NY-ESO-1 is highly expressed in poor-prognosis multiple myeloma and induces spontaneous humoral and cellular immune responses

Frits van Rhee, Susann M. Szmania, Fenghuang Zhan, Sushil K. Gupta, Mindy Pomtree, Pei Lin, Ramesh B. Batchu, Amberly Moreno, Giulio Spagnoli, John Shaughnessy, and Guido Tricot

The presence of a metaphase cytogenetic abnormality (CA) is the key negative predictor of outcome in patients with multiple myeloma (MM). Gene expression profiling (GEP) of such patients showed increased expression of NY-ESO-1 compared to patients with normal cytogenetics (60% versus 31%; \( P = .004 \)). NY-ESO-1 was also highly expressed in relapsing MM especially patients with CA (100% versus 60.7%; \( P < .001 \)). GEP findings were confirmed at the protein level by immunostaining of marrow biopsies for NY-ESO-1. We detected spontaneous NY-ESO-1–specific antibodies by enzyme-linked immunosorbent assay in 33% of patients with NY-ESO-1–positive MM, especially in CA patients (9 of 13; 70%), but in none of the NY-ESO-1–negative patients with MM (27) or healthy donors (21). Spontaneous NY-ESO-1–specific T cells (0.2%-0.6% of CD8+ T cells) were found in the peripheral blood of NY-ESO-1–positive MM with HLA-A*0201/NY-ESO-1157-165 tetramers. These NY-ESO-1–specific T cells, when expanded, killed primary MM cells (50% lysis, effector-target [E/T] ratio, 10:1). Our data demonstrate that NY-ESO-1 is frequently expressed in MM with CA and is capable of eliciting spontaneous humoral and T-cell immunity. The pool of NY-ESO-1–specific cytotoxic T cells expands easily on NY-ESO-1 peptide stimulation and is functionally active. NY-ESO-1 should therefore be an ideal tumor target antigen for immunotherapy of patients with poor-prognosis MM. (Blood. 2005;105:3939-3944)

**Introduction**

During the past 10 years, numerous human tumor-associated antigens (Ags) have been identified, either by screening cDNA libraries with sera derived from cancer patients containing an antibody (Ab) to a tumor-associated Ag (SEREX) or by using T lymphocytes specific for tumor peptides presented in the context of specific HLA alleles.1-11 The most rapidly expanding group of tumor Ags are the cancer/testis (C/T) Ags, which are either not expressed or are present at very low levels in normal tissues except the testes and perhaps the placenta.12,13 Because the testes are not immunogenic with not only well-documented spontaneous14-20 and vaccine-induced immunity, but also clinical responses in a broad-spectrum tumor-specific cancer vaccine.

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at significantly higher levels in patients with CAs. We determined by immunohistochemistry (IHC) that NY-ESO-1 Ag is also present at the protein level in malignant plasma cells (PCs). We next established that patients with NY-ESO-1+ and CA MM have spontaneous humoral and cytotoxic immune responses to NY-ESO-1. Moreover, expanded NY-ESO-1–specific T lymphocytes were capable of killing primary NY-ESO-1+ MM cells.

Patients, materials, and methods

Patients

NY-ESO-1 expression was studied in 374 patients with MM at diagnosis or relapse by GEP (n = 335) or IHC (n = 39). Twenty of these patients were studied by both GEP and IHC for comparison between NY-ESO-1 RNA and protein expression. Of 19 patients studied by IHC, 13 were studied both at diagnosis and at relapse. Enzyme-linked immunosorbent assay (ELISA) studies for NY-ESO-1 Abs were performed on 66 patients with MM. Informed consent was obtained according to the Declaration of Helsinki and the study was approved by the University of Arkansas for Medical Sciences Institutional Review Board.

Detection of NY-ESO-1 gene expression by GEP

Testing for NY-ESO-1 RNA expression in highly purified (> 95%) CD138+ MM PCs by GEP was performed and analyzed as reported previously.19

Immunohistochemical staining for NY-ESO-1 protein

To detect NY-ESO-1 protein, bone marrow (BM) biopsies were fixed in Zenker fluid at the time of collection and stained by IHC using the NY-ESO-1–specific murein IgGl monoclonal Ab (mAb) B9.8 and the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) per the manufacturer’s instructions. Briefly, sections were deparaffinized with xylene and hydrated after passing through descending grades of ethanol, immersed in preheated Ag retrieval solution, steamed for 30 minutes, cooled, and washed. Endogenous peroxidase activity was abolished using peroxidase block (Dako, Carpinteria, CA) for 10 minutes at room temperature (RT) and 10% goat serum in Tris (tris(hydroxymethyl)aminomethane)–buffered saline, pH 7.6, which was used for nonspecific protein blocking. After incubation with undiluted primary mAb B9.8 in a humid chamber for 2 hours at RT, the sections were washed and biotinylated using a goat antiserum secondary Ab (Pel-Freez, Rogers, AR) diluted 1:500. After incubation with avidin-biotin-peroxidase complex (Vector Laboratories), the chromogenic substrate 3, 3’-diaminobenzidine tetrahydrochloride (ResGen; Invitrogen, Carlsbad, CA) in 2% BSA/PBS was added and incubated for 1 hour at RT. The sections were washed, treated with aminoethylocyanin-2 (Richard Allan Scientific, Kalamazoo, MI). A tumor-free testicular biopsy (Richard Allan Scientific, Kalamazoo, MI). A tumor-free testicular biopsy

NY-ESO-1157-C165V/A2 phycoerythrin (PE)–labeled tetramers (NIAID Tetramer Facility, Bethesda, MD and Beckman Coulter, Fullerton, CA), which stained NY-ESO-1–specific CTLs equally well. Influenza matrix peptide58-66–specific CTLs were stained with influenza matrix peptide58-66/A2 PE tetramers (Beckman Coulter).

Intracellular staining of CTLs

Intracellular staining for interferon γ (IFN-γ), IL-4, perforin, and granzyme production was performed as previously described28 with the exception that the CTLs were stained by NY-ESO-1157-C165V/pulsed autologous DCs for 4 hours prior to staining as opposed to nonspecific stimulation. Quadrant assignments were based on isotype controls designed specifically for intracellular staining.

Cytotoxicity assays with NY-ESO-1–specific T cells

NY-ESO-1–specific CTLs were tested for cytotoxicity in triplicate in standard 4-hour 51CrO4 release assays. Targets were more than 95% purified CD138+, A2+, and NY-ESO-1+ primary MM cells, the A2+ and NY-ESO-1+ MM cell line U266, autologous phytohemagglutinin-induced blasts (PHA-blasts) pulsed with NY-ESO-1157-C165V peptide (positive control) or PHA-blasts pulsed with the HLA-A2–binding MAGE-312-120 peptide (negative control), and the natural killer (NK)–sensitive cell line K562.

Results

NY-ESO-1 is more frequently expressed in patients with CA or in relapse

GEP analysis of cRNA of more than 95% pure PCs from 335 patients showed that high NY-ESO-1 expression was more frequent
increased expression of NY-ESO-1.

in monoclonal gammopathy of undetermined significance (MGUS; 16%; *P* < .001; Table 1; Figure 1). Patients with newly diagnosed NY-ESO-1+ MM were more likely to have chromosome 13 deletion or hypodiploidy (or both) than NY-ESO-1− patients (31% versus 16%; *P* = .045). The expression frequency of NY-ESO-1 was low in monoclonal gammapathy of undetermined significance (MGUS; n = 22) and smoldering MM (SMM; n = 34), at 4.5% and 5.9%, respectively (*P* < .001) compared with newly diagnosed MM requiring intensive chemotherapy or relapsed MM.

**NY-ESO-1 expression by GEP correlates well with protein expression by IHC**

Immunostaining of MM cells by IHC for NY-ESO-1 revealed that NY-ESO-1 was located in the cytoplasm (Figure 2). Simultaneous GEP and IHC data from the same BM aspirate and biopsy were available on 20 patients. All biopsies positive for NY-ESO-1 by GEP also tested positive by IHC, and vice versa. Thus, there is complete concordance between NY-ESO-1 expression at RNA and protein levels.

Because NY-ESO-1 RNA expression by GEP is more frequently increased at relapse than at diagnosis, we next performed IHC to confirm these GEP findings at the protein level. A total of 55 biopsies from 39 patients were available for IHC. NY-ESO-1 protein was detected in all 38 relapse samples compared to 9 of 17 biopsies from 39 patients were available for IHC. NY-ESO-1 expression at RNA and protein levels.

**Spontaneous humoral responses are present in poor-prognosis NY-ESO-1+ MM**

Ab responses to NY-ESO-1 were detected by ELISA in 13 of 39 (33%) patients who tested NY-ESO-1+ by GEP (Table 2). The antibody responses were titered and remained positive at 1:1600, the highest dilution tested. In contrast, Ab responses to NY-ESO-1 were not detected in any of the 27 NY-ESO-1− patients (*P* < .001). The mean OD titers in the NY-ESO-1+ GEP+ and ELISA+ group, the GEP+ and ELISA+ group, and the GEP-NY-ESO-1 group differed significantly at 2.9, 1.2, and 1.2 (*P* < .001). All 21 healthy donors tested negative. Nine of 13 patients with Ab to NY-ESO-1 had CAs, indicating that patients with poor-prognosis MM were also able to mount a CD4+ T-cell and humoral response to NY-ESO-1.

Of the 19 patients studied in the presence of frank MM (diagnosis, n = 11; progressive/relapsed disease, n = 8), 9 patients (47%) had Ab to NY-ESO-1. In contrast, only 4 of 20 (20%) NY-ESO-1+ patients achieving either a major tumor reduction after therapy (n = 13) or (near) complete remission (CR; n = 7) had NY-ESO-1 Abs detectable (*P* = .08). This suggests a relationship between tumor load and the presence of NY-ESO-1 aAs. There was no relationship between ELISA positivity and absolute CD3+, CD4+, and CD19+ counts per microliter (data not shown).

**Spontaneous NY-ESO-1–specific T cells to NY-ESO-1 are present in patients with NY-ESO-1+ poor-prognosis MM**

We investigated banked PBMCs from 3 patients who had NY-ESO-1+ MM with CA and who were HLA-A2+. This allowed for the enumeration in the peripheral blood by tetramer analysis of spontaneously present CD8+ T cells specific for the immunodominant peptide NY-ESO-1_{157-165} in the context of HLA-A2. Spontaneous NY-ESO-1–specific T cells comprised 0.2% and 0.6%, respectively, of the CD8+ population in 2 patients. The third patient had been heavily pretreated including tandem auto-PBSCT. Control cultures with the HLA-A2–binding influenza matrix peptide (IM_{55-64}) were performed in all 3 patients both to check the specificity of the tetramers and to examine the immunocompetence of the patients. In patients 1 and 2 we could readily expand IM_{55-64}–specific T cells, but this was not possible in patient 3 indicating that the immune system of this patient may have been exhausted.

**Table 1. Expression of NY-ESO-1**

<table>
<thead>
<tr>
<th>MM stage</th>
<th>Normal cytogenetics</th>
<th>Abnormal cytogenetics</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Patients studied</td>
<td>Percent positive</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>126</td>
<td>31</td>
</tr>
<tr>
<td>Relapse</td>
<td>28</td>
<td>61</td>
</tr>
</tbody>
</table>

*Newly diagnosed and relapsed patients with cytogenetically abnormal MM have increased expression of NY-ESO-1. *P* was determined with the Pearson *χ*² method.*

**Table 2. Antibody responses to NY-ESO-1**

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No. tested</th>
<th>NY-ESO-1+ ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM, GEP NY-ESO-1+</td>
<td>39</td>
<td>13 (39%)</td>
</tr>
<tr>
<td>MM, GEP NY-ESO-1-</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Healthy donors</td>
<td>21</td>
<td>0</td>
</tr>
</tbody>
</table>

A humoral response to NY-ESO-1 protein is detected in one third of patients with NY-ESO-1+ myeloma (*P* = .003).
NY-ESO-1–specific CTLs kill primary MM cells

We expanded NY-ESO-1–specific T cells by restimulation with autologous antigen-presenting cells (APCs) pulsed with the NY-ESO-1157-C165V analog peptide, which is more immunogenic due to better binding to HLA-A2 and is also easier to work with in vitro due to its superior stability. The expansion of NY-ESO-1–specific CTLs allowed for detailed investigation of their functional capacity. After expansion, CD8+ NY-ESO-1157-C165V–specific T cells comprised 98% of the CD8+ T-cell population. HLA-A2 tetramers loaded with either wild-type NY-ESO-1157,165 or analog NY-ESO-1157,C165V peptide recognized the NY-ESO-1–specific CTLs equally well (Figure 3A). We were unable to generate NY-ESO-1+ CTLs from 3 NY-ESO-1+ healthy donors suggesting that pre-existing immunity to NY-ESO-1 is a prerequisite for expansion of NY-ESO-1–specific CTLs.

The CD8+ NY-ESO-1–specific CTLs killed primary MM, the NY-ESO-1+ and HLA-A2+ MM cell line U266, and the patient’s normal cells pulsed with NY-ESO-1157,165 peptide but not the patient’s normal cells pulsed with the HLA-A2–binding MAGE-3112,123 (irrelevant peptide) or the NK cell target K562 (Figure 3B). The CTLs were of Tc1 type (IFN-γ+ and IL-4−), contained cytolytic granules, and are of memory-effector type. VLA indicates very late antigen; CD31 is an excellent surrogate marker for the presence of a CD4+ T-cell population. HLA-A2 tetramers loaded with either wild-type NY-ESO-1157,165 or analog NY-ESO-1157,C165V peptide recognized the NY-ESO-1–specific CTLs equally well (Figure 3A). We were unable to generate NY-ESO-1+ CTLs from 3 NY-ESO-1+ healthy donors suggesting that pre-existing immunity to NY-ESO-1 is a prerequisite for expansion of NY-ESO-1–specific CTLs.

The presence of spontaneous antibody responses to NY-ESO-1 is important because this suggests that there is already a reservoir of NY-ESO-1–specific CD4+ T cells relatively early in the disease that could be expanded and used for the immunotherapy of NY-ESO-1+ MM patients. These patients attain a CR or near CR with the same frequency as patients with normal cytogenetics, but these responses are not durable.

We examined BM biopsies at the protein level and found that all samples that were positive for NY-ESO-1 RNA by GEP were also positive by IHC. It was important to confirm RNA findings at the protein level because there have been reports that NY-ESO-1 expression by reverse transcription-polymerase chain reaction (RT-PCR) is not always confirmed by IHC, presumably due to the low level of RNA expression in these patients. NY-ESO-1–specific Ab responses to NY-ESO-1 were also frequently expressed in MM with CA, including patients who have deletion of chromosome 13. At diagnosis approximately 60% of patients with CA express NY-ESO-1, whereas at relapse 100% of patients with CA expressed NY-ESO-1. In contrast, NY-ESO-1 was expressed in only 5% to 11% of patients with MGUS or smoldering MM.

We and others have documented that NY-ESO-1 is not expressed, or is at low levels, in normal tissues, including CD34+ cells and normal PCs (data not shown). NY-ESO-1 is therefore an obvious candidate Ag for true MM-specific immunotherapy to supplement the effects of tandem transplants in poor-prognosis patients. These patients attain a CR or near CR with the same frequency as patients with normal cytogenetics, but these responses are not durable.

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specific T-cell response, which will help to prime, expand, and most importantly, sustain CD8+ NY-ESO-1–specific T-cell responses. The CD4+ population may also exert effector functions both via macrophage and eosinophil activation and via cytokines such as IFN-γ that up-regulate expression of MHC class I and II complexes on many tumor cells.56-59 A caveat is that IgG subclass ELISAs were not performed and it can therefore not be completely excluded that some Ab+ patients may have type 2 cellular immune responses.

A high frequency of NY-ESO-1–specific Ab has also been detected in patients with advanced stage IV, NY-ESO-1+ melanoma.6,66 Interestingly, NY-ESO-1–specific Abs were detected less frequently in our patients who had obtained an excellent response to therapy. Our data are consistent with others who found in melanoma that a specific humoral immune response appears to be Ag-driven and removal or regression of NY-ESO-1 tumor results in loss of Ab.60

Spontaneous NY-ESO-1–specific CD8+ T cells were detected by tetramer staining of the blood of NY-ESO-1+ patients with MM, at levels from 0.2% to 0.6% prior to any restimulation. The NY-ESO-1–specific cells were detected both by NY-ESO-1157-165/HLA-A2 and HLA-A2 tetramers loaded with the more immunogenic NY-ESO-1157-165SV analog peptide. Substitution of cysteine for valine at position 165 of the NY-ESO-1 peptide reduces dimerization of the carboxy terminus cysteine and improves binding affinity for HLA-A2, resulting in enhanced antigenicity.51,62 We were able to significantly expand NY-ESO-1–specific CTLs by repeated stimulation with autologous mature DCs or PBMCs pulsed with NY-ESO-1 peptide from NY-ESO-1+/HLA-A2 patients with MM. These expanded CTLs killed primary MM cells effectively, which indicates that CTLs were not only present but also functional and able to recognize naturally processed and presented NY-ESO-1–protein and displayed NY-ESO-1157-165 peptide in the context of HLA-A2 at the cell surface. We were unable to expand and expand NY-ESO-1–specific CTLs lines in vitro from NY-ESO-1+ patients with MM or from healthy individuals. This suggests that stimulation with NY-ESO-1–peptide does not lead to de novo priming of NY-ESO-1–specific CTLs. A good correlation between humoral and cellular immune responses has been observed mainly in advanced melanoma both spontaneously and after peptide vaccination.8,15 Vaccine-induced T-cell responses to NY-ESO-1 have been observed and in one study all patients with humoral responses to NY-ESO-1 showed a strong CD8+ T-cell response, with not only strong concordance between enzyme-linked immunospot assay (ELISPOT), tetramer, and cytotoxicity assays for CD8+ T-cell reactivity to NY-ESO-1, but also stabilization of disease or regression of a single metastasis.21,63

Our data support the suitability of NY-ESO-1 immunotherapy in patients with poor-prognosis MM, preferably in combination with chemotherapy and auto-PBSCT to attain a state of minimal residual disease, which should be optimal for effective immunotherapy. We already have data showing the feasibility of combining auto-PBSCT with immunotherapy, by early (ie, prior to transplantation) vaccination with DCs loaded with tumor lysate and collecting and freezing of specific T cells prior to transplantation. After transplantation, the primed T cells are infused and immediately boosted with further vaccinations. Early vaccination expands memory cells, which are much more resistant even to high-dose chemotherapy than naive T cells and recover more promptly after transplantation. We will test the novel strategy of priming anti-MM T cells before auto-PBSCT and protecting the primed T cells from high-dose chemotherapy by apheresis and cryopreservation of the primed cells in a new clinical trial for high-risk MM using vaccinations with NY-ESO-1 Ag.

Finally, we expect that analysis of the MM transcriptome or protein profile or both will allow us to predict in the near future with high confidence which patients with MGUS or smoldering MM are likely to progress to frank MM and thus require high-dose chemotherapy. We are considering prophylactic vaccination with an optimized NY-ESO-1 vaccine64,65 for these patients to prevent disease progression by eradicating emerging more malignant MM clones, which are likely to be NY-ESO-1+.66

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References


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