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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

Takemasa Tsuji,* Junko Matsuuki,† Otavia L. Caballero,*1 Achim A. Junghbluth,* Gerd Ritter,* Kunle Odunsi,† Lloyd J. Old,* and Sacha Gnjatic*

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.

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Abbreviations used in this article: DC, dendritic cell; HSP, heat shock protein; MHC-II, MHC class II; PFT-μ, pifithrin-μ; RPMI-10%FCS, RPMI 1640 medium supplemented with 10% FCS, streptomycin/penicillin, and L-glutamine; siRNA, small interfering RNA; Treg, regulatory T cell.

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NY-ESO-1<sup>+</sup> melanoma cell lines. In contrast, despite the same HLA restriction and an epitope in a proximal region, NY-ESO-1<sub>187-198</sub>-specific CD4<sup>+</sup> T cell line was incapable of recognizing tumor cell lines, which indicated epitope-selective presentation of endogenous tumor Ags to CD4<sup>+</sup> 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 cytosolic proteasome and endosomal/lysosomal protease inhibitors, but not by autophagy or HLA-recycling inhibitors. Importantly, cytosolic 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<sup>+</sup> 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<sup>+</sup> T cell culture was performed in RPMI 1640 medium supplemented with 10% human AB serum (Gemini Bio Products), streptomycin/penicillin (Gemini Bio Products) and L-glutamine. CD4<sup>+</sup> 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<sup>+</sup> T cells were further depleted for CD8<sup>+</sup> 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<sup>+</sup> T cells, and peptide-pulsed T cell-depleted PBMCs were cocultured for 20 d in the presence of 10 U/ml IL-2 (Roche Diagnostics) and 20 ng/ml IL-7 (R&D Systems) to allow expansion of NY-ESO-1-specific CD4<sup>+</sup> T cell lines. NY-ESO-1 peptide-specific T cell lines were established as described (15), CD154-expressing CD4<sup>+</sup> T cells were isolated after restimulation with PHA-activated CD4<sup>+</sup> 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<sup>+</sup> T cell line, NY-ESO-1<sub>187-198</sub> peptide-specific CD4<sup>+</sup> T cell line (SB87) was obtained by cell sorting using HLA-DRB<sub>1</sub>*0101/NY-ESO-1<sub>187-198</sub> tetramer and expansion by PHA (data not shown). NY-ESO-1<sub>187-198</sub>-specific CD4<sup>+</sup> T cells was detected using IFN-γ catch reagents (Miltenyi Biotec) after restimulation with T-APC pulsed with NY-ESO-1<sub>187-198</sub> peptide. After isolation of IFN-γ<sup>+</sup> cells by cell sorting, they were expanded with PHA to generate NY-ESO-1<sub>187-198</sub> peptide-specific CD4<sup>+</sup> T cell line (SB87). Both CD4<sup>+</sup> T cell lines were maintained by periodical 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-DAGA, SU11652, and pifithrin-µ (PFT-µ) or 16–20 h (other reagents) in 2 ml RPMI+10%FCS in six-well plates (BD Falcon); lactacystin and epoxomicin from Boston Biochem; chloroquine, leupeptin, 3-methyl adenine, brefeldin A (BFA), PFT-µ and primase from Sigma-Aldrich; 17-dimethylaminoethanalamine-17-dimethoxy-geldanamycin (17-DAGA) from Invivogen; 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<sup>+</sup> 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 Esherichia 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 GFE, 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 µM siRNA and were applied with two pulses of 3 kV/cm for 200 µs 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 HSP90α, HSP90 β, gp96, and HSC70 from Enzo Life Sciences, and an mAb against LAMP2 from eBioscience.

**Intracellular cytokine staining**

Melanoma cell line (50,000 cells) and NY-ESO-1 peptide-specific CD4<sup>+</sup> T cell line (100,000 cells) were cocultured for 6 h in 96-well round-bottom plate in 250 µM RPMI+10%FCS in the presence of GolgiStop (BD Biosciences). Cells were fixed and permeabilized using a BD Cytofix/Cytoperm kit and stained with PE-conjugated anti–GM-CSF and allophycocyanin-conjugated anti–IFN-γ mAbs (BD Biosciences). T cells were analyzed by FACS-Calibur instrument and CellQuest software (BD Biosciences) by gating lymphocyte population in an FCS/SSC plot.

**ELISPOT assay**

Indicated number of effector CD4<sup>+</sup> T cell lines were cocultured with target melanoma cell lines (3 × 10<sup>5</sup>) or peptide-pulsed EBV-transformed B cell lines (5 × 10<sup>4</sup>) on nitrocellulose-coated microtiter plates (Millipore, Bedford, MA) precoated overnight with 2 µg/ml anti–IFN-γ mAb (1-D1K; Mabtech) and blocked with 10% human serum (Sigma-Aldrich) in RPMI 1640 medium. After 20–24 h, plates were developed using 0.2 µg/ml biotinylated anti–IFN-γ mAb (7-B6-1; Mabtech), 1 µM streptavidin–alkaline phosphate 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 µg/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<sup>+</sup> T cells was 10 µg/ml.

**Measurement of cytokine levels**

Melanoma cell line (3 × 10<sup>6</sup>) and NY-ESO-1 peptide-specific CD4<sup>+</sup> T cell line (5 × 10<sup>5</sup>) were cocultured for 20–24 h in a 96-well round-bottom plate in 250 µM RPMI+10%FCS. Supernatant was harvested and stored at −20°C. IFN-γ 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 ICp47<sup>+</sup>-expressing SK-MEL-37 clones**

HSV ICp47 gene was cloned into pcDNA3.1/V5-His TOPO plasmid with a stop codon (pcDNA-ICp47). SK-MEL-37 was transfected with pcDNA-ICp47 using Lipofectamine (Invitrogen). Clones growing in the presence of 500 µg/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 µg/ml G418. Functional expression of ICp47 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 µM Melan-A<sub>27-35</sub> peptide and 3 µg/ml β2-microglobulin (Sigma-Aldrich) and reduced recognition by NY-ESO-1–specific CD8<sup>+</sup> 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<sup>+</sup> T cell lines**

To characterize spontaneous NY-ESO-1–specific CD4<sup>+</sup> T cell responses in ovarian cancer patients who had serum Ab against NY-ESO-1, CD4<sup>+</sup> 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 cells, one recognizing NY-ESO-1–97–98 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 that were specific for NY-ESO-1–87–98 and NY-ESO-1–95–106 peptides 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.

FIGURE 1. Characterization of spontaneous CD4+ T cell responses against NY-ESO-1 in an ovarian cancer patient. (A) CD4+ T cells were stimulated once with a pool of 17 NY-ESO-1 overlapping peptides (#1–#17). After 20 d, NY-ESO-1–specific CD4+ T cells were detected by IFN-γ ELISPOT assay against the subpools of peptides (#1–#4, #5–#8, #9–#12, and #13–#17) using PHA-activated CD4+ T cells as APCs. (B) NY-ESO-1–peptide-specific CD4+ T cell line was generated by sorting CD154-expressing cells after restimulation with a #9–#12 peptide pool and polyclonally expanding with PHA. Reactivity of CD4+ T cell lines against individual peptide in the peptide pools was evaluated by IFN-γ ELISPOT assay. (C) Recognition of NY-ESO-1–expressing (SK-MEL-37 and SK-MEL-139) or NY-ESO-1–nonexpressing (SK-MEL-81) and MHC-II–expressing melanoma cell lines by NY-ESO-1 #9–#12 peptide-specific CD4+ T cell lines was evaluated by intracellular cytokine staining. Data are expressed as mean ± SD in (A).
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

FIGURE 2. Characterization of the tumor recognition by DR01-restricted NY-ESO-1–specific CD4+ T cell lines. (A) Recognition of DR01+NY-ESO-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.

FIGURE 3. Characterization of the recognition of naturally-processed NY-ESO-1 by DR01-restricted CD4+ T cell lines. (A) DR01+NY-ESO-1+ SK-MEL-12 was pulsed overnight with 10 μg/ml NY-ESO-1 (ESO) protein or transduced overnight with NY-ESO-1 (Ad-ESO) or control (Ad-Cont) adenovirus vectors at a multiplicity of infection of 1000. Recognition by SB87 or SB95 was evaluated by IFN-γ ELISA. (B) Dose-dependence of recognition of exogenous NY-ESO-1 protein by SB87 and SB95. SK-MEL-12 was incubated 16–20 h with the indicated concentration of NY-ESO-1 or 3 μg/ml control (SSX-2) protein. Recognition by SB87 or SB95 was evaluated by IFN-γ ELISPOT assay. (C) SK-MEL-12 was cultured overnight in the culture supernatant (SUP) of SK-MEL-37, and recognition by SB95 was evaluated by IFN-γ ELISPOT assay. (D) CFSE-labeled SK-MEL-12 was cocultured with PKH26-labeled SK-MEL-37 for 2 d to allow potential molecular exchange. After separation by cell sorting, recognition by SB95 was evaluated by IFN-γ ELISPOT assay. All experiments were repeated at least twice with similar results. Data are expressed as mean ± SD.
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 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. 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. 4A, 4B). 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 parental 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).

**Role of previously characterized pathways for endogenous MHC-II presentation**

It is known that MHC-II on APCs is constitutively loaded with peptides from endogenous proteins (19, 20). Recent studies of endogenous MHC-II presentation of viral or ectopically overexpressed model Ags identified several mechanisms such as intercellular Ag transfer, macroautophagy, chaperone-mediated autophagy, and presentation by recycling MHC-II molecules (11–14). Although TCR affinity of tumor Ag-specific T cells is in general lower than those of foreign or model Ag-specific T cells, it is important to investigate their role in the present endogenous MHC-II presentation by tumor cells. Results shown in Fig. 3C and 3D indicate that intercellular Ag transfer was not the mechanism for the presentation of cytosolic NY-ESO-1 to SB95.

Recent studies demonstrated that macroautophagy is involved in the presentation of intracellular viral Ag to CD4^+ T cells. However, recognition of SK-MEL-37 by SB95 was not inhibited by pretreatment of SK-MEL-37 with a macroautophagy inhibitor, 3-methyladenine, excluding the involvement of macroautophagy in the presentation (Fig. 5A). Interestingly, measurement of cytokine levels in supernatant by ELISA showed that inhibition of macroautophagy in SK-MEL-37 enhanced the recognition by SB95 (Fig. 5A), possibly indicating that macroautophagy in tumor cells constitutively processed NY-ESO-1 and reduced the amount of the Ag available for the presentation to SB95. Pretreatment of SK-MEL-37 by primaquine, which inhibits recycling of MHC-II, did not inhibit the recognition by SB95 (Fig. 5B). Interestingly, primaquine treatment significantly increased direct tumor recognition, especially at lower doses, suggesting that inhibition of MHC recycling enhanced endogenous MHC-II presentation in our experimental system. At higher doses, primaquine was found to reduce cell surface MHC-II expression (Supplemental Fig. 1A), which may be canceling the enhancement effect seen at lower doses. Efficient inhibition by brefeldin A also indicated that the peptides were loaded on newly synthesized MHC-II (Fig. 4D).

Involvement of chaperone-mediated autophagy in the endogenous MHC-II presentation of NY-ESO-1 was tested by siRNA-based silencing of LAMP-2 and HSC70 (Fig. 5C), which are critical for chaperone-mediated autophagy. As shown in Fig. 5D, silencing of
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-dimethylaminomethylaminol-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. This result that gp96 is 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 peptide expression by electroporation of siRNA, HSP90α, HSP90β, or gp96-specific siRNA or scrambled siRNA were electroporated to SK-MEL-37; 3 d later, protein levels of HSP90α, HSP90β, gp96, and NY-ESO-1 were evaluated by Western blot analyses. (D) 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 target cells in (B) and (D).
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 cell 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, breakpoint 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 provide a basis for the current development of HSP-based vaccine 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 for 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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