complexes induce the maturation of human monocyte-derived DCs *in vitro*, and that 12D7 significantly enhances the therapeutic efficacy of chemotherapy using a preclinical syngeneic mouse model.

Table 1

Binding of human monoclonal anti-NY-ESO-1 antibodies to NY-ESO-1. Comparison of EC_{50} and equilibrium affinity constants for the binding between NY-ESO-1 and different anti-NY-ESO-1 antibodies.

Antibody	EC_{50} [pM] (prok. NY-ESO-1)	K_D [M] (prok. NY-ESO-1)	K_D [M] (euk. NY-ESO-1)
12D7	1.14	$2.08x10^{-10}$	$1.56x10^{-10}$
1D4	2.23	$1.62x10^{-9}$	$2.24x10^{-10}$
30D6	1.09	$4.35x10^{-9}$	$2.65x10^{-9}$
31E4	9.52	$1.9x10^{-8}$	$2.23x10^{-8}$
15B12	72.6	---	---
E978 control	6.66	$2.56x10^{-8}$	$1.56x10^{-11}$

Results

Cloning of human-derived monoclonal antibodies from cancer patients

We cloned eight different NY-ESO-1-specific human-derived monoclonal antibodies (HD mAbs) from a melanoma patient, of which the following five were selected for further analysis based on their affinity to the target: 1D4, 12D7, 15B12, 30D6, and 31E4. All HD mAbs were of IgG1 isotype.

In vitro characterization of HD mAbs

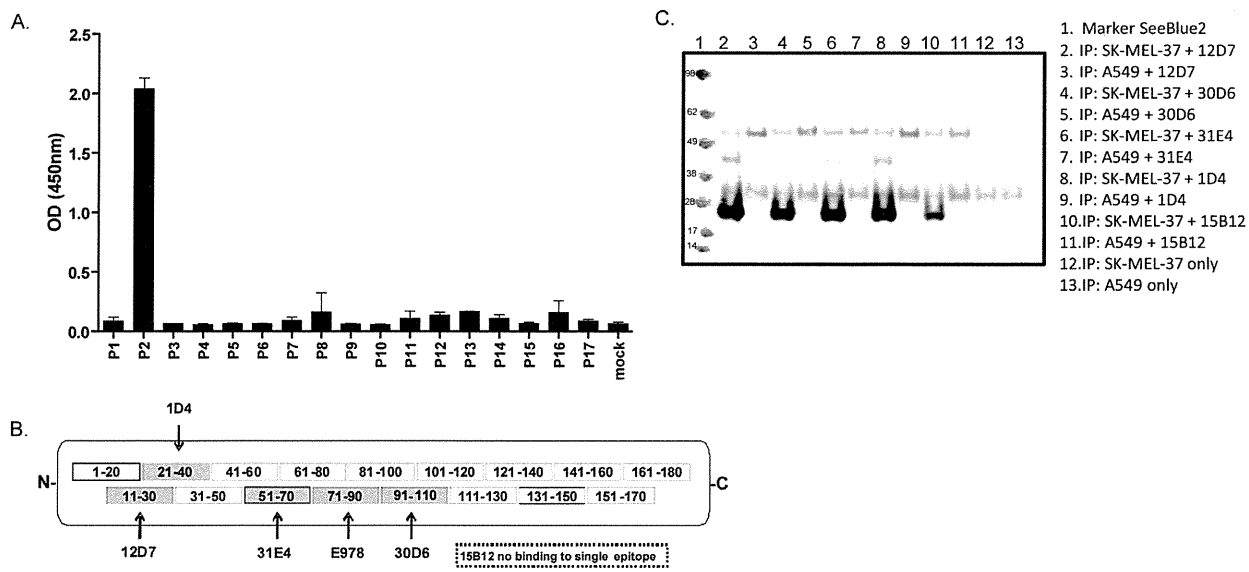
To compare the binding properties of five different anti-NY-ESO-1 HD mAbs to recombinant NY-ESO-1 protein, we determined the half-maximal effective concentration (EC_{50}) using a protein ELISA. All antibodies bound recombinant NY-ESO-1 produced in bacteria in the low pM range. Actual binding constants to recombinant NY-ESO-1 produced in bacteria and in eukaryotic cells were determined by surface plasmon resonance (Biacore Systems) (Table 1).

To determine the epitopes recognized by the different mAbs, we used a set of overlapping peptides spanning the complete NY-ESO-1 protein as coating antigen in ELISA. As shown in Figure 1A, 12D7 binds to a peptide representing the amino acids 11 to 30 from the NY-ESO-1 protein, but not to the two adjacent peptides that span amino acids 1-20 or 21-40. This suggests that the epitope recognized by 12D7 lies at the junction of these two peptides around amino acid 20 of NY-ESO-1. Figure 1B summarizes the epitope-specificity of all five anti-NY-ESO-1 antibodies. In addition, all antibodies were tested for binding to endogenous NY-ESO-1 from the human melanoma cell line SK-MEL-37 by immunoprecipitation. All antibodies precipitate NY-ESO-1 from a cell lysate of an NY-ESO-1+ cell line (SK-MEL-37) (Figure 1C). Because 12D7 had the highest affinity for eukaryotic NY-ESO-1, we performed further experiments with this mAb.

12D7 facilitates cross-presentation of NY-ESO-1 by DCs and induces concomitant DC maturation

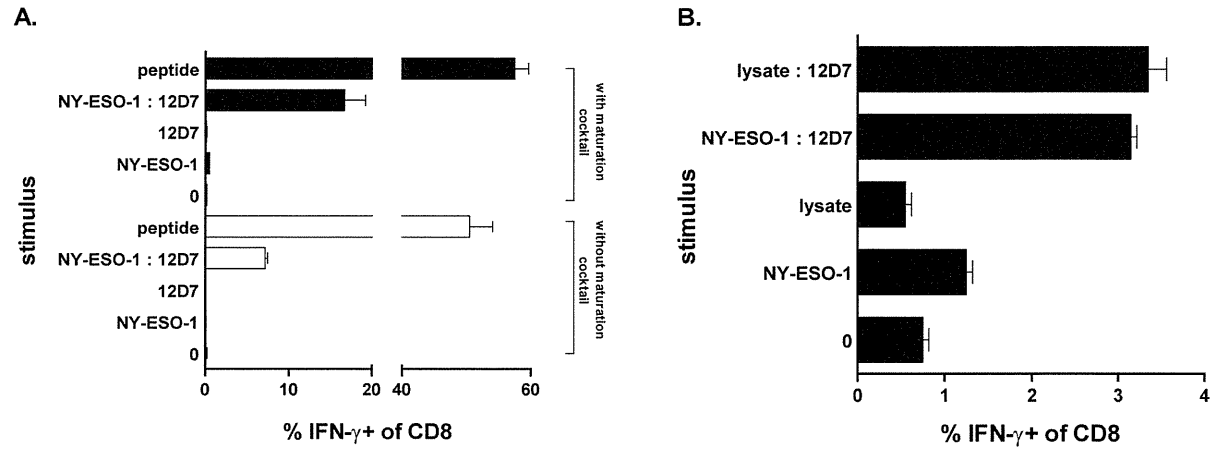
To test whether 12D7 facilitates the cross-presentation of NY-ESO-1-derived epitopes *in vitro*, we generated monocyte-derived, HLA-A*0201+ DCs and fed them with 12D7:NY-ESO-1 immune complexes, NY-ESO-1, or 12D7. DCs were subsequently incubated with cloned NY-ESO-$1_{157-165}$/HLA-A*0201-specific CD8+ T cells, and the percentage of T cells that produced IFN-γ was used as readout for antigen recognition. Mature DCs fed with NY-ESO-1 protein induced IFN-γ production in a low but discernible percentage of T cells (Figure 2A, black bars), which did not occur when DCs were not matured (Figure 2A, white bars). DCs fed with 12D7:NY-ESO-1 immune complexes induced the production of IFN-γ in a much

Figure 1



Epitope mapping of anti-NY-ESO-1 human monoclonal antibodies. (A) Representative peptide ELISA for antibody 12D7, where P1-P17 represent overlapping NY-ESO-1 peptides. (B) Overview of the specificities of different NY-ESO-1 specific human-derived mAbs. (C) Immunoprecipitation of NY-ESO-1 from a cell lysate of a NY-ESO-1+ cell line SK-MEL-37 or a NY-ESO-1- cell line A549 by human anti-NY-ESO-1 mAbs.

Figure 2



Human monoclonal anti-NY-ESO-1 antibody (12D7) facilitates cross-presentation of a NY-ESO-1-derived, HLA-A2-restriced epitope (NY-ESO-$1_{157-165}$). (A) HLA-A2+, monocyte-derived DCs were incubated with 20 μg NY-ESO-1 protein, 200 μg human monoclonal anti-NY-ESO-1 antibody (12D7), with immune complexes (12D7:NY-ESO-1) or with media for 3 h, were washed and cultured for 36 h with (black bars) or without (white bars) 25 ng/mL TNF-α + 1 μg/mL sCD40L (maturation cocktail). 6 x 10^4 cloned, NY-ESO-$1_{157-165}$ /HLA-A2-specific CD8+ T cells were added to 10^5 DCs in the presence of 10 μg/mL Brefeldin A, followed by a 5 h incubation and subsequent surface staining for CD8 and intracellular staining for IFN-γ. 10^{-6} M peptide was added to DCs as positive control. All cultures were performed in triplicate. (B) HLA-A2+, monocyte-derived DCs were incubated with 20 μg NY-ESO-1 protein, 200 μg human monoclonal anti-NY-ESO-1 antibody (12D7), with lysate of 10^7 NY-ESO-1+ SK-MEL-37 cells (lysate), with immune complexes (NY-ESO-1:12D7 or lysate:12D7), or with media (0) for 3 h, were washed and cultured for 36 h with 25 ng/mL TNF-α + 1 μg/mL sCD40L (maturation cocktail). 6 x 10^4 cloned, NY-ESO-$1_{157-165}$ /HLA-A2-specific CD8+ T cells were added to 10^5 DCs in the presence of 10 μg/mL Brefeldin A, followed by a 5 h incubation and subsequent surface staining for CD8 and intracellular staining for IFN-γ. All cultures were performed at least in duplicate.

higher percentage of T cells and, importantly, also did so when DCs that were not deliberately matured were used (Figure 2A, compare black and white bars). None of the negative controls—DCs fed with 12D7, mock immune complexes, or medium—induced IFN-γ production (Figure 2A and data not shown). To exclude that our observations are a peculiarity of recombinant NY-ESO-1, we incubated 12D7 with a cell lysate of SK-MEL-37 cells, which naturally express NY-ESO-1, and subsequently fed this mixture to DCs. DCs fed with the 12D7:lysate or with 12D7:NY-ESO-1 presented NY-ESO-1-derived epitopes approximately equally well (Figure 2B).

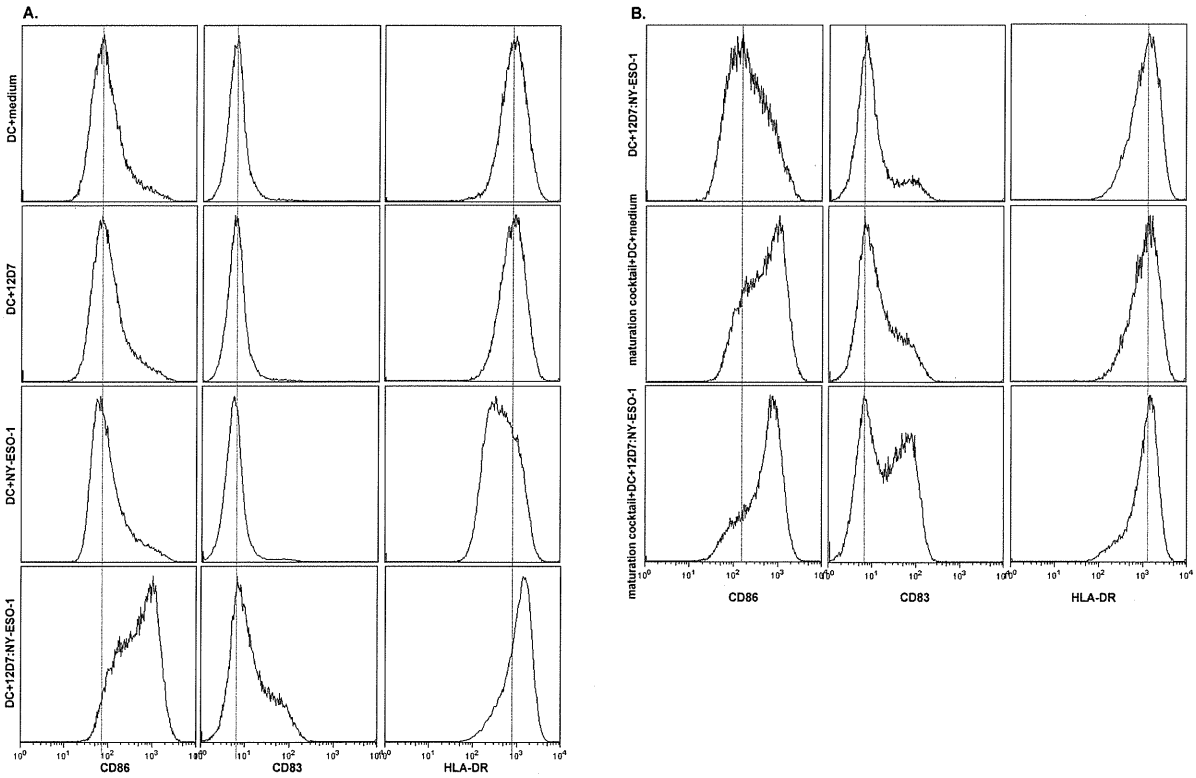
Because presentation of 12D7:NY-ESO-1 immune complexes seemed not to require deliberate DC maturation, we addressed whether the uptake of immune complexes, but not the uptake of uncomplexed protein, induced DC maturation *in vitro*. We therefore compared the expression of three surface molecules that are upregulated on mature DCs (CD83, CD86, and MHC class II) after incubation with media, 12D7, NY-ESO-1, or with 12D7:NY-ESO-1 immune complexes in the absence of maturation cocktail. We found that only immune complexes induced an upregulation of CD86, CD83, and MHC II (Figure 3A; left, middle, and right panels, respectively). We then compared the expression of CD83, CD86, and MHC II on DCs that were incubated with the maturation cocktail, with immune complexes, or with both, in order to determine the relative potency of immune complexes with respect to DC maturation. We found that immune complexes were almost as potent in inducing DC maturation as the classical maturation cocktail (sCD40L plus TNF-α) (Figure 3B). A combination of immune complexes plus maturation cocktail resulted in the most pronounced upregulation of CD86 and CD83 (Figure 3B; left and middle panels, respectively), whereas MHC II was not further upregulated compared to any of the two treatments alone (Figure 3B, right panels).

12D7 increases the therapeutic efficacy of chemotherapy in mice with NY-ESO-1+ tumors

To test the therapeutic efficacy of 12D7 *in vivo*, we injected 10^6 syngeneic, NY-ESO-1-transfected CT26 tumor cells s.c. in BALB/c mice. To induce release of intracellular NY-ESO-1, mice were treated with 75 mg/kg 5-FU when tumors reached a size of approximately 25 mm^2, which was typically around 2 weeks after injection of tumor cells. The treatment with 5-FU was repeated one week later and, in some groups, was combined with 100 μg 12D7 given systemically 2 d after each 5-FU injection. As can be seen from the growth curves, 5-FU has the expected therapeutic effect. Importantly, this was enhanced by 12D7 (Figure 4A). Treatment with 12D7 alone had no effect, presumably because the amount of spontaneously released antigen is not sufficient in this particular model. A compilation of end-point tumor sizes from 4 independent experiments shows a highly significant difference between mice treated with 5-FU plus 12D7, and mice treated with 5-FU alone (Figure 4B).

To investigate whether treatment with 5-FU plus 12D7 supported tumor-specific immunity, we injected mice with Brefeldin A 4 h before euthanasia, followed by staining for CD45.2, CD8, and intracellular IFN-γ. This way of analysis shows which cells actually are making IFN-γ *in vivo* and not which cells potentially can do this upon *in vitro* restimulation with peptide. This method obviously does not allow discrimination between single peptide specificities, but it is of higher biological relevance (25) particularly because we envisaged that DC activation, which we have shown to occur upon cross-presentation (Figure 3), may also support the presentation of other epitopes besides those derived from NY-ESO-1. Treatment with 5-FU plus 12D7 supported CD8+ and

Figure 3



Immune complexes induce maturation of monocyte-derived DCs *in vitro*. (A) CD14-derived DCs were incubated with media, 200 μg NY-ESO-1, 20 μg 12D7 or preformed immune complexes of 20 μg 12D7 + 200 μg NY-ESO-1, and were analyzed 36 h later for surface expression of CD86, CD83, or MHC II. (B) CD14-derived DCs were incubated with preformed immune complexes of 20 μg 12D7 + 200 μg NY-ESO-1, maturation cocktail (sCD40L + TNF-α) or with preformed immune complexes plus maturation cocktail, and were analyzed 36 h later for surface expression of CD86, CD83, or MHC II.

effector function in the tumor (Figure 4C). Treatment with 5-FU (Figure 4C) or 12D7 (data not shown) did not have this effect.

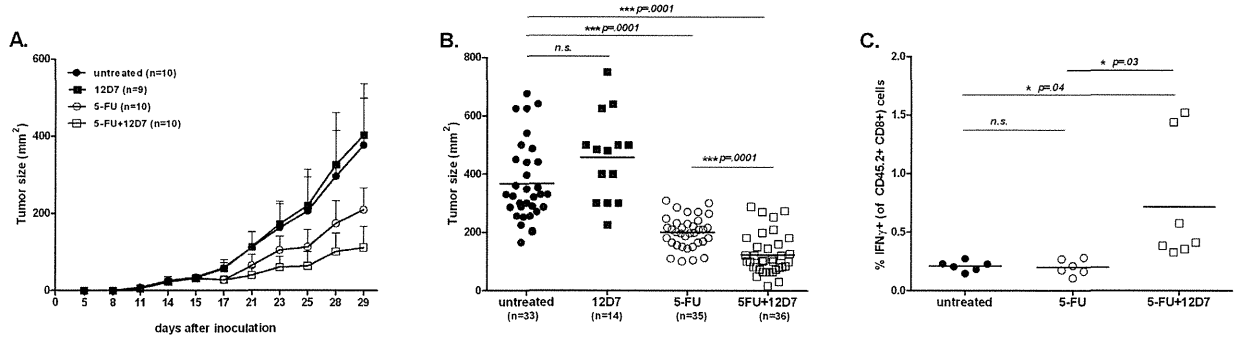
Discussion

We hypothesized that antibodies against intracellular, tumor-associated antigens support tumor-specific immunity when used in combination with a therapy that induces cell death such as chemo- or radiotherapy. We envisaged that such antibodies form immune complexes with the released tumor antigens. These immune complexes are subsequently taken up with higher efficiency compared to protein (fragments) by DCs (26), which then cross-present relevant epitopes to local CD8+, tumor-specific T cells. This presumed sequence of events may be of particular interest as evidence is accumulating that both chemo- and radiotherapy support tumor-specific immunity (27), and we therefore reasoned that additional stimulation of tumor-specific immunity could further improve the efficacy of these standard therapies.

For this purpose, we have cloned the first fully human mAbs to NY-ESO-1 using Epstein-Barr virus (EBV)-transformed B cells from a melanoma patient and subjected those to preclinical experiments to obtain proof of principle. We found that 12D7, a fully human IgG1 mAb specific for the immunogenic CT antigen NY-ESO-1, supported cross-presentation of NY-ESO-1 *in vitro* resulting in an approximate 15-fold increase of the number of responding CD8+ T cells. Of the other four NY-ESO-1-specific mAbs we generated here, 1D4 and 30D6 improved cross-presentation of NY-ESO-1 (data not shown), whereas 15B12 and 31E4 seemed not effective (data not shown). This difference may be explained by the difference in affinity, as 15B12 did not show binding to NY-ESO-1 by Biacore—although it did bind weakly to NY-ESO-1 in ELISA—and 31E4 had at least a 1-log lower affinity than 12D7, 1D4, and 30D6. At present, we have no reason to think that the epitope recognized by the mAb impacts on its ability to support cross-presentation. Our observation that 12D7:NY-ESO-1 immune complexes are considerably less efficient than peptide-loaded DCs in stimulating IFN-γ production illustrates that cross-presentation is a rather inefficient process, but underscores the therapeutic potential of antibodies against tumor-associated antigens.

It is well accepted now that activation of T cells *in vivo* crucially depends on antigen presentation by mature or activated DCs (14, 28). Many cues, including inflammation and infection but also endogenous signals, can induce DC maturation (29), and the lack of such signals in the tumor environment may be one reason why tumor-infiltrating T cells often have compromised functions (16, 30). Because the uptake of immune complexes was shown to result in DC maturation (19), we specifically addressed this issue here. We found that the *in vitro* uptake of immune complexes resulted in DC maturation that was comparable to sCD40L plus TNF-α, which is a classical maturation cocktail. Therefore, the use of mAbs against CT antigens may serve both purposes: DC activation and enhanced

Figure 4



A human, monoclonal anti-NY-ESO-1 antibody (12D7) increases the therapeutic efficacy of 5-FU chemotherapy in mice bearing NY-ESO-1+ syngeneic tumors. Female BALB/c mice were injected s.c. with 10^6 CT26/NY-ESO-1+ cells and treatment was started when tumors reached a surface of approximately 25 mm^2 (~ d13-15). (A) Mice received 75 mg/kg 5-FU i.p. at days 15 and 22 and/or 100 μg 12D7 i.p. on days 17 and 24. The results are shown as mean±SD. A representative experiment of 4 experiments is shown. (B) Compilation of 4 independent experiments, each symbol represents the tumor surface of an individual mouse at the end of the experiment (d 29). (C) Mice were injected i.p. 1 week after the last injection with 12D7 (d 29) with 250 μg Brefeldin A and were euthanized 4 h later. Processing of tumors and staining with antibodies for CD45.2, CD8 (surface), and IFN-γ (intracellular) was performed in the presence of 10 μg/mL Brefeldin A. Each symbol represents values from individual mice at the end of the experiment.

cross-presentation at the relevant anatomic location. This is not trivial, as systemic activation of DCs may not be without risk as systemic side effects such as the release of cytokines or autoimmunity may ensue (31, 32).

We found that 12D7 improved the efficacy of chemotherapy in a preclinical mouse model of transplanted, syngeneic NY-ESO-1-expressing tumors, thus supporting our concept. Further support comes from the fact that more CD8+ T cells infiltrate the tumor and that those cells have increased effector function. By itself, however, 12D7 had no therapeutic effect, suggesting that the amount of released tumor antigen is limiting without deliberate destruction of the tumor. Our *in vivo* experiments require the binding of human IgG to mouse Fcγ receptors (FcγR), which was previously described (33, 34). Improved efficacy of chemotherapy by the use of tumor-associated antigen-specific antibodies will presumably work for chemotherapies especially, which are not immunosuppressive or—even more important—promote immunogenic cell death (35).

We propose the concept of antibody-facilitated T cell induction in cancer (AFTIC) as a novel type of immunotherapy. AFTIC is based on the application of mAbs against tumor-associated antigens, including CT antigens, plus a treatment that promotes the local release of those antigens, such as chemo- or radiotherapy. The locally released antigens and the mAb form immune complexes, which facilitate the uptake and subsequent presentation of antigen-derived peptides by tumor-associated DCs. As the uptake of immune complexes induces concomitant maturation of DCs, AFTIC supports boosting as well as *de novo* activation of tumor-specific CD8+ T cells. Furthermore, administration of antibodies against a particular tumor-associated antigen may promote the presentation of the same antigen when administrated as a cancer vaccine, thereby improving the efficacy of immunotherapy. Alternatively, better antigen presentation of immune complexes and concomitant DC maturation may support the activity of adoptively transferred T cells provided they have the same antigen specificity as the therapeutic antibodies.

Abbreviations

CT, Cancer/Testis; DC, dendritic cell; mAb, monoclonal antibody

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References

1. Jäger D, Jäger E, Knuth A. Immune responses to tumour antigens: implications for antigen specific immunotherapy of cancer. *J Clin Pathol* 2001; **54:** 669-674. (PMID: 11533070)

2. Scanlan MJ, Güre AO, Jungbluth AA, Old LJ, Chen YT. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunol Rev* 2002; **188:** 22-32. (PMID: 12445278)

3. Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. *Cancer Immun* 2004; **4:** 1. (PMID: 14738373)

4. Jäger E, Gnjatic S, Nagata Y, Stockert E, Jäger D, Karbach J, Neumann A, Rieckenberg J, Chen YT, Ritter G, Hoffman E, Arand E, 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:** 12198-12203. (PMID: 11027314)

5. Davis ID, Chen W, Jackson H, Parente P, Shackleton M, Hopkins W, Chen Q, Dimopoulos N, Luke T, Murphy R, Scott AM, Maraskovsky E, McArthur G, MacGregor D, Sturrock S, Tai TY, Green S, Cuthbertson A, Maher D, Miloradovic L, Mitchell SV, Ritter G, Jungbluth AA, Chen YT, Gnjatic S, Hoffman EW, Old LJ, Cebon JS.

Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans. *Proc Natl Acad Sci U S A* 2004; **101:** 10697-10702. (PMID: 15252201)

6. Gnjatic S, Nishikawa H, Jungbluth AA, Güre AO, Ritter G, Jäger E, Knuth A, Chen YT, Old LJ. NY-ESO-1: review of an immunogenic tumor antigen. *Adv Cancer Res* 2006; **95:** 1-30. (PMID: 16860654)

7. Jäger E, Karbach J, Gnjatic S, Neumann A, Bender A, Valmori D, Ayyoub M, Ritter E, Ritter G, Jäger D, Panicali D, Hoffman E, Pan L, Oettgen H, Old LJ, Knuth A. Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients. *Proc Natl Acad Sci U S A* 2006; **103:** 14453-14458. (PMID: 16984998)

8. Bender A, Karbach J, Neumann A, Jäger D, Al-Batran SE, Atmaca A, Weidmann E, Biskamp M, Gnjatic S, Pan L, Hoffman E, Old LJ, Knuth A, Jäger E. LUD 00-009: phase 1 study of intensive course immunization with NY-ESO-1 peptides in HLA-A2 positive patients with NY-ESO-1-expressing cancer. *Cancer Immun* 2007; **7:** 16. (PMID: 17944437)

9. Nicholaou T, Ebert LM, Davis ID, McArthur GA, Jackson H, Dimopoulos N, Tan B, Maraskovsky E, Miloradovic L, Hopkins W, Pan L, Venhaus R, Hoffman EW, Chen W, Cebon J. Regulatory T-cell-mediated attenuation of T-cell responses to the NY-ESO-1 ISCOMATRIX vaccine in patients with advanced malignant melanoma. *Clin Cancer Res* 2009; **15:** 2166-2173. (PMID: 19276262)

10. Karbach J, Gnjatic S, Bender A, Neumann A, Weidmann E, Yuan J, Ferrara CA, Hoffmann E, Old LJ, Altorki NK, Jäger E. Tumor-reactive CD8+ T-cell responses after vaccination with NY-ESO-1 peptide, CpG 7909 and Montanide ISA-51: association with survival. *Int J Cancer* 2010; **126:** 909-918. (PMID: 19728336)

11. Karbach J, Neumann A, Atmaca A, Wahle C, Brand K, von Boehmer L, Knuth A, Bender A, Ritter G, Old LJ, Jäger E. Efficient *in vivo* priming by vaccination with recombinant NY-ESO-1 protein and CpG in antigen naive prostate cancer patients. *Clin Cancer Res* 2011; **17:** 861-870. (PMID: 21163871)

12. Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, O'Neill D, Pavlick A, Escalon JB, Cruz CM, Angiulli A, Angiulli F, Mears G, Vogel SM, Pan L, Jungbluth AA, Hoffmann EW, Venhaus R, Ritter G, Old LJ, Ayyoub M. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proc Natl Acad Sci U S A* 2007; **104:** 8947-8952. (PMID: 17517626)

13. Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, Ravetch JV, Steinman RM, Nussenzweig MC. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions *in vivo*. *J Exp Med* 2001; **194:** 769-779. (PMID: 11560993)

14. Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol* 2005; **6:** 280-286. (PMID: 15685176)

15. Vicari AP, Chiodoni C, Vaure C, Ait-Yahia S, Dercamp C, Matsos F, Reynard O, Taverne C, Merle P, Colombo MP, O'Garra A, Trinchieri G, Caux C. Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody. *J Exp Med* 2002; **196:** 541-549. (PMID: 12186845)

16. Middel P, Brauneck S, Meyer W, Radzun HJ. Chemokine-mediated distribution of dendritic cell subsets in renal cell carcinoma. *BMC Cancer* 2010; **10:** 578. (PMID: 20969772)

17. Otahal P, Hutchinson SC, Mylin LM, Tevethia MJ, Tevethia SS, Schell TD. Inefficient cross-presentation limits the CD8+ T cell response to a subdominant tumor antigen epitope. *J Immunol* 2005; **175:** 700-712. (PMID: 16002665)

18. Petersen TR, Dickgreber N, Hermans IF. Tumor antigen presentation by dendritic cells. *Crit Rev Immunol* 2010; **30:** 345-386. (PMID: 20666707)

19. Regnault A, Lankar D, Lacabanne V, Rodriguez A, Théry C, Rescigno M, Saito T, Verbeek S, Bonnerot C, Ricciardi-Castagnoli P, Amigorena S. Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med* 1999; **189:** 371-380. (PMID: 9892619)

20. Cioca DP, Deak E, Cioca F, Paunescu V. Monoclonal antibodies targeted against melanoma and ovarian tumors enhance dendritic cell-mediated cross-presentation of tumor-associated antigens and efficiently cross-prime CD8+ T cells. *J Immunother* 2006; **29:** 41-52. (PMID: 16365599)

21. Nimmerjahn F, Ravetch JV. Fc-receptors as regulators of immunity. *Adv Immunol* 2007; **96:** 179-204. (PMID: 17981207)

22. Jäger E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Jäger 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:** 265-270. (PMID: 9432985)

23. Maio M, Coral S, Sigalotti L, Elisei R, Romei C, Rossi G, Cortini E, Colizzi F, Fenzi G, Altomonte M, Pinchera A, Vitale M. Analysis of cancer/testis antigens in sporadic medullary thyroid carcinoma: expression and humoral response to NY-ESO-1. *J Clin Endocrinol Metab* 2003; **88:** 748-754. (PMID: 12574209)

24. Fratta E, Coral S, Covre A, Parisi G, Colizzi F, Danielli R, Marie Nicolay HJ, Sigalotti L, Maio M. The biology of cancer testis antigens: putative function, regulation and therapeutic potential. *Mol Oncol* 2011; **5:** 164-182. (PMID: 21376678)

25. Liu F, Whitton JL. Cutting edge: re-evaluating the *in vivo* cytokine responses of CD8+ T cells during primary and secondary viral infections. *J Immunol* 2005; **174:** 5936-5940. (PMID: 15879085)

26. Melief CJ. Cancer immunotherapy by dendritic cells. *Immunity* 2008; **29:** 372-383. (PMID: 18799145)

27. Lugade AA, Moran JP, Gerber SA, Rose RC, Frelinger JG, Lord EM. Local radiation therapy of B16 melanoma tumors increases the generation of tumor antigen-specific effector cells that traffic to the tumor. *J Immunol* 2005; **174:** 7516-7523. (PMID: 15944250)

28. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392:** 245-252. (PMID: 9521319)

29. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010; **11:** 373-384. (PMID: 20404851)

30. Zippelius A, Batard P, Rubio-Godoy V, Bioley G, Lienard D, Lejeune F, Rimoldi D, Guillaume P, Meidenbauer N, Mackensen A, Rufer N, Lubenow N, Speiser D, Cerottini JC, Romero P, Pittet MJ. Effector function of human tumor-specific CD8 T cells in melanoma lesions: a state of local functional tolerance. *Cancer Res* 2004; **64:** 2865-2873. (PMID: 15087405)

31. Beatty GL, Chiorean EG, Fishman MP, Saboury B, Teitelbaum UR, Sun W, Huhn RD, Song W, Li D, Sharp LL, Torigian DA, O'Dwyer PJ, Vonderheide RH. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science* 2011; **331:** 1612-1616. (PMID: 21436454)

32. Rüter J, Antonia SJ, Burris HA, Huhn RD, Vonderheide RH. Immune modulation with weekly dosing of an agonist CD40 antibody in a phase I study of patients with advanced solid tumors. *Cancer Biol Ther* 2010; **10:** 983-993. (PMID: 20855968)

33. Junttila TT, Parsons K, Olsson C, Lu Y, Xin Y, Theriault J, Crocker L, Pabonan O, Baginski T, Meng G, Totpal K, Kelley RF, Sliwkowski MX. Superior *in vivo* efficacy of afucosylated trastuzumab in the treatment of HER2-amplified breast cancer. *Cancer Res* 2010; **70:** 4481-4489. (PMID: 20484044)

34. Loisel S, Ohresser M, Pallardy M, Daydé D, Berthou C, Cartron G, Watier H. Relevance, advantages and limitations of animal models used in the development of monoclonal antibodies for cancer treatment. *Crit Rev Oncol Hematol* 2007; **62:** 34-42. (PMID: 17197192)

35. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, André F, Delaloge S, Tursz T, Kroemer G, Zitvogel L. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 2007; **13:** 1050-1059. (PMID: 17704786)

36. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science* 2003; **301:** 1374-1377. (PMID: 12920303)

37. Rader C, Ritter G, Nathan S, Elia M, Gout I, Jungbluth AA, Cohen LS, Welt S, Old LJ, Barbas CF 3rd. The rabbit antibody repertoire as a novel source for the generation of therapeutic human antibodies. *J Biol Chem* 2000; **275:** 13668-13676. (PMID: 10788485)

38. Stockert E, Jäger 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:** 1349-1354. (PMID: 9547346)

39. Nuber N, Curioni-Fontecedro A, Matter C, Soldini D, Tiercy JM, von Boehmer L, Moch H, Dummer R, Knuth A, van den Broek M. Fine analysis of spontaneous MAGE-C1/CT7-specific immunity in melanoma patients. *Proc Natl Acad Sci U S A* 2010; **107:** 15187-15192. (PMID: 20696919)

40. Mitsui J, Nishikawa H, Muraoka D, Wang L, Noguchi T, Sato E, Kondo S, Allison JP, Sakaguchi S, Old LJ, Kato T, Shiku H. Two distinct mechanisms of augmented antitumor activity by modulation of immunostimulatory/inhibitory signals. *Clin Cancer Res* 2010; **16:** 2781-2791. (PMID: 20460483)

Materials and methods

Patient material

Serum and peripheral blood was collected from cancer patients. All patients were admitted at the University Hospital Zürich and provided written informed consent in accordance with the Declaration of Helsinki. The local ethics committee approved the study.

Memory B cell culture

PBMC were incubated with anti-CD22 coupled to magnetic beads (Miltenyi Biotec), PE-conjugated anti-IgD, and APC-conjugated antibodies to IgM, CD3, CD8, and CD56 (Becton Dickinson). B cells were isolated by positive selection of CD22+ cells using a midi-MACS device and LS columns (Miltenyi Biotec), followed by sorting PE-APC- cells using a MoFlo cell sorter (Beckman Coulter). CD22+ IgD- IgM- memory B cells were incubated with 10% EBV-containing supernatant from B95-8 cells (from European Collection of Cell Cultures, ECACC) in the presence of 2.5 μg/mL CpG 2006 at 37°C for 4 h. Cells were seeded in 96-well U-bottom plates at 10 cells per well plus 3 x 10^4 irradiated allogeneic PBMCs in RPMI 1640 medium supplemented with 10% human serum, antibiotics, 10% supernatant from B95-8 cells, and 2.5 μg/mL CpG 2006. Supernatants were tested for NY-ESO-1-specific antibodies after 2 weeks by ELISA.

Single cell-RT-PCR

B cell cultures were harvested and single cells were deposited into a 96-well PCR plate (Applied Biosystems) using a MoFlo XDP cell sorter (Beckman Coulter). RT-PCR was performed using random hexamer primers for cDNA synthesis and specific primers to amplify the immunoglobulin variable and constant regions. Immunoglobulin heavy and light chain variable regions were amplified using a nested PCR approach as described (36). Primer-encoded amino acid sequences and J-C regions of the antibodies were corrected to represent the authentic amino acid sequence as it occurred in the patient in a subsequent step prior to antibody production.

Antibody production and purification

293-T human embryonic kidney cells were transfected with 25 kDa branched polyethylenimine (PEI, Polysciences, Warrington, PA) plus DNA plasmids (heavy and light chain in equal ratios) in a 1.3:1 ratio and were incubated for 15 min at room temperature. Following transfection, the cells were cultured in serum free Opti-MEM I + GlutaMAX-I (Invitrogen) supplemented with 10 U penicillin-streptomycin (Lonza, Switzerland). After 72 h supernatants were collected and IgG was purified on a protein A column (GE Healthcare, Sweden) using FPLC (GE Healthcare, Sweden).

Biacore analysis

Antibody binding kinetics with NY-ESO-1 proteins derived from *E. coli* (LICR New York Branch) and HEK293 cells (OriGene Technologies, Inc.) were determined by Biacore technology (model Biacore 2000; Biacore AB) using CM5 sensor

chips, EDC-NHS conjugation, and BIAevaluation software. Technical details have been described previously (37).

ELISA

- Protein or peptide ELISA

96-well half-area microtiter plates (Costar, USA) were coated with 30 µL/well of 1 µg/mL recombinant NY-ESO-1 protein, or 10 µg/mL 20-mer peptides spanning the entire NY-ESO-1 protein (Peptides & Elephants, Germany) diluted in PBS overnight at 4°C. After coating, plates were washed with PBS + 0.05% Tween-20 (PBS-T) and blocked for 1 h at room temperature with 2% BSA/PBS (Sigma). B cell-conditioned medium, patient serum, or recombinant antibody was incubated for 2 h at room temperature (RT) at indicated concentrations or dilutions in PBS. Plates were washed with PBS-T and incubated for 1 h at RT with HRPO-conjugated goat-anti-human Fcγ antibody (Jackson ImmunoResearch), diluted 1:4000 in 0.5% BSA/PBS, followed by measurement of the HRPO activity using a TMB substrate solution (Sigma, Buchs, Switzerland). The mouse IgG1 monoclonal anti-NY-ESO-1 antibody E978 (38) and HRPO-conjugated goat-anti-mouse Fcγ antibody (Jackson ImmunoResearch) at 1:4000 dilution in 0.5% BSA/PBS served as positive control.

- Cellular ELISA

4 x 10^4 SK-MEL-37 cells were seeded in a 96-well flat bottom plate and cultured under standard conditions overnight. Cells were fixed in ice-cold ethanol/acetone mix (1:1) for 15 min on ice. After two wash steps with PBS, cells were blocked and permeabilized with 100 µL of PBS + 0.5% BSA + 0.5% Triton X 100 for 2 h at 4°C. B cell-conditioned medium or recombinant antibody was incubated at indicated concentrations for 2 h at 4°C. Bound antibodies were detected after 1 h incubation at 4°C with HRPO-labeled goat anti-human Fc secondary antibody (Jackson ImmunoResearch).

Immunoprecipitation

SK-MEL-37 tumor cells were lysed with Triton X 100/ Glycerol-based lysis buffer for 15 min at 4°C. Cell debris was separated by centrifugation at maximum speed in a table centrifuge and protein concentration of the supernatant analyzed by standard Bradford assay. 300 ng of antibody was used to precipitate NY-ESO-1 from 250 µg of SK-MEL-37 cell lysate in a 16 h incubation at 4°C. The immune complex was isolated by adding magnetic Protein G beads (New England Biolabs, Ipswich, MA) for 1 h at 4°C under constant agitation. Beads were washed, resuspended in NuPAGE LDS sample buffer (Invitrogen) and boiled prior to Gradient SDS Polyacrylamide Gel Electrophoresis (NuPAGE 4-12% Bis-Tris Gel, Invitrogen). NY-ESO-1 protein was detected by Western blot using murine antibody E978 (38).

***In vitro* cross-presentation assay**

Human, HLA-A*0201/NY-ESO-$1_{157-165}$ specific CD8+ T cells were cloned as previously described (39). To generate DCs, CD14+ cells were MACS-purified according to the manufacturer's instructions (Miltenyi Biotech) from PBMC from HLA-A*0201+ healthy donors and cultured at 10^6 cells/mL in serum-free CellGro DC media (CellGenix), supplemented with 800 U/mL GM-CSF and 500 U/mL IL-4 (R&D Systems) to generate DCs. Medium was exchanged the following day and DCs were harvested on d 4 of culture and resuspended at 10^6/ mL in Opti-MEM (Gibco). Immune complexes were generated by incubating 20 µg recombinant NY-ESO-1 with 200 µg 12D7 in a total volume of 500 µL Opti-MEM (Gibco) for 30 min at 37°C. Human IgG1 (Sigma Aldrich), 12D7 alone, or NY-ESO-1 alone were used as controls. Alternatively, 200 µg 12D7 was incubated with a lysate of an equivalent of 10^7 NY-ESO-1+ SK-MEL-37 cells in 500 µL Opti-MEM. DCs (5x10^5 in 0.5 mL Opti-MEM) were added to the immune complexes and controls. The mixture was incubated at 37°C for 3 h. DCs were then centrifuged and resuspended in CellGro DC media at 10^6/mL. Hundred µL (10^5 DCs) were cultured in 96-well flatbottom plates at 37°C in the presence or absence of maturation cocktail (1 µg/mL soluble CD40L (sCD40L) trimer (PeproTech) plus TNF-α (25 ng/mL; R&D Systems)). After 36 h, approximately 6 x 10^5 HLA-A*0201/NY-ESO-$1_{157-165}$ specific CD8+ T cells in 100 µL RPMI + 10% human serum + antibiotics + 20 µg/mL Brefeldin A (Sigma Chemicals) were added to the different DC-cultures. After 4 h, cultures were harvested in FACS buffer (PBS + 2% FCS + 2 mM EDTA + 0.05% NaN_3) and surface stained with anti-CD8 followed by intracellular staining for IFN-γ as previously described (39). CD8+ T cells plus unloaded DCs served as negative control, and CD8+ T cells plus DCs with 10^{-6} M of NY-ESO-$1_{157-165}$ (SLLMWITQC, Thermo Fisher Scientific) served as positive control. All cultures were performed at least in duplicates.

Mice and cell lines

BALB/c mice were originally obtained from Jackson Laboratories and were bred and kept under specific pathogen-free conditions in the Institute of Laboratory Animal Sciences (University of Zürich). Age- and sex-matched mice of 9-12 weeks old were used for all experiments. Mice were housed under specific pathogen-free conditions at University Hospital Zürich. All experiments were performed in agreement with the federal and cantonal laws on animal protection.

The colon carcinoma cell line CT26 was transfected to stably express intracellular NY-ESO-1 (40) and was cultured in RPMI + 10% FCS + antibiotics + 10 µg/mL puromycin under standard tissue culture conditions. CT26/NY-ESO-1 and the human melanoma cell line SK-MEL-37 were cultured in RPMI + 10% FCS + antibiotics under standard tissue culture conditions. 293-T cells were cultured in DMEM (Lonza, Switzerland) supplemented with 10% FCS (Linaris) and 10 U penicillin-streptomycin (Lonza, Switzerland) under standard tissue culture conditions.

Treatment of mice

Mice were injected s.c. into the right flank with 10^6 CT26/NY-ESO-1+ cells in 100 µL RPMI. The tumor surface was measured at least twice a week with a calliper. Treatment was started (d 0) when tumors reached a size of approximately 25 mm^2. 5-Fluorouracil (5-FU, TEVA Pharma, Aesch, Switzerland) was diluted in saline and were given i.p. on d 0 and d 7 at 75 mg/kg, respectively. 12D7 (100 µg in 100 µL PBS) was given i.p. on d 2 and d 9. All animal experiments were performed in accordance with the Swiss federal and cantonal law on animal protection.

Flow cytometry

At the end of the experiment (1 week after the last injection of 12D7), mice were injected i.p. with 250 µg Brefeldin A and were euthanized 4 h later. Subsequent processing and staining was performed in the presence of 10 µg/mL Brefeldin A (25). Tumors were cut into small pieces and subsequently digested with 1.5 mg/mL collagenase + 100 µg/mL DNase for 1 h at 37°C followed by filtration through a 50 µm cell strainer. Single cell suspensions were surface stained in FACS buffer (FB, PBS + 2% FCS + 0.03% NaN_3 + 20 mM EDTA) with anti-CD45.2 pacific

blue and anti-CD8bPE. For intracellular staining to detect IFN-γ, cells were permeabilized with permeabilization buffer (PB, FB + 0.1% saponin) and stained intracellularly with anti-IFN-γ APC. All antibodies were obtained from BioLegend, San Diego, CA, USA. Samples were measured with a CyAn ADP9 (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo Analysis Software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

Statistics were done using an unpaired Student two-tailed *t*-test. Error bars represent SD. p values less than 0.05 were considered significant.

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Induction of CD8 T-cell responses restricted to multiple HLA class I alleles in a cancer patient by immunization with a 20-mer NY-ESO-1f (NY-ESO-1 91-110) peptide

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Immunogenicity of a long 20-mer NY-ESO-1f peptide vaccine was evaluated in a lung cancer patient TK-f01, immunized with the peptide with Picibanil OK-432 and Montanide ISA-51. We showed that internalization of the peptide was necessary to present CD8 T-cell epitopes on APC, contrasting with the direct presentation of the short epitope. CD8 T-cell responses restricted to all five HLA class I alleles were induced in the patient after the peptide vaccination. Clonal analysis showed that B*35:01 and B*52:01-restricted CD8 T-cell responses were the two dominant responses. The minimal epitopes recognized by A*24:02, B*35:01, B*52:01 and C*12:02-restricted CD8 T-cell clones were defined and peptide/HLA tetramers were produced. NY-ESO-1 91-101 on A*24:02, NY-ESO-1 92-102 on B*35:01, NY-ESO-1 96-104 on B*52:01 and NY-ESO-1 96-104 on C*12:02 were new epitopes first defined in this study. Identification of the A*24:02 epitope is highly relevant for studying the Japanese population because of its high expression frequency (60%). High affinity CD8 T-cells recognizing tumor cells naturally expressing the epitopes and matched HLA were induced at a significant level. The findings suggest the usefulness of a long 20-mer NY-ESO-1f peptide harboring multiple CD8 T-cell epitopes as an NY-ESO-1 vaccine. Characterization of CD8 T-cell responses in immunomonitoring using peptide/HLA tetramers revealed that multiple CD8 T-cell responses comprised the dominant response.

The NY-ESO-1 antigen was originally identified in esophageal cancer by serological expression cloning (SEREX) using autologous patient serum.[1,2] NY-ESO-1 expression is observed in a wide range of human malignancies, but the expression is restricted to germ cells in the testes in normal adult tissues.[1–4] Therefore, NY-ESO-1 has emerged as a prototype of a class of cancer/testis (CT) antigens.[5] The efficacy of the NY-ESO-1 antigen as a cancer vaccine has been studied extensively using various preparations, *e.g.*, peptide, protein or DNA, etc. of the antigen with various adjuvants.[6–14] These studies established the safety of the NY-ESO-1 vaccine and demonstrated its immunogenicity.

In a phase I clinical trial, we immunized cancer patients with a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 whole protein (CHP-NY-ESO-1) and showed that the vaccine had potent capacity to induce the NY-ESO-1 antibody in vaccinated patients.[13,14] The most dominant serological antigenic epitope was NY-ESO-1 91-108. The CHP-NY-ESO-1 vaccine also elicited CD4 and CD8 T-cell responses in immunized patients.[14] Analysis of T cell responses against overlapping peptides (OLPs) spanning the NY-ESO-1 molecule revealed that two dominant NY-ESO-1 regions, regions II (73-114) and III (121-144), were recognized by either CD4 or CD8 T-cells in most patients irrespective of their HLA type. Essentially similar findings were obtained by studies using other preparations of NY-ESO-1 protein vaccine.[11,12,15]

Key words: cancer vaccine, NY-ESO-1, long peptide, CD8 T-cell response
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What's new?

An antigen called NY-ESO-1 is expressed by a wide range of human cancers, and has shown promise as a cancer vaccine. In this study, the authors studied a peptide derived from that antigen, and analyzed the cellular and molecular mechanisms that allow the peptide to provoke an immune response. They found that the peptide must be internalized by antigen-presenting cells (APCs) in order to yield T-cells that can attack tumours via the NY-ESO-1 antigen. These data increase our understanding of the requirements for an effective therapeutic cancer vaccine. (This section added after initial online publication.)

CD8 T-cells induced by immunization with NY-ESO-1 class I short epitope peptides have been shown to be of low affinity and do not recognize naturally processed NY-ESO-1 on tumor cells.[16] However, the advantage of synthetic long peptides over short peptides for use as vaccines has been reported.[17] Long peptides do not bind to MHC class I molecules directly, and require antigen processing by dendritic cells to be presented. Therefore, the use of long peptides prevents the antigen peptides from direct binding to MHC class I molecules on nonprofessional antigen-presenting cells (APC), which may cause transient activation and subsequent anergy of CTLs in the absence of appropriate costimulatory signals.[17–19] Based on these findings, we recently used a long peptide spanning a peptide region NY-ESO-1 91-110 (NY-ESO-1f peptide) which included the dominant serological antigenic epitope and overlapped one of the two dominant regions recognized by CD4 and CD8 T-cells for a vaccine in a clinical trial.[20] Ten patients received the NY-ESO-1f peptide vaccine. The NY-ESO-1f peptide vaccine was well tolerated and elicited humoral, CD4 and CD8 T-cell responses in immunized patients.

In this study, we demonstrated that internalization of the peptide was necessary to present CD8 T-cell epitopes on APC treated with the long 20-mer NY-ESO-1f peptide. Analysis of the CD8 T-cell response in an NY-ESO-1f peptide-immunized patient revealed occurrence of responses restricted to all five HLA class I alleles defined in the patient. The frequency of A*24:02, B*35:01, B*52:01, C*03:03 and C*12:02-restricted CD8 T-cells in PBMCs was defined by clonal analysis revealing B*35:01- and B*52:01-restricted CD8 T-cell responses as dominant. By establishing clones from those HLA-restricted CD8 T-cells, new epitopes on A*24:02, B*35:01, B*52:01 and C*12:02 were defined and peptide/HLA tetramers were prepared. Clonal analysis showed that CD8 T-cells that recognize natural epitopes on tumor cells were induced in a significant proportion by immunization with the NY-ESO-1f peptide. Immunomonitoring using the tetramers revealed that multiple CD8 T-cell responses comprised the dominant response.

Material and Methods

Clinical trial

A phase I clinical trial of the NY-ESO-1f peptide vaccine was conducted to evaluate the safety, immune response and tumor response.[20] Patients with advanced cancers that were refractory to standard therapy and expressed NY-ESO-1 as assessed by immunohistochemistry (IHC) were eligible. The protocol was approved by the Ethics Committee of Tokyo, Osaka and Okayama Universities in light of the Declaration of Helsinki. Written informed consent was obtained from each patient before enrolling in the study. The study was performed in compliance with Good Clinical Practice. The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000001260) on July 24, 2008 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/ index.htm).

Blood samples

Patient TK-f01 was a lung cancer patient who received a right middle lobectomy in October, 2004.[20] As the tumor continued to grow despite chemotherapy, he was enrolled in the study in June, 2008. The patient received 12 vaccinations once every 3 weeks. Peripheral blood was drawn from patient TK-f01 with informed consent for immunological monitoring. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation using a Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). CD4-, CD8- and CD19-positive cells were purified by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). The residual cells were kept for use as APC. The cells were stored in liquid N_2 until use. HLA tying was done with PBMCs by a sequence-specific oligonucleotide probe and sequence-specific priming of genomic cDNA using a standard procedure.

Cell lines

LC99A and OU-LC-OK are lung cancer cell lines. SK-OV3 is an ovarian cancer cell line and SK-MEL37 is a melanoma cell line. These cell lines were kept by serial passage in tissue culture. EBV-B cells were generated from CD19-positive peripheral blood B cells using a culture supernatant from EBV-producing B95-8 cells. The medium used to maintain these cell lines was RPMI1640 supplemented with 10% FCS (JRM, Bioscience, Lenexa, KA), 2 mmol/l Glutamax, antibitotics, and 10 mmol/l HEPES (Invitrogen, Carlsbad, CA).

Antibodies

Anti-human CD4, anti-human CD8, anti-HLA class I and anti-HLA class II mAbs were purchased from BD Bioscience (San Jose, CA).

Peptides

The following series of 28 18-mer OLPs and a C-terminal 30-mer peptide spanning the entire NY-ESO-1 protein were used: 18.1 (1-18), 18.2 (7-24), 18.3 (13-30), 18.4 (19-36), 18.5 (25-42), 18.6 (31- 48), 18.7 (37-54), 18.8 (43-60), 18.9 (49-

66), 18.10 (55-72), 18.11 (61-78), 18.12 (67-84), 18.13 (73-90), 18.14 (79-96), 18.15 (85-102), 18.16 (91-108), 18.17 (97-114), 18.18 (103-120), 18.19 (109-126), 18.20 (115-132), 18.21 (121-138), 18.22 (127-144), 18.23 (133-150), 18.24 (139-156), 18.25 (145-162), 18.26 (149-166), 18.27 (153-170), 18.28 (156-173) and 30.9 (151-180). Various N- and C-termini truncated peptides in the NY-ESO-1f peptide were also used. These peptides were synthesized using standard solid-phase methods based on *N*-(9-fluorenyl)-methoxycarbonyl (Fmoc) chemistry on an ABIMED Multiple Peptide Synthesizer (AMS422, ABIMED, Langenfeld, Germany) at Okayama University (Okayama, Japan). The carboxyfluorescein (FAM)-conjugated NY-ESO-1f peptide (5(6)-FAM-YLAMPFATPMEAELARRSLA) was synthesized by Operon (Tokyo, Japan).

Recombinant NY-ESO-1 protein

Recombinant NY-ESO-1 protein was prepared as described earlier.[13] NY-ESO-1 cDNA was cloned into the *Sph*I/*Sal*I and *Bam*HI/*Sph*I sites of the pQE-30 vector. N-His tagged protein was purified by nickel-ion affinity chromatography under denaturing conditions.

Preparation of immature dendritic cells

Monocytes were isolated from PBMCs using anti-CD14 mAb-coated magnetic beads (Miltenyi Biotec) and cultured in AIM-V medium supplemented with 5% heat-inactivated pooled human serum, 10 ng/ml rhGM-CSF (Kyowa Hakko Kirin, Tokyo, Japan) and 10 ng/ml rhIL-4 (PeproTech) for 7 days at 37°C in a 5% CO_2 atmosphere.

In vitro stimulation of bulk CD4 and CD8 T-cells

CD4 and CD8 T-cells (2×10^6/well) were cultured with a mixture of 29 NY-ESO-1 OLPs (10^{-6} M) in the presence of an equal number of irradiated (40 Gy), autologous CD4- and CD8-depleted cells as APC in a 24-well culture plate (BD Bioscience) for 12 days at 37°C in a 5% CO_2 atmosphere. The medium was AIM-V (Invitrogen) supplemented with 5% heat-inactivated pooled human serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 units/ml recombinant human (rh) IL-2 (Takeda Chemical Industries, Osaka, Japan) and 10 ng/ml rhIL-7 (Peprotech, London, UK).

Cloning of CD8 T-cells

CD8 T-cells were cloned by limiting dilution after *in vitro* stimulation in round-bottomed 96-well plates in the presence of irradiated (40 Gy) allogeneic PBMCs freshly prepared from the healthy donor as feeder cells. The medium used was AIM-V (Invitrogen) supplemented with 5% heat-inactivated pooled human serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 units/ml rhIL-2 (Takeda Chemical Industries), 10 ng/ml rhIL-7 (Peprotech) and 1 μg/ml phytohemagglutinin (PHA)-L (Sigma-Aldrich).

Clonal expansion

Cloned CD8 T-cells (1×10^3) obtained by limiting dilution were expanded in a round-bottomed 96 well plate in the presence of irradiated (40 Gy) PBMCs (5×10^4) freshly prepared from allogeneic healthy donors as feeder cells. Fresh medium was added every 3 days. After culture for 14 days, the cells were harvested and kept frozen at 2×10^6/tube. The medium used was AIM-V supplemented with 5% heat-inactivated pooled human serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 units/ml rhIL-2, 10 ng/ml rhIL-7 and 1 μg/ml phytohemagglutinin (PHA)-L.

IFNγ capture assay

Bulk CD4 or CD8 T-cells (1×10^5) from the *in vitro* stimulation culture were cultured with autologous or allogeneic EBV-B cells (1×10^5) pulsed with OLPs for 4 hr. The cells were then treated with a bi-specific CD45 and IFNγ antibody (IFNγ catch reagent) (2 μl) for 5 min on ice. The cells were diluted in AIM-V medium (3 ml) and placed on a slow rotating device (Miltenyi Biotec) to allow IFNγ secretion at 37°C in a 5% CO_2 atmosphere. After incubation for 45 min, the cells were washed with cold buffer and treated with PE-conjugated anti-IFNγ (detection reagent), and FITC-conjugated anti-CD4 or anti-CD8 mAb. After incubation for 10 min at 4°C, the cells were washed and analyzed by a FACS Canto II (BD Bioscience).

Tetramer construction and staining

Peptide/HLA tetramers were produced as described earlier.[21,22] NY-ESO-1 91-101/A*24:02, NY-ESO-1 92-100/B*35:01, NY-ESO-1 92-102/B*35:01, NY-ESO-1 94-104/B*35:01 and NY-ESO-1 96-106/C*12:02 tetramers were used. The HIV Env/A*24:02 tetramer was used as a control. For staining, cells were incubated with tetramer at a concentration of 20 μg/ml for 15 min at 37°C, followed by incubation with an FITC-conjugated anti-CD8 mAb (Miltenyi Biotec) on ice for 15 min and analyzed by a FACS Canto II (Becton Dickinson).

Cytotoxicity assay

Cytotoxicity was assayed by a luminescent method using the aCella-Tox kit (Cell Technology, Mountain View, CA). Effector cells were incubated with 5,000 target cells at various ratios in 96-well round bottomed culture plates for 12 hr at 37°C in a 5% CO_2 atmosphere. The plate was read by a luminometer (multi-detection microplate reader, DS Pharma, Osaka, Japan).

IFNγ ELISA

CD8 T-cell clones (5×10^3) were cultured with autologous or allogeneic EBV-B cells (5×10^3) pulsed with the peptides in a 96-well round bottomed culture plate for 24 hr at 37°C in a 5% CO_2 atmosphere. Culture supernatants were then collected and the amount of IFNγ was measured by sandwich ELISA. For antibody blocking experiments, each mAb (5 μg/ml) was added to the assay culture. To inhibit internalization of the antigens to DCs, cytochalasin B (10 μM) was added to the culture.

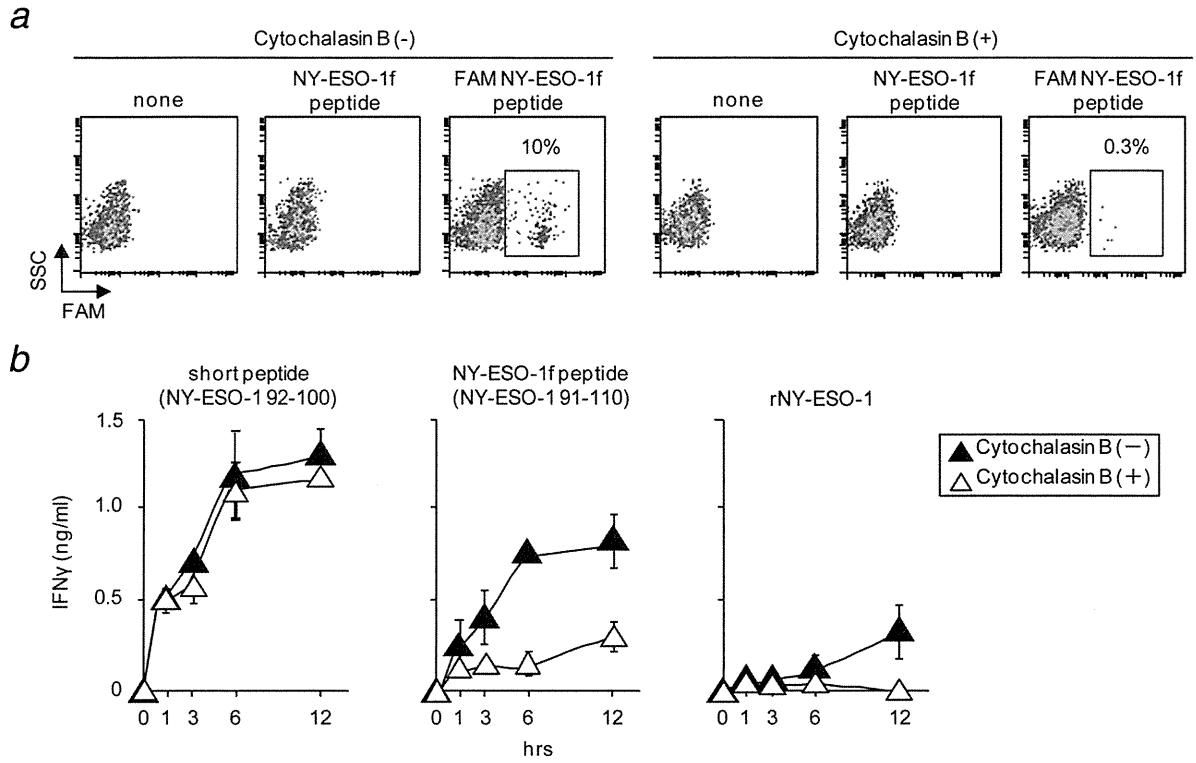


Figure 1. Internalization and processing of the long 20-mer NY-ESO-1f-peptide by APC in CD8 T-cell recognition. In (*a*), immature dendritic cells (iDCs) from a healthy donor (HD) NO PBMCs (A*02:07/*26:02, B*35:01/*46:01, C*08:03/*14:03) were cultured with an NY-ESO-1f peptide (10 μM) or a FAM NY-ESO-1f peptide (10 μM) for 12 hr in the presence or absence of cytochalasin B (10 μM). After culture, the internalization of the FAM NY-ESO-1f peptide was analyzed by FACS Canto II. In (*b*), the CD8 T-cell clone 2H10 (5×10^3) was stimulated with iDCs (5×10^3) pulsed with the short peptide (NY-ESO-1 92-100: LAMPFATPM, 9-mer; 1 μM), the NY-ESO-1f peptide (NY-ESO-1 91-110: YLAMPFATPMEAELARRSLA, 20-mer; 1 μM) or recombinant NY-ESO-1 protein (1 μM) for the indicated time in the presence or absence of cytochalasin B (10 μM). The amount of IFNγ in the culture supernatant was determined by ELISA. One representative of three independent experiments is shown.

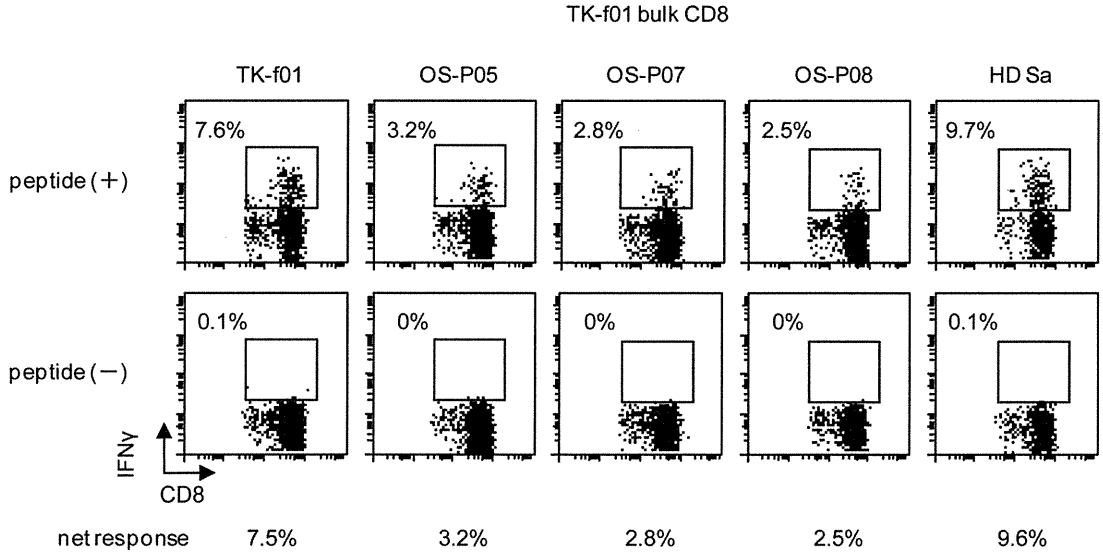
Results

Internalization of 20-mer NY-ESO-1f peptide to present CD8 T-cell epitopes on antigen presenting cells

We investigated the need for internalization of the 20-mer NY-ESO-1f peptide (NY-ESO-1 91-110: YLAMPFATPMEAELARRSLA) to present CD8 T-cell epitopes on APC. Immature dendritic cells (iDCs) were prepared from purified CD14-positive cells from a B*35:01 healthy donor by treating them with IL-4 and GM-CSF for 7 days and used as APC. As shown in Figure 1*a*, internalization of the FAM-conjugated NY-ESO-1f peptide was observed in approximately 10% of DCs after culture with the peptide for 12 hr. Treatment of DCs with the peptide in the presence of cytochalasin B diminished internalization. Recognition by a B*35:01-restricted CD8 T-cell clone 2H10 of DCs treated with the peptides was investigated. As shown in Figure 1*b*, clone 2H10 recognized DCs pulsed with the short peptide NY-ESO-1 92-100 in the presence of cytochalasin B. On the other hand, recognition of DCs pulsed with NY-ESO-1f peptide was inhibited in the presence of cytochalasin B. Only marginal recognition was observed with DCs pulsed with recombinant NY-ESO-1 protein.

Multiple HLA class I-restricted CD8 T-cell responses in PBMCs from patient TK-f01 immunized with the 20-mer NY-ESO-1f peptide

TK-f01 was a lung adenocarcinoma patient immunized with the NY-ESO-1f peptide (600 μg) with Picibanil OK-432 and Montanide ISA-51 12 times once every 3 weeks.[20] We investigated multiple HLA class I-restricted CD8 T-cell responses in PBMCs from patient TK-f01. The patient's HLA class I was A*24:02, B*35:01, B*52:01, C*03:03 and C*12:02. Purified CD8 T-cells from the patient's PBMCs obtained at day 64 after the third vaccination were stimulated once with a mixture of 29 18-mer series of NY-ESO-1 OLPs spanning the entire protein for 12 days using CD4 and CD8-depleted PBMCs as APC. The cells were harvested and stimulated with NY-ESO-1f peptide for 4 hrs using autologous and allogeneic EBV-B cells as APC and the response was assayed by IFNγ capture assay. As shown in Figure 2, TK-f01 CD8 T-cells responded strongly to NY-ESO-1f peptide-pulsed autologous EBV-B cells. Use of various allogeneic EBV-B cells as APC showed TK-f01 CD8 T-cell responses against NY-ESO-1f peptide presented on multiple HLA class I molecules shared with patient TK-f01.



HLA class I

EBV-B	A	B	C
TK-f01	***24:02**	***35:01**, ***52:01**	***03:03**, ***12:02**
OS-P05	***24:02**, *24:20	*51:01, ***52:01**	***12:02**, *14:02
OS-P07	*02:06, ***24:02**	*46:01, *51:01	*01:02, *14:02
OS-P08	***24:02**	*40:02, *59:01	*01:02, ***03:03**
HD Sa	***24:02**, *33:03	***35:01**, *44:03	***03:03**

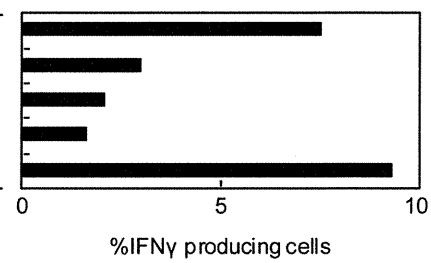


Figure 2. Multiple HLA class I-restricted CD8 T-cell responses in patient TK-f01 immunized with the 20-mer NY-ESO-1f peptide. CD8 T-cells (2×10^6) were stimulated with a mixture of NY-ESO-1 OLPs (10^{-6} M) in the presence of APC for 12 days. The cells were harvested and stimulated with NY-ESO-1f peptide for 4 hr using autologous and allogeneic EBV-B cells as APC and the response was assayed by an IFNγ capture assay.

Clonal analysis of the multiple HLA class I-restricted TK-f01 CD8 T-cell responses against the NY-ESO-1f peptide

The multiple HLA class I-restricted TK-f01 CD8 T-cell responses against the NY-ESO-1f peptide were investigated by clonal analysis. Purified CD8 T-cells from the patient's PBMCs obtained at day 64 after the third vaccination were stimulated once with a mixture of NY-ESO-1 OLPs for 12 days using CD4 and CD8-depleted PBMCs as APC as above. The cells were then collected and seeded at 3 cells per well in 96-well culture plates to expand clones. After culture for 14 days, 300 clones were obtained. Each of the 300 clones were split into 7 and the responses against NY-ESO-1f peptide on autologous, OS-P06, OS-P08, HD NM, HD NO, OY-P04 and OY-P02 EBV-B cells shown in Figure 3*b* were investigated by IFNγ ELISA for the culture supernatant to determine restriction molecules. As shown in Table 1, the frequencies of B*35:01-, B*52:01-, A*24:02-, C*12:02- and C*03:03-restricted CD8 T-cell clones were 11.0, 9.3, 2.3, 1.3 and 0.7%, respectively. The frequencies of CD8 T-cell clones determined after clonal expansion were comparable to the results shown in Figure 2.

The frequency of NY-ESO-1f peptide-reactive CD8 T-cells in PBMCs restricted to each allele was calculated assuming the doubling time of the cells to be 24 hr during the culture period for 12 days as listed in Table 1.

Determination of minimal epitopes derived from the 20-mer NY-ESO-1f peptide recognized by TK-f01 CD8 T-cell clones in restriction to A*24:02, B*35:01, B*52:01 and C*12:02

We established stably proliferating TK-f01 CD8 T-cell clones: 4E10, 2H10, 8D5, 10-10U and 7B recognizing the 20-mer NY-ESO-1f peptide in restriction to A*24:02, B*35:01, B*35:01, B*52:01 and C*12:02, respectively, from the culture shown in Table 1 and determined the minimal epitopes. Figure 3 shows the confirmation of the restriction molecule and determination of a minimal epitope in recognition of the NY-ESO-1f peptide by the CD8 T-cell clone 4E10. As shown in Figure 3*a*, the response was blocked by anti-CD3 mAb, anti-CD8 mAb and anti-HLA class I mAb, but not anti-CD4 mAb or anti-class II mAb, confirming that the response was HLA class I restricted. As shown in Figure 3*b*, use of a panel

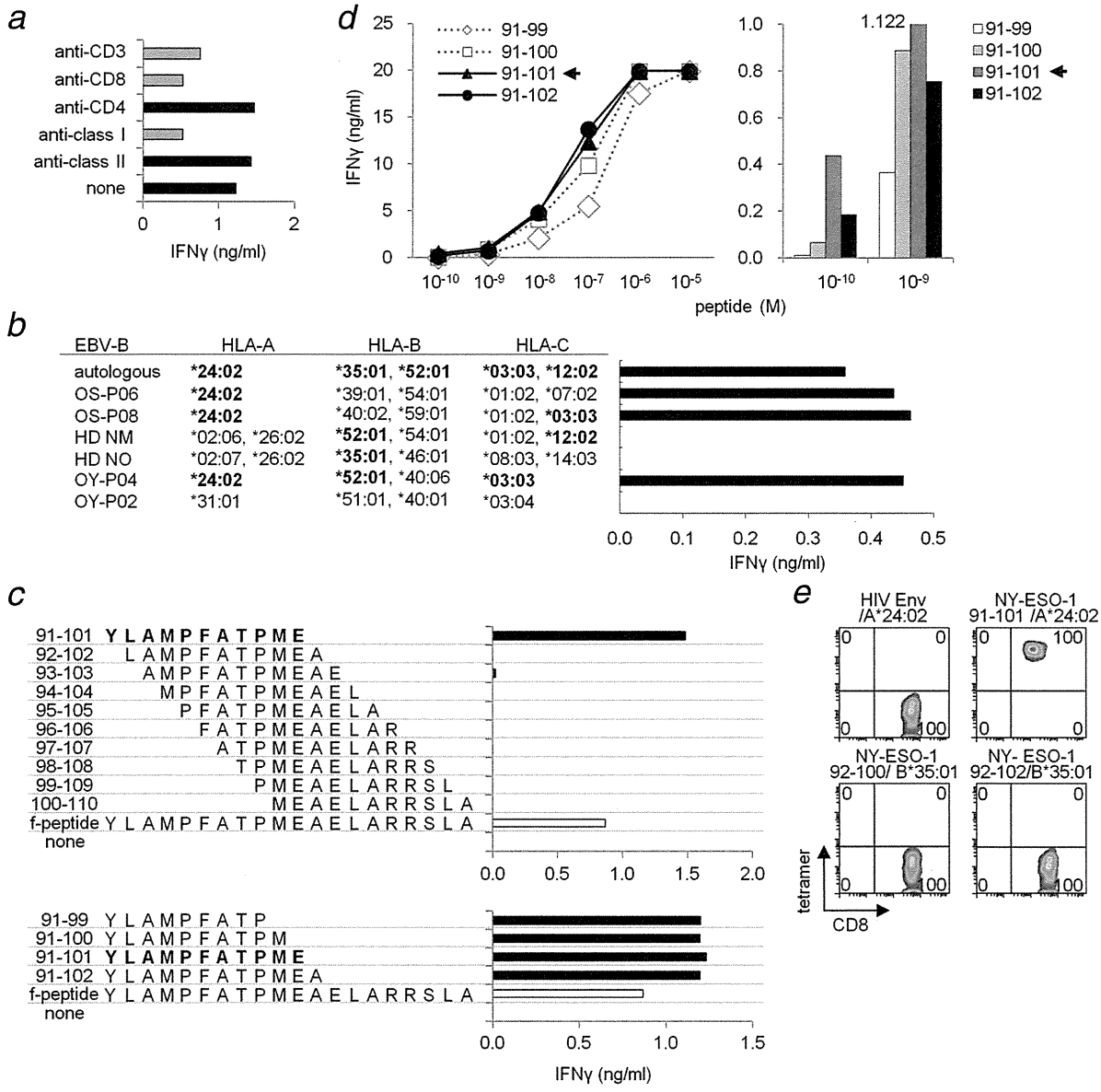


Figure 3. The restriction molecule and the minimal epitope in recognition of NY-ESO-1f peptide by the CD8 T-cell clone 4E10. A CD8 T-cell clone 4E10 (5×10^3) obtained from the culture shown in Table 1 was cultured with autologous EBV-B cells pulsed with the NY-ESO-1f-peptide (1 μM). The restriction molecule was analyzed by antibody blocking (5 μg/ml) (*a*) and using various EBV-B cells as APC (*b*). The minimal epitope was determined by using various N- and C-termini truncated peptides (1 μM) (*c*) and by titration of the peptide concentration (*d*). In (*e*), the binding of the NY-ESO-1 91-101/A*24:02 tetramer to the clone 4E10 is shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1. Clonal analysis of multiple HLA class I-restricted CD8 T cell responses against the NY-ESO-1f peptide

HLA	Positives/total number of clones	Calculated frequency of CD8 T-cells in PBMCs
A*24:02	7/300 (2.3%)	5.7×10^{-6}
B*35:01	33/300 (11.0%)	2.7×10^{-5}
B*52:01	28/300 (9.3%)	2.3×10^{-5}
C*03:03	2/300 (0.7%)	1.6×10^{-6}
C*12:02	4/300 (1.3%)	3.3×10^{-6}

The other 226 clones (75.4%) showed no reaction against NY-ESO-1f peptide on seven EBV-B cells (see Text).

of allogeneic EBV-B cells as APC to present the NY-ESO-1f peptide confirmed the response of clone 4E10 was A*24:02-restricted. As shown in Figures 3*c* and 3*d*, use of various N- and C-termini truncated peptides and titration of the peptide concentration revealed that the minimal epitope was NY-ESO-1 91-101 (11-mer). Figure 3*e* shows the binding of the tetramer constructed using the epitope peptide to the clone 4E10.

Similarly, as shown in Supporting Information Figures S1–S4, we confirmed B*35:01-, B*35:01-, B*52:01- and C*12:02-restricted recognition of the NY-ESO-1f peptide by clones 2H10, 8D5, 10-10U and 7B, respectively, determined minimal epitopes and successfully produced tetramers.

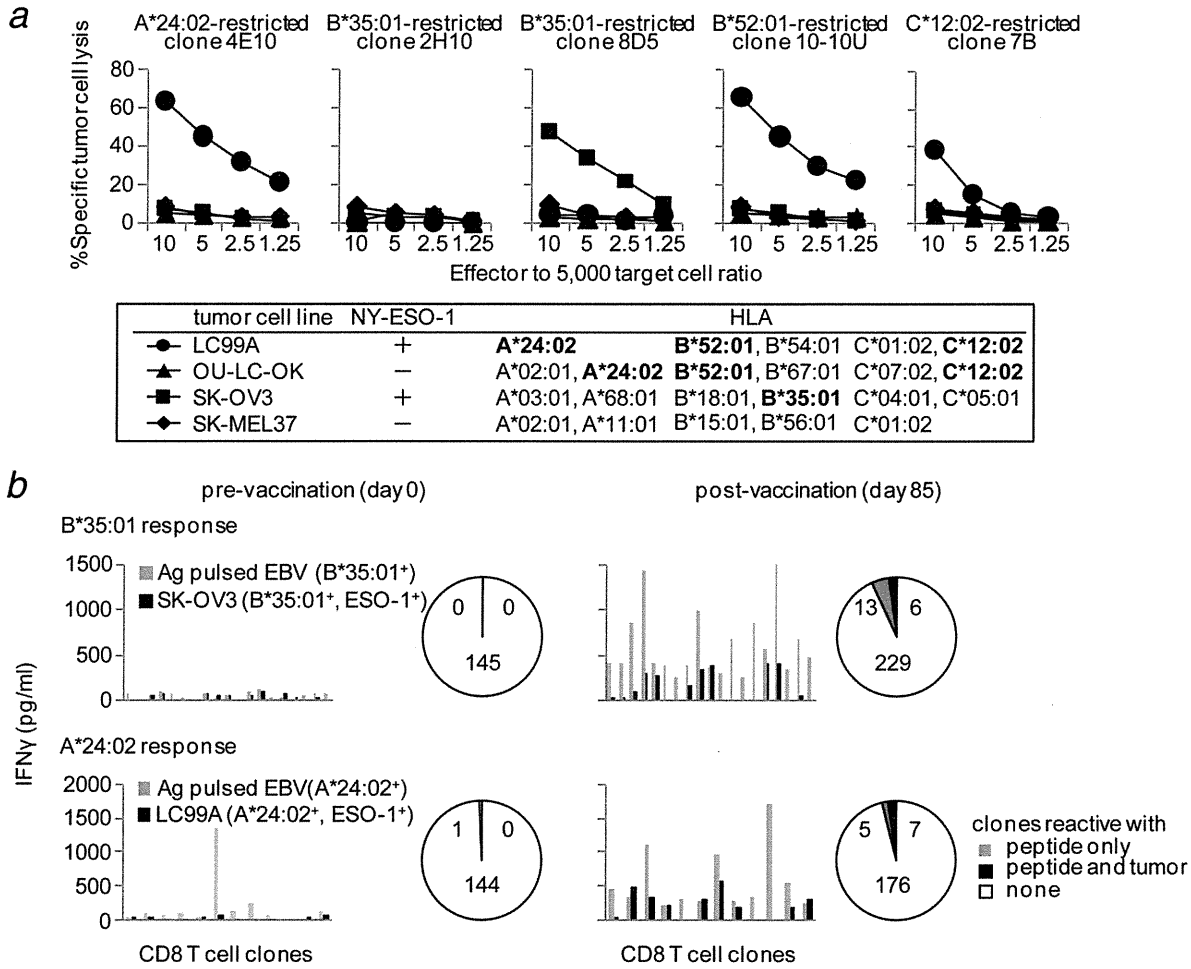


Figure 4. Clonal analysis of CD8 T-cells recognizing tumor cells induced by NY-ESO-1f peptide vaccination. In (*a*), cytotoxicity of the CD8 T-cell clones shown in Figure 3, and Supporting Information Figures S1–S4 was assayed by a luminescent method using the aCella-Tox kit. Shared HLA class I alleles in target tumors are shown in bold. In (*b*), purified CD8 T-cells (2×10^6) from TK-f01 PBMCs obtained at day 0 and at day 85 (after the fourth vaccination) were stimulated once with a mixture of NY-ESO-1 OLPs for 12 days in the presence of APC for 12 days. The cells were collected and seeded at 3 cells per well in 96-well culture plates to expand clones. After culture for 14 days, 290 clones were obtained from a culture at day 0 and 436 clones were obtained from a culture at day 85. Those clones were split into two groups and cultured with the NY-ESO-1f peptide (1 μM) pulsed-HD NO EBV-B cells or NY-ESO-1 positive SK-OV3 both sharing only B*35:01 with the patient to determine the B*35:01-restricted response and with NY-ESO-1f (1 μM) peptide pulsed-OS-P06 EBV-B cells or NY-ESO-1-positive LC99A both sharing A*24:02 with the patient to determine the A*24:02-restricted response. The amount of IFNγ in the culture supernatant was determined by ELISA.

We investigated the cytotoxicity of CD8 T-cell clones using an aCella-Tox system to examine G3PDH release from target tumor cell lines. As shown in Figure 4*a*, cytotoxicity against tumor cell lines expressing NY-ESO-1 and an appropriate HLA class I allele was observed with the A*24:02-restricted clone 4E10, B*35:01-restricted clone 8D5, B*52:01-restricted clone 10-10U and C*12:02-restricted clone 7B, but not with the B*35:01-restricted clone 2H10. With those clones, no cytotoxicity was observed against tumor cell lines which lacked either NY-ESO-1 or a matched HLA class I allele.

Frequency of CD8 T-cells recognizing tumor cells

We then investigated the frequency of CD8 T-cells recognizing tumor cells by clonal analysis. Purified CD8 T-cells from TK-f01 PBMCs obtained at day 0 and at day 85 (after the fourth vaccination) were stimulated once with a mixture of NY-ESO-1 OLPs for 12 days using CD4 and CD8-depleted PBMCs as APC as above. The cells were collected and seeded at 3 cells per well in 96-well culture plates to expand clones. After culture for 14 days, 290 clones were obtained from a culture of CD8 T-cells at day 0 and 436 clones were obtained from a culture of CD8 T-cells at day 85. Those clones were split into two groups and the B*35:01- and A*24:02-restricted responses were investigated. As shown in Figure 4*b*, in CD8 T-cells obtained at day 0, no B*35:01-restricted clone reactive against NY-ESO-1f peptide was obtained. There was only one A*24:02-restricted clone reactive against the NY-ESO-1f peptide (1/145). On the other hand, in CD8 T-cells obtained at

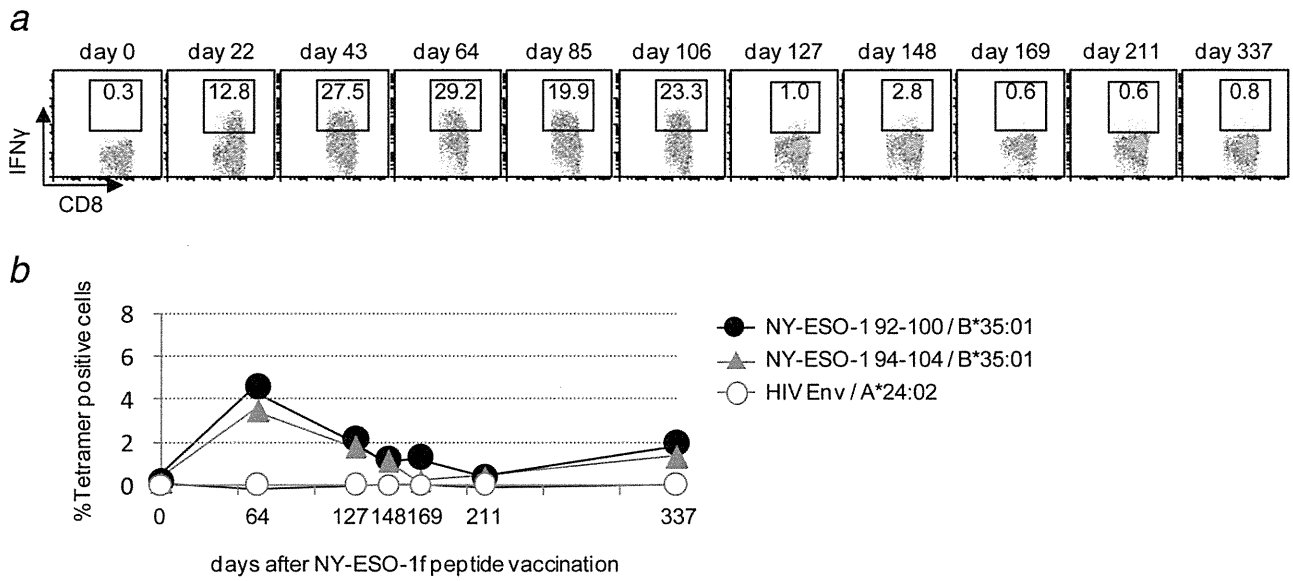


Figure 5. Immunomonitoring of the CD8 T-cell response by IFNγ capture assay and by peptide/HLA class I tetramers in PBMCs from TK-f01 during vaccination. CD8 T-cells (2×10^6) were cultured with a mixture of NY-ESO-1 OLPs (1 μM) in the presence of APC for 12 days. In (*a*), the response was assayed by an IFNγ capture assay. In (*b*), staining by PE-labeled NY-ESO-1 92-100/B*35:01 and NY-ESO-1 94-104/B*35:01 tetramers is shown. An HIV Env/A*24:02 tetramer was used as control.

day 85, the number of B*35:01-restricted clones reactive against the NY-ESO-1f peptide was 19 (7.7%), and 6 of 19 (31.6%) clones were also reactive against the NY-ESO-1-positive and B*35:01-positive tumor SK-OV3. The number of A*24:02-restricted clones reactive against the NY-ESO-1f peptide was 12 (6.4%), and 7 of 12 (58.3%) clones were also reactive against the NY-ESO-1-positive and A*24:02-positive tumor LC99A. Recognition of tumor cells was also confirmed by an aCella-Tox system (data not shown).

Immunomonitoring of CD8 T-cell responses by IFNγ capture assay and by peptide/HLA class I tetramers in PBMCs from TK-f01 during vaccination

After initiating the vaccine, the tumor remained stable for 6 months and was classed as SD at the end of the sixth vaccination in this patient.[20] However, the tumor started to grow after the eighth vaccination, consistent with an accelerated elevation in the serum CEA level.

Figure 5*a* shows the results of the IFNγ capture assay demonstrating the CD8 T-cell response against NY-ESO-1f peptide after stimulation with a mixture of NY-ESO-1 OLPs for 12 days. A vigorous CD8 T-cell response was observed even after the first vaccination (day 22), continuing until after the fifth vaccination (day 106). Thereafter, the response decreased suddenly. As shown in Figure 5*b*, the number of CD8 T-cells in the same culture positively stained with NY-ESO-1 92-100 and NY-ESO-1 94-104/B*35:01 tetramers was parallel with that of CD8 T-cells in an IFNγ capture assay. The findings indicated that CD8 T-cell responses against the peptides on B*35:01 comprised the dominant response, consistent with the clonal analysis shown in Table 1.

Discussion

In this study, we showed that internalization of the peptide was necessary for presenting CD8 T-cell epitopes derived from the long 20-mer NY-ESO-1f peptide on APC. Labeled NY-ESO-1f peptide was detected in approximately 10% of DCs after incubation with the peptide for 12 hr. No CD8 T-cell recognition of NY-ESO-1f peptide-pulsed DCs was observed after blocking internalization with cytochalasin B. Thus, the CD8 T-cell epitope was presented following internalization with a long 20-mer NY-ESO-1f peptide, contrasting with the direct presentation of the short epitope. Recognition by CD8 T-cells of NY-ESO-1f peptide-pulsed DCs, but not the recombinant NY-ESO-1 protein-pulsed DCs, indicated the occurrence of efficient cross presentation on MHC class I molecules after internalization of the 20-mer NY-ESO-1f peptide. Cross presentation of the long peptides on MHC class I molecules after internalization was previously shown with a peptide derived from HPV.[23] Processing of the peptide in APC may facilitate the presentation of natural epitopes. This study showed a high frequency of CD8 T-cells recognizing natural epitopes on tumor cells (see below). It has been shown previously that immunization with the short peptide induced CD8 T-cells with low affinity incapable of recognizing natural epitopes on tumor cells.[16] Use of the long peptide for immunization is beneficial to induce CD8 T-cells that recognize tumor cells.

We showed multiple HLA class I-restricted CD8 T-cell responses against a long 20-mer NY-ESO-1f peptide in the lung cancer patient TK-f01, who was immunized with the peptide with Picibanil OK-432 and Montanide ISA-51. Clonal analysis showed that the frequencies of A*24:02, B*35:01,

B*52:01, C*03:03 and C*12:02-restricted CD8 T-cells were 2.3, 11.0, 9.3, 0.7 and 1.3%, respectively, in the bulk CD8 T-cell population stimulated with NY-ESO-1 OLPs, indicating that B*35:01 and B*52:01-restricted CD8 T-cell responses were the two dominant CD8 T-cell responses. The minimal epitopes recognized by A*24:02, B*35:01, B*52:01 and C*12:02, but not C*03:03-restricted CD8 T-cell clones, were defined and the peptide/HLA tetramers were produced using those epitopes. NY-ESO-1 91-101 (11-mer) on A*24:02, NY-ESO-1 92-102 (11-mer) on B*35:01, NY-ESO-1 96-104 (9-mer) on B*52:01 and NY-ESO-1 96-106 (9-mer) on C*12:02 were new epitopes first defined in this study. Particularly, identification of this A*24:02 binding epitope and successful production of a tetramer with the epitope will be highly relevant when studying Japanese populations because of its high expression frequency (60%). Interestingly, within 25 HLA class I epitopes in the NY-ESO-1 molecule defined to date,[24–26] 15 epitopes including the epitopes shown in this study clustered in the peptide region NY-ESO-1 89-104. These findings suggested the usefulness of a long 20-mer NY-ESO-1f peptide harboring multiple CD8 T-cell epitopes for an NY-ESO-1 vaccine and this study indeed showed that vaccination with a long 20-mer NY-ESO-1f peptide elicited multiple HLA class I-restricted CD8 T-cell responses against the respective epitopes.

We also estimated the frequency of B*35:01- and A*24:02-restricted CD8 T-cells recognizing only the peptide-pulsed DCs, or both the peptide-pulsed DCs and the tumor cells naturally expressing the epitope and the matched HLA class I. At day 0, no B*35:01-restricted CD8 T-cell clones were detected from 145 clones obtained from 960 wells in total of the bulk CD8 T-cells stimulated with NY-ESO-1 OLPs for 12 days. Only a single A*24:02-restricted CD8 T-cell clone reactive against the peptide, but not the tumor, was obtained from the same number of clones. On the other hand, at day 85 after the fourth vaccination, 19 B*35:01-restricted and 12 A*24:02-restricted CD8 T-cell clones reactive against the peptide were detected from 248 and 188 clones, respectively, obtained from 960 wells for each of the bulk CD8 T-cells stimulated as above. 31.6% (6 of 19) of B*35:01-restricted and 58.3% (7 of 12) of A*24:02-restricted CD8 T-cell clones were also reactive against the tumors naturally expressing the epitope and the matched HLA class I. Thus, a significant frequency of CD8 T-cells recognizing tumor cells naturally expressing the epitopes and matched HLA was induced. This was likely to be related to the internalization and processing of the peptide in APC.

It has been reported that vaccination with NY-ESO-1 protein with CpG and Montanide elicited detectable CD8 T-cell responses in half of the immunized patients (9/18) and that vaccine-induced CD8 T-cells mostly recognized NY-ESO-1 81-110 in restriction to either HLA-B35 or HLA-Cw3.[27] B35 binding peptide epitopes 94-102 and 94-104 and Cw3 binding peptide epitopes 92-100 and 96-104 have been described.[25,28] In our previous study, we showed that an NY-ESO-1f peptide (NY-ESO-1 91-110) vaccine elicited a response to peptide 16 (NY-ESO-1 91-108) in all six patients analyzed with or without B35 and/or Cw3. The fact that the NY-ESO-1f peptide vaccine elicited CD8 T cell responses in patients with various HLA types suggests the advantage of a long peptide over the whole protein for vaccination.

Patient TK-f01 showed stable disease until the eighth vaccination and the disease exacerbated thereafter. In immunomonitoring CD8 T-cell responses following 20-mer NY-ESO-1f peptide vaccination, we observed vigorous CD8 T-cell responses, even after the first vaccination (day 22), lasting until the fifth vaccination (day 106) by IFNγ capture assay with a decrease thereafter, consistent with the clinical course. Characterization of CD8 T-cell responses using the various peptide/HLA tetramers showed that vigorous CD8 T-cell responses against the peptides restricted to B*35:01 were observed at day 64 after the third vaccination. These findings suggested that the multiple B*35:01-restricted CD8 T-cell responses comprised the dominant response.

Because Th cells help CTL induction, introduction of a Th epitope into the vaccine or physical linking of Th and CTL epitope peptides facilitated an increase in the immunogenicity of CTL vaccines.[29,30] The synthetic long peptide containing overlapping CD4 and CD8 T cell epitope sequences in the antigens is expected to generate both CD4 and CD8 T cell responses as a vaccine. In our study, the NY-ESO-1f peptide was shown to contain multiple HLA class II epitopes, as well as class I epitopes,[20] and is considered to be beneficial to elicit CD8 T-cell responses efficiently.

Acknowledgements

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References

1. Chen YT, Scanlan MJ, Sahin U, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A* 1997;94:1914–8.
2. Gnjatic S, Nishikawa H, Jungbluth AA, et al. NY-ESO-1: review of an immunogenic tumor antigen. *Adv Cancer Res* 2006;95:1–30.
3. Jungbluth AA, Chen YT, Stockert E, et al. Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. *Int J Cancer* 2001;92:856–60.
4. Scanlan MJ, Gure AO, Jungbluth AA, et al. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunol Rev* 2002;188:22–32.
5. Simpson AJ, Caballero OL, Jungbluth A, et al. Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 2005;5:615–25.
6. Jager E, Gnjatic S, Nagata Y, et al. 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.
7. Gnjatic S, Jager E, Chen W, et al. CD8(+) T cell responses against a dominant cryptic HLA-A2 epitope after NY-ESO-1 peptide immunization of cancer patients. *Proc Natl Acad Sci U S A* 2002;99:11813–8.
8. Valmori D, Dutoit V, Ayyoub M, et al. Simultaneous CD8+ T cell responses to multiple tumor antigen epitopes in a multipeptide melanoma vaccine. *Cancer Immun* 2003;3:15.

9. Shackleton M, Davis ID, Hopkins W, et al. The impact of imiquimod, a Toll-like receptor-7 ligand (TLR7L), on the immunogenicity of melanoma peptide vaccination with adjuvant Flt3 ligand. *Cancer Immun* 2004;4:9.
10. Davis ID, Chen W, Jackson H, et al. Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans. *Proc Natl Acad Sci U S A* 2004;101:10697–702.
11. Valmori D, Souleimanian NE, Tosello V, et al. Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proc Natl Acad Sci U S A* 2007;104: 8947–52.
12. Jager E, Karbach J, Gnjatic S, et al. Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients. *Proc Natl Acad Sci U S A* 2006;103:14453–8.
13. Kawabata R, Wada H, Isobe M, et al. Antibody response against NY-ESO-1 in CHP-NY-ESO-1 vaccinated patients. *Int J Cancer* 2007;120: 2178–84.
14. Uenaka A, Wada H, Isobe M, et al. T cell immunomonitoring and tumor responses in patients immunized with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein. *Cancer Immun* 2007;7:9.
15. Chen Q, Jackson H, Parente P, et al. Immunodominant CD4+ responses identified in a patient vaccinated with full-length NY-ESO-1 formulated with ISCOMATRIX adjuvant. *Proc Natl Acad Sci U S A* 2004;101: 9363–8.
16. Le Gal FA, Ayyoub M, Dutoit V, et al. Distinct structural TCR repertoires in naturally occurring versus vaccine-induced CD8+ T-cell responses to the tumor-specific antigen NY-ESO-1. *J Immunother* 2005;28:252–7.
17. Melief CJ, van der Burg SH. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. *Nat Rev Cancer* 2008;8: 351–60.
18. Bijker MS, van den Eeden SJ, Franken KL, et al. CD8+ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. *J Immunol* 2007;179: 5033–40.
19. van der Burg SH, Bijker MS, Welters MJ, et al. Improved peptide vaccine strategies, creating synthetic artificial infections to maximize immune efficacy. *Adv Drug Deliv Rev* 2006;58: 916–30.
20. Kakimi K, Isobe M, Uenaka A, et al. A phase I study of vaccination with NY-ESO-1f peptide mixed with Picibanil OK-432 and Montanide ISA-51 in patients with cancers expressing the NY-ESO-1 antigen. *Int J Cancer* 2011;129:2836–46.
21. Altman JD, Moss PA, Goulder PJ, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996;274:94–6.
22. Morishima S, Akatsuka Y, Nawa A, et al. Identification of an HLA-A24-restricted cytotoxic T lymphocyte epitope from human papillomavirus type-16 E6: the combined effects of bortezomib and interferon-gamma on the presentation of a cryptic epitope. *Int J Cancer* 2007;120:594–604.
23. Zwaveling S, Ferreira Mota SC, Nouta J, et al. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J Immunol* 2002;169:350–8.
24. Jager E, Chen YT, Drijfhout JW, et al. 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.
25. Gnjatic S, Nagata Y, Jager E, et al. 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.
26. Jager E, Karbach J, Gnjatic S, et al. Identification of a naturally processed NY-ESO-1 peptide recognized by CD8+ T cells in the context of HLA-B51. *Cancer Immun* 2002; 2:12.
27. Bioley G, Guillaume P, Luescher I, et al. HLA class I – associated immunodominance affects CTL responsiveness to an ESO recombinant protein tumor antigen vaccine. *Clin Cancer Res* 2009;15:299–306.
28. Jackson H, Dimopoulos N, Mifsud NA, et al. Striking immunodominance hierarchy of naturally occurring CD8+ and CD4+ T cell responses to tumor antigen NY-ESO-1. *J Immunol* 2006;176:5908–17.
29. Ossendorp F, Mengede E, Camps M, et al. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J Exp Med* 1998;187: 693–702.
30. Shirai M, Pendleton CD, Ahlers J, et al. Helper-cytotoxic T lymphocyte (CTL) determinant linkage required for priming of anti-HIV CD8+ CTL in vivo with peptide vaccine constructs. *J Immunol* 1994;152:549–56.

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Case Report

Stevens–Johnson Syndrome associated with mogamulizumab treatment of adult T-cell leukemia/lymphoma

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We report an adult T-cell leukemia/lymphoma patient suffering from Stevens–Johnson Syndrome (SJS) during mogamulizumab (humanized anti-CCR4 monoclonal antibody) treatment. There was a durable significant reduction of the $CD4^+CD25^{high}FOXP3^+$ regulatory T (Treg) cell subset in the patient's PBMC, and the affected inflamed skin almost completely lacked FOXP3-positive cells. This implies an association between reduction of the Treg subset by mogamulizimab and occurrence of SJS. The present case should contribute not only to our understanding of human pathology resulting from therapeutic depletion of Treg cells, but also alert us to the possibility of immune-related severe adverse events such as SJS when using mogamulizumab. We are currently conducting a clinical trial of mogamulizumab for CCR4-negative solid cancers (UMIN000010050), specifically aiming to deplete Treg cells. (*Cancer Sci* 2013; 104: 647–650)

Adult T-cell leukemia/lymphoma (ATL) is an aggressive peripheral T-cell neoplasm caused by HTLV-1. The disease is resistant to conventional chemotherapeutic agents, and has a very poor prognosis.[1] Mogamulizumab (KW-0761) is a defucosylated humanized monoclonal antibody targeting CC chemokine receptor 4 (CCR4).[2] A phase I clinical trial for relapsed CCR4-positive peripheral T-cell neoplasms, including ATL, and a phase II study for relapsed ATL have been conducted with mogamulizumab.[3,4] This agent was subsequently approved for the treatment of relapsed or refractory ATL in Japan, the first country in the world to do so, in March 2012. Mogamulizimab went on sale on 29 May 2012. The interim report for the post-marketing surveillance from 29 May to 28 September 2012 revealed skin-related severe adverse events (SAE), as defined by the Medical Dictionary for Regulatory Activities Terminology/Japan, in nine patients. Thus, during only the first 4 months of use, 9 skin-related SAE, including 4 cases of Stevens–Johnson Syndrome (SJS)/toxic epidermal necrolysis (TEN) were reported, with 1 SJS/TEN fatality. These skin-related, potentially fatal SAE are certainly a challenge to the free use of this agent and clearly require investigation. Therefore, here we report an informative ATL patient suffering from SJS on mogamulizumab treatment, focusing on the reduction of the regulatory T (Treg) cell subset ($CD4^+CD25^{high}FOXP3^+$) caused by the antibody.

Case Report

A 71-year old woman was admitted due to elevation of her lymphocyte count. She had been diagnosed as suffering from acute-type ATL nearly 5 months prior to admission. She had received VCAP-AMP-VECP chemotherapy[5] followed by oral sobuzoxane in another hospital, and achieved a transient partial remission. We started mogamulizumab to treat the flare-up of ATL disease (Fig. 1). Grade 1 skin eruptions appeared around her neck after three antibody infusions. Because we were also giving her antibacterial (ciprofloxacin hydrochloride), fungal (itraconazole), pneumocystic (sulfamethoxazole-trimethoprim) and viral (aciclovir) prophylaxes in addition to stomach medicine (lansoprazole), we judged the skin event to be due to drug eruption caused by one of these concomitant drugs. Therefore, we stopped all five, but continued with mogamulizumab. Despite their discontinuation and treatment with topical steroids, the skin rashes continued to worsen. We started the patient on 30 mg oral prednisolone, which improved the skin symptoms. The patient was then able to complete the eight planned infusions, and oral prednisolone was tapered off. She was discharged from hospital 8 days after her eighth infusion (day 65), and thereafter seen as an outpatient. However, she had to be readmitted as an emergency patient at day 75 because of fulminant skin rashes. These included erythemas, scale-like plaques, vesicles, blisters and erosions over many areas of the body. Her lips were swollen and oral mucosa was erosive (Fig. 2a). Skin biopsy revealed marked liquefaction, degeneration and perivascular inflammation with dominant CD8-positive cells but almost complete lack of FOXP3-positive cells (Fig. 2b). We diagnosed her as a SJS, and immediately started steroid pulse therapy (methylprednisolone 500 mg/day ×3 days), followed by oral prednisolone. Her skin and mucosal lesions improved gradually, and became inactive. At the same time, her general condition improved. Thus, we again tapered the steroid dose, and she was discharged at day 144. However, she had to come back yet again as an emergency patient on day 151 for the same reason as before, with fulminant skin rashes. We prescribed her mini-steroid pulse therapy (methylprednisolone 125 mg/day ×1 day), followed by oral prednisolone. Once more, her skin lesions improved gradually. Over this whole period, complete ATL remission was maintained by mogamulizumab. The HTLV-1 provirus load in PBMC pre-treatment, and at days 121 and 162 was 750.1, 0.0 (under the limit of detection) and 0.8 copies/1000 cells, respectively. These post-treatment values are strikingly low, considering that median HTLV-1

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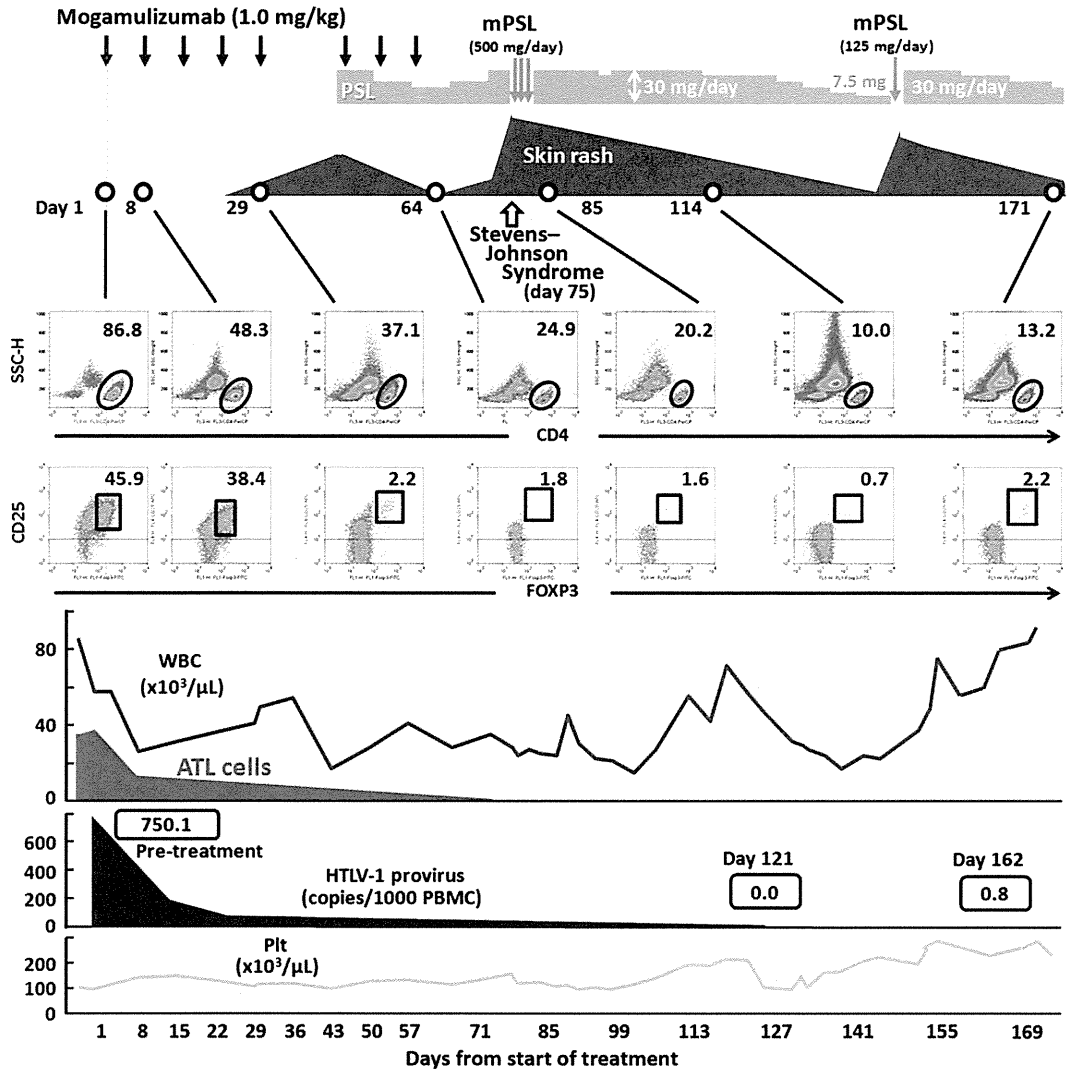

Fig. 1. Clinical course of an ATL patient receiving mogamulizumab monotherapy. ATL; adult T-cell leukemia/lymphoma; mPSL, methylprednisolone; Plt, platelet PSL; prednisolone; WBC, white blood cell.

load in asymptomatic carriers reported by other investigators is 18.0 copies/1000 cells.[6]

We also analyzed CD4, CD25 and FOXP3 expression by PBMC during and after antibody treatment (Fig. 1, middle panels). Before treatment, the majority of the patient's PBMC consisted of CD4-positive and CD25-positive ATL cells. Just before the 5th antibody infusion (day 29), around the time when her skin rash first appeared, the proportion of $CD25^{high}$-$FOXP3^{+}/CD4^{+}$ cells was markedly reduced, to 2.2%. This is low even compared to healthy individuals ($CD25^{high}$-$FOXP3^{+}/CD4^{+}$ cells, mean 3.3%, median 3.3%, range 2.6–4.4%) (Fig. 3). Around the time of SJS onset, the proportion of cells in the Treg subset was further reduced. The proportion of $CD25^{high}FOXP3^{+}/CD4^{+}$ cells at days 64, 85 and 114 was 1.8%, 1.6% and 0.7%, respectively. The striking reduction of the Treg subset persisted until 4 months after the last of the eight antibody infusions (day 171).

Discussion

Drugs often induce adverse cutaneous reactions of varying severity, ranging from simple uncomplicated eruptions to potentially fatal eruptions, such as SJS and TEN, within the spectrum of severe adverse reactions affecting skin and mucosa. Although many factors that might cause variability in the clinical course of such adverse reactions have been suggested, it remains unknown which factors are predominantly involved in these processes. The most prevalent severe drug eruptions are thought to be mediated by drug-reactive T-cells,[7] although we also need to be aware of the alternative view that severe drug eruptions are due to a dysregulated immune system. In this regard, an effect mediated by Treg cells is a likely candidate in severe drug eruptions. Indeed, it is reported that Treg cells can prevent experimentally-induced epidermal injury mimicking TEN in an animal model.[8] Furthermore, Takahashi *et al.* (2009) report that Treg cell function is profoundly impaired in patients with TEN.[9] Consistent with these reports, a marked reduction of the Treg subset was observed in the present case.

Mogamulizumab is the first therapeutic agent targeting CCR4, which is expressed on Treg cells,[10,11] to receive marketing approval anywhere in the world. The reduction of the Treg subset seen here was not specific to the present case, but is commonly observed in ATL patients receiving mogamulizumab. In fact, skin rashes were observed as a frequent non-hematologic adverse event (AE) (63%), mostly occurring

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