CD8\(^+\) T-Cell Response to NY-ESO-1: Relative Antigenicity and \textit{in Vitro} Immunogenicity of Natural and Analogue Sequences\(^1\)

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Abstract

We have shown previously that HLA-A*0201 melanoma patients can frequently develop a CTL response to the cancer testis antigen NY-ESO-1. In the present study, we have analyzed in detail the relative antigenicity and \textit{in vitro} immunogenicity of natural and modified NY-ESO-1 peptide sequences. The results of this analysis revealed that, although suboptimal for binding to the HLA-A*0201 molecule, peptide NY-ESO-1 157–165 is, among natural sequences, very efficiently recognized by specific CTL clones derived from three melanoma patients. In contrast, peptides NY-ESO-1 157–167 and NY-ESO-1 155–163, which bind very strongly to HLA-A*0201, are recognized less efficiently. In agreement with previous data, substitution of peptide NY-ESO-1 157–165 COOH-terminal C with various other amino acids resulted in a significantly increased binding to HLA-A*0201 molecules as well as in an increased CTL recognition, although variable at the clonal level. Among natural peptides, NY-ESO-1 157–165 and NY-ESO-1 155–167 exhibited good \textit{in vitro} immunogenicity, whereas peptide NY-ESO-1 155–163 was poorly immunogenic. The fine specificity of interaction between peptide NY-ESO-1 157–167 and HLA-A*0201, and T-cell receptor was analyzed at the molecular level using a series of variant peptides containing single alanine substitutions. The findings reported here have significant implications for the formulation of NY-ESO-1-based vaccines as well as for the monitoring of either natural or vaccine-induced NY-ESO-1-specific CTL responses in cancer patients.

Introduction

The recent identification of molecularly defined human tumor antigens recognized by autologous CTLs has opened new opportunities for the development of antigen-specific cancer vaccines. Most of the known antigens have been identified in melanoma. Among these, differentiation antigens (e.g., Melan-A, tyrosinase, and gp100) are exclusively expressed by melanoma cells as well as by normal melanocytes but not by tumors of other histological types. In contrast, CT\(^3\) antigens are expressed in several cancer types but not in normal tissues, except male germ-line cells and placental trophoblasts, which do not express MHC class I molecules (1). Thus, CTL reactivity to CT antigen can be considered both tumor specific and potentially beneficial for many cancer types. For this reason, CT antigens are particularly interesting candidates for the development of cancer vaccines. Six CT antigen families (including MAGE, BAGE, and GAGE) have been identified thus far, either by using tumor-reactive CTL clones from melanoma patients (2) or by immunoscreening of cDNA expression libraries from tumors with autologous sera from cancer patients (SEREX; Ref. 3).

T-cell reactivity to some CT antigens, such as those derived from the MAGE family, has been in general detected only rarely in a limited number of cancer patients, even after vaccination (4, 5). In contrast, we and others have shown recently that CD8\(^+\) T-cell responses to the SEREX-defined antigen NY-ESO-1 can be frequently detected in peptide-stimulated PBMCs from HLA-A*0201 melanoma patients (6, 7). Although this observation encourages the development of NY-ESO-1-based vaccines, knowledge of the molecular details of the interaction between NY-ESO-1-derived peptides, HLA-presenting molecules, and specific T-cell receptors is required. Several overlapping antigenic peptide sequences have been identified in the NY-ESO-1 protein region spanning amino acid residues 155–167 (8). By using a single NY-ESO-1-specific CTL clone, we have shown recently that the peptide NY-ESO-1 157–165 was the most efficiently recognized among the three NY-ESO-1 natural sequences. Moreover, the efficiency of recognition could be further improved by replacing the COOH-terminal C (cysteine; amino acids are indicated in the single-letter code) with L or A (6). In the present study, we have confirmed and extended these observations by analyzing in detail the binding of both natural overlapping NY-ESO-1 sequences and enhanced peptide analogues to HLA-A*0201, as well as their relative antigenicity and \textit{in vitro} immunogenicity.

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\(^{3}\) The abbreviations used are: CT, cancer-testis; PBMC, peripheral blood mononuclear cell; TIL, tumor-infiltrating lymphocyte; hIL-2, human recombinant interleukin; PBL, peripheral blood lymphocyte.
Materials and Methods

Cell Cultures. Melanoma cell lines Me 275 and Me 242 were established in our laboratory from surgically excised melanoma metastases from patients LAU 50 and LAU 92, respectively. NY-ESO-1-specific CTL clone LAU 156/5 was derived from TILs of melanoma patient LAU 156 by limiting dilution in the presence of irradiated allogeneic PBMCs, phytohemagglutinin, and hrIL-2 (150 units/ml; Glaxo, Geneva, Switzerland; kindly provided by Dr. M. Nabholz, Institut Suisse de Recherche Experimenterale sur le Cancer, Epalinges, Switzerland) as described elsewhere (6). PBMCs from two HLA-A*O201 melanoma patients (LAU 50 and LAU 155) were used in this study. For peptide stimulation experiments, CD8+ lymphocytes were positively selected by magnetic cell sorting using a miniMACS device (Miltenyi Biotec, Sunnyvale, CA). Cells from the CD8~ fraction were irradiated (3000 rads) and used as autologous antigen-presenting cells. CD8~ highly enriched lymphocytes (1 x 10^6/well) were stimulated with the indicated peptide (1 μM) and irradiated autologous antigen-presenting cells (2-4 x 10^6/well) in 2 ml of Iscove’s Dulbecco’s medium (Life Technologies, Inc., Basel, Switzerland) supplemented with 0.24 mM Asn, 0.55 mM Arg, 1.5 mM Gin, 8% pooled human A+ serum (CTL medium), hrIL-2 (100 units/ml), and hrIL-7 (10 ng/ml; R&D System Europe, Oxon, United Kingdom). Cells were cultured for the indicated period of time prior to ELISPOT or tetramer analysis.

HLA-A2 Binding and Antigen Recognition Assays. Peptide binding to HLA-A2 was assayed in a functional competition assay based on inhibition of recognition of the antigenic peptide tyrosinase 368-376 (YMDGTMSQV) by the HLA-A2-restricted CTL clone LAU132/2 (9). Briefly, T2 cells were labeled with ^51Cr in the presence of the anti-class I monoclonal antibody W6/32 for 1 h at 37°C and washed three times. Various concentrations of competitor peptides were incubated with ^51Cr-labeled T2 cells (1000 cells/well) for 15 min at room temperature. A suboptimal dose (1 nM) of the antigenic peptide was then added together with specific CTLS (10,000 cells/well) in a final volume of 200 μl. Chromium release was measured after 4 h incubation at 37°C. The concentration of each competitor peptide required to achieve 50% inhibition of target cell lysis was then determined as [nM] 50%. To facilitate comparison, the relative competitor activity of each peptide was calculated as the [nM] 50% of the reference peptide divided by the [nM] 50% of the competitor peptide. Antigen recognition was assessed using chromium-release assays. Labeled target cells (1000 cells in 50 μl) were incubated in the presence of various concentrations of peptide (50 μl) for 15 min at room temperature before the addition of effector cells (10,000 cells in 100 μl). Chromium release was measured in supernatant harvested after 4 h incubation at 37°C. The percentage of specific lysis was calculated as:

% specific lysis = (Experimental - spontaneous release) / (Total - spontaneous release) x 100

The relative antigenic activity of each peptide was calculated as the [nM] 50% of the parental NY-ESO-1 nonapeptide 157-165 SLLMWITQC divided by the [nM] 50% of the index peptide.

Tetramers, Monoclonal Antibodies, and Flow Cytometry Immunofluorescence Analysis. Two HLA-A2/peptide tetramers were synthesized as described (10, 11). The NY-ESO-1 157-165 natural peptide (SLLMWITQC) and the NY-ESO-1 157-165 C165A analogue (SLLMWITQA) were used. Cells were stained with tetramers in 20 μl of PBS (5-10 μg/ml), 0.2% BSA, and 0.2% sodium azide for 1 h at room temperature; then 2 μl of anti-CD8FITC (Becton Dickinson) were added, and incubation continued for additional 30 min at 4°C. Cells were washed once in the same buffer and analyzed by flow cytometry. Data analysis was performed using Cell Quest software.

IFN-γ ELISPOT Assay. IFN-γ ELISPOT assay (12) was performed in nitrocellulose-lined, 96-well microplates (Milipore MAHA S45, Bedford, MA) using a IFN-γ ELISPOT kit (Mabtech, Stockholm, Sweden) according to the manufacturer’s instructions, with minor modifications. Plates were coated overnight with antibody to human IFN-γ and washed six times. T2 cells (5 x 10^4/well) were then added together with responder T cells (2 x 10^5/well) and peptide (1 μM, where indicated) in duplicate cultures and incubated for 20 h at 37°C. Cells were then removed, and plates were developed with a second antibody to human IFN-γ (biotinylated) and streptavidin-alkaline phosphatase. Spots were counted using a stereomicroscope with a magnification of ×15.

Results

Assessment of A2/NY-ESO-1 Tetramer Reactivity. The use of peptide analogues with improved binding to MHC molecules for the preparation of tetramers has proven useful in the case of suboptimal natural antigenic peptides (10). We have shown previously that substitution of the COOH-terminal C on NY-ESO-1 157-165 with A improves HLA-A2 binding (6). We thus synthesized A2/NY-ESO-1 peptide tetramers containing either the natural NY-ESO-1 157-165 or the analogue NY-ESO-1 157-165 containing the C165A substitution. We first compared the ability of these tetramers to stain NY-ESO-1-reactive CTL clone LAU 156/5. Both tetramers stained efficiently the CTL clone in a dose-dependent manner (Fig. 1A). At the highest dose tested, fluorescence intensity was 3 logs above background. The concentration of tetramers required to obtain a fluorescent signal of intermediate intensity (~700 units) was of 12 μg/ml for the natural peptide containing tetramer and of 3 μg/ml for the peptide analogue containing tetramer. Thus, there was no major quantitative difference between the two tetramers. In addition, staining with the two A2/NY-ESO-1 tetramers of a 14-day bulk culture of PBLs from patient LAU 50 after a single stimulation with the NY-ESO-1 157-165 natural peptide gave identical percentages of CD8+ NY-ESO-1 tetramer+ lymphocytes. The mean fluorescence intensity was 809 and 1164 for the natural and peptide analogue-containing tetramers, respectively (Fig. 1B). Similar results were obtained with several other polyclonal populations. On the basis of these results, the natural peptide containing tetramers were used in the rest of this study.

Isolation and Characterization of NY-ESO-1 157-165-specific CTL Clones. We have described previously the isolation and characterization of a NY-ESO-1-specific CTL clone LAU 156/5. This clone was isolated from A2/NY-ESO-1 tetramer+ sorted cells from 2-week-cultured TILs from melanoma...
Recall the antigenic peptide analogues of NY-ESO-1. Importantly, all three clones also efficiently lysed the melanoma cell line Me 242, which also expresses high levels of NY-ESO-1 and LAGE-1, but is HLA-A2 negative (Fig. 1B and data not shown). Together, these data demonstrate the antigen specificity and tumor reactivity of the three independent NY-ESO-1-specific CTL clones.

Relative HLA-A*0201 Binding of NY-ESO-1-derived Peptides. Binding of different NY-ESO-1-derived peptides to the HLA-A2 molecule was measured in a functional competition assay (13). Although ~0.3 μM of the NY-ESO-1 157–165 peptide was required to obtain 50% of competitor activity, 9- and 13-fold lower concentrations were sufficient to obtain the same effect with the two HLA-A2 binding overlapping peptides identified initially (8), i.e., NY-ESO-1 155–163 and 157–167, respectively (Table 1). As we have reported recently, substitution of the COOH-terminal amino acid C in the NY-ESO-1 157–165 nonapeptide by either A or L resulted in a marked increase (10–100-fold of magnitude) in peptide binding to the HLA-A2 molecule (Ref. 6 and Table 1). Similarly, substitutions of the same amino acid residue with amino acids S and V also resulted in a significantly increased peptide binding activity (13-fold for S and ~4000-fold for V).

To analyze in detail NY-ESO-1 antigen recognition by human CTLs, a panel of single A-substituted NY-ESO-1 157–165 peptides was synthesized. This type of analysis allows us to gain information on the role of single amino acids in both peptide binding to the MHC molecule and interaction with the specific T-cell receptors. To avoid the deleterious influence of C on peptide binding, the single A substitutions were introduced in the context of the NY-ESO-1 157–165 C165A enhanced peptide analogue. As expected, substitution of A for L at position two, which is the main anchor position for binding to HLA-A2, led to 100-fold reduction in peptide binding. Similarly, substitution at two other positions (P5 and P6) significantly reduced peptide binding to HLA-A2, whereas substitution of M at P4 had a positive effect on peptide binding. Substitution of amino acids at the remaining positions with alanine only minimally affected peptide binding.

Antigenicity of NY-ESO-1 Natural and Analogue Peptides. To assess the efficiency of peptide antigen recognition by NY-ESO-1-specific CTL clones, peptide titrations were performed using a standard 4-h chromium release assay and the T2 cell line as target cell. This cell line is convenient because it is highly susceptible to CTL-mediated lysis and, because of its defect in transporter associated with antigen processing-dependent transportation of endogenously synthesized peptides (14), efficiently presents exogenous peptides binding to HLA-A2. The three CTL clones recognized the parental NY-ESO-1 157–165 peptide with similar efficiency. Indeed, the peptide concentration required to reach 50% of the maximal lysis was 3–12 nM, corresponding to intermediate-avidity CTLs. Two other natural peptide sequences (NY-ESO-1 157–167 and 155–163), identified previously as potential CTL epitopes (8), were less efficient as antigens for all three CTL clones. In particular,
recognition of peptide 155–163 was not detectable with clone LAU 156/5 (Fig. 3, left panels). NY-ESO-1 157–165 analogue results from substitution of the COOH-terminal C were generally better antigens than the parental peptide. Of note, the C165A replacement appeared to be the most efficient antigenic peptide analogue, with concentrations required to obtain 50% of maximal lysis three to four orders of magnitude lower than the parental nonapeptide for all three clones (Fig. 3, left panels). Peptides with the C165L and C165S substitutions were also recognized more efficiently by the three CTL clones, with a variable increase in relative antigenic activity. The most dramatic effect was observed with the C165L analogue and clone LAU 155/18 (10^2-fold increase in relative antigenic activity). In contrast, the same substitution had a minimal effect on clone LAU 156/5. Finally, the C165V amino acid substitution was recognized more efficiently (two to three logs) by only two of the three CTL clones.

The recognition patterns of the set of NY-ESO-1 157–165 C165A single A-substituted peptide variants are shown in the panels to the right in Fig. 3. The relative antigenic activity was calculated using the peptide NY-ESO-1 157-165 C165A as reference. Each clone exhibited a unique pattern of fine specificity of recognition. The peptide variant more clearly showing the distinct specificities of these clones was the one containing the Q164A substitution at P8. Indeed, although this peptide was not detectably recognized by the clone LAU 155/18, it was recognized as efficiently as the reference peptide by clone LAU 156/5 and 100-fold more efficiently than the reference peptide by the clone LAU 50/13. The pattern of recognition of the other A-substituted peptides was quite similar for two of the three clones (clone LAU 50/13 and clone LAU 156/5). Moreover, all of the amino acid residues located in the middle region of the antigenic peptide (P3 to P7) appeared to be critical for recognition by all of the three CTL clones. The most dramatic effects on recognition were apparent for clone LAU 155/18.

In Vitro Immunogenicity of NY-ESO-1 Natural and Analogue Peptides. To evaluate the ability of either natural or modified NY-ESO-1 peptides to elicit specific responses, we measured the frequency of tetramer-reactive lymphocytes resulting from stimulation of CD8^+ enriched PBLs with the individual peptides. As shown in Fig. 4, 1 week after stimulation with NY-ESO-1 157–165 peptide, tetramer^+ lymphocytes constituted approximately 0.1–0.2% of the CD8^+ lymphocytes present in the cultures. The 155–163 peptide was a weak stimulator, whereas stimulation with the 157–167 peptide as well as with the COOH-terminal substituted 157–165 analogues led to an expansion of tetramer^+ lymphocytes comparable with that obtained upon stimulation with the 157–167 nonapeptide. Because testing peptide analogue-driven T-cell expansion with tetramers may fail to detect peptide-specific T cells that do not cross-react with the parental sequence, cultures were also tested using the IFN-γ ELISPOT assay. As illustrated in Table 2, a
Table 1  Relative competitor activity of NY-ESO-1 natural and analogue peptides

<table>
<thead>
<tr>
<th>NY-ESO-1 peptide</th>
<th>Sequence</th>
<th>Competitor activity[a]</th>
<th>Relative competitor activity to peptide</th>
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<td>Natural peptides</td>
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<td>157-165</td>
<td>SLLMWITQC</td>
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<td>155-163</td>
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<td>157-167</td>
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<td>13</td>
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<td>157-165 analogues</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C165A</td>
<td>SLLMWITQQA</td>
<td>0.0003</td>
<td>900</td>
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<tr>
<td>C165L</td>
<td>SLLMWITQL</td>
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<td>130</td>
</tr>
<tr>
<td>C165S</td>
<td>SLLMWITQS</td>
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<td>13</td>
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<tr>
<td>C165V</td>
<td>SLLMWITQY</td>
<td>0.00006</td>
<td>4500</td>
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<td>S157A, C165A</td>
<td>ALLMWITQA</td>
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<td>208</td>
</tr>
<tr>
<td>L158A, C165A</td>
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<td>27</td>
</tr>
<tr>
<td>L159A, C165A</td>
<td>SLAMNWITQA</td>
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<td>270</td>
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<tr>
<td>M160A, C165A</td>
<td>SLLAATQA</td>
<td>&lt;0.0001</td>
<td>&gt;2700</td>
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<tr>
<td>W161A, C165A</td>
<td>SLLMAITQA</td>
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<tr>
<td>I162A, C165A</td>
<td>SLLMWATQA</td>
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<tr>
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</tr>
<tr>
<td>Q164A, C165A</td>
<td>SLLMWITAA</td>
<td>0.00018</td>
<td>1500</td>
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</table>

[a] Competitor activity was measured on the basis of the inhibition of recognition of the antigenic peptide tyrosinase 368-376 (YMDGTMSQV) in the context of HLA-A*0201 by the specific clone LAU 156/2.
[b] The relative competitor activity was calculated as described in "Materials and Methods" using the NY-ESO-1 157-165 peptide SLLMWITQC as the reference peptide with an arbitrary competitor activity of 1.
[c] Substitutions are underlined.

comparable number of specific cells were obtained in the presence of either the stimulating peptide analogue or the parental 157–165 peptide. These results confirm the poor immunogenicity of the 155–163 peptide and indicate that the majority of specific T cells elicited upon stimulation with COOH-terminal substituted 157–165 peptide analogues fully cross-react with the natural peptide.

Discussion

In this study, we have analyzed in detail the reactivity and fine specificity of CTL clones isolated from three metastatic melanoma patients and specific for an HLA-A2-restricted NY-ESO-1 tumor-associated antigen. The results showed that the natural antigen recognized by these CTLs was optimally mimicked by the synthetic peptide 157–165. In addition, several enhanced antigenic peptide analogues generated by single substitution of the COOH-terminal amino acid were identified.

The NY-ESO-1 tumor-associated antigen has been shown to be the target of both naturally occurring antibody (15) and CTL (8, 16) responses in cancer patients. Moreover, studies with relatively large numbers of patients with advanced metastatic melanoma revealed that about half of those with NY-ESO-1-expressing tumors had high titers of specific serum IgG that fell upon reduction of tumor burden, suggesting that NY-ESO-1 seropositivity is dependent on persistent antigen stimulation (7, 17, 18). The identification of HLA-A2-restricted T-cell epitopes derived from NY-ESO-1 has allowed the assessment of the extent to which NY-ESO-1 is also targeted spontaneously by specific CTLs in cancer patients. These studies showed that melanoma patients who are both HLA-A2[2] and NY-ESO-1 seropositive have naturally occurring CTL responses directed against the HLA-A2-restricted NY-ESO-1 T-cell site(s) (6, 7), Thus, the tumor-specific NY-ESO-1 antigen appears to be highly immunogenic in melanoma.

Our results of NY-ESO-1 antigen recognition by CTL clones obtained from different melanoma patients confirmed previous observations with polyclonal CTL lines, indicating that three overlapping NY-ESO-1 peptides can be efficiently recognized at saturating peptide concentrations (6, 8). Although the extent of cross-reactivity between CTLs elicited upon stimulation with peptides NY-ESO-1 157–165 or 155–163 remains to be established, analysis of their relative immunogenicity suggested that peptide NY-ESO-1 155–163 is poorly immunogenic, at least in vitro. The availability of NY-ESO-1-specific and tumor-reactive CTL clones may in the future aid in the identification of the naturally processed NY-ESO-1 peptide(s) present at the surface of tumor cells and isolated by biochemical approaches (19). This should help in understanding the nature of the endogenously processed NY-ESO-1 peptide(s) that are presented by tumor cells to the immune system.

The evaluation of the efficiency of peptide antigen recognition by the panel of CTL clones showed that their relative avidity for antigen is similar, both by intensity of tetramer staining and peptide titration in functional recognition assays. Despite their common ability to optimally recognize the 157–165 nonapeptide as well as tumor cell lines expressing the naturally processed NY-ESO-1 antigen, individual clones exhibited unique patterns of fine specificity, as revealed by the experiments with a panel of single A-substituted NY-ESO-1 peptides. Previous studies of the T-cell receptor repertoire of
Fig. 3  Relative antigenicity of NY-ESO-1 natural and analogue peptides. The antigenic activity of natural peptides and their analogues was assessed in a 4-h chromium release assay as detailed in “Materials and Methods.” The nanomolar concentration required for 50% maximal lysis by each CTL clone and for each peptide was calculated from peptide titration curves. For clones LAU 50/13, LAU 155/18, and LAU 156/5, the nanomolar concentration required for 50% maximal lysis of the reference natural peptide NY-ESO-1 157–165 was of 7, 12, and 3 nM, respectively. These values were used for calculating the relative potency of all of the other peptides analyzed, for each clone, as described in “Materials and Methods.”

Table 2  Number of IFN-γ producing cells in 20,000 cultured cells

<table>
<thead>
<tr>
<th>Stimulating peptide</th>
<th>No peptide</th>
<th>SLLMWITQC</th>
<th>Stimulating peptide</th>
</tr>
</thead>
<tbody>
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<td>SLLMWITQC</td>
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<td>75</td>
<td>NA</td>
</tr>
<tr>
<td>QLSLLMWIT</td>
<td>40</td>
<td>38</td>
<td>29</td>
</tr>
<tr>
<td>SLLMWITQCL</td>
<td>11</td>
<td>74</td>
<td>66</td>
</tr>
<tr>
<td>SLLMWITQQA</td>
<td>19</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SLLMWITQL</td>
<td>14</td>
<td>72</td>
<td>77</td>
</tr>
<tr>
<td>SLLMWITQS</td>
<td>10</td>
<td>&gt;100</td>
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<tr>
<td>SLLMWITQV</td>
<td>9</td>
<td>66</td>
<td>65</td>
</tr>
</tbody>
</table>

* Numbers in bold, values 3-fold higher than the corresponding background value.

NA, not applicable.

Fig. 4  In vitro immunogenicity of NY-ESO-1 natural peptides and analogues. Highly enriched CD8<sup>+</sup> cells from PBMCs of melanoma patients LAU 50 and LAU 155 were stained with A2/NY-ESO-1 157–165 tetramers together with anti-CD8 1 week after in vitro stimulation with the indicated peptides. Percentages of A2/NY-ESO-1 157–165 tetramer<sup>+</sup> cells were calculated with Cell Quest software. Results are shown for gated CD8<sup>+</sup> cells.

study a series of COOH-terminal substitutions (L, V, and S) in the 157–165 antigenic peptide that exhibit enhanced binding to the HLA-A2 molecule. These peptide analogues also exhibited increased antigenicity and similar or higher in vitro immunogenicity compared with the parental sequence. The relative antigenicity was variable at the clonal level for enhanced analogues containing a L or V substitution, whereas analogues containing a COOH-terminal A or S were recognized at least 100-fold more efficiently than the parental sequence by all CTLs analyzed. Enhanced NY-ESO-1 157–165 analogues may prove useful in future clinical trials of peptide-based tumor
vaccination. Indeed, the C residue at the COOH-terminal of the parental peptide is not only suboptimal for binding to the A2 molecule but can also be easily oxidized, which may render appropriate peptide purification and formulation difficult. Thus, NY-ESO-1 157–165 COOH-terminal substituted analogues might be better peptide candidates, based not only on their increased immunological activity but also because they should lend themselves for efficient large-scale production under GMP conditions.

In conclusion, the results of this study support the use of COOH-terminally substituted enhanced NY-ESO-1 157–165 analogues in clinical trials of peptide vaccination in patients with NY-ESO-1 expressing tumors and underline the usefulness of A2/NY-ESO-1 157–165 tetramers for the monitoring of NY-ESO-1-specific responses in A2+ cancer patients.

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