Cancer Immunity, Vol. 4, p. 15 (16 December 2004)
Submitted: 2 September 2004. Accepted: 20 October 2004.
Contributed by: A Knuth

CANCER
IMMUNITY
Article

HLA-DP4 expression and immunity to NY-ESO-1: correlation and characterization of cytotoxic CD4+ CD25- CD8- T cell clones

Eduardo Huarte[1], Julia Karbach[1], Sacha Gnjatic[2], Armin Bender[1], Dirk Jäger[3], Michael Arand[4], Djordje Atanackovic[2], Jonathan Skipper[2], Gerd Ritter[2], Yao-Tseng Chen[2], Lloyd J. Old[2], Alexander Knuth[3], and Elke Jäger[1]

[1]II. Medizinische Klinik, Hämatologie-Onkologie, Krankenhaus Nordwest, Frankfurt, Germany
[2]Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, New York, USA
[3]Abteilung für Onkologie, Universitätsspital Zürich, Switzerland
[4]Institut für Pharmakologie und Toxikologie, Universität Würzburg, Germany

Keywords: human, cancer, NY-ESO-1, humoral immunity, HLA-DP4, cytotoxic T cells

NY-ESO-1 is one of the most immunogenic cancer antigens known to date, eliciting spontaneous immune responses in approximately 50% of patients with NY-ESO-1+ cancers. Spontaneous CD4+ and CD8+ T cell responses were found in patients with detectable NY-ESO-1 serum antibody, indicating an integrated type of immune response induced by NY-ESO-1+ malignancies. A close association between spontaneous NY-ESO-1 immunity and the HLA-DP4 allele was suggested in a recent study. To address these results, we assessed the NY-ESO-1 antibody and HLA-DP4 status of 102 patients with NY-ESO-1+ malignancies. However, no correlation between HLA-DP4 and NY-ESO-1 immunity was found. To explore the role of HLA-DP4-restricted CD4+ T cells in cancer immunity, we established HLA-DP4-restricted NY-ESO-1-specific CD4+ T cell clones by limiting dilution and repeated stimulation with NY-ESO-1 peptide p157-170 from NY-ESO-1 seropositive patients. A subset of CD4+ T cell clones was reactive with naturally processed NY-ESO-1 presented by autologous DCs that were pulsed with recombinant NY-ESO-1 protein, lysates of NY-ESO-1-expressing tumor cell lines, or transduced with recombinant NY-ESO-1 viral constructs in ELISPOT assays. Three different CD4+ T cell clones were used to mediate the specific lysis of allogeneic HLA-DP4+ Epstein-Barr virus-transformed B cells (EBV-B) pulsed with NY-ESO-1 p157-170. The Th1 phenotype and effector functions of the CD4+ T cell clones described here provide an important rationale for the activation of antigen-specific CD4+ T cells along with CD8+ T cells in cancer vaccination strategies.

Introduction

NY-ESO-1 was identified by serological analysis of a recombinant cDNA expression library (SEREX) from a squamous cell cancer of the esophagus (1). NY-ESO-1 is a cancer-testis antigen that is expressed in different types of cancer, including hematological malignancies, and in normal germ cells (2, 3, 4, 5). Spontaneous humoral and cellular immune responses against NY-ESO-1 are detected in approximately 50% of patients with NY-ESO-1-expressing cancers (6, 7) and are strictly dependent on the presence of NY-ESO-1+ disease. Changes in NY-ESO-1-specific immune responses are closely correlated with the clinical development of NY-ESO-1+ cancers (7, 8).

We identified a number of NY-ESO-1 epitopes recognized by CD4+ and CD8+ T cells that are used to monitor spontaneous and vaccine-induced T cell responses against NY-ESO-1 (9, 10, 11, 12). Spontaneous NY-ESO-1-specific CD4+ and CD8+ T cell responses were found exclusively in patients with detectable NY-ESO-1 antibody (13, 14, 15, 16, 17). Since CD8+ T cells are considered the most potent effectors controlling cancer growth *in vivo*, the majority of clinical cancer vaccine studies have focused on the induction of antigen-specific CD8+ T cell responses (18, 19, 20). The role of MHC class II-restricted CD4+ T cells in mediating tumor regression *in vivo*, however, has been less extensively studied. A number of studies have indicated that antigen-specific CD8+ T cell responses were significantly enhanced and maintained over extended periods of time in the presence of CD4+ T helper cells (21, 22, 23, 24).

Integrated spontaneous immune responses against NY-ESO-1 that involve cellular and humoral effectors of the immune system (13, 16) are often observed in patients with advanced NY-ESO-1-expressing cancer. Therefore, NY-ESO-1 may be considered a model system for studying the correlation between immune response parameters and defined patient and disease characteristics, such as tumor type, extent of disease, HLA type, antigen expression levels, and intratumoral localization of CD4+ and CD8+ T cells. A recent study suggests that HLA-DP4 is closely associated with spontaneous NY-ESO-1 immunity (11). To address this observation, we assessed the NY-ESO-1 serum antibody and HLA-DP4 status of 102 patients with NY-ESO-1-expressing cancers. To better understand the role of NY-ESO-1-specific CD4+ T cells in orchestrating integrated immune responses against NY-ESO-1, we established NY-ESO-1-specific, HLA-DP4-restricted CD4+ T clones from two HLA-DP4+ patients with detectable NY-ESO-1 antibody and characterized these with respect to phenotype, pattern of antigen recognition, and effector function. The results provide important perspectives for new strategies of cancer vaccination focusing on the induction of integrated immune responses against defined cancer antigens *in vivo*.

Results

Lack of correlation between HLA-DP4 status and spontaneous NY-ESO-1 immunity

We tested 102 patients with advanced cancers of different types that expressed NY-ESO-1 for the presence of NY-ESO-1 serum antibody and the HLA-DP4 allele. Fifty patients were NY-ESO-1

antibody positive, and 52 were NY-ESO-1 antibody negative. The NY-ESO-1 antibody-positive and -negative patients were comparable with respect to previous treatments, extent of disease, metastatic sites, and duration of metastatic disease. Seventy-nine patients were HLA-DP4-positive and 23 were HLA-DP4-negative. Of 79 HLA-DP4-positive patients, 39 were NY-ESO-1 antibody positive, and 40 were NY-ESO-1 antibody negative. Of 23 HLA-DP4-negative patients, 11 were NY-ESO-1 antibody positive, and 12 were NY-ESO-1 antibody negative. In this extended analysis, we found no correlation between HLA-DP4 status and the presence of spontaneous NY-ESO-1 serum antibody (Table 1).

CD4+ and CD8+ NY-ESO-1-specific T cell responses

CD4+ and CD8+ T cells obtained from two NY-ESO-1 antibody-positive patients (NW1454 and NW1662) and from two NY-ESO-1 antibody-negative patients (NW1045 and NW1691) were stimulated separately with autologous CD4- CD8- PBMCs, which were either pulsed with NY-ESO-1 peptide p157-170 or transduced with NY-ESO-1 recombinant adenovirus (Ad2/ESO). After 2-3 weeks of presensitization, CD4+ effector cells were tested for the specific recognition of the NY-ESO-1 epitope p157-170 presented by the allogeneic HLA-DP4+ NW1539-EBV-B cell line. CD8+ effector cells were tested for the specific recognition of the NY-ESO-1 epitope p157-165 presented by T2 cells in ELISPOT assays. Strong NY-ESO-1-specific CD4+ and CD8+ T cell responses were induced in NY-ESO-1 antibody-positive patients by presensitization with NY-ESO-1 p157-170 and Ad2/ESO, whereas no reactivity was observed in NY-ESO-1 antibody-negative patients (Figure 1, panels a and b). Efficient stimulation of CD8+ T cells was achieved using the HLA-DP4-restricted NY-ESO-1 peptide p157-170, which includes the HLA-A2-restricted NY-ESO-1 epitopes p157-165 and p157-167, as documented in ELISPOT assays against T2 cells pulsed with NY-ESO-1 p157-165 and p157-167 (Figure 1c).

Generation of NY-ESO-1-specific, HLA-DP4-restricted CD4+ T cell clones

CD4+ T cells, obtained from the NY-ESO-1 antibody-positive patients NW1454 and NW1662, were presensitized with NY-ESO-1 peptide p157-170 and further stimulated under limiting dilution conditions. Four NY-ESO-1-specific CD4+ T cell clones were obtained from patient NW1454, and 34 were obtained from patient NW1662. All CD4+ T cell clones were tested for the specific recognition of NY-ESO-1 p157-170 in the context of four allogeneic HLA-DP4-positive (NW1539-EBV, MZ070782, NW115-EBV, MZ1802-EBV) and two HLA-DP4-negative (Ducaf-EBV, MZ1851-EBV) EBV-B cell lines. Reactivity against NY-ESO-1 p157-170 was documented for all of the CD4+ T cell clones in the context of HLA-DP4. Figure 2 shows a representative experiment with the CD4+ T cell clone NW1662-CD4-12.

Subsequently, the reactivity of these CD4+ T cell clones against graded doses of NY-ESO-1 p157-170 added to EBV-B target cells was evaluated. Figure 3 shows that peptide concentrations between 600 ng/ml (NW1662-CD4-12) and 3000 ng/ml (NW1662-CD4-54) were required to achieve half-maximal reactivity with the CD4+ T cell clones tested.

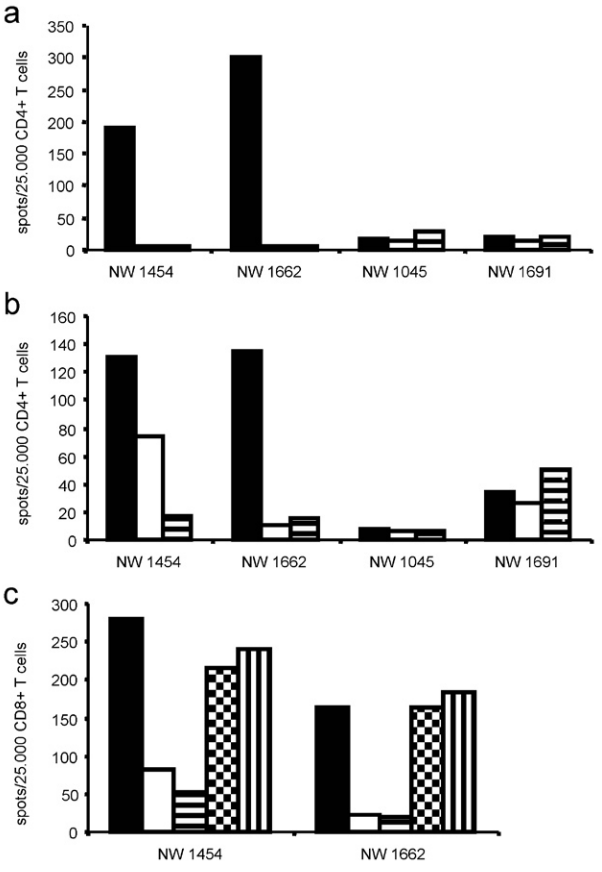
Recognition of naturally processed NY-ESO-1

To confirm the HLA-DP4-restricted NY-ESO-1 p157-170 reactivity of CD4+ T cell clones against naturally processed NY-ESO-1 epitopes, autologous DCs, either pulsed with recombinant NY-ESO-1 protein or SSX protein as a control, or

Table 1
NY-ESO-1 antibody and HLA-DP4 status of patients with NY-ESO-1-expressing cancers

HLA-DP4 status	NY-ESO-1 Ab status	
	Ab+ patients (n=50)	Ab- patients (n=52)
HLA-DP4+	39	40
HLA-DP4-	11	12

Figure 1



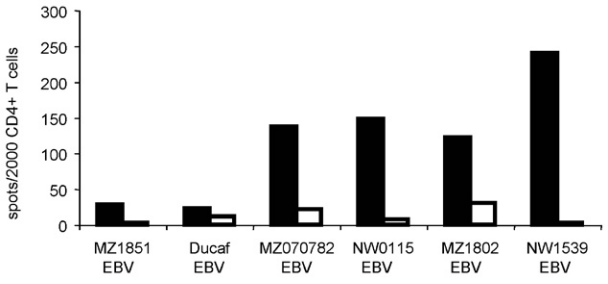
Induction of NY-ESO-1-specific CD4+ and CD8+ T cell responses after *in vitro* presensitization with NY-ESO-1 p157-170 or Ad2/ESO. CD4+ and CD8+ T cells were presensitized with (a) NY-ESO-1 p157-170 or (b) Ad2/ESO. Two patients, NW1454 and NW1691, showed nonspecific reactivity against the irrelevant Melan A peptide p29-37 (white bars) after Ad2/ESO stimulation (a and b). (c) CD8+ T cell responses were induced after presensitization with NY-ESO-1 p157-170 against the stimulating peptide (black bar), and against T2 cells pulsed with NY-ESO-1 p157-165 (checkered bars) and p157-167 (vertical stripes). No significant reactivity was observed against the irrelevant Melan A peptide p29-37 (white bars) or against T2 cells alone (horizontal stripes).

infected with Ad2/ESO, vaccinia/*NY-ESO-1* recombinant virus (v.v. ESO) or wild-type vaccinia virus (v.v. WT) as a control, were used as target APCs in ELISPOT assays. Three of four NW1454-CD4 clones and two of four NW1662-CD4 clones reacted with autologous DCs pulsed with recombinant NY-ESO-1 protein, whereas only one of these, NW1662-CD4-2, reacted with Ad2/ESO- or v.v. ESO-infected autologous DCs. Autologous DCs pulsed with control SSX protein or transduced with v.v. WT were not recognized (Figure 4a). Clone NW1662-CD4-2 was further tested for recognition of autologous DCs pulsed with lysates of NY-ESO-1-positive and NY-ESO-1-negative melanoma cell lines. As shown in Figure 4b, reactivity was only documented against DCs pulsed with lysates of the NY-ESO-1-expressing tumor cell lines NW-MEL-38 and SK-MEL-37.

Cytotoxicity of NY-ESO-1-specific HLA-DP4-restricted CD4+ T cell clones

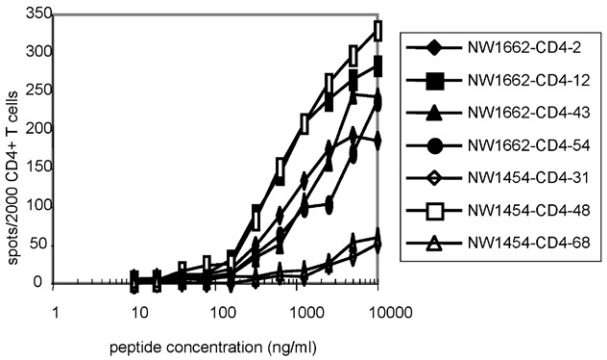
Three of the seven CD4+ T cell clones that were tested lysed the NY-ESO-1 p157-170-pulsed HLA-DP4+ EBV-B cell line NW-1539-EBV efficiently; Figure 5a shows representative results of two different CD4+ T cell clones. In addition, T cell clone NW1454-CD4-68 showed significant lytic activity against the NY-ESO-1-expressing HLA-DP4+ melanoma cell lines NW-MEL-38, NW-MEL-634, and NW-MEL-450 after treatment with 100 U/ml IFN-gamma, but not against the NY-ESO-1-negative melanoma cell line NW-MEL-8 or the HLA-DP4-negative melanoma cell lines Mel624 and SK-MEL-29 (Figure 5b).

Figure 2



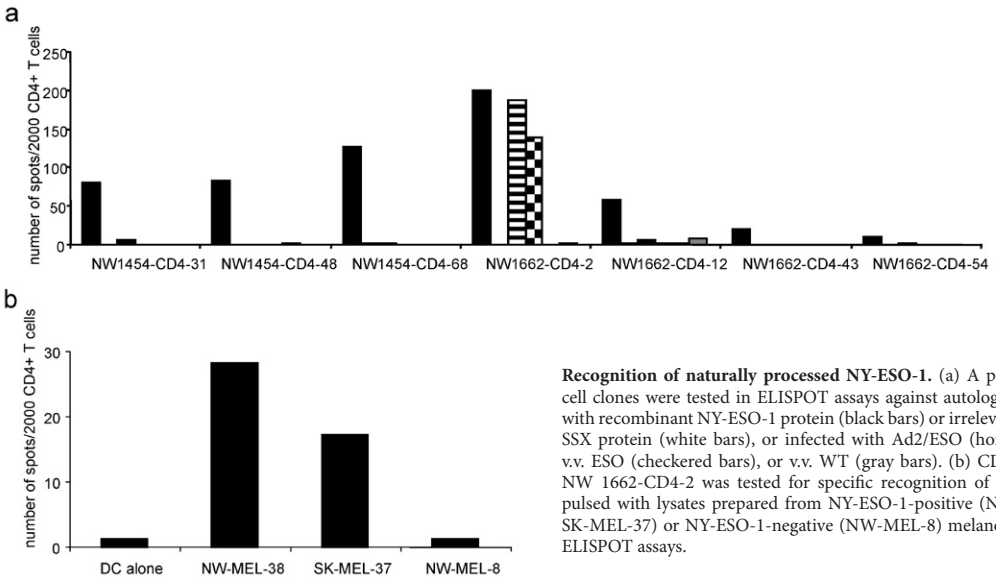
NY-ESO-1 p157-170-specific CD4+ T cell reactivity is restricted by HLA-DP4. The CD4+ T cell clone NW 1662-CD4-12 was tested against a panel of HLA-DP4-positive and HLA-DP4-negative allogeneic EBV-B cell lines pulsed with NY-ESO-1 p157-170 (black bars) in ELISPOT assays. NY-ESO-1 p157-170-specific reactivity was documented against peptide-pulsed HLA-DP4+ EBV-B cells only. The white bars represent the background reactivity against EBV-B cells alone.

Figure 3



Reactivity of HLA-DP4-restricted NY-ESO-1-specific CD4+ T cells clones against different concentrations of NY-ESO-1 p157-170. Seven CD4+ T cell clones obtained from patients NW1454 and NW1662 were tested against different concentrations of NY-ESO-1 p157-170 in ELISPOT assays. Depending upon the interclonal avidity, different levels of peptide concentration are required for detectable reactivity with the respective CD4+ T cell clone.

Figure 4



Recognition of naturally processed NY-ESO-1. (a) A panel of CD4+ T cell clones were tested in ELISPOT assays against autologous DCs pulsed with recombinant NY-ESO-1 protein (black bars) or irrelevant recombinant SSX protein (white bars), or infected with Ad2/ESO (horizontal stripes), v.v. ESO (checkered bars), or v.v. WT (gray bars). (b) CD4+ T cell clone NW 1662-CD4-2 was tested for specific recognition of autologous DCs pulsed with lysates prepared from NY-ESO-1-positive (NW-MEL-38 and SK-MEL-37) or NY-ESO-1-negative (NW-MEL-8) melanoma cell lines in ELISPOT assays.

Immunophenotyping of NY-ESO-1-specific HLA-DP4-restricted CD4+ T cell clones

Immunophenotyping of the CD4+ T cell clones showed a CD4+ CD25- CD8- phenotype for all clones. After restimulation with NY-ESO-1 p157-170, all the CD4+ T cell clones were negative for the suppressor marker CTLA-4 and positive for the activation marker CD69 (data not shown).

Discussion

NY-ESO-1 is one of the most immunogenic cancer antigens, leading to spontaneous humoral and cellular immune responses in approximately 50% of patients with NY-ESO-1-expressing malignancies (1, 7, 8, 13, 14). Much attention has been paid to monitoring spontaneous and vaccine-induced NY-ESO-1-specific CD8+ T cell responses, which directly mediate the lysis of antigen-expressing tumor cells *in vivo* (16, 18, 25). However, several studies have indicated that active immunization may be made more efficient by the simultaneous stimulation of antigen-specific CD4+ T cells (21, 26). A number of NY-ESO-1 peptide epitopes presented by different MHC class II molecules have been identified and can be used to explore the role of NY-ESO-1-specific CD4+ T cells in the induction and maintenance of antibody and CD8+ T cell responses against NY-ESO-1 (9, 10, 11, 12).

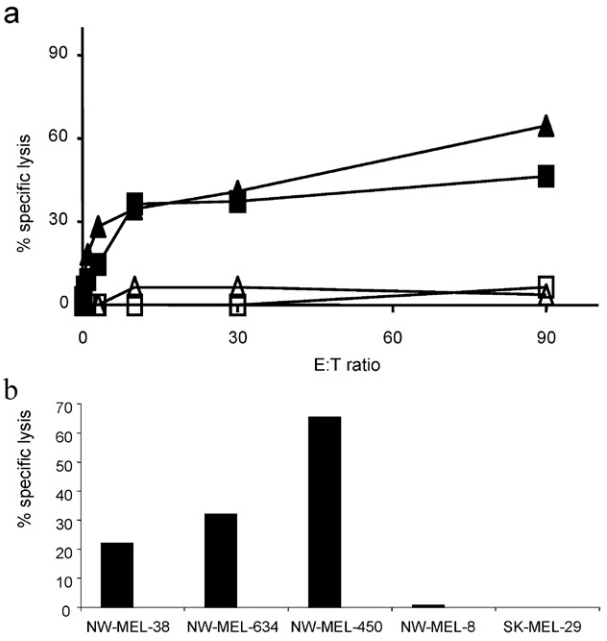
Spontaneous immunity against NY-ESO-1 was found to be associated with a more favorable clinical outcome in a number of patients analyzed over an extended period of time, suggesting that NY-ESO-1 immunity may play a role in the control of cancer growth *in vivo*. A relationship between spontaneous NY-ESO-1 immunity and the presence of the HLA-DP4 allele had been suggested in a previous study of 17 patients, in which 16/17 NY-ESO-1 antibody-positive patients expressed the HLA-DP4 allele (11). This relationship was not confirmed in our analysis of 102 patients with NY-ESO-1-expressing cancers. Of 79 HLA-DP4+ patients, NY-ESO-1 antibody was detected in 39 patients (49.8%). Of 23 HLA-DP4- patients, NY-ESO-1 serum antibody was found in 11 (47.8%).

To further characterize the phenotype and different effector functions of HLA-DP4-restricted NY-ESO-1-specific CD4+ T cells, we generated CD4+ T cell clones from two HLA-DP4+, NY-ESO-1 antibody-positive patients. The majority of CD4+ T cell clones tested were reactive with processed recombinant NY-ESO-1 protein, and one CD4+ T cell clone, NW1662-CD4-2, reacted with autologous DCs pulsed with a lysate of NY-ESO-1-expressing melanoma cells and with APCs virally transduced with NY-ESO-1, indicating a less efficient processing of MHC class II-presented epitopes after viral gene transfer.

The effector functions of NY-ESO-1-specific CD4+ T cell clones were further characterized in cytotoxicity assays. A subset of CD4+ T cell clones was tested for specific lysis of HLA-DP4+ APCs pulsed with NY-ESO-1 p157-170, and of NY-ESO-1-expressing tumor cell lines. Specific lysis of peptide-pulsed APCs was demonstrated for three CD4+ T cell clones, and one of them (NW1454-CD4-68) also lysed the NY-ESO-1-expressing HLA-DP4+ melanoma cell lines NW-MEL-38, NW-MEL-634, and NW-MEL-450 after treatment with IFN-gamma.

The central role of CD4+ T cells in the regulation of antibody and CD8+ T cell responses is well established. Influenced by different cytokine profiles, CD4+ T cells may be modulated toward helper or suppressor functions, as is indicated by the expression of different surface markers (21, 27, 28). The NY-ESO-1-specific CD4+ T cell clones established in this study were characterized by the CD4+ CD25- CD8- phenotype.

Figure 5



NY-ESO-1-specific cytotoxic reactivity of CD4+ T cell clones. (a) The CD4+ T cell clones NW 1454-CD4-68 (triangles) and NW 1662-CD4-2 (squares) were tested against the allogenic HLA-DP4+ EBV-B cell line NW1539-EBV-B, either pulsed with NY-ESO-1 p157-170 or not (closed and open symbols, respectively) in a 4-h chromium release assay at E/T ratios of 90, 30, 10, and 1:1. (b) The CD4+ T cell clone NW 1454-CD4-68 was tested against the HLA-DP4+ NY-ESO-1-positive melanoma cell lines NW-MEL-38, NW-MEL 634, and NW-MEL-450, against the NY-ESO-1-negative melanoma cell line NW-MEL-8, and against the HLA-DP4-negative melanoma cell line SK-MEL-29 in a 4-h chromium release assay.

CD4+ CD25+ suppressor T cells, which are known to have downregulating effects on antigen-specific CD8+ T cell responses (29, 30), were not identified, possibly as a result of the culture conditions. A subset of the NY-ESO-1-specific CD4+ T cell clones that were generated exhibited strong lytic reactivity against NY-ESO-1 p157-170-pulsed APCs and NY-ESO-1-expressing HLA-DP4+ melanoma cell lines, making them attractive effectors for cancer immunotherapy. New strategies for cancer vaccination, including protein, DNA, and recombinant viral vector constructs, focus on the induction of integrated immune responses involving specific CD4+ and CD8+ T cells. Monitoring vaccine-induced immune responses will provide new insights into the interplay between CD4+ T cells and other specific effectors of the immune system and to their capacity to directly modulate the clinical development of disease in cancer patients.

Abbreviations

Ad2/ESO, NY-ESO-1 recombinant adenovirus; EBV-B, Epstein-Barr virus-transformed B cells; v.v. ESO, vaccinia/*NY-ESO-1* recombinant virus; v.v. WT, wild-type vaccinia virus

Acknowledgements

This work was supported by the Cancer Research Institute/ Partridge Foundation, New York, USA, and by Krebsforschung Rhein Main, Frankfurt, Germany.

References

1. Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, Williamson B, Stockert E, Pfreundschuh M, Old LJ. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A* 1997; **94**: 1914-8. (PMID: 9050879)

2. Chen CH, Chen GJ, Lee HS, Huang GT, Yang PM, Tsai LJ, Chen DS, Sheu JC. Expressions of cancer-testis antigens in human hepatocellular carcinomas. *Cancer Lett* 2001; **164**: 189-95. (PMID: 11179834)

3. Kurashige T, Noguchi Y, Saika T, Ono T, Nagata Y, Jungbluth A, Ritter G, Chen YT, Stockert E, Tsushima T, Kumon H, Old LJ, Nakayama E. NY-ESO-1 expression and immunogenicity associated with transitional cell carcinoma: correlation with tumor grade. *Cancer Res* 2001; **61**: 4671-4. (PMID: 11406534)

4. Jungbluth AA, Chen YT, Stockert E, Busam KJ, Kolb D, Iversen K, Coplan K, Williamson B, Altorki N, Old LJ. Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. *Int J Cancer* 2001; **92**: 856-60. (PMID: 11351307)

5. Lee L, Wang RF, Wang X, Mixon A, Johnson BE, Rosenberg SA, Schrump DS. NY-ESO-1 may be a potential target for lung cancer immunotherapy. *Cancer J Sci Am* 1999; **5**: 20-5. (PMID: 10188057)

6. Soling A, Schurr P, Berthold F. Expression and clinical relevance of NY-ESO-1, MAGE-1 and MAGE-3 in neuroblastoma. *Anticancer Res* 1999; **19**: 2205-9. (PMID: 10472332)

7. Stockert E, Jager E, Chen YT, Scanlan MJ, Gout I, Karbach J, Arand M, Knuth A, Old LJ. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med* 1998; **187**: 1349-54. (PMID: 9547346)

8. Jager E, Stockert E, Zidianakis Z, Chen YT, Karbach J, Jager D, Arand M, Ritter G, Old LJ, Knuth A. Humoral immune responses of cancer patients against "Cancer-Testis" antigen NY-ESO-1: correlation with clinical events. *Int J Cancer* 1999; **84**: 506-10. (PMID: 10502728)

9. Zarour HM, Storkus WJ, Brusic V, Williams E, Kirkwood JM. NY-ESO-1 encodes DRB1*0401-restricted epitopes recognized by melanoma-reactive CD4+ T cells. *Cancer Res* 2000; **60**: 4946-52. (PMID: 10987311)

10. Zeng G, Touloukian CE, Wang X, Restifo NP, Rosenberg SA, Wang RF. Identification of CD4+ T cell epitopes from NY-ESO-1 presented by HLA-DR molecules. *J Immunol* 2000; **165**: 1153-9. (PMID: 10878395)

11. Zeng G, Wang X, Robbins PF, Rosenberg SA, Wang RF. CD4(+) T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: association with NY-ESO-1 antibody production. *Proc Natl Acad Sci U S A* 2001; **98**: 3964-9. (PMID: 11259659)

12. Jager E, Jager D, Karbach J, Chen YT, Ritter G, Nagata Y, Gnjatic S, Stockert E, Arand M, Old LJ, Knuth A. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101-0103 and recognized by CD4(+) T lymphocytes of patients with NY-ESO-1-expressing melanoma. *J Exp Med* 2000; **191**: 625-30. (PMID: 10684854)

13. Jager E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Jager D, Arand M, Wada H, Noguchi Y, Stockert E, Old LJ, Knuth A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med* 1998; **187**: 265-70. (PMID: 9432985)

14. Gnjatic S, Nagata Y, Jager E, Stockert E, Shankara S, Roberts BL, Mazzara GP, Lee SY, Dunbar PR, Dupont B, Cerundolo V, Ritter G, Chen YT, Knuth A, Old LJ. Strategy for monitoring T cell responses to NY-ESO-1 in patients with any HLA class I allele. *Proc Natl Acad Sci U S A* 2000; **97**: 10917-22. (PMID: 11005863)

15. Gnjatic S, Atanackovic D, Jager E, Matsuo M, Selvakumar A, Altorki NK, Maki RG, Dupont B, Ritter G, Chen YT, Knuth A, Old LJ. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. *Proc Natl Acad Sci U S A* 2003; **100**: 8862-7. (PMID: 12853579)

16. Jager E, Nagata Y, Gnjatic S, Wada H, Stockert E, Karbach J, Dunbar PR, Lee SY, Jungbluth A, Jager D, Arand M, Ritter G, Cerundolo V, Dupont B, Chen YT, Old LJ, Knuth A. Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. *Proc Natl Acad Sci U S A* 2000; **97**: 4760-5. (PMID: 10781081)

17. Atanackovic D, Matsuo M, Ritter E, Mazzara G, Ritter G, Jager E, Knuth A, Old LJ, Gnjatic S. Monitoring CD4(+) T cell responses against viral and tumor antigens using T cells as novel target APC. *J Immunol Methods* 2003; **278**: 57-66. (PMID: 12957396)

18. Jager E, Gnjatic S, Nagata Y, Stockert E, Jager D, Karbach J, Neumann A, Rieckenberg J, Chen YT, Ritter G, Hoffman E, Arand M, Old LJ, Knuth A. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. *Proc Natl Acad Sci U S A* 2000; **97**: 12198-203. (PMID: 11027314)

19. Marchand M, van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, Vanwijck R, Lienard D, Beauduin M, Dietrich PY, Russo V, Kerger J, Masucci G, Jager E, De Greve J, Atzpodien J, Brasseur F, Coulie PG, van der Bruggen P, Boon T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene *MAGE-3* and presented by HLA-A1. *Int J Cancer* 1999; **80**: 219-30. (PMID: 9935203)

20. Thurner B, Haendle I, Roder C, Dieckmann D, Keikavoussi P, Jonuleit H, Bender A, Maczek C, Schreiner D, von den Driesch P, Brocker EB, Steinman RM, Enk A, Kampgen E, Schuler G. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999; **190**: 1669-78. (PMID: 10587357)

21. Toes RE, Ossendorp F, Offringa R, Melief CJ. CD4 T cells and their role in antitumor immune responses. *J Exp Med* 1999; **189**: 753-6. (PMID: 10049938)

22. Husmann LA, Bevan MJ. Cooperation between helper T cells and cytotoxic T lymphocyte precursors. *Ann N Y Acad Sci* 1988; **532**: 158-69. (PMID: 2972241)

23. von Herrath MG, Yokoyama M, Dockter J, Oldstone MB, Whitton JL. CD4-deficient mice have reduced levels of memory cytotoxic T

lymphocytes after immunization and show diminished resistance to subsequent virus challenge. *J Virol* 1996; **70**: 1072-9. (PMID: 8551565)

24. Keene JA, Forman J. Helper activity is required for the *in vivo* generation of cytotoxic T lymphocytes. *J Exp Med* 1982; **155**: 768-82. (PMID: 6801178)

25. Romero P, Dutoit V, Rubio-Godoy V, Lienard D, Speiser D, Guillaume P, Servis K, Rimoldi D, Cerottini JC, Valmori D. CD8+ T-cell response to NY-ESO-1: relative antigenicity and *in vitro* immunogenicity of natural and analogue sequences. *Clin Cancer Res* 2001; **7 Suppl 3**: 766s-72s. (PMID: 11300471)

26. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 2003; **421**: 852-6. (PMID: 12594515)

27. Romerdahl CA, Kripke ML. Role of helper T-lymphocytes in rejection of UV-induced murine skin cancers. *Cancer Res* 1988; **48**: 2325-8. (PMID: 2965612)

28. Fischer WH, thor Straten P, Terheyden P, Becker JC. Function and dysfunction of CD4(+) T cells in the immune response to melanoma. *Cancer Immunol Immunother* 1999; **48**: 363-70. (PMID: 10501848)

29. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002; **2**: 389-400. (PMID: 12093005)

30. Somasundaram R, Jacob L, Swoboda R, Caputo L, Song H, Basak S, Monos D, Peritt D, Marincola F, Cai D, Birebent B, Bloome E, Kim J, Berencsi K, Mastrangelo M, Herlyn D. Inhibition of cytolytic T lymphocyte proliferation by autologous CD4+/CD25+ regulatory T cells in a colorectal carcinoma patient is mediated by transforming growth factor-beta. *Cancer Res* 2002; **62**: 5267-72. (PMID: 12234995)

31. Zeng G, Li Y, El-Gamil M, Sidney J, Sette A, Wang RF, Rosenberg SA, Robbins PF. Generation of NY-ESO-1-specific CD4+ and CD8+ T cells by a single peptide with dual MHC class I and class II specificities: a new strategy for vaccine design. *Cancer Res* 2002; **62**: 3630-5. (PMID: 12097265)

32. Knuth A, Danowski B, Oettgen HF, Old LJ. T-cell-mediated cytotoxicity against autologous malignant melanoma: analysis with interleukin 2-dependent T-cell cultures. *Proc Natl Acad Sci U S A* 1984; **81**: 3511-5. (PMID: 6610177)

Materials and methods

Patients

We typed 102 patients with metastatic NY-ESO-1-expressing cancers of different kinds for the presence of the HLA-DP4 allele and tested them for detectable NY-ESO-1 serum antibody using Western blot assays. Of these, four HLA-A2+ DP4+ patients were selected for the evaluation of NY-ESO-1-specific CD4+ and CD8+ T cell responses. At the time lymphocytes were collected for analysis, two patients (melanoma patient NW1454 and non-small cell lung cancer patient NW1662) had detectable NY-ESO-1 serum antibody and NY-ESO-1-specific CD8+ T cell reactivity, and two patients (melanoma patient NW1045 and ovarian cancer patient NW1691) were NY-ESO-1 antibody negative.

Expression of NY-ESO-1 in tumor tissues

Expression of *NY-ESO-1* mRNA in tumor specimens frozen immediately after biopsy at -80°C was assessed by RT-PCR, using PCR conditions and primers as previously described (1).

NY-ESO-1 serum antibody

NY-ESO-1 serum antibody was assessed by standard Western blot, using NY-ESO-1 recombinant protein purified from *Escherichia coli* as described (7, 13).

Cell lines

EBV-B cells were derived from PBMCs obtained from individuals of different HLA types using culture supernatant from the EBV-producing B95-8 cell line. EBV-B cell lines and the mutant TAP-deficient lymphoblastoid cell line T2 (CEMx721.174.T2) were maintained in RPMI 1640 medium supplemented with 10% FCS, L-asparagine (50 mg/l), L-arginine (24 mg/l), and L-glutamine (300 mg/l) as described (13). The EBV-B cell lines MZ070782 and DUCAF were a kind gift from Dr. Ghislaine Sterkers, Hopital Robert Debre, Paris, France.

The NY-ESO-1-expressing melanoma cell lines NW-MEL-38, NW-MEL-450, NW-MEL-634, SK-MEL-29, Mel624, and SK-MEL-37, and the NY-ESO-1-negative melanoma cell line NW-MEL-8, were established from surgically resected melanoma metastases and cultured in DMEM (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% FCS, L-asparagine (50 mg/l), L-arginine (24 mg/l), and L-glutamine (300 mg/l) as described (13). For the cytotoxicity assay, the tumor cells were pretreated with IFN-gamma (100 U/ml, Bioferon, Laupheim, Germany) for 24 h.

Peptides

The HLA-DP4-restricted NY-ESO-1 peptide p157-170 (SLLMWITQCFLPVF) was selected to analyze the CD4+ T cell response to NY-ESO-1 in HLA-DP4+ patients (31). Two HLA-A2-restricted NY-ESO-1 peptides, p157-167 (SLLMWITQCFL) and p157-165 (SLLMWITQC), were selected to analyze the CD8+ T cell response to NY-ESO-1 (13). The HLA-A2-binding Melan A peptide p29-37 (GILTVILGV) was included for control purposes. All peptides were synthesized by Multiple Peptide Systems (San Diego, CA, USA) with a purity of >90%, as determined by reverse-phase HPLC.

Viral constructs

Adenovirus encoding the full-length *NY-ESO-1* cDNA (Ad2/ESO) was provided by Genzyme Corporation (Framington, MA, USA), and v.v. WT and v.v. ESO were provided by Therion Biologics (Cambridge, MA, USA). Viral constructs were used for the preparation of NY-ESO-1-expressing APCs as described (14).

Presensitization of CD4+ and CD8+ effector T cells

CD4+ and CD8+ T lymphocytes were separated from PBMCs by antibody-coated magnetic beads (MiniMACS, Miltenyi Biotec, Auburn, CA, USA) and seeded into 24-well plates (Greiner) at 5×10^5 cells/well in RPMI 1640 medium supplemented with 10% human serum and L-glutamine (300 mg/l). PBMCs depleted of CD4+ and CD8+ T cells were either pulsed with peptide (10 µg/ml) or infected with recombinant Ad2/ESO at 1000 infection units/cell and used as APCs. These

APCs were then irradiated and added to plates containing CD4+ or CD8+ T cells at a concentration of 1 x 10^6 cells/well. IL-2 (2.5 ng/ml, Biotest, Dreieich, Germany) and IL-4 (10 U/ml, Strathmann Biotech, Hannover, Germany) were added to CD4+ T cell cultures, and IL-2 (2.5 ng/ml) was added to CD8+ T cell cultures (16).

Generation of NY-ESO-1-specific CD4+ T cell clones

NY-ESO-1-specific CD4+ T cell clones were obtained from limiting dilution cultures. Selected CD4+ T cells were stimulated with irradiated autologous APCs pulsed with the HLA-DP4-restricted NY-ESO-1 peptide p157-170 (10 μg/ml) in the presence of irradiated HLA-DP4+ EBV-B cells as feeder cells, and recombinant interleukin-2 (rIL-2) at 10 ng/ml and rIL-4 at 10 U/ml. Clones were maintained by weekly stimulation with irradiated HLA-DP4+ EBV-B cells pulsed with NY-ESO-1 peptide p157-170 (10 μg/ml) and supplemented with rIL-2 and rIL-4 (10 ng/ml and 10 U/ml, respectively).

Antigen-presenting target cells

DCs were generated from plastic-adherent peripheral blood monocytes that were cultured for 6 d in the presence of IL-4 (1000 U/ml) and GM-CSF (1000 U/ml, Leukomax, Sandoz, Nurnberg, Germany). On day 6 of *in vitro* culture, APCs were treated with IL-4 (1000 U/ml), IL-6 (1000 U/ml), IL-1-beta (10 ng/ml), TNF-alpha (10 ng/ml, all obtained from Pharma Biotechnologie, Hannover, Germany), GM-CSF (1000 U/ml), and prostaglandin/E2 (1 μg/ml, Sigma Chemical, St. Louis, MO, USA).

PHA-transformed blast cells were generated by stimulation of CD4+ selected T cells with PHA (1 μg/ml), and maintained in culture by repeated addition of IL-2 (10 ng/ml/wk).

DC or PHA blasts were either infected with Ad2/ESO or v.v. ESO at 1000 infection units/cell at 37°C in 250 μl serum-free medium (X-VIVO-15, BioWhittaker, Walkersville, MD, USA), or pulsed with peptides at 10 μg/ml, with recombinant proteins at 20 μg/ml, or with tumor cell lysates prepared by three freeze-thaw cycles of 1 x 10^6 tumor cells, and cultured for 24 h. APCs were then washed twice and used as targets in ELISPOT assays at 3 x 10^4 cells/well.

ELISPOT assay

Flat-bottomed, 96-well nitrocellulose-lined plates (Millipore MultiScreen, Millipore, Bedford, MA, USA) were coated with IFN-gamma mAb (2 μg/ml, 1-D1K, MABTECH, Stockholm, Sweden) and incubated overnight at 4°C. After washing with PBS, plates were blocked with 10% human AB serum for 1 h at 37°C. Typically, 2.5 x 10^4 CD4+ or CD8+ presensitized effector T cells (see above) were incubated with 5 x 10^4 target cells, which were pulsed with NY-ESO-1 peptide, recombinant protein, NY-ESO-1+ tumor cells lysates, or transduced with the viral vector constructs Ad2/ESO or v.v. ESO. Effector and target cells were incubated for 20 h in RPMI medium 1640 supplemented with 10% human serum. After incubation, the plates were thoroughly washed with 0.05% Tween 20 in PBS to remove cells, and biotinylated secondary IFN-gamma mAb (0.2 μg/ml, 7-B6-1-biotin, MABTECH) was added to each well. After incubation for 2 h at 37°C, the plates were washed and developed with streptavidin-alkaline phosphatase (1 μg/ml, MABTECH) for 1 h at room temperature. After washing, substrate (5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium, Sigma) was added and the plates incubated for 5-10 min. After washing, the dark-violet spots were counted under the microscope (14, 16).

Cytotoxicity assay

Cytotoxicity against NY-ESO-1 peptide-pulsed NW1539 EBV-B cells and tumor cell lines before and after treatment with IFN-gamma was determined in standard chromium release assays as described (32).

Immunophenotyping of CD4+ T cell clones

The expression of cell surface markers was determined by FACS® analysis using FITC-conjugated anti-CD4, anti-CD8, anti-CD25, anti-CD69, and anti-CTLA4 antibodies (Beckman Coulter, Marseille, France). FITC-conjugated antimouse IgG1 was used as a negative control. 1-5 x 10^5 CD4+ T cells were labeled with the appropriate antibody in PBS for 20 min (4°C), washed, and analyzed using a FACS® (Epics XL, Beckman Coulter).

Contact

Address correspondence to:
Prof. Dr. med. Elke Jäger
II. Medizinische Klinik
Hämatologie-Onkologie
Krankenhaus Nordwest
Steinbacher Hohl 2-26
60488 Frankfurt
Germany
Tel.: + 49 69 7601-3319
Fax: + 49 69 769932
E-mail: elke.jaeger@licr.org