healthy donors expressing various HLA types. CD8 T cells induced in HD4 carrying HLA-A*0206, but not in HD2 carrying HLA-A*0203, also recognized exogenously added NY-ESO-1157-165. The results indicated that immature DCs loaded with CHP/ESO processed NY-ESO-1 protein through the MHC class I pathway and efficiently presented antigenic peptides on MHC class I molecules without in vitro treatment to induce DC maturation.

Mature DCs have the capacity to process and present exogenous protein antigens on MHC class I molecules through cross-presentation (22, 23). Loading DCs with CHP/ESO neither up-regulated costimulatory molecules and CD40 on their surface, nor induced IL-12 production (data not shown), suggesting that DCs loaded with CHP/ESO retained an immature phenotype and cross-presented NY-ESO-1 antigen on MHC class I. CHP nanoparticles form complexes with denatured protein and release complexed proteins in their refolded naïve form upon the addition of cyclodextrin, which disrupts the hydrophobic interaction of cholesterol branches (24, 25), suggesting that CHP nanoparticles behave like...
Determination of the minimal peptide sequence recognized by HD1 CD4 T cells. Peptides truncated at either the NH2 or COOH terminus of NY-ESO-137-54 were synthesized. Production of IFNγ ELISpot by HD1 CD4 T cells (2 × 10^4) stimulated twice with autologous CHP/ESO-loaded DCs was determined against autologous DCs (2 × 10^3; experiments 1 and 2) or irradiated autologous EBV-B cells (2 × 10^4; experiment 3) pulsed with 10^{-7} mol/L peptide or loaded with 1 μg/mL NY-ESO-1 protein. Background IFNγ ELISpot by CD4 T cells (2 × 10^4) cultured with DCs or EBV-B cells alone have been subtracted (51, 62, and 60 spots in experiments 1, 2, and 3, respectively). Assays were done in duplicates. Columns, means; ND, not done.

Fig. 5. Determination of the minimal peptide sequence recognized by HD1 CD4 T cells. Peptides truncated at either the NH2 or COOH terminus of NY-ESO-137-54 were synthesized. Production of IFNγ ELISpot by HD1 CD4 T cells (2 × 10^4) stimulated twice with autologous CHP/ESO-loaded DCs was determined against autologous DCs (2 × 10^3; experiments 1 and 2) or irradiated autologous EBV-B cells (2 × 10^4; experiment 3) pulsed with 10^{-7} mol/L peptide or loaded with 1 μg/mL NY-ESO-1 protein. Background IFNγ ELISpot by CD4 T cells (2 × 10^4) cultured with DCs or EBV-B cells alone have been subtracted (51, 62, and 60 spots in experiments 1, 2, and 3, respectively). Assays were done in duplicates. Columns, means; ND, not done.

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References


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