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Heat Shock Protein 90-Mediated Peptide-Selective Presentation of Cytosolic Tumor Antigen for Direct Recognition of Tumors by CD4⁺ T Cells

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Tumor Ag-specific CD4⁺ T cells play important functions in tumor immunosurveillance, and in certain cases they can directly recognize HLA class II-expressing tumor cells. However, the underlying mechanism of intracellular Ag presentation to CD4⁺ T cells by tumor cells has not yet been well characterized. We analyzed two naturally occurring human CD4⁺ T cell lines specific for different peptides from cytosolic tumor Ag NY-ESO-1. Whereas both lines had the same HLA restriction and a similar ability to recognize exogenous NY-ESO-1 protein, only one CD4⁺ T cell line recognized NY-ESO-1⁻ HLA class II-expressing melanoma cells. Modulation of Ag processing in melanoma cells using specific molecular inhibitors and small interfering RNA revealed a previously undescribed peptide-selective Ag-presentation pathway by HLA class II⁺ melanoma cells. The presentation required both proteasome and endosomal protease-dependent processing mechanisms, as well as cytosolic heat shock protein 90-mediated chaperoning. Such tumor-specific pathway of endogenous HLA class II Ag presentation is expected to play an important role in immunosurveillance or immunosuppression mediated by various subsets of CD4⁺ T cells at the tumor local site. Furthermore, targeted activation of tumor-recognizing CD4⁺ T cells by vaccination or adoptive transfer could be a suitable strategy for enhancing the efficacy of tumor immunotherapy. The Journal of Immunology, 2012, 188: 3851–3858.
NY-ESO-1\(^{-}\) melanoma cell lines. In contrast, despite the same HLA restriction and an epitope in a proximal region, NY-ESO-1\(_{187-198}\)–specific CD4\(^{+}\) T cell line was incapable of recognizing tumor cell lines, which indicated epitope-selective presentation of endogenous tumor Ags to CD4\(^{+}\) T cells. By studying the Ag-presenting pathway for the direct presentation of intracellular NY-ESO-1 by tumor cells, we have identified a previously undescribed pathway that was disrupted by both cytotoxic proteasome and endosomal/lysosomal protease inhibitors, but not by autophagy or HLA-recycling inhibitors. Importantly, cytotoxic heat shock protein (HSP) 90 was required for the endogenous MHC-II presentation pathway, as shown by specific inhibitors and silencing experiments.

Materials and Methods

**Generation of NY-ESO-1–specific CD4\(^{+}\) T cell lines**

PBMCs from ovarian cancer patients were obtained with informed consent at the Roswell Park Cancer Institute (Buffalo, NY) under an approved protocol from the institutional review board. CD4\(^{+}\) T cell culture was performed in RPMI 1640 medium supplemented with 10% human AB serum (Gemini Bio Products), streptomycin/penicillin (Gemini Bio Products), performin, and L-glutamine. CD4\(^{+}\) T cells were isolated from PBMCs from an ovarian cancer patient who had spontaneous serum Abs against NY-ESO-1 using a Dynal CD4\(^{+}\) positive isolation kit (Invitrogen). CD4\(^{+}\) T cells were further depleted for CD8\(^{+}\) T cells using a Dynal CD8\(^{+}\) positive isolation kit, and T cell-depleted PBMCs were pulsed overnight with 17 overlapping peptides (#1–#17) for NY-ESO-1. CD4\(^{+}\) T cells, and peptide-pulsed T cell-depleted PBMCs were cocultured for 20 d in the presence of 10\(\mu\)l IL-2 (Roche Diagnostics) and 20 ng/ml IL-7 (R&D Systems) to allow expansion of NY-ESO-1–specific CD4\(^{+}\) T cells. NY-ESO-1–peptide-specific T cell lines were established as described (15). CD154-expressing CD4\(^{+}\) T cells were isolated after restimulation with PHA and HLA-DRB1*0101/NY-ESO-187–98 tetramer and expansion of NY-ESO-1–specific CD4\(^{+}\) T cells (T-APC) pulsed with a subpool (#9–#12 peptides) of NY-ESO-1 overlapping peptides using a FACSaria instrument and expanded with PHA (Remel). From the parental #9–#12-specific CD4\(^{+}\) T cell line, NY-ESO-1\(_{187-198}\) peptide-specific CD4\(^{+}\) T cell line (SB87) was obtained by cell sorting using HLA-DRB1*0101/NY-ESO-1\(_{187-198}\) tetramer and expansion by PHA (data not shown). NY-ESO-1\(_{185-196}\)/specific CD4\(^{+}\) T cells was detected using IFN-\(\gamma\) capture reagents (Miltenyi Biotec) after restimulation with T-APC pulsed with NY-ESO-1\(_{185-196}\) peptide. After isolation of IFN-\(\gamma\)^+ cells by cell sorting, they were expanded with PHA to generate NY-ESO-1\(_{185-196}\) peptide-specific CD4\(^{+}\) T cell line (SB85). Both CD4\(^{+}\) T cell lines were maintained by periodic stimulations with PHA in the presence of irradiated allogeneic PBMCs.

Reagents and culture of cell lines

EBV-transformed B cell lines and melanoma cell lines were from our cell bank. All cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, streptomycin/penicillin, and L-glutamine (RPMI+10%FCS). Melanoma cell lines were treated with the following reagents at indicated concentration for 40–44 h (3-methyladenine, 17-DMAg, SU11652, and pifithrin-\(\mu\) (PFT-\(\mu\)) or 16–20 h (other reagents) in 2 ml RPMI+10%FCS in six-well plates (BD Falcon); lactacytin and epoxomicin from Boston Biochem; chloroquine, leupeptin, 3-methyl adenine, brefeldin A (BFA), PFT-\(\mu\) and primaquine from Sigma-Aldrich; 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAg) from Invitrogen; and SU11652 from EMD Chemicals. After treatment, cells were harvested and extensively washed, and live cells were counted by trypan blue exclusion test and used as target cells in T cell recognition assays. Functional cell surface expression of MHC-II after pharmacologic inhibition of Ag processing was confirmed by flow cytometric evaluation of HLA-DR (Supplemental Fig. 1A) and presentation of a synthetic peptide to a control CD4\(^{+}\) T cell line (Supplemental Fig. 1B). All peptides were purchased from Bio-Synthesis or GenScript. In some experiments, melanoma cells were incubated overnight with indicated concentration of NY-ESO-1 peptide or recombinant NY-ESO-1 protein, or infected overnight with adenoviruses encoding NY-ESO-1 or empty vector at multiplicity of infection of 1000. Recombinant NY-ESO-1 protein was expressed in E. coli and purified by a standard method.

Small interfering RNA transduction

Dicer substrate small interfering RNA (siRNA) for NY-ESO-1 and the control siRNA for MAGE and GFP, or scrambled siRNA were designed and synthesized by Integrated DNA Technologies. All other 27-mer siRNAs were purchased from Origene. For the electroporation, SK-MEL-37 was washed once in culture medium and resuspended in siPORT siRNA Electroporation Buffer (Applied Biosystems-Ambion). Cells were mixed with the final concentration of 1.4 \(\mu\)M siRNA and were applied with two pulses of 3 kV/cm for 200 \(\mu\)s using ECM 830 Electroporation System and electroporation cuvettes (Harvard Apparatus-BTX, Holliston, MA). Cells were harvested 3 d after electroporation and were used as target cells in T cell recognition assays. In some experiments, the same treatment was repeated at day 2, and cells were used at day 4. Protein levels after transduction were evaluated by intracellular staining of NY-ESO-1 using E978 or 219–510 (16) anti–NY-ESO-1 mAbs and PE-conjugated anti-mouse IgG1 mAb (BD Biosciences) or Western blot analyses using commercial mAbs against HSP90\(\alpha\), HSP90\(\beta\), gp96, and HSC70 from Enzo Life Sciences, and a mAb against LAMP2 from BioGeorgia. By gating lymphocyte population in an FCS/SSC plot.

**ELISPOT assay**

Indicated number of effector CD4\(^{+}\) T cell lines was cocultured with target melanoma cell lines (3 \(\times\) 10\(^{5}\)) or peptide-pulsed EBV-transformed B cell lines (5 \(\times\) 10\(^{5}\)) on nitrocellulose-coated microtiter plates (Millipore, Bedford, MA) precoated overnight with 2 \(\mu\)g/ml anti–IFN-\(\gamma\) mAb (1-DIK, Mabtech) and blocked with 10% human serum (Sigma-Aldrich) in RPMI 1640 medium. After 20–24 h, plates were developed using 0.2 \(\mu\)g/ml biotinylated anti–IFN-\(\gamma\) mAb (7-B6-1; Mabtech), 1 \(\mu\)l streptavidin-alkaline phosphatase conjugate (Roche Diagnostics) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT) (Sigma-Aldrich). The number of spots was evaluated using C.T.L. Immunospot analyzer and software (Cellular Technologies). In some experiments, melanoma cell lines were pretreated with 20 \(\mu\)g/ml anti–HLA-DR, -DQ, or -DP mAb (Leinco Technologies) for 30 min at room temperature and added without wash. The final concentration of anti–HLA-class II mAb during coculture of melanoma cell lines and CD4\(^{+}\) T cells was 10 \(\mu\)g/ml.

**Measurement of cytokine levels**

Melanoma cell line (3 \(\times\) 10\(^{5}\)) and NY-ESO-1 peptide-specific CD4\(^{+}\) T cell line (5 \(\times\) 10\(^{5}\)) were cocultured for 20–24 h in a 96-well round-bottom plate in 250 \(\mu\)l RPMI+10%FCS. Supernatant was harvested and stored at –20°C. IFN-\(\gamma\) level was measured by ELISA using unlabeled and biotinylated mAb pairs (BD Biosciences), HRP-labeled avidin D (eBioscience), and TMB substrate solution (eBioscience).

**Generation of ICPI47-expressing SK-MEL-37 clones**

HSV ICPI47 gene was cloned into pDNA3.1/V5-His TOPO plasmid with a stop codon (pDNA-ICPI47). SK-MEL-37 was transfected with pDNA-ICPI47 using Lipofectamine (Invitrogen). Clones growing in the presence of 500 \(\mu\)g/ml G418 (Invitrogen) were tested for intracellular NY-ESO-1 expression, as well as surface HLA class I and HLA class II expression using FITC-conjugated HLA-ABC and PE-conjugated anti-HLA-DR mAbs (BD Biosciences) by flow cytometry. Clones expressing comparable levels of NY-ESO-1 and HLA-DR to parental SK-MEL-37, but with reduced HLA class I expression, were selected and further expanded in the presence of 500 \(\mu\)g/ml G418. Functional expression of ICPI47 was tested by recovery of surface HLA-A*0201 expression using supernatant from MA2.1 hybridoma and PE-conjugated anti-mouse IgG1 mAb after overnight culture with 100 \(\mu\)M Melan-A\(_{27-35}\) peptide and 3 \(\mu\)g/ml \(\beta\)-microglobulin (Sigma-Aldrich) and reduced recognition by NY-ESO-1–specific CD8\(^{+}\) T cell clone.

**Statistical analysis**

Statistical significance was determined by performing a two-tailed Student \(t\) test using Prism 5 software (GraphPad Software); \(p < 0.05\) was considered significant.

**Results**

**Establishment of NY-ESO-1–specific CD4\(^{+}\) T cell lines**

To characterize spontaneous NY-ESO-1–specific CD4\(^{+}\) T cell responses in ovarian cancer patients who had serum Ab against NY-ESO-1, CD4\(^{+}\) T cells were stimulated once with a pool of 17
NY-ESO-1 overlapping peptides (#1–#17) (for peptide sequences, see 16) pulsed on T cell-depleted PBMCs and cultured for 20 d in the presence of low-dose IL-2 and IL-7. Specificity of CD4+ T cells was first determined by IFN-γ ELISPOT assays against subpools of peptides. As shown in Fig. 1A, CD4+ T cells from patient SB showed specific IFN-γ production against #9–#12 and #13–#17 peptides pools. To fully characterize these responses, SB/#9–#12 NY-ESO-1–specific CD4+ T cell line reactive against a #9–#12 peptide subpool was generated by isolating CD154-expressing cells after restimulation with the subpool of peptides followed by polyclonal expansion with PHA. Peptide reactivity of the CD4+ T cell line was determined against individual NY-ESO-1 peptides (Fig. 1B). They were further tested for the recognition of tumor cell lines that expressed cell surface MHC-II by intracellular cytokine staining (Fig. 1C). A small but significantly higher fraction of the SB/#9–#12 CD4+ line produced IFN-γ after stimulation with NY-ESO-1–expressing SK-MEL-37 compared with SB/#9–#12 stimulated with other melanoma cell lines.

By testing MHC-II tetramer staining and reactivity against shorter peptides, SB/#9–#12 was found to be a mixture of two DR01-restricted CD4+ T cell lines, one recognizing NY-ESO-1 #9–98 contained in #9 peptide and the other NY-ESO-1 #95–106 in #10 peptide (data not shown). To characterize tumor recognition by NY-ESO-1–specific CD4+ T cells in detail, two CD4+ T cell lines were established from the parental SB/#9–#12 line and were named SB87 and SB95, respectively. Both SB87 and SB95 were restricted by the same DR01 for the recognition of cognate peptides (Supplemental Fig. 2A), in agreement with the DR01 allele expressed on histocompatible tumor cell line SK-MEL-37 and its direct recognition by the parental SB/#9–#12 CD4+ T cell line.

Recognition of naturally processed NY-ESO-1 by CD4+ T cells

We next tested the tumor recognizing ability of CD4+ T cell lines specific against a single peptide by IFN-γ ELISPOT assays. It was found that SB95 but not SB87 strongly recognized SK-MEL-37 (Fig. 2A). In accordance with DR01 restriction of SB95 to recognize NY-ESO-1 #95–106 peptide (Supplemental Fig. 2A), recognition of SK-MEL-37 by SB95 was blocked by anti-HLA-DR mAb (Fig. 2B). Furthermore, SB95 efficiently recognized another DR01*NY-ESO-1* tumor cell line (MZ-MEL-19), but not DR01*NYY-ESO-1* lines (SK-MEL-52 and SK-MEL-139; Fig. 2C). To test whether the recognition of SK-MEL-37 by SB95 was NY-ESO-1 specific, NY-ESO-1 protein expression was silenced by electroporation of SK-MEL-37 with NY-ESO-1 siRNA (Fig. 2D). Silencing of NY-ESO-1 expression was confirmed by the reduced recognition of HLA-A*02 (A02)* SK-MEL-37 by HLA-A*02–restricted NY-ESO-1–specific CD8+ T cell clone, which is used as a control for direct SK-MEL-37 recognition by T cells (Supplemental Fig. 3A). NY-ESO-1 siRNA electroporation specifically inhibited the recognition by SB95 (Fig. 2E), indicating SB95 CD4+ T cell line recognized peptide generated from intracellular NY-ESO-1 and presented on DR01.

To gain insights into the differential recognition of cytosolic NY-ESO-1 by SB87 and SB95 (Fig. 2A), NY-ESO-1 protein was introduced in a DR01*NY-ESO-1* melanoma cell line, SK-MEL-12, as exogenously added protein or as endogenous protein produced by recombinant adenoviral vector and the recognition by SB87 and SB95 was compared. As shown in Fig. 3A, both SB87 and SB95 efficiently recognized exogenous NY-ESO-1 protein-pulsed SK-MEL-12, indicating that both epitopes for SB87 and SB95 were generated and presented from exogenous NY-ESO-1 protein. Presentation of exogenous NY-ESO-1 protein required intracellular processing, because fixing target cells 1 h after addition of NY-ESO-1 protein completely abolished the presentation (data not shown). In contrast, recognition of intracellular NY-ESO-1 protein ectopically expressed by adenoviral vector was more efficient by SB95 than SB87, indicating that intracellular NY-ESO-1 was preferentially presented to SB95 (Fig. 3A). Titration curves for the recognition of exogenous NY-ESO-1 protein pulsed on SK-MEL-12 were similar for SB87 and SB95 (Fig. 3B). In addition, apparent avidities of SB87 and SB95 for the recognition of 12-mer peptide (NY-ESO-1 #97–98 and NY-ESO-1 #95–106, respectively) were similar (Supplemental Fig. 2B), suggesting that the differential tumor-recognizing ability was not ascribed to the different avidity or other characteristics, such as expression of costimulatory molecules of T cells.

Recognition of protein-loaded target cells by SB95 (Fig. 3A, 3B) raised the possibility that SK-MEL-37 could take up NY-ESO-1 protein that was released into the culture supernatant and presented via exogenous pathway. However, SK-MEL-12 pulsed with culture supernatant of SK-MEL-37 was not recognized by SB95, suggesting the absence of NY-ESO-1 protein in the supernatant (Fig. 3C). In addition, CFSE-labeled DR01*NY-ESO-1* SK-MEL-12, which was cocultured with PKH26-labeled DR01*NY-ESO-1* SK-MEL-37 for 2 d and separated by cell sorting, was not recognized by SB95 (Fig. 3D), excluding the possibility of intercellular transfer of NY-ESO-1.

Ag-processing pathway for the presentation of intracellular NY-ESO-1 to CD4+ T cells

To address pathways for endogenous MHC-II presentation of naturally expressed NY-ESO-1 by tumor cells, SK-MEL-37 was pre-treated with inhibitors of protein degradation before T cell recognition was tested. As shown in Fig. 4A, recognition by SB95 was inhibited by the protein synthesis inhibitors cycloheximide (CHX) and actinomycin D (ActD), indicating that both cytosolic and endogenousny-ESO-1 were presented by SK-MEL-37 to CD4+ T cells. Recognition of SK-MEL-37 pulsed with culture supernatant of SK-MEL-37 was abrogated by actinomycin D (ActD), indicating that both cytosolic and endogenousny-ESO-1 were presented by SK-MEL-37 to CD4+ T cells. Recognition of SK-MEL-37 pulsed with culture supernatant of SK-MEL-37 was abrogated by actinomycin D (ActD), indicating that both cytosolic and endogenousny-ESO-1 were presented by SK-MEL-37 to CD4+ T cells. Recognition of SK-MEL-37 pulsed with culture supernatant of SK-MEL-37 was abrogated by actinomycin D (ActD), indicating that both cytosolic and endogenousny-ESO-1 were presented by SK-MEL-37 to CD4+ T cells.
was strongly inhibited by pretreatment of SK-MEL-37 with proteasome inhibitors, lactacystin, and epoxomicin, whereas the same treatment did not inhibit the peptide presentation to SB87 by SK-MEL-37 (Supplemental Fig. 1B). As expected, recognition of A02+ SK-MEL-37 by control A02-restricted NY-ESO-1–specific CD8+ T cell clone was inhibited by both inhibitors (Supplemental Fig. 3B). In addition, treatment of SK-MEL-37 with endosomal protease inhibitors, chloroquine and leupeptin, significantly inhibited the recognition by SB95 (Fig. 4B). Although treatment with 100 μM chloroquine partially inhibited cell surface MHC-II expression on SK-MEL-37 and peptide presentation to SB87 (Supplemental Fig. 1), the inhibition appeared too weak to explain the complete inhibition of direct tumor recognition by SB95. In contrast, leupeptin treatment showed no inhibition in MHC-II expression and peptide presentation (Supplemental Fig. 1), indicating that processing by endosomal protease was required for endogenous MHC-II presentation. By the use of these protease inhibitors, it is impossible to determine whether endosomal or lysosomal proteases processed NY-ESO-1 peptides in addition to degradation of invariant chain. However, these results suggested that NY-ESO-1 peptides were presented to CD4+ T cells by DR01-restricted T cell lines. These results are summarized in FIGURE 2.

FIGURE 2. Characterization of the tumor recognition by DR01-restricted NY-ESO-1–specific CD4+ T cell lines. (A) Recognition of DR01+NYPESO-1+ SK-MEL-37 by SB87 and SB95 was evaluated by IFN-γ ELISPOT assay. (B) SK-MEL-37 was cocultured with SB87 in the presence or absence of Abs against MHC-II for the measurement of IFN-γ production by ELISPOT assay. (C) A panel of DR01+ and DR01− NY-ESO-1–expressing melanoma cell lines was tested for the recognition by SB95 by IFN-γ ELISPOT assay. (D) NY-ESO-1 expression in SK-MEL-37 was evaluated by intracellular staining after electroporation with siRNA against NY-ESO-1 (ESO) or control genes. Results are shown as histograms and mean fluorescent intensities (MFIs) of NY-ESO-1 expression. (E) Recognition of siRNA-electroporated SK-MEL-37 by SB95 was evaluated by IFN-γ ELISPOT assay. All experiments were repeated at least three times with similar results. Data are expressed as mean ± SD. Asterisks indicate a significant difference (p < 0.05) compared with untreated (in B) or scrambled siRNA electroporated (in E) target cells.
were loaded on MHC-II in endosomes or lysosomes to be presented to CD4\(^+\) T cells. As expected, recognition of SK-MEL-37 by NY-ESO-1–specific CD8\(^+\) T cell clone was unaffected by chloroquine treatment (Supplemental Fig. 3B). From these results, MHC-II-binding NY-ESO-1\(_{105-116}\) peptide was likely generated by the cytosolic proteasome and loaded on MHC-II in acidic endosomal or lysosomal compartments in a protease-dependent manner.

TAP molecules, which transport peptides from cytosol to the endoplasmic reticulum, have a critical role in the Ag presentation to CD8\(^+\) T cells, although some peptides are also presented to CD8\(^+\) T cells in a TAP-independent manner (17). To assess the involvement of TAP in the endogenous MHC-II presentation of intracellular NY-ESO-1 to SB95, SK-MEL-37 clones stably expressing a viral inhibitory sequence for TAP, ICP47, were established. ICP47-expressing clones were confirmed to express comparable levels of intracellular NY-ESO-1 and cell surface HLA-DR with parental SK-MEL-37 (Supplemental Fig. 4A, 4B). The functional expression of ICP47 was confirmed by down-modulation of cell surface HLA class I expression compared with the parental cell line, recovery of cell surface HLA class I expression after incubating with class I-binding peptides in the presence of β2-microglobulin, and reduced recognition of ICP47-expressing clones by A02-restricted NY-ESO-1–specific CD8\(^+\) T cell clone (Fig. 4C, Supplemental Fig. 4B, 4C). It was found that TAP was not involved in the endogenous MHC-II presentation of NY-ESO-1 to CD4\(^+\) T cells by SK-MEL-37 because ICP47-expressing SK-MEL-37 stimulated SB95 with similar efficacy to NY-ESO-1–expressing SK-MEL-37 (Fig. 4C). These results suggested that NY-ESO-1 peptides generated by proteasomal degradation are loaded on MHC-II in endosomes or lysosomes without entering the endoplasmic reticulum. Vesicular transport through Golgi apparatus is required in Ag presentation to CD8\(^+\) T and CD4\(^+\) T cells (18).

The endogenous MHC-II presentation to SB95 was efficiently inhibited by brefeldin A, which inhibits vesicular trafficking (Fig. 4D), whereas the same treatment did not affect surface MHC-II expression and peptide presentation to SB87 by SK-MEL-37 (Supplemental Fig. 1).
LAMP-2, HSC70, or both proteins did not affect the recognition by SB95, suggesting that chaperone-mediated autophagy was not involved in the endogenous MHC-II presentation to SB95. Recently it was reported that LAMP-2-independent microautophagy translocates cytoplasmic Ags to endosome in DC in an HSC70-dependent manner (21); however, efficient recognition of HSC70-silenced SK-MEL-37 by SB95 (Fig. 5D) indicated that substrate-selective microautophagy was not involved in the presentation in our experimental system.

**Involvement of cytosolic HSP90 in the endogenous MHC-II presentation**

Because HSPs are known to be involved in various aspects of Ag presentation (22), we asked whether HSP played a role in the endogenous MHC-II presentation of NY-ESO-1. To test the role of HSP in the presentation, SK-MEL-37 was treated with specific inhibitors for chaperoning by HSP90 family (17-dimethylaminoethylamino-17-demethoxygeldanamycin; 17-DMAG) (23) or HSP70 (PFT-α) (24) and recognition by SB95 was evaluated. As shown in Fig. 6A, 17-DMAG but not PFT-α completely inhibited the presentation. Geldanamycin is also known to inhibit various kinases; however, the effect of a control tyrosine kinase inhibitor, SU11652 (25), on the recognition by SB95 was negligible (Fig. 6B), strongly indicating the involvement of HSP90-chaperoning in the presentation. Interestingly, treatment of SK-MEL-37 with SU11652 enhanced the presentation to SB95 in a dose-dependent manner, possibly indicating an inhibitory role of tyrosine kinases in the processing or presentation of NY-ESO-1 by tumor cells. Among HSP90 family members, cytosolic HSP90α (HSP90αA1 and HSP90αA2) and HSP90β (HSP90B1) and endoreticular gp96 (HSP90B1, also known as GRP94) are known to play a role in Ag presentation of chaperoned peptides. To identify which HSP90 members were involved in the presentation, each HSP90 member was silenced by electroporation of gene sequence-specific siRNA (Fig. 6C). As shown in Fig. 6D, downmodulation of HSP90α, but not endoreticular gp96, significantly inhibited the presentation. This result that gp96 was not involved was consistent with the observation that peptide transport into endoplasmic reticular through TAP was not involved in the presentation (Fig. 4C). Based on our results, we hypothesized the mechanism for the presentation of MHC-II binding peptides from endogenous protein Ags as follows. First, endogenous proteins such as NY-ESO-1 are digested via cytosolic proteasome to small peptides, and the peptides are selectively chaperoned by cytosolic HSP90α/β. Next, the HSP90/peptide complex is translocated into endosomes or lysosomes by an autophagy-independent manner, in which the peptides are loaded on MHC-II in a protease-dependent manner.

**Discussion**

In the classical view of the Ag-presentation pathway, CD4+ T cells recognize exogenous proteins that are processed by APCs, whereas the recognition of intracellular proteins by CD4+ T cells is inefficient unless the protein is localized in or targeted to endosome or lysosome (26, 27). In addition, it was clearly shown that MHC-II-transfected tumors that lack invariant chain expression could efficiently present intracellular Ags and activate tumor Ag-specific CD4+ T cells, which demonstrates that MHC-II on tumors are fully functional for endogenous tumor Ag peptide presentation, but indicates a critical role of the invariant chain to
protect endogenous peptide loading on MHC-II in the endoplasmic reticular (28, 29). Nevertheless, direct recognition of MHC-II–expressing tumors by tumor Ag-specific CD4+ T cells has been reported, although the mechanism for the direct presentation of physiologically expressed tumor Ag on MHC-II remains largely unclear except for a melanosomal Ag, gp100, which is efficiently presented to gp100-specific CD4+ T cells. The presentation of gp100 to CD4+ T cells was completely abolished by removing a melanosomal targeting sequence in gp100, indicating the critical role of melanosomal localization for the MHC-II presentation (30). Because melanosomes are closely related to lysosomes and gp100 is localized to late endosomes in melanosome-negative tumors, it is reasonable that melanosomal Ags are naturally processed via an MHC-II processing pathway. In the current study, we have characterized direct recognition of tumor cells by CD4+ T cells against a nonmelanosomal but cancer-testis Ag, NY-ESO-1. NY-ESO-1 is a cytosolic protein expressed in a wide variety of tumor types, and NY-ESO-1–expressing tumors induce spontaneous immune responses in cancer patients (31). NY-ESO-1–specific CD4+ T cells used in the current study were derived from a cancer patient who had spontaneous immune responses against NY-ESO-1, indicating that our CD4+ T cells were primed during a natural course of tumor immunosurveillance in the patient. In addition, melanoma cell lines recognized by our CD4+ T cells (SB95) are not genetically modified and express a physiologic level of NY-ESO-1. Thus, it is expected that the MHC-II positive tumors present tumor Ag peptides to CD4+ T cells via the present Ag pathway in cancer patients.

Importantly, only SB95 but not SB87 could recognize tumor cells, implying epitope selective mechanism for endogenous MHC-II presentation. The mechanism for the endogenous MHC-II presentation of endogenous NY-ESO-1 to CD4+ T cells by melanoma was found to be different from previously characterized pathways, including intercellular Ag transfer, macroautophagy, presentation by recycling HLA, and chaperone-mediated autophagy (Fig. 5). In previous studies of endogenous MHC-II presentation, intracellular viral Ags or ectopically expressed model Ags were used, for which specific CD4+ T cells have strong TCR affinity and where Ag expression is relatively high. In contrast, TCR affinity for self or tumor Ags, except for mutated Ags, break point of fusion proteins, or Ags with tumor-specific modification such as phosphorylation (32), is considered to be lower because of the expression of these Ags in the thymus (33). Therefore, analyses of tumor-recognizing CD4+ T cells are required for the evaluation of the role of previously characterized pathways in the tumor immunosurveillance by tumor-recognizing CD4+ T cells. The endogenous MHC-II presentation of NY-ESO-1 to SB95 required chaperoning by cytosolic HSP90. Cytosolic HSP90α and HSP90β as well as their endoreticular family member, gp96, were shown to have a critical role in the induction of tumor Ag-specific CD8+ T cells by chaperoning of peptides from tumor Ags and targeting them to APCs (34–36). These observations provided a basis for the current development of HSP-based vaccines purified from tumor cells (37). In addition, HSP90α/β has a role in cross-presentation of exogenous proteins and in endogenous MHC-II presentation via chaperone-mediated autophagy by immune APCs (38, 39). The present Ag presentation pathway shares many features with chaperone-mediated autophagy-dependent endogenous MHC-II presentation, such as dependence on proteasome, endosomal proteases, and HSP90 (13, 38, 40). In addition, increased recognition of SK-MEL-37 by inhibition of macroautophagy resembles increased chaperone-mediated autophagy after inhibition of macroautophagy (41). The current pathway, however, appears distinct from chaperone-mediated autophagy because HSC70/LAMP-2 appeared to have a negligible role in the presentation of NY-ESO-1 to SB95. We postulate that HSP90α/β is involved in the translocation of chaperoned peptide between cytosol and endosome/lysosome without the need for HSC70/LAMP-2. It is known that chaperoning by HSP is substrate specific, although the motif for the chaperoning is not known. Selective chaperoning of tumor Ag peptides by HSP90α/β can explain the epitope selectivity in the endogenous MHC-II presentation by tumor cells. Comprehensive analysis of HSP-binding peptides or identification of motif of HSP binding could be an efficient approach to identify the immunogenic peptides that are presented to CD4+ T cells by endogenous MHC-II presentation.

Although the role of tumor-recognizing CD4+ T cells for tumor immunosurveillance remained to be addressed, it is expected that in addition to CD8+ T cells, tumor-recognizing CD4+ T cells have a distinct role in tumor immunosurveillance by producing cytokines or by cytotoxicity at tumor sites, or both. Recently, efficient tumor eradication by tumor-recognizing CD4+ T cells was demonstrated in several mouse models (42–44). In addition, adoptive transfer of NY-ESO-1–specific CD4+ T cell clone could induce complete tumor eradication in a melanoma patient (45). Selective activation of tumor-recognizing CD4+ T cells by vaccination or adoptive transfer could be a good approach to harness the anti-tumor role of CD4+ T cells.

Another implication of the present findings in the understanding of anti-tumor immunity is that CD4+Foxp3+ regulatory T cells (Tregs) could also directly recognize tumor cells. Tregs are known to inhibit antitumor immunity (46). If Tregs express tumor-recognizing TCR, they can be activated at tumor sites and suppress immune reaction without the need for APCs. Staining of tumor-infiltrating lymphocytes using the MHC-II tetramers to detect both tumor-recognizing and non–tumor-recognizing TCRs will help to address this issue.

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Disclosures

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