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Antibody-Targeted NY-ESO-1 to Mannose Receptor or DEC-205 In Vitro Elicits Dual Human CD8+ and CD4+ T Cell Responses with Broad Antigen Specificity

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Immunization of cancer patients with vaccines containing full-length tumor Ags aims to elicit specific Abs and both CD4+ and CD8+ T cells. Vaccination with protein Ags, however, often elicits only CD4+ T cell responses without inducing Ag-specific CD8+ T cells, as exogenous protein is primarily presented to CD4+ T cells. Recent data revealed that Ab-mediated targeting of protein Ags to bound to their respective targets on APC. Whereas nontargeted and Ab-targeted NY-ESO-1 proteins similarly activated CD4+ human mannose receptor and DEC-205, both internalizing molecules expressed on APC. These targeting proteins were evaluated for their ability to activate NY-ESO-1–specific human CD4+ and CD8+ T cells in vitro. Both targeted NY-ESO-1–proteins rapidly bound to their respective targets on APC. Whereas nontargeted and Ab-targeted NY-ESO-1–proteins similarly activated CD4+ T cells, cross-presentation to CD8+ T cells was only efficiently induced by targeted NY-ESO-1. In addition, both mannose receptor and DEC-205 targeting elicited specific CD4+ and CD8+ T cells from PBLs of cancer patients. Receptor-specific delivery of NY-ESO-1 to APC appears to be a promising vaccination strategy to efficiently generate integrated and broad Ag-specific immune responses against NY-ESO-1 in cancer patients. The Journal of Immunology, 2011, 186: 1218–1227.

Cancer-testis Ag NY-ESO-1 is expressed in a wide range of different tumor types, but its expression in normal adult tissues is limited to the testis (1, 2). We have shown that NY-ESO-1 is highly immunogenic, inducing spontaneous immune responses in patients with NY-ESO-1–expressing tumor but not in healthy individuals (3). In addition, the presence of a spontaneous anti–NY-ESO-1 Ab response is typically associated with spontaneous NY-ESO-1–specific CD8+ and CD4+ T cell responses in melanoma patients (4–6). Because of its frequent expression in tumors and high immunogenicity, NY-ESO-1 is an attractive target for cancer immunotherapy and has been used in a series of clinical vaccine trials. Extensive immune monitoring revealed that NY-ESO-1–specific immune responses were inducible by a variety of NY-ESO-1–based vaccines such as HLA class I and/or class II-binding peptides, recombinant viruses, and recombinant protein with or without adjuvants or delivery systems, even in patients without spontaneous immunity (7–12). Compared to vaccination with HLA-binding short peptides, vaccination with full-length tumor Ag such as recombinant proteins or viruses is considered to be more attractive because of the potential to induce a wider repertoire of specific Ab and T cell responses. Furthermore, recombinant proteins or viruses are expected to induce high-avidity T cells, whereas short peptide vaccination often induces low avidity T cells that fail to recognize naturally processed Ag (13, 14). Full-length tumor Ags intrinsically contain all possible CD4+ and CD8+ T cell epitopes for any type of HLA allele and thus they should potentially activate both T cell subsets. However, it was found that vaccination with recombinant NY-ESO-1 protein in the presence of vaccine adjuvant, bacillus Calmette-Guerin plus GM-CSF or imiquimod, induced CD4+ T cell responses in most immunized patients, whereas cross-priming of NY-ESO-1–specific CD8+ T cells was rare unless NY-ESO-1 protein was formulated in ISCOMATRIX or strong adjuvant like CpG was used (7, 9, 12, 15). In contrast, vaccination with recombinant viruses inducing intracellular NY-ESO-1 expression elicited frequent CD8+ T cell responses, though not always accompanied by the induction of specific CD4+ T cells (10). These observations are consistent with distinct pathways for MHC class I and class II Ag presentation and thus for separate processing and presentation of endogenous and exogenous protein via MHC class I and class II, respectively. Because both tumor Ag-specific CD8+ and CD4+ T cells cooperatively play important roles in eradicating cancer (16), using vaccine constructs that can simultaneously activate NY-ESO-1–specific CD8+ and CD4+ T cells is a much desired strategy to immunologically reverse the clinical course of cancer.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; EBV-B cell, EBV-transformed B cell; hCG, human chorionic gonadotropin β-chain; mo-DC, monocYTE-derived dendritic cell; MR, mannose receptor; T-APC, PHA-activated CD4+ T APC.

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Recently, we and others have shown that exogenous protein targeted to dendritic cells (DCs) by certain cell-surface molecule-specific Abs was efficiently cross-presented to Ag-specific CD8\(^+\) T cells (17–23). In previous studies, we evaluated induction of Ag-specific T cell responses by targeting a tumor Ag to the mannose receptor (MR), whereby an anti-MR mAb was fused to the tumor Ags human chorionic gonadotropin β-chain (hCG\(β\)) or pmel17. The genes for the mAb against MR were derived from hybridomas generated from human Ig-transgenic mice to minimize the risk of an undesired immunogenicity of the Ab itself. It was found that these fusion proteins targeted DC and were presented to both CD4\(^+\) and CD8\(^+\) T cells in vitro (21, 22). Furthermore, the efficient stimulation of Ag-specific CD4\(^+\) and CD8\(^+\) T cells and antitumor response by MR-targeted Ag were demonstrated in vivo in a human MR-transgenic mouse model (20). Thus, this strategy could be the basis for an efficient cancer vaccine to induce both CD4\(^+\) and CD8\(^+\) T cells against other human tumor Ags and clinical development of MR-targeted hCG\(β\) has been initiated. In addition to MR, the DEC-205 receptor was also demonstrated to be an efficient mAb-based target to enhance the induction of strong Ag-specific immune responses in mice (24). DEC-205 is a specific DC marker in the mouse and is thought to be implicated in Ag cross-presentation, although its specific ligand is not known (25). However, in humans, it is more broadly expressed on circulating lymphocytes including T, NK, and B cells in addition to DC and monocytes/macrophages, although the highest expression is found on professional APCs (25, 26). In a recent report, it was shown that HIV gag protein targeted by anti–DEC-205 mAb was efficiently cross-presented to multiple epitope-specific CD8\(^+\) T cells in vitro (18).

To translate these findings into potential clinical applications, we have generated two novel fusion proteins consisting of full-length NY-ESO-1 fused to human mAbs with specificity for MR (MR-ESO) and DEC-205 (DEC-ESO). In the current study, both reagents were evaluated for their activity to stimulate NY-ESO-1–specific T cells in vitro. These two fusion constructs retained their capacity to avidly bind to APCs via their respective surface receptors, and, in vitro sensitization of autologous lymphocytes with Ag-mediated NY-ESO-1–targeted APCs resulted in efficient expansion of NY-ESO-1–specific CD4\(^+\) and CD8\(^+\) T cells. Thus, engineered Ab-based immunotherapy combined with synergizing adjuvants (e.g., TLR activators) may overcome limitations observed with many current cancer vaccines.

Materials and Methods

Preparation of targeting constructs

Generation of Ab–Ag fusion proteins with human anti-human MR mAb (clone B11) was described previously (21, 22). For the NY-ESO-1 construct, the entire coding cDNA sequence for NY-ESO-1 was cloned into the same vectors were used, but the NY-ESO-1 sequence was replaced with the H chain C region sequence. The Ab–Ag fusion protein was expressed by transfection of Chinese hamster ovary cells and purified by protein A chromatography. For targeting to DEC-205, a novel human Ab was generated by immunization of mice engineered to express human IgGs with recombinant human DEC-205 (provided by R.M. Steinman, Rockefeller University, New York, NY). The human anti-human DEC-205 mAb (clone 3G9) was chosen for developing a targeting vector to DEC-205. The H and L chain variable regions of 3G9 were used to replace the B11 variable regions in the vector described above. As control targeting proteins, the H chain variable regions of 3G9 were used to replace the B11 variable regions of the targeting vector. The H chain C region sequence. The Ab–Ag fusion protein was expressed by transfection of Chinese hamster ovary cells and purified by protein A chromatography. For targeting to DEC-205, a novel human Ab was generated by immunization of mice engineered to express human IgGs with recombinant human DEC-205 (provided by R.M. Steinman, Rockefeller University, New York, NY). The human anti-human DEC-205 mAb (clone 3G9) was chosen for developing a targeting vector to DEC-205. The H and L chain variable regions of 3G9 were used to replace the B11 variable regions in the vector described above. As control targeting proteins, the H chain variable regions of 3G9 were used to replace the B11 variable regions of the targeting vector.
and were periodically tested for Ag expression and the absence of mycoplasma by RT-PCR. In some experiments, EBV-B cells were electroporated with in vitro-transcribed NY-ESO-1 or GFP mRNA prepared by mMESSAGE mMACHINE T7 Ultra kit (Ambion, Austin, TX) using the ECM 830 Electroporation System and electroporation cuvettes (Harvard Apparatus-BTX, Holliston, MA).

Generation of NY-ESO-1–specific T cell lines

NY-ESO-1–specific IFN-γ–producing CD8+ T cells were purified from presensitized CD8+ T cells using IFN-γ secretion assays reagents (Miltenyi Biotec). Briefly, presensitized CD8+ T cells were restimulated for 4 h with T-APCs that were pulsed with NY-ESO-1 overlapping peptides and labeled with CFSE (Invitrogen-Molecular Probes, Eugene, OR). Sorted cells were stimulated with PHA in the presence of irradiated allogeneic PBMCs and were expanded for ∼20 d in the presence of 10 U/ml IL-2 and 20 ng/ml IL-7. Reactivity of the T cell lines was tested against autologous or partially HLA-compatible allogeneic EBV-B cells pulsed with peptides.

Statistical analyses

Statistical analyses were performed using the Student t test. Results were considered significant at p < 0.05.

Results

Generation and characterization of targeted NY-ESO-1 fusion proteins

The targeted NY-ESO-1 fusion proteins were analyzed by SDS-PAGE and Western blot and showed the expected molecular shifts for a human IgG and an added NY-ESO-1 moiety (∼20 kDa for the H chain under reducing conditions and ∼40 kDa for intact Ab) (Fig. 1A). The NY-ESO-1 protein moiety in the fusion protein was detectable by Western blot using Abs against NY-ESO-1 (Fig. 1B). $K_D$ of DEC-ESO and MR-ESO against their respective target was determined to be 0.99 and 20.3 nM, respectively, by Biacore analysis, showing that both targeting constructs Ab were binding with high affinity (data not shown). The binding affinity of DEC-ESO was higher than the binding affinity of the MR-ESO construct.

To demonstrate that the targeted NY-ESO-1 proteins bind to the cell surface of APCs that express MR and DEC-205, mo-DCs generated from healthy donors were incubated with the targeted NY-ESO-1 or nontargeted recombinant protein and analyzed by flow cytometry using an anti–NY-ESO-1 mAb. As shown in Fig. 1C, modest or high levels of NY-ESO-1 protein were detected on the cell surface of immature mo-DC after incubation with DEC-ESO or MR-ESO, respectively. DEC-ESO or MR-ESO binding was mediated through target-specific Ab because the binding was specifically blocked by competitive binding of a corresponding control DEC-hCGβ or MR-hCGβ, respectively (Supplemental Fig. 1A). In contrast, binding of the nontargeted NY-ESO-1 protein on immature mo-DCs was negligible. Efficient binding of MR-ESO was also observed on mature mo-DCs. In addition, DEC-ESO binding was enhanced after maturation of mo-DCs (Fig. 1C), consistent with the report that mature mo-DCs express a higher level of DEC-205. Similar binding of DEC-ESO was also observed on EBV-B cells that only express DEC-205 but not MR, and the binding was blocked by competitive binding of control DEC-hCGβ, indicating specificity of binding (Fig. 1C and data not shown).

Next, we asked whether binding of the targeted NY-ESO-1 fusion protein to cell-surface DEC-205 or MR induced phenotypic changes in APCs per se or altered activation of APCs by other stimuli. To explore this, immature mo-DCs were treated with targeted NY-ESO-1 fusion protein in the presence or absence of optimal or suboptimal concentrations of LPS (1–100 ng/ml) or polyinosinic-polycytidylic acid (1–10 μg/ml) for mo-DC maturation. After 24 h, expression of activation/maturation molecules and cytokine levels in the supernatant were measured. Addition of NY-ESO-1 protein or the targeted NY-ESO-1 fusion proteins resulted in no significant changes relative to controls in markers such as CD86 and CD83 or cytokines including TNF-α, IL-10, and IL-12 (Supplemental Figs. 2, 3).
Cross-presentation of targeted NY-ESO-1 fusion proteins

To investigate cross-presentation of the targeted NY-ESO-1 to CD8+ T cells, mo-DCs were pulsed with targeted fusion proteins or nontargeted recombinant NY-ESO-1 protein, and the recognition of NY-ESO-1 by NY-ESO-1–specific CD8+ T cells was evaluated using IFN-γ ELISPOT assays. As shown in Fig. 2A, HLA-B*2705-restricted NY-ESO-194–102 peptide-specific CD8+ T cell clone UC98F11 (31) recognized mo-DCs that were pulsed with DEC-ESO or MR-ESO. Both targeted NY-ESO-1 fusion proteins induced comparable numbers of IFN-γ spots with peptide-pulsed mo-DCs. In contrast, mo-DCs pulsed with nontargeted recombinant NY-ESO-1 protein were inefficient to stimulate the UC98F11 CD8+ T cell clone. The recognition of targeted NY-ESO-1 fusion proteins by UC98F11 was NY-ESO-1 specific, as the control DEC-hCGβ and MR-hCGβ fusion proteins were not recognized. In contrast, NY-ESO-1–specific CD4+ T cells were similarly stimulated by mo-DCs coincubated with either the targeted NY-ESO-1 fusion proteins or the recombinant NY-ESO-1 protein at the concentrations used (Fig. 2B). These results indicate that although both targeted and nontargeted NY-ESO-1 were taken up by mo-DCs and processed via an HLA class II presentation pathway, only the cross-presentation to NY-ESO-1–specific CD8+ T cells was enhanced by targeting to cell-surface DEC-205 or MR. However, one of the CTL clones tested, the HLA-Cw*03-restricted NY-ESO-192–100 peptide-specific CTL clone C5 (32), did not recognize the targeted NY-ESO-1 fusion proteins pulsed on mo-DCs, even though it efficiently recognized adenovirus-induced NY-ESO-1 (Fig. 2C) and even though DEC-ESO and MR-ESO bound efficiently to mo-DCs (Fig. 1C). Although these results suggest that cross-presentation by the targeted NY-ESO-1 appears to be dependent on the epitope, it is plausible that targeting of NY-ESO-1 protein to MR or DEC-205 molecules enhances cross-presentation of selected NY-ESO-1 peptides on HLA class I molecules.

To demonstrate that targeting NY-ESO-1 protein to cell-surface DEC-205 or MR was essential for the cross-presentation to CD8+ T cells, we tested whether the cross-presentation of targeting NY-ESO-1 protein is inhibited by blocking the binding by preincubation with competitive control targeting protein (hCGβ construct). As shown in Supplemental Fig. 1B, preincubation of mo-DC with DEC-hCGβ and MR-hCGβ significantly reduced the cross-presentation of DEC-ESO and MR-ESO, respectively, indicating that targeting of NY-ESO-1 to the cell-surface DEC-205 or MR significantly enhanced the cross-presentation of NY-ESO-1. Furthermore, we tested EBV-B cells that express DEC-205 but not MR as APCs for the UC98F11 clone. As shown in Fig. 2D, DEC-ESO but not MR-ESO or recombinant NY-ESO-1 was cross-presented by DEC-205/MR−EBV-B cells.

Activation of NY-ESO-1–specific T cells in PBMCs of cancer patients

The targeting ability of NY-ESO-1 proteins to APCs was further investigated for the capacity to activate NY-ESO-1–specific T cells from PBMCs of cancer patients. NY-ESO-1 is highly immunogenic in patients with ovarian cancer, eliciting both cellular and humoral immune responses in a proportion of patients with advanced NY-ESO-1–expressing tumors (33, 34). We reported that both NY-ESO-1–specific CD8+ and CD4+ T cell responses were detectable in PBMCs from NY-ESO-1–seropositive patients following in vitro presensitization (34, 35). CD8+ or CD4+ T cells from PBMCs of seropositive ovarian cancer patients were stimulated with T cell-depleted autologous PBMCs after pulsing with the targeted NY-ESO-1 fusion proteins or NY-ESO-1 overlapping peptides. Protein-spanning overlapping peptides were used as a positive control, as they are useful in vitro reagents to detect broad T cell responses. After 10 or 20 d culture in the presence of IL-2 and IL-7, the frequency of NY-ESO-1–specific IFN-γ–producing CD8+ or CD4+ T cells, respectively, was evaluated by ELISPOT assays. Because APCs were mixture of various types of cells, we evaluated the binding of targeted fusion protein on T cell-depleted PBMCs, as shown in Fig. 3A. After pulsing on PBMCs, DEC-ESO rapidly bound on CD14+ monocytes, especially on a CD16− subset, and CD303+ plasmacytoid DCs, as detected by staining surface NY-ESO-1 in the fusion proteins. Consistent with previous reports, CD19+ B cells and CD56+ NK cells were also targeted by low levels of DEC-ESO (25, 26). MR-ESO bound on monocytes, especially on a CD14+CD16+ subset, and CD1c+ myeloid DCs. In contrast to DEC-ESO, MR-ESO binding to B cells was negligible. Interestingly, detectable levels of MR-ESO bound on CD56+ cells. The expression of MR on uterine NK cells was recently reported (36). The binding of MR-ESO on peripheral NK cells suggested that MR is also expressed on peripheral NK cells. No significant cell-surface binding of nontargeted NY-ESO-1 protein was detected on any population in T cell-depleted PBMCs.

Characterization of CD8+ T cell responses

A comparison of the number of IFN-γ–producing CD8+ T cells for three different patients after a single round of presensitization with targeted NY-ESO-1 proteins or NY-ESO-1 overlapping peptides is
shown in Fig. 3B–D. DEC-ESO expanded NY-ESO-1–specific CD8+ T cells in NY-ESO-1–seropositive ovarian cancer patient SB as indicated by IFN-γ spots against NY-ESO-1 overlapping peptide-pulsed target cells (Fig. 3B). The magnitude of the response, however, appeared less than after stimulation with a pool of overlapping NY-ESO-1 peptides. An NY-ESO-1–specific CD8+ T cell response was also detectable after presensitization with MR-ESO in this patient. A similar induction of NY-ESO-1–specific CD8+ T cells by the DEC-ESO was observed in another seropositive cancer patient, AA (Fig. 3C). The MR-ESO was not tested because of the limited number of cells from this patient. In another patient, CB, a CD8+ T cell response was not observed after presensitization with DEC-ESO, although a weak NY-ESO-1–specific CD8+ T cell response was elicited by overlapping peptides (Fig. 3D).

We compared the epitope(s) recognized by NY-ESO-1–specific CD8+ T cells induced by NY-ESO-1 overlapping peptides and targeted NY-ESO-1 proteins. NY-ESO-1–specific CD8+ T cells were further expanded by isolating specific T cells using an IFN-γ capture cell-sorting technique and polyclonal expansion with PHA. The expanded NY-ESO-1–specific CD8+ T cell lines were then tested for their reactivity against each peptide in the overlapping peptide pool. We found that in the case of patient SB, all CD8+ T cell lines generated from CD8+ T cells presensitized with NY-ESO-1 overlapping peptides or targeted NY-ESO-1 fusion proteins strongly recognized peptides #10 and #11 and weakly recognized peptides #13 and #16 (Fig. 4). These results indicate that targeted NY-ESO-1 can stimulate multiple epitope-specific CD8+ T cells, including all epitope-specific CD8+ T cells elicited by the peptide pool.

Because the number of spots against peptides #10 and #11 were approximately equal to spots against the entire overlapping peptide pool #1–17, the immunodominant epitopes driving the CD8+ T cell response was expected to be within the overlapping NY-ESO-1–specific peptide region of #10 and #11 peptides. To determine the minimal epitope, three peptides NY-ESO-1 101–110, NY-ESO-1 101–109, and NY-ESO-1 102–110 were tested separately for their recognition by the CD8+ T cell lines. As shown in Fig. 5A, two 9-mer peptides, NY-ESO-1 101–109 and NY-ESO-1 102–110, were similarly recognized, whereas the recognition of 10-mer peptide NY-ESO-1 101–110 was less efficient. These two 9-mer peptides were further tested to determine the avidity of CD8+ T cell lines. As shown in Fig. 5B, NY-ESO-1 101–109 peptide was ~10 times more efficient to stimulate the CD8+ T cell lines than NY-ESO-1 102–110 peptide, indicating that NY-ESO-1 101–110 peptide was the minimum epitope. The HLA restriction of the recognition of NY-ESO-1 101–110 and NY-ESO-1 102–110 was determined to be HLA-B*4002 by allogeneic EBV-B cell lines partially sharing the patient’s HLA alleles as shown in Fig. 5C. To test if NY-ESO-1 101–110 peptide was naturally processed from endogenously expressed NY-ESO-1 protein, the recognition of HLA-B*4002 EBV-B cells electro-porated NY-ESO-1 mRNA by NY-ESO-1–specific CD8+ T cell lines was tested. As shown in Fig. 5D, all CD8+ T cell lines specifically produced IFN-γ against NY-ESO-1 mRNA-transfected EBV-B cells but not control GFP mRNA-transfected cells. Finally, these CD8+ T cells recognized NY-ESO-1–HLA-B*4002 melanoma cell line, SK-MEL-52, but not NY-ESO-1–HLA-B*4002 SK-MEL-139 (Fig. 5E). Interestingly, the same NY-ESO-1 101–109 peptide was recognized by NY-ESO-1–specific CD8+ T cell line established from another HLA-B*4002 cancer patient, AA, who was tested in this study (Fig. 3C) and indeed was presented by HLA-B*4002 (Supplemental Fig. 4). Thus, this NY-ESO-1 101–109 peptide presented by HLA-B*4002 appears to be immunodominant in cancer patients who have HLA-B*4002. This
immunological monitoring carried out in our laboratories of patients immunized with recombinant NY-ESO-1 protein revealed that vaccination with NY-ESO-1 protein induced CD8+ T cell and Ab responses against NY-ESO-1 but the induction of CD8+ T cells was rather uncommon (12, 15). However, when NY-ESO-1 protein was formulated in delivery systems such as cholesterol-bearing hydrophobized pullulan or ISCOMATRIX, which facilitate cross-presentation of a formulated protein Ag (9, 11), or was administered with CpG 7909 in montanide (7), CD4+ and CD8+ T cell responses were observed, suggesting that immunization with protein Ags alone or combined with a weak adjuvant are inefficiently presented to the immune system. Even with these approaches, not all patients developed strong CD8+ T cell responses, indicating that in vivo cross-priming may not be optimal. Recently, several groups that focused on delivering proteins to APCs have reported that protein Ags targeted by receptor-specific Abs to APCs were efficiently cross-presented to specific CD8+ T cells (18–23). In the current study, we have investigated if NY-ESO-1 protein, when targeted to internalizing receptors on APCs such as the MR or DEC-205, would be a valid vaccination strategy to prime both NY-ESO-1–specific CD4+ and CD8+ T cells in cancer patients. Selective delivery is achieved through targeting fusion proteins consisting of human mAbs to MR (MR-ESO) and DEC-205 (DEC-ESO) and the full-length NY-ESO-1 protein fused to the C terminus of the IgG H chain by molecular engineering. The genes for anti–DEC-205 and anti-MR mAbs were derived from NY-ESO-1 seropositive ovarian cancer patient SB by an IFN-γ catch cell sorting after restimulation with overlapping peptides and were poly-specific against each peptide in 17 overlapping peptides and an overlapping peptides pool (#1–17) by ELISPOT assay. Autologous T-APCs were used as APCs. Each column indicates the number of spots per 10,000 CD8+ T cells without background subtraction. Experiments were repeated twice to confirm the reactivity against peptides.

epitope was similarly recognized by CD8+ T cell lines induced from overlapping peptides or from targeted proteins.

Characterization of CD4+ T cell responses

NY-ESO-1–specific CD4+ T cell responses after presensitization with targeted and nontargeted NY-ESO-1 protein and overlapping peptides were evaluated at day 20. Both targeted and nontargeted NY-ESO-1 proteins were found to efficiently induce multiple epitopes recognizing NY-ESO-1–specific CD4+ T cells in all seropositive patients tested (Fig. 6).

When the recognition of each peptide in the overlapping peptides were compared with CD4+ T cells obtained after stimulation with targeted NY-ESO-1 protein or a peptide pool, a difference in the relative strength of the responses was observed (Fig. 6). For patient SB, a much stronger CD4+ T cell response against peptide #11 was observed by the DEC-ESO and MR-ESO than that induced by overlapping peptides. In contrast, responses against peptides #13 and #14 were stronger in CD4+ T cells stimulated with overlapping peptides than after stimulation with MR-ESO. Similarly, CD4+ T cells from patient AS and VZ were presensitized with recombinant NY-ESO-1 protein to compare NY-ESO-1 protein with the targeted NY-ESO-1 fusion proteins and overlapping peptides pools. CD4+ T cell responses against peptides #7 and #12 peptides in patient AS and against peptide #9 in patient VZ were more effectively induced by targeted NY-ESO-1 protein than by the nontargeted NY-ESO-1 protein.

Discussion

Full-length tumor Ags are becoming increasingly available and are now being investigated in human cancer vaccine trials for their ability to induce T cell and humoral responses against tumors in patients regardless of a patient’s HLA haplotype. Extensive
DEC-ESO bound to EBV-B cells in addition to mature DC. Consistent with this finding, we observed that DEC-ESO, but not MR-ESO, was cross-presented to a NY-ESO-1–specific CD8+ T cell clone by EBV-B cells. It would be interesting to investigate the Ag-cross-presentation also by other lymphocyte subsets after targeting Ags to their cell-surface receptors. However, our preliminary experiments indicated that isolation of pure lymphocyte subsets by flow cytometric cell sorting significantly reduced the cross-presentation capability and did not allow determination of the relative contribution of each subset in Ag cross-presentation.

In the current study, DEC-ESO and MR-ESO were compared for their capability to stimulate NY-ESO-1–specific CD4+ and CD8+ T cells in PBMCs of cancer patients, who had spontaneous Abs against NY-ESO-1. Both DEC-ESO and MR-ESO were able to stimulate NY-ESO-1–specific CD8+ T cells, which recognized multiple epitopes presumably presented by diverse HLA class I and II molecules. DEC-ESO, in particular, appeared to be more efficient to induce CD8+ T cell response than MR-ESO in patient SB. In these experiments, T cell-depleted PBMCs were used as APCs that contained DC, monocyte/macrophages, B, and NK cells. Because DEC-205 is expressed on a wider range of cells in contrast to myoid DC and NK-specific expression of MR (Fig. 3A), it would be interesting to analyze the different leukocyte subsets for the presentation of DEC-205–targeted proteins to explain the different ability to prime specific CD8+ T cells by MR-ESO and DEC-ESO. In addition to CD8+ T cells, both DEC-ESO and MR-ESO induced strong CD4+ T cell responses in all patients tested. These NY-ESO-1–specific CD4+ T cells recognized multiple epitopes, and, in contrast to CD8+ T cells, they were induced by targeted and nontargeted NY-ESO-1 proteins as well as NY-ESO-1 overlapping peptides. However, several differences were observed. Some epitopes for NY-ESO-1–specific CD4+ T cells appeared to be preferentially presented by the APC-targeting NY-ESO-1 protein compared with a synthetic NY-ESO-1 peptide pool or nontargeted NY-ESO-1 protein. In addition, targeted NY-ESO-1 proteins clearly showed stronger and broader CD4+ T cell responses than recombinant nontargeted protein. These observations suggest that protein tumor Ags taken up via the cell-surface molecules DEC-205 and MR are processed by a different pathway, resulting in CD4+ T cells recognizing a wider spectrum of epitopes or that an enhanced Ag uptake via those receptors induces the generation of T cells recognizing more epitopes.

Although NY-ESO-1 epitopes for CD8+ T cells have been reported for relatively many HLA class I types compared with other tumor Ags, it is still desirable to identify more epitopes for the various HLA class I molecules to broaden the applicability of vaccination. In addition, it would allow expanded monitoring of spontaneous and/or vaccine-induced CD8+ T cell responses. In the
FIGURE 6. Induction of multiepitope-specific CD4+ T cells by DEC-ESO or MR-ESO. CD4+ T cells from NY-ESO-1 seropositive ovarian cancer patients were cultured for 20 d after presensitization with overlapping peptides, targeted DEC-ESO or MR-ESO, or nontargeted NY-ESO-1 protein. The specificity of CD4+ T cells was tested against each peptide in overlapping peptides by IFN-γ ELISPOT assay. Autologous T-APCs were used as target cells. Experiments were repeated twice to confirm the peptide reactivity. *CD4+ T cell responses against #1–4 peptides and #5–8 peptides pools in patient SB were shown to be negative in a separate experiment and were not tested in this assay.
current study, a spontaneously induced novel epitope for HLA-B*4002-restricted NY-ESO-1–specific CD8+ T cells were identified in cancer patients with NY-ESO-1–expressing tumor. The CD8+ T cell response against the epitope (NY-ESO-1101–109) was found to be immunodominant in two HLA-B*4002+ patients. Importantly, NY-ESO-1101–109-specific CD8+ T cells were able to recognize a B*4002+ and NY-ESO-1+ tumor cell line, indicating that this epitope was naturally processed in cancer cells. The finding that both HLA-B*4002+ patients showed strong CD8+ T cell response against NY-ESO-1101–109 suggests that this epitope was highly immunogenic in individuals who are HLA-B*4002+.

In conclusion, both targeted fusion proteins, DEC-ESO and MR-ESO, were shown to efficiently deliver and cross-present NY-ESO-1 protein in humans, providing a strong immunological rationale for exploring these constructs in clinical cancer vaccine trials in patients with NY-ESO-1–expressing tumors. A clinical trial of DEC-ESO in combination with the TLR agonist resiquimod has recently been initiated. In contrast to other studies using TLR agonists, it is suggested that this epitope was naturally processed in cancer cells.

References


Figure Legends for Supplemental Figures

**Fig. S1.** Blocking of targeting NY-ESO-1 protein binding to the cell surface of mo-DC by competitive antibody. (a) Binding of DEC-ESO and MR-ESO on mo-DC was investigated by flowcytometry. Mo-DC were preincubated with or without 10 μg/ml DEC-hCGβ or MR-hCGβ on ice for 30 minutes and then incubated with 4 μg/ml DEC-ESO (left) or MR-ESO (right). After extensive washes, NY-ESO-1 protein bound on mo-DC was detected by anti-NY-ESO-1 mAb and PE-conjugated anti-mouse IgG1 mAb. Similar results were obtained in mo-DCs from two donors. (b) Inhibition of cross-presentation of DEC-ESO and MR-ESO by blocking their binding to the cell surface molecules. Mo-DCs were preincubated with or without 100 μg/ml DEC-hCGβ or MR-hCGβ on ice for 30 minutes and then incubated with 4 μg/ml DEC-ESO or MR-ESO on ice for 15 minutes. After extensive washes, cells were incubated overnight at 37°C to allow the antigen processing and used as target cells in ELISPOT assay. Experiments were repeated twice and the result from one experiment was shown as mean + SD from duplicate samples. *, p<0.05.

**Fig. S2.** Absence of stimulatory/costimulatory effect of targeted NY-ESO-1 proteins on monocyte-derived dendritic cells (mo-DCs). Mo-DCs were prepared as described in Materials and Methods. At day 6, mo-DCs were incubated with indicated targeted NY-ESO-1 fusion protein (DEC-ESO or MR-ESO) or recombinant NY-ESO-1 protein for 30 minutes before adding indicated toll like receptor ligands. After 24 hours, cells were harvested and stained with PE-conjugated anti-CD83 and FITC-conjugated anti-CD86 mAbs. Fluorescent intensity was measured and analyzed by a FACSCalibur instrument and CellQuest software, respectively.

**Fig. S3.** Absence of stimulatory/costimulatory effect of targeted NY-ESO-1 proteins on monocyte-derived dendritic cells (mo-DCs). Mo-DCs were prepared and stimulated as in Fig. S1. After 24 hours, supernatant was harvested and cytokine levels in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA). The limit of detection in these assays was 78 pg/ml for IL-12, 9.4 pg/ml for IL-10, and 390 pg/ml for TNF. Experiments
were performed using mo-DCs from two different healthy donors. The results were shown as mean + SD from duplicate samples.

**Fig. S4.** Characterization of NY-ESO-1-specific CD8\(^+\) T cell responses from patient AA. CD8\(^+\) T cells from patient AA were presensitized and NY-ESO-1-specific CD8\(^+\) T cell line was generated as described in Materials and Methods. (A-B) Peptide specificity of NY-ESO-1-specific CD8\(^+\) T cell lines was evaluated using autologous EBV-B cells as APCs by ELISPOT assay. (C) Avidity of CD8\(^+\) T cell line against short peptides was evaluated by ELISPOT assay using autologous EBV-B cells pulsed with an indicated concentration of short peptides. (D) HLA-restriction of NY-ESO-1\(_{101-109}\) and NY-ESO-1\(_{102-110}\) was determined as HLA-B*4002 by HLA-compatible allogeneic EBV-B cells. (E) Tumor recognition of HLA-B*4002-restricted and NY-ESO-1\(_{101-109}\)-specific CD8\(^+\) T cell line. SK-MEL-52: NY-ESO-1\(^+\)HLA-B*4002\(^+\); SK-MEL-139: NY-ESO-1\(^+\)HLA-B4002\(^+\); SK-MEL-149: NY-ESO-1\(^+\)HLA-B*4002\(^+\); SK-MEL-37: NY-ESO-1\(^+\)HLA-B4002\(^+\).