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-YLAMPFATPMEAE-LARRSLA) 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 *SphI/SalI* and *BamHI/SphI* 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.
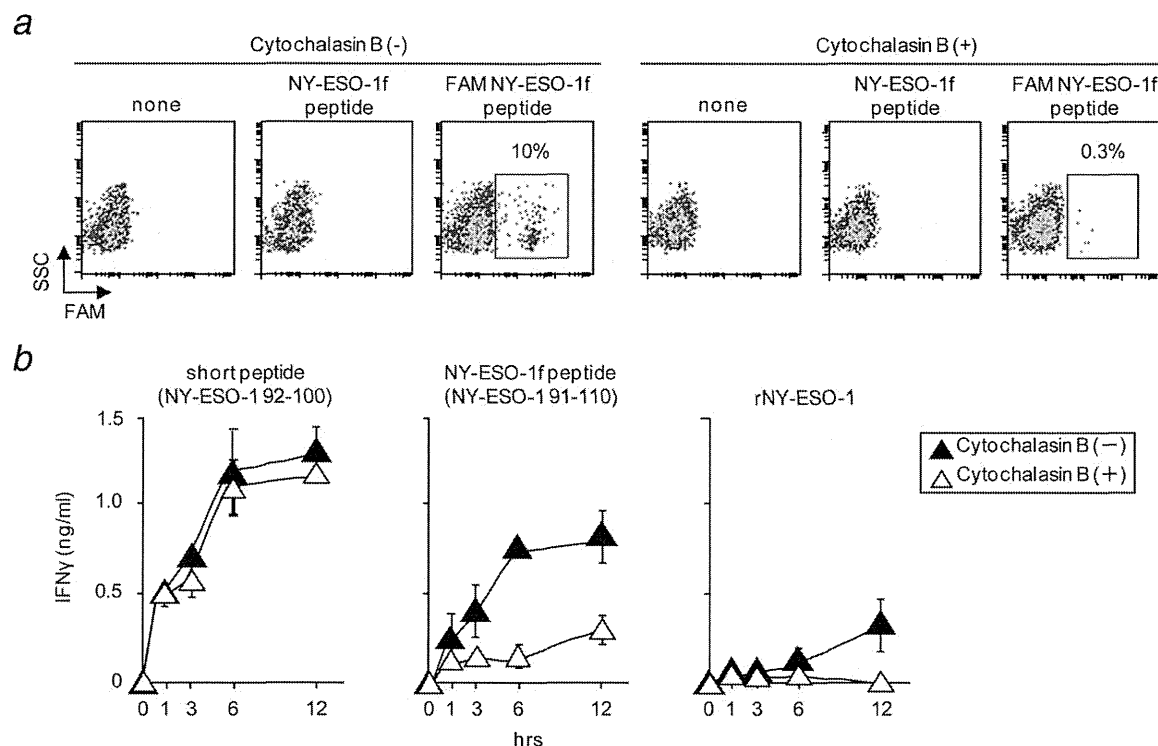
Figure 1. Internalization and processing of the long 20-mer NY-ESO-1f-peptide by APC in CD8 T-cell recognition. In (a), immature dendritic cells (iDCs) from a healthy donor (HD) NO PBMCs (A*02:07/*26:02, B*35:01/*46:01, C*08:03/*14:03) were cultured with an NY-ESO-1f peptide (10 μM) or a FAM NY-ESO-1f 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 × 10³) was stimulated with iDCs (5 × 10³) 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: YLAMPFATPMEAE-LARRSLA) 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 1a, 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 1b, 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.
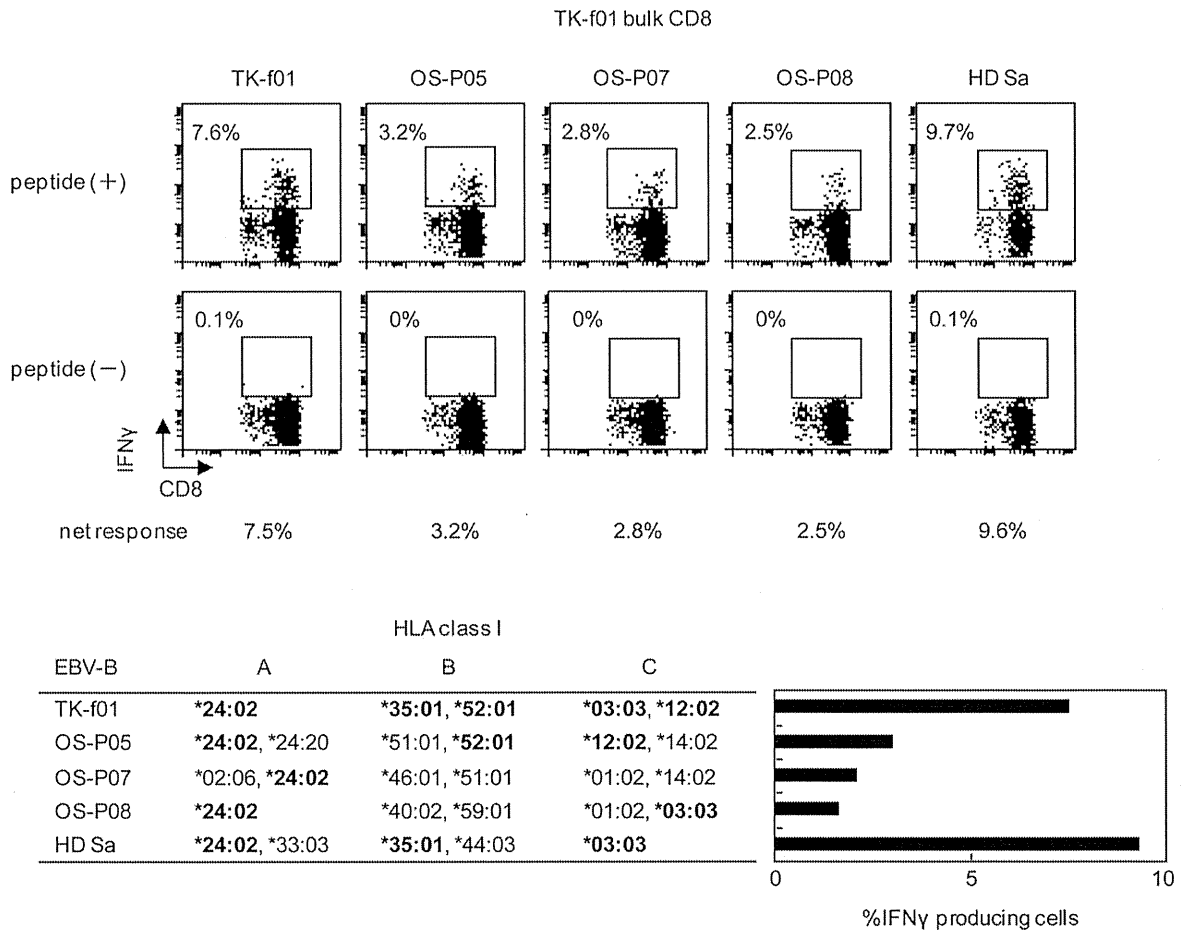
TK-f01 bulk CD8

	TK-f01	OS-P05	OS-P07	OS-P08	HD Sa
peptide(+)	7.6%	3.2%	2.8%	2.5%	9.7%
peptide(−)	0.1%	0%	0%	0%	0.1%
net response	7.5%	3.2%	2.8%	2.5%	9.6%

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

0   5   10

%IFNγ producing cells

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 3b 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 3a, 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 3b, use of a panel
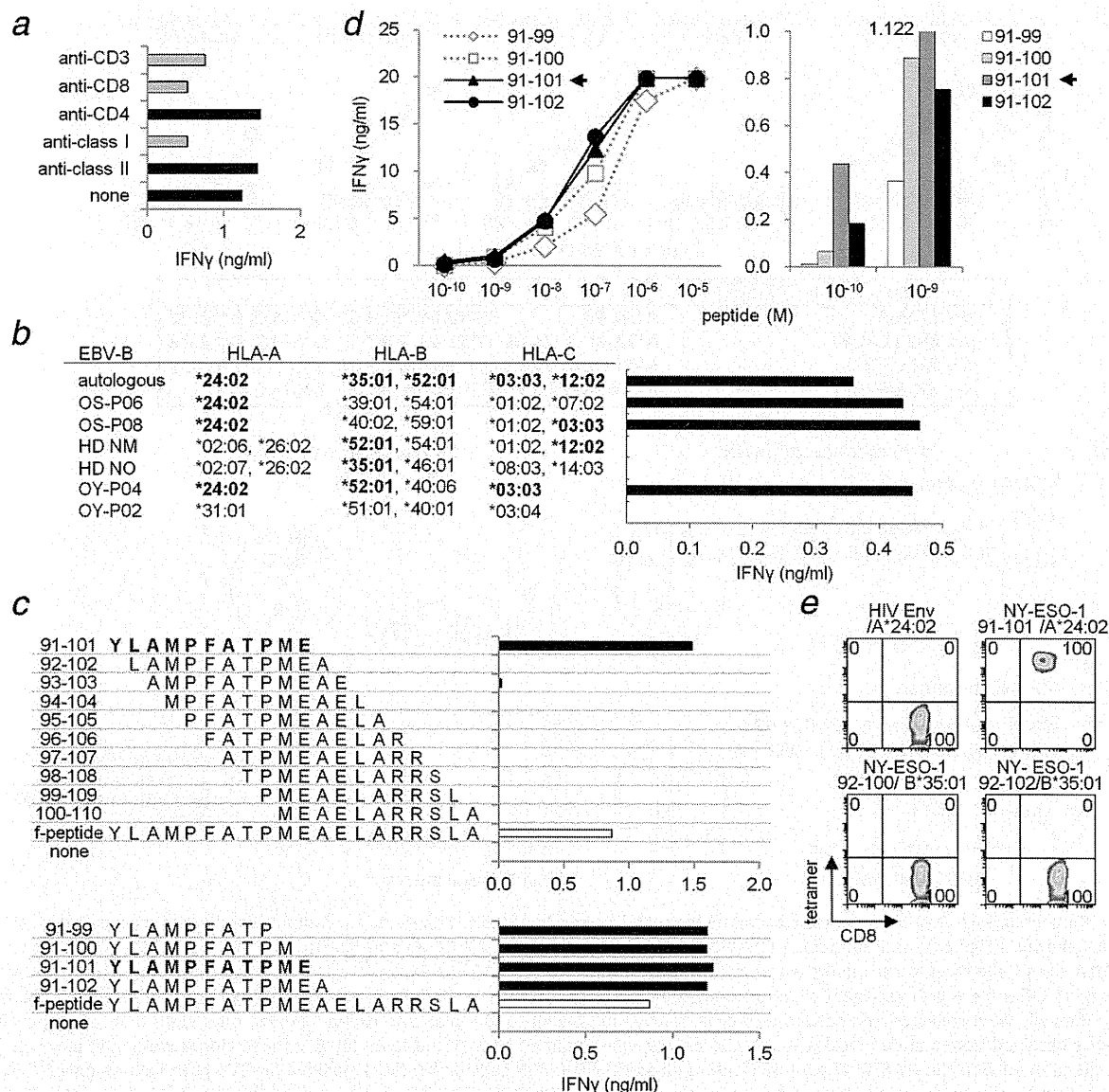
Figure 3. The restriction molecule and the minimal epitope in recognition of NY-ESO-1f peptide by the CD8 T-cell clone 4E10. A CD8 T-cell clone 4E10 (5×10^3) obtained from the culture shown in Table 1 was cultured with autologous EBV-B cells pulsed with the NY-ESO-1f-peptide (1 μM). The restriction molecule 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 3c and 3d, 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 3e 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.
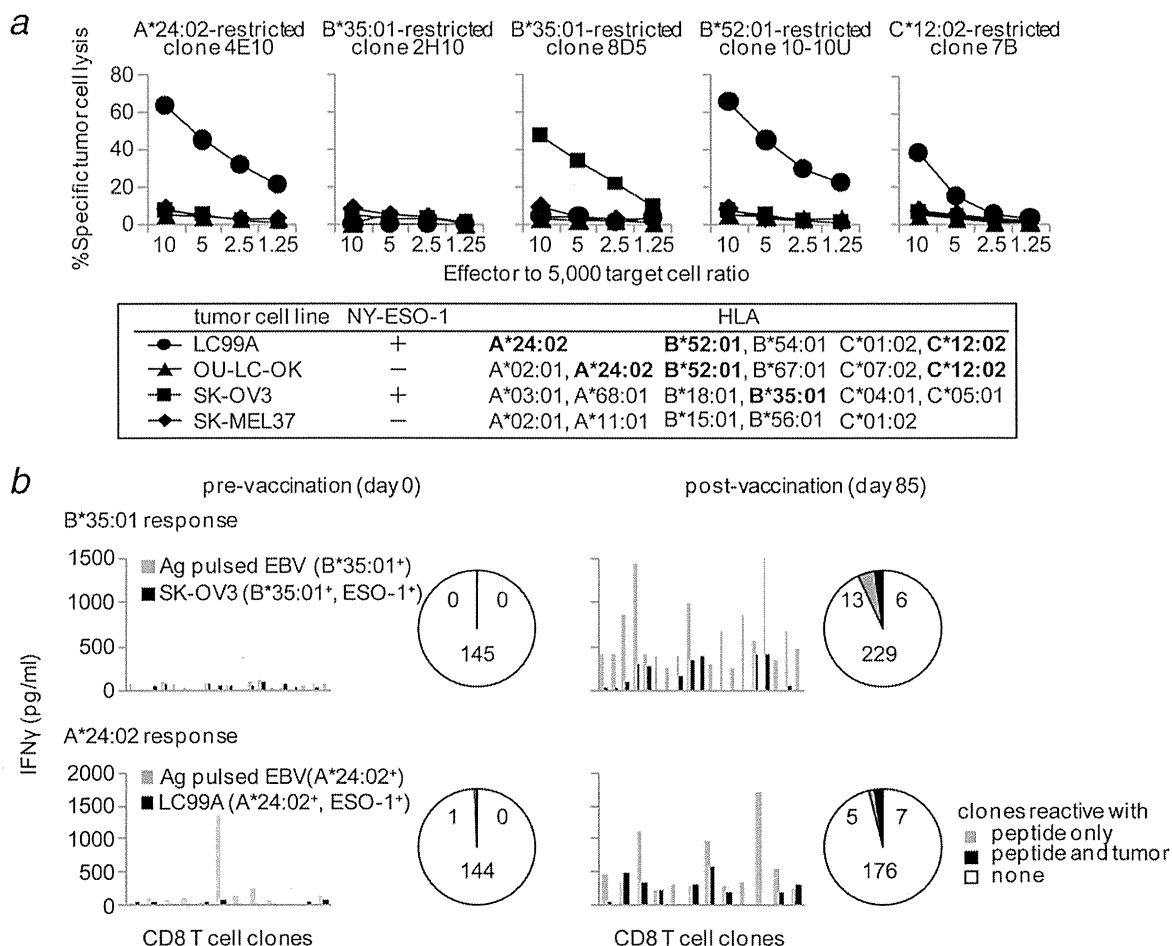
Figure 4. Clonal analysis of CD8 T-cells recognizing tumor cells induced by NY-ESO-1f peptide vaccination. In (*a*), cytotoxicity of the CD8 T-cell clones shown in Figure 3, and Supporting Information Figures S1–S4 was assayed by a luminescent method using the aCella-Tox kit. Shared HLA class I alleles in target tumors are shown in bold. In (*b*), purified CD8 T-cells (2 × 10⁶) 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
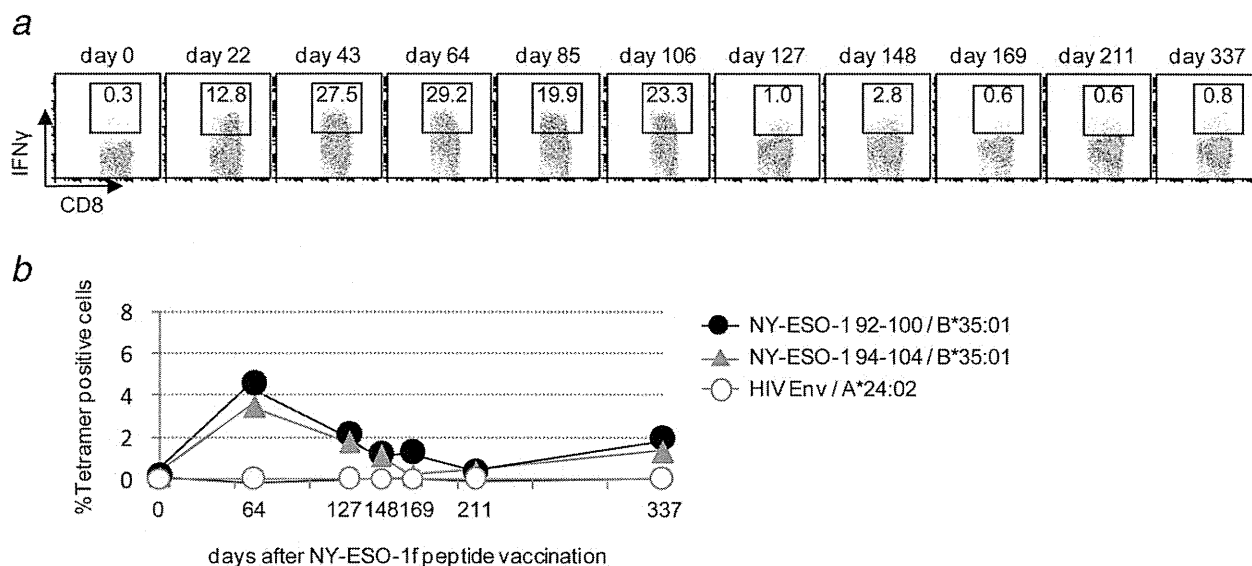
Figure 5. Immunomonitoring of the CD8 T-cell response by IFNγ capture assay and by peptide/HLA class I tetramers in PBMCs from TK-f01 during vaccination. CD8 T-cells (2 × 10^{6}) were cultured with a mixture of NY-ESO-1 OLPs (1 μM) in the presence of APC for 12 days. In (a), the response was assayed 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 5a 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 5b, 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

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.

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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.

NY-ESO-1 antibody as a novel tumour marker of gastric cancer

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Background: NY-ESO-1 antibodies are specifically observed in patients with NY-ESO-1-expressing tumours. We analysed whether the NY-ESO-1 humoral immune response is a useful tumour marker of gastric cancer.

Methods: Sera from 363 gastric cancer patients were screened by enzyme-linked immunosorbent assay (ELISA) to detect NY-ESO-1 antibodies. Serial serum samples were obtained from 25 NY-ESO-1 antibody-positive patients, including 16 patients with curative resection and 9 patients who received chemotherapy alone.

Results: NY-ESO-1 antibodies were detected in 3.4% of stage I, 4.4% of stage II, 25.3% of stage III, and 20.0% of stage IV patients. The frequency of antibody positivity increased with disease progression. When the NY-ESO-1 antibody was used in combination with carcinoembryonic antigen and CA19-9 to detect gastric cancer, information gains of 11.2% in stages III and IV, and 5.8% in all patients were observed. The NY-ESO-1 immune response levels of the patients without recurrence fell below the cutoff level after surgery. Two of the patients with recurrence displayed incomplete decreases. The nine patients who received chemotherapy alone continued to display NY-ESO-1 immune responses.

Conclusion: When combined with conventional tumour markers, the NY-ESO-1 humoral immune response could be a useful tumour marker for detecting advanced gastric cancer and inferring the post-treatment tumour load in seropositive patients.

Gastric cancer is the second most common cause of cancer-related death worldwide (Health and Welfare Statistics Association: Tokyo, 2006; Katanoda and Yako-Suketomo, 2009). Although complete removal of the tumour by surgical resection is an ideal treatment option for patients with gastric cancer, many patients with advanced-stage gastric cancer need to be treated with intensive chemotherapy. Gastric cancer patients exhibit high relapse rates even after curative surgery and unresponsiveness to chemotherapy, resulting in dismal survival rates (Sasako et al, 2011). Several methods for the prediction and early detection of

subclinical 'minimal residual cancer' after surgery (Austrup et al, 2000; Klein et al, 2002) or relapse have been developed, for example, peritoneal lavage, positron emission tomography, gene profiling, and so on. (Motoori et al, 2006; Makino et al, 2010; Graziosi et al, 2011), reliable markers that can specifically reflect gastric cancer disease status have not been determined.

Analysing serum level of tumour markers is employed for cancer detection, monitoring patients' disease status, and prognosis prediction. Several organ-specific tumour markers are used in the clinic, for example, prostate-specific antigen and prostatic acid

phosphatase for prostate cancer (Seamonds et al, 1986; Ferro et al, 1987) and protein induced by vitamin K absence-II for liver cancer (Fujiyama et al, 1986). As no gastric cancer-specific markers have been determined, a combination of several nonspecific tumour markers, for example, carcinoembryonic antigen (CEA), CA19-9,and so on, is merely applicable for monitoring treatment efficacy, but not the diagnosis of gastric cancer (Takahashi et al, 1995, 2003). Carcinoembryonic antigen and CA19-9 are found in the sera of 20–60% of gastric cancer patients, and their expression levels in gastric cancer are related to clinical events, such as relapse (Kodera et al, 1996). Carcinoembryonic antigen value, in particular, is indicative of the formation of a large tumour, liver or peritoneal metastasis, and/or a high risk of relapse and poor prognosis (Ikeda et al, 1993; Yamamoto et al, 2004). However, as CEA, a cell surface-anchored glycoprotein, is expressed in normal cell membranes, 5% of CEA-positive cases are pseudopositives, that is, caused by heavy smoking, endometriosis, and ageing, and so on. (Alexander et al, 1976), suggesting the importance of novel markers for gastric cancer.

NY-ESO-1 antigen, a cancer/testis (CT) antigen, was originally identified in oesophageal cancer by serological expression cloning using autologous patient serum and has been shown to be strongly immunogenic. Spontaneous NY-ESO-1 antibody production is often observed in patients with NY-ESO-1-expressing tumours, for example, 9.4% of melanoma patients, 12.5% of ovarian cancer patients, 7.7–26.5% of breast cancer patients, 4.2–20.0% of lung cancer patients, and 52% of prostate cancer patients, but has not been detected in non-cancerous donors (Stockert et al, 1998; Nakada et al, 2003; Türeci et al, 2006; Chapman et al, 2007; Isobe et al, 2009; Gati et al, 2011). Thus, it is possible that the NY-ESO-1 humoral immune response could be used as a serological marker for detecting these cancers and to facilitate the clinical management of some patients with particular types of cancer (Gnjatic et al, 2006). Jäger et al (1999) found that the change in the NY-ESO-1 humoral immune response reflected the overall tumour load in 10 out of 12 patients with various cancers. However, there is ongoing controversy regarding the association between the NY-ESO-1 immune response and prognostic criteria (Yuan et al, 2011). To address these issues in gastric cancer, we investigated the clinical usefulness of the NY-ESO-1 humoral immune response for diagnosis, monitoring, and relapse prediction in gastric cancer patients.

MATERIALS AND METHODS

Serum sample and tissue specimen collection from gastric cancer patients. In all, 363 patients with histologically confirmed gastric cancer, who underwent surgical resection or chemotherapy at one of four institutions between 2004 and 2011, were included in this study after providing written informed consent. Serum samples were obtained from the 363 patients during their admission to hospital for surgical treatment and/or chemotherapy, and afterwards, serial serum samples were obtained at each follow-up visit from 25 patients who displayed NY-ESO-1 humoral immune responses. All serum samples were collected as surplus samples after routine blood tests and stored. Fixed and frozen gastric cancer tissue samples were obtained from 60 out of 363 patients during surgery and stored. The samples were subsequently subjected to expression analysis. Information regarding blood test results, tumour stage, histological type, depth of invasion, lymph node metastasis, and distant metastasis, which were obtained from pathological examinations and CT scans, were collected from the relevant patient databases. Serum samples obtained from 50 healthy donors were used as controls. This study was approved by the institutional review boards of Osaka University Hospital,

Toyonaka Municipal Hospital, Ikeda City Hospital, and Minoh City Hospital.

Reverse transcription–polymerase chain reaction. Total cellular RNA was extracted from the frozen tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The total RNA (1 μg) was subjected to the reverse transcription (RT) in 20 μl buffer with oligo-(dT)$_{15}$ primer using a RT system (Promega, Madison, WI, USA). Conventional polymerase chain reaction (PCR) was performed in a 25-μl reaction mixture containing 1 μl of cDNA template, 500 nM of each primer, and 1 U of Taq DNA polymerase (AmpliTaq Gold, Roche Molecular Systems, Pleasanton, CA, USA) in the following conditions: one cycle of 95 °C for 12 min; followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min; and then a final step of 72 °C for 10 min. The sequences of the primers for NY-ESO-1 were as follows: ESO1-1, 5′-AGTTC TACCTCGCCATGCCT-3′; and ESO1-2, 5′-TCCTCCTCCAGC GACAAACAA-3′. The integrity of each RNA sample was verified by performing RT–PCR for porphobilinogen deaminase (PBGD). The PCR products were subjected to electrophoresis on a 2% agarose gel and visualised with ethidium bromide.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were used for the immunohistochemistry (IHC) analyses. Slides were incubated with the primary antibody overnight at 4 °C. The monoclonal antibody E978, which was previously generated by our group, was used to detect NY-ESO-1. The slides were then subjected to a heat-based antigen retrieval technique by immersing them in a preheated buffer solution (hipH solution; Dako, Carpinteria, CA, USA). A polymer-based antibody detection system (PowerVision; Leica Microsystems, Buffalo Grove, IL, USA) was used as the secondary reagent, and 3,3-diaminobenzi-dine tetrahydrochloride (Liquid DAB; Biogenex, San Ramon, CA, USA) was used as the chromogen. Normal adult testis tissue as a positive control and appropriate negative controls were included for each case.

Enzyme-linked immunosorbent assay. A measure of 100 μl of 1 μg ml^{-1} recombinant protein in coating buffer (pH 9.6) were added to each well of 96-well PolySorp immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. The plates were then washed with PBS and blocked with 200 μl per well of 5% FCS/PBS for 1 h at room temperature. After being washed again, 100 μl of serially diluted serum were added to each well and incubated for 2 h at room temperature. Then, after extensive washing, goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells as a secondary antibody, and the plates were incubated for 1 h at room temperature. The plates were washed again, and the signals were developed with 100 μl per well of 0.03% o-phenylene diamine dihydrochloride, 0.02% hydrogen peroxide, and 0.15 M citrate buffer, and absorbance was read at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA). Ovalbumin (OVA; Sigma, St Louis, MO, USA) was used as the control protein. Levels of NY-ESO-1 humoral response were assessed using optical density (OD) values.

CEA and CA19-9. Serum CEA and CA19-9 levels were measured at each hospital's clinical laboratory department. Carcinoembryonic antigen and CA19-9 positivity were defined as serum levels of CEA and CA19-9 of >5.0 ng ml^{-1} and >37 U ml^{-1}, respectively.

Statistical analysis. Fisher's exact test was used to assess the associations between NY-ESO-1 antibody expression and clinico-pathological parameters. Kaplan–Meier curves were plotted to assess the effect of the NY-ESO-1 antibody on overall survival. Survival curves were compared using the log-rank test.

Table 1. Frequencies of NY-ESO-1 antibody, CEA, and CA19-9 in gastric cancer patients

Stage	NY-ESO-1 Ab	CEA	CA19-9	CEA and/or CA19-9	CEA and/or CA19-9 and/or NY-ESO-1 Ab
I	6/176 (3.4)	24/176 (13.6)	6/176 (3.4)	27/176 (15.3)	31/176 (17.6)
II	2/45 (4.4)	8/45 (17.8)	7/45 (15.6)	11/45 (24.4)	12/45 (26.6)
III	17/67 (25.3)	22/67 (32.9)	11/67 (16.4)	25/67 (37.3)	35/67 (52.2)
IV	16/75 (20.0)	23/75 (30.7)	30/75 (40.0)	40/75 (53.3)	46/75 (61.3)
I+II	8/221 (3.6)	32/221 (14.5)	13/221 (5.9)	38/221 (17.2)	43/221 (19.5)
III+IV	33/142 (23.2)	45/142 (31.7)	41/142 (28.9)	65/142 (45.8)	81/142 (57.0)
Total	41/363 (11.1)	77/363 (21.2)	54/363 (14.9)	103/363 (28.4)	124/363 (34.2)

Abbreviations: Ab = antibody; CA = carbohydrate antigen; CEA = carcinoembryonic antigen. Values within parentheses are percentages.

RESULTS

Determination of NY-ESO-1 humoral immune response positivity. We first determined the OD cutoff value for NY-ESO-1 humoral immune response positivity. When the serum samples from the 50 healthy donors were examined for reactivity to the NY-ESO-1 recombinant protein by ELISA, their OD values ranged from 0.08 to 0.20, and their mean and standard deviation values were 0.15 and 0.05, respectively, at a dilution of 1:200. Thus, NY-ESO-1 humoral immune response positivity was defined as an OD value of >0.25 at a dilution of 1:200 (95% accuracy level) and >3 times of the OD value against control protein (OVA).

NY-ESO-1 humoral immune responses of gastric cancer patients. Serum samples were obtained from 363 gastric cancer patients, including 176 stage I, 45 stage II, 67 stage III, and 75 stage IV patients at admission (Table 1). The NY-ESO-1 antibody was detected in 3.4% (6 of 176) of stage I, 4.4% (2 of 45) of stage II, 25.3% (17 of 67) of stage III, and 20.0% (16 of 75) of stage IV gastric cancer patients, resulting in an overall detection rate of 11.1% (41 of 363). An analysis of the gastric cancer patients' characteristics found that NY-ESO-1 antibody positivity was significantly correlated with gender (male>female) and tumour progression (Table 2). In particular, the patients with progressive gastric cancer involving deeper tumour invasion, positive lymph node metastasis, positive distant metastasis, or a higher clinical stage tended to produce the NY-ESO-1 antibody.

Analysis of NY-ESO-1 antigen expression. *NY-ESO-1* mRNA and NY-ESO-1 protein expression were analysed by RT–PCR and IHC, respectively, in gastric cancer tissues obtained from 60 patients for whom both frozen and formalin-fixed specimens were available, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients (Table 3). *NY-ESO-1* mRNA was detected in six specimens. NY-ESO-1 was immunohistochemically detected in 19 specimens, including 6 and 13 that were positive and negative for *NY-ESO-1* mRNA, respectively. Most of the specimens displayed a heterogeneous staining pattern (data not shown).

NY-ESO-1 antibody and antigen expression. We analysed the frequency of NY-ESO-1 antibody positivity in gastric cancer patients in whom NY-ESO-1 antigen expression was or was not detected by RT–PCR or IHC. As shown in Table 3, 9 out of the 60 gastric cancer patients whose specimens were available for expression analysis possessed the NY-ESO-1 antibody in their sera. The NY-ESO-1 antibody was detected in 8 of 19 (42.1%) patients with IHC-positive gastric cancer and 5 of 6 (83.3%) patients with RT–PCR (and IHC)-positive gastric cancer, whereas only 1 of 41 patients in whom both RT–PCR and IHC analysis

Table 2. Relationship between NY-ESO-1 antibody positivity and clinicopathological features in gastric cancer patients

Variable	NY-ESO-1 Ab		P-value*
	Negative	Positive	
Gender			
Male	223 (86.4)	35 (13.6)	0.04307
Female	99 (94.3)	6 (5.7)	
Age (years)			
>65	178 (88.6)	23 (11.4)	0.9209
<65	144 (88.9)	18 (11.1)	
Histological type			
Differentiated	143 (89.4)	17 (10.6)	0.5605
Undifferentiated	132 (87.4)	19 (12.6)	
Depth of tumour invasion			
cT1–T2	193 (92.8)	15 (7.2)	0.0044
cT3–T4	129 (83.2)	26 (16.8)	
Lymph node metastasis			
Negative	196 (97.0)	6 (3.0)	<0.001
Positive	126 (78.3)	35 (21.7)	
Distant metastasis			
Negative	277 (91.1)	27 (8.9)	<0.001
Positive	45 (76.3)	14 (23.7)	
Stage			
I–II	213 (96.4)	8 (3.6)	<0.001
III–IV	109 (76.8)	33 (23.2)	

Abbreviations: Ab = antibody. Fisher's exact test was used for the statistical analysis. Values within parentheses are percentages.

produced negative results displayed an NY-ESO-1 humoral immune responses.

Frequencies of NY-ESO-1 humoral immune responses and conventional tumour markers in gastric cancer patients. The frequency of the NY-ESO-1 humoral immune response was compared with those of conventional tumour markers in gastric

Table 3. Frequency of NY-ESO-1 antibody positives in gastric cancer patients in whom the NY-ESO-1 antigen was or was not detected by IHC or RT–PCR

	IHC		
	Positive	Negative	Total
mRNA			
Positive	5/6 (83.3)	0/0 (0.0)	5/6 (83.3)
Negative	3/13 (23.1)	1/41 (2.4)	4/54 (7.4)
Total	8/19 (42.1)	1/41 (2.4)	9/60 (15.0)

Abbreviations: IHC = immunohistochemistry; RT–PCR = reverse transcription–polymerase chain reaction. Frozen and formalin-fixed tissue specimens from 60 patients, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients, were analysed. All stage IV patients had previously undergone surgical treatment. Values within parentheses are percentages.
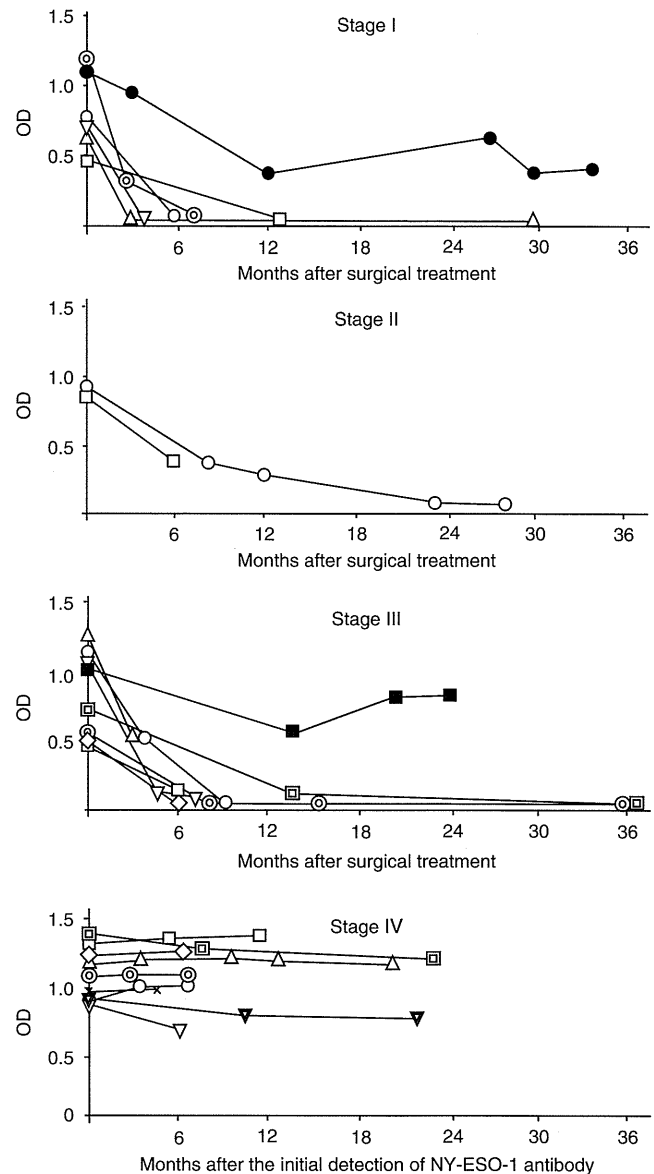

Figure 1. Change in the NY-ESO-1 humoral immune responses of gastric cancer patients after treatment. The serum NY-ESO-1 humoral immune responses of patients with stage I, II, III, or IV gastric cancer in whom NY-ESO-1 antibody production was detected before surgical treatment or chemotherapy were serially analysed. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgery, and only 2 patients (●, ■) suffered recurrence. Other 14 patients did not suffer recurrence. Nine patients with stage IV gastric cancer received chemotherapy alone after the initial detection of NY-ESO-1 antibody. Each mark represents a patient. Optical density (OD) values were measured at a serum dilution of 1 : 200.

cancer patients. The serum CEA and CA19-9 levels of 363 gastric cancer patients were measured at admission (Table 1). Carcinoembryonic antigen and CA19-9 positivity were observed in 21.2% (77 of 363) and 14.9% (54 of 363) of the gastric cancer patients, respectively, and, except for CA19-9 in the stage III patients, they displayed higher frequencies than the NY-ESO-1 humoral immune response in all stages of the disease. We then analysed whether the addition of the NY-ESO-1 humoral immune response to CEA and CA19-9 increased the diagnostic frequency of gastric cancer. The combined use of CEA and CA19-9 tests produced positivity rates of 15.3% (27 of 176) in stage I, 24.4% (11 of 45) in stage II, 37.3% (25 of 67) in stage III, and 53.3% (40 of 75) in stage IV gastric cancer patients, resulting in an overall positivity rate of 28.4% (103 of 363). When the NY-ESO-1 humoral immune response was added to these two conventional tumour markers, the positivity rates of all stages increased, resulting in information gains of 14.9% (from 25 to 35 patients; 10 of 67) in stage III and 11.2% (from 65 to 81 patients; 16 of 142) in stage III and IV gastric cancer patients.

Changes in the NY-ESO-1 humoral immune responses of the patients during their clinical courses. Serial serum samples were obtained from 25 gastric cancer patients who displayed positive NY-ESO-1 antibody at admission, and the changes in their NY-ESO-1 humoral immune responses were examined throughout their clinical courses. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgical treatment, and 14 did not suffer recurrence. The NY-ESO-1 immune response levels of the patients who did not suffer recurrence decreased after treatment and had fallen below the cutoff level by 9 months after surgery in most cases and did not subsequently increase (Figure 1). The half-lives of their NY-ESO-1 humoral immune response levels were 1.5, 1.6, 2.1, 3.2, and 6.6 months in the stage I patients; 3.0 and 4.0 months in the stage II patients; and 1.6, 1.9, 2.3, 3.0, 3.2, 4.1, and 6.7 months in the stage III patients (mean: 3.0 months). On the other hand, the two patients who underwent curative surgery but subsequently suffered recurrence, M-2 (stage I) and M-11 (stage III), displayed not only incomplete decreases in their NY-ESO-1 humoral immune response levels but also their subsequent restoration to pretreatment levels (Figure 1 and Figure 2A and B). In a comparison between the patients' conventional tumour marker levels and their NY-ESO-1humoral immune response levels, we found that the changes in their CEA and CA19-9 levels were consistent with their NY-ESO-1 immune response levels in patient M-2, whereas patient M-11 was negative for both CEA and CA19-9 throughout their clinical course. Nine stage IV patients who received chemotherapy alone maintained high NY-ESO-1 humoral immune response levels throughout their clinical courses,

including some patients who achieved partial tumour responses after chemotherapy (Figure 1).

Prognostic value of the NY-ESO-1 humoral immune response in gastric cancer. The prognostic value of the NY-ESO-1 immune response was evaluated in gastric cancer patients. An analysis of the cumulative overall survival of the gastric cancer patients indicated that there was no difference in the survival rates of the patients who did and did not display positive NY-ESO-1 humoral immune responses (Figure 3A). However, among the patients with higher stage gastric cancer, overall survival was better in the patients in whom NY-ESO-1humoral immune responses were

detected, although the difference was not significant (Figure 3B). NY-ESO-1 protein expression, as detected by IHC, did not affect the overall survival rate (data not shown).

DISCUSSION

NY-ESO-1 antibody was detected in 23.2% of stage III and IV gastric cancer patients, and the combinatorial use of the NY-ESO-1
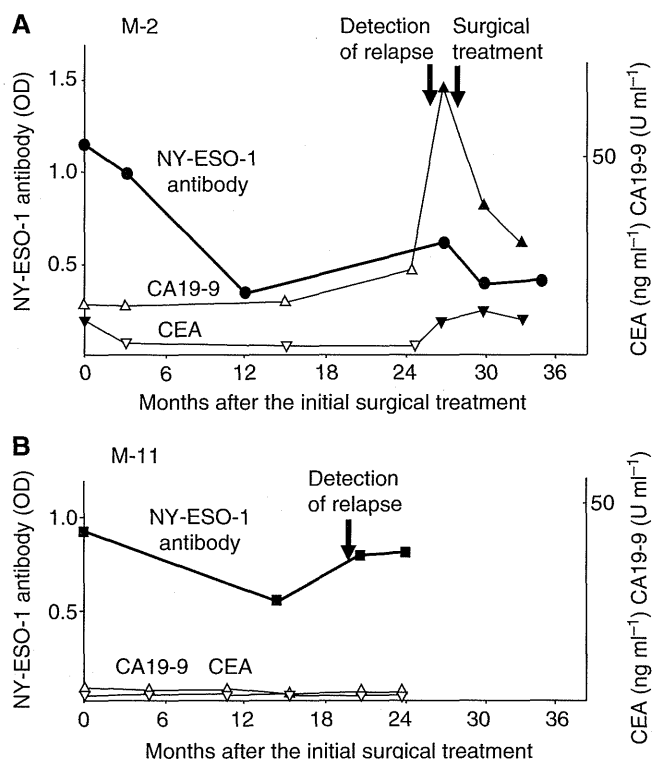

Figure 2. NY-ESO-1 humoral immune response, CEA, and carbohydrate antigen (CA)19-9 levels of patients who relapsed after curative surgery. The NY-ESO-1 humoral immune response (●, ■; Figure 1), CEA (▽), and CA19-9 (△) levels of two patients, M-2 (stage I) (A) and M-11 (stage III) (B), who underwent curative surgery but subsequently suffered recurrence, were serially analysed. OD values were measured at a serum dilution of 1 : 200. The closed marks indicate CEA or CA19-9 positivity.

antibody with CEA and CA19-9 as tumour markers increase the percentage of tumour detection from 45.8 to 57.0%. As the frequency of NY-ESO-1 humoral immune response was relatively low in the patients with early-stage gastric cancer, analysing serum NY-ESO-1 antibody levels alone might not be useful for screening for early-stage gastric cancer. Nevertheless, the expression of NY-ESO-1, a CT antigen, is restricted to tumour tissues and NY-ESO-1 antibody is only detectable in patients with NY-ESO-1-expressing tumours (Stockert et al, 1998), indicating the highly specific nature of NY-ESO-1 humoral immune responses in cancer patients. Given that NY-ESO-1 expression by malignant cells is required for antibody induction (Stockert et al, 1998), the detection of NY-ESO-1 antibody would be helpful for diagnosing malignancy, although extensive analysis of serum samples from patients with non-cancerous disease, for example, liver or renal disorders, autoimmune diseases, and so on, would be necessary to confirm. In our expression analysis, more NY-ESO-1-positive cases were detected by IHC (19 of 60) than by RT–PCR (6 of 60). This was probably due to the heterogeneous expression of NY-ESO-1 in gastric cancer and the fact that a limited number of biopsy samples were used for the RT–PCR, whereas multiple slices from whole tumour specimens were used for the IHC. Extensive IHC analysis should be used for NY-ESO-1 expression studies of gastric cancer.

We detected a correlation between the NY-ESO-1 humoral immune response levels and the clinical outcome after therapy in gastric cancer patients. The patients who underwent surgery and did not suffer a subsequent relapse displayed consistent decreases in their NY-ESO-1 humoral immune response levels or even the complete disappearance of the NY-ESO-1 antibody from their sera. It is generally accepted that constant immunological stimulation is necessary to maintain a strong humoral immune response (Jager et al, 1999). Thus, reduction of antigen doses by the removal of NY-ESO-1-expressing tumour is one possible reason for the observed decreases in these patients' NY-ESO-1 humoral immune response levels after surgery. Patients M-2 and M-11, in whom NY-ESO-1 humoral immune responses remained high for 1 year after surgery and increased thereafter, may have a subclinical residual disease of the so-called 'minimal residual cancer' (Austrup et al, 2000; Klein et al, 2002) after curative surgery. Local recurrent tumours of 23 and 25 mm in diameter subsequently developed in M-2 and M-11, respectively, suggesting that even a small tumour burden is sufficient to stimulate antibody production. Patient M-2 showed a partial decrease in their NY-ESO-1 humoral immune response levels after the resection of the relapsed tumour, and we are carefully observing the progression of this tumour.

Nine patients with stage IV gastric cancer received chemotherapy alone. Among them, six patients displayed stable disease, two
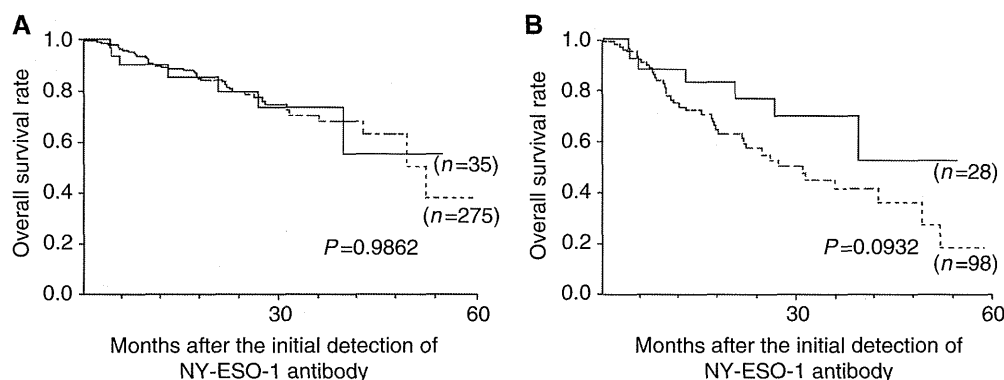

Figure 3. Prognostic role of NY-ESO-1 antibody in gastric cancer patients. The cumulative overall survival rate was analysed in all patients (n = 310; A) and stage III and IV (n = 126; B) gastric cancer patients in whom NY-ESO-1 antibodies were (continuous line) and were not detected (dotted line). The detection of NY-ESO-1 protein by IHC analysis did not affect the overall survival rate (data not shown). Survival curves were plotted using the Kaplan–Meier method. The log-rank test was used for comparisons between groups. P-values <0.05 were considered significant.

patients displayed progressive disease, and one patient (M-19) achieved a partial response. Serial analysis of the NY-ESO-1 humoral immune responses of these nine patients including M-19 showed that they barely changed throughout their clinical courses, suggesting that even small tumours are enough to provoke strong NY-ESO-1 humoral immune responses. In this regard, the NY-ESO-1 humoral immune response might not be suitable as a clinical marker for palliative therapy.

We have performed serial cancer vaccine clinical trials with NY-ESO-1 because of its strong immunogenicity and high specificity (Uenaka et al, 2007; Wada et al, 2008; Kakimi et al, 2011). The NY-ESO-1humoral immune response could be a reliable marker of the induction of immune response, as well as for predicting clinical responses in these trials. Furthermore, antibody-based examinations detected both intra- and intermolecular antigen spreading in the sera of patients who had been vaccinated with NY-ESO-1 protein (Kawada et al, 2012), suggesting the possible correlation of NY-ESO-1 humoral immne responses and clinical status. In addition, we have started a phase I study of vaccination with NY-ESO-1 protein mixed with Hiltonol (Poly ICLC), Picibanil (OK-432), and Montanide (ISA-51) in patients with NY-ESO-1-expressing cancers (UMIN000007954). Furthermore, NY-ESO-1 vaccine involving modulators of immune checkpoints, for example, anti-CTLA4 antibody and anti-PD-1 antibody, and reagents that are antagonistic to regulatory T cells, for example, anti-CCR4 antibody (Pardoll, 2012) should be considered.

Recently, the antibody against p53, another tumour antigen, has been recognised as a useful tumour marker (Lubin et al, 1995). Shimada et al (2000)) reported that p53 antibody was detected in 35% of serum samples from patients with in situ oesophageal cancer and that it disappeared after endoscopic mucosal resection, proposing that p53 antibody is useful for the early detection and subsequent monitoring of oesophageal cancer. In addition, Müller et al (2006) reported that p53 antibody was found in 23.4% of serum samples from cancer patients with 100% accuracy and was correlated with poor prognosis in hepatocellular carcinoma and breast cancer.

Here, we have demonstrated that the NY-ESO-1 humoral immune response could also be valuable as a marker for detecting advanced gastric cancer and inferring whether residual tumour cells remain after treatment, although its frequency in gastric cancer is not very high. We have started a prospective multi-institutional clinical study of NY-ESO-1 humoral immune responses in higher stage gastric cancer patients. In this new study, the NY-ESO-1 humoral immune responses of approximately 100 patients who relapsed after curative surgery will be serially analysed and then followed up. This trial has been registered as UMIN000007925 in Japan.

REFERENCES

Alexander JC, Silverman NA, Chretien PB (1976) Effect of age and cigarette smoking on carcinoembryonic antigen levels. JAMA 235: 1975–1979.

Austrup F, Uciechowski P, Eder C, Böckmann B, Suchy B, Driesel G, Jäckel S, Kusiak I, Grill HJ, Giesing M (2000) Prognostic value of genomic alterations in minimal residual cancer cells purified from the blood of breast cancer patients. Br J Cancer 83: 1664–1673.

Chapman C, Murray A, Chakrabarti J, Thorpe A, Woolston C, Sahin U, Barnes A, Robertson J (2007) Autoantibodies in breast cancer: their use as an aid to early diagnosis. Ann Oncol 18: 868–873.

Ferro MA, Barnes I, Roberts JB, Smith PJ (1987) Tumor markers in prostatic carcinoma. A comparison of prostate-specific antigen with acid phosphatase. Br J Urol 60: 69–73.

Fujiyama S, Morishita T, Sagara K, Sato T, Motohara K, Matsuda I (1986) Clinical evaluation of plasma abnormal prothrombin (PIVKA-II) in patients with hepatocellular carcinoma. Hepatogastroenterology 33: 201–205.

Gati A, Lajmi N, Derouiche A, Marrakchi R, Chebil M, Benammar-Elgaaied A (2011) NY-ESO-1 expression and immunogenicity in prostate cancer patients. Tunis Med 89: 779–783.

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

Graziosi L, Bugiantella W, Cavazzoni E, Cantarella F, Porcari M, Baffa N, Donini A (2011) Role of FDG-PET/CT in follow-up of patients treated with resective gastric surgery for tumour. Ann Ital Chir 82: 125–129.

Health and Welfare Statistics Association: Tokyo (2006) Statistics and Information Department, Ministry of Health, Labour, and Welfare Vital Statistics of Japan 2004.

Ikeda Y, Mori M, Adachi Y, Matsushima T, Sugimachi K, Saku M (1993) Carcinoembryonic antigen (CEA) in stage IV gastric cancer as a risk factor for liver metastasis: a univariate and multivariate analysis. J Surg Oncol 53: 235–238.

Isobe M, Eikawa S, Uenaka A, Nakamura Y, Kanda T, Kohno S, Kuzushima K, Nakayama E (2009) Correlation of high and decreased NY-ESO-1 immunity to spontaneous regression and subsequent recurrence in a lung cancer patient. Cancer Immun 9: 8.

Jäger E, Stockert E, Zidianakis Z, Chen YT, Karbach J, Jäger D, Arand M, Ritter G, Old LJ, Knuth A (1999) Humoral immune responses of cancer patients against 'Cancer-Testis' antigen NY-ESO-1: correlation with clinical events. Int J Cancer 84: 506–510.

Kakimi K, Isobe M, Uenaka A, Wada H, Sato E, Doki Y, Nakajima J, Seto Y, Yamatsuji T, Naomoto Y, Shiraishi K, Takigawa N, Kiura K, Tsuji K, Iwatsuki K, Oka M, Pan L, Hoffman EW, Old LJ, Nakayama E (2011) 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 129: 2836–2846.

Katanoda K, Yako-Suketomo H (2009) Comparison of time trends in stomach cancer incidence (1973–2002) in Asia, from Cancer Incidence in Five Continents, Vols IV–IX. Jpn J Clin Oncol 39: 71–72.

Kawada J, Wada H, Isobe M, Gnjatic S, Nishikawa H, Jungbluth AA, Okazaki N, Uenaka A, Nakamura Y, Fujiwara S, Mizuno N, Saika T, Ritter E, Yamasaki M, Miyata H, Ritter G, Murphy R, Venhaus R, Pan L, Old LJ, Doki Y, Nakayama E (2012) Heteroclitic serological response in esophageal and prostate cancer patients after NY-ESO-1 protein vaccination. Int J Cancer 130: 584–592.

Klein CA, Blankenstein TJ, Schmidt-Kittler O, Petronio M, Polzer B, Stoecklein NH, Riethmüller G (2002) Genetic heterogeneity of single disseminated tumour cells in minimal residual cancer. Lancet 360: 683–689.

Kodera Y, Yamamura Y, Torii A, Uesaka K, Hirai T, Yasui K, Morimoto T, Kato T, Kito T (1996) The prognostic value of preoperative serum levels of CEA and CA19-9 in patients with gastric cancer. Am J Gastroenterol 91: 49–53.

Lubin R, Schlichtholz B, Teillaud JL, Garay E, Bussel A, Wild CP (1995) P53 antibodies in patients with various types of cancer: assay, identification, and characterization. Clin Cancer Res 1: 1463–1469.

Makino T, Fujiwara Y, Takiguchi S, Miyata H, Yamasaki M, Nakajima K, Nishida T, Mori M, Doki Y (2010) The utility of pre-operative peritoneal lavage examination in serosa-invading gastric cancer patients. Surgery 148: 96–102.

Motoori M, Takemasa I, Doki Y, Saito S, Miyata H, Takiguchi S, Fujiwara Y, Yasuda T, Yano M, Kurokawa Y, Komori T, Yamasaki M, Ueno N, Oba S, Ishii S, Monden M, Kato K (2006) Prediction of peritoneal metastasis in advanced gastric cancer by gene expression profiling of the primary site. Eur J Cancer 42: 1897–1903.

Müller M, Meyer M, Schilling T, Ulsperger E, Lehnert T, Zentgraf H, Stremmel W, Volkmann M, Galle PR (2006) Testing for anti-p53 antibodies increases the diagnostic sensitivity of conventional tumor markers. Int J Oncol 29: 973–980.

Nakada T, Noguchi Y, Satoh S, Ono T, Saika T, Kurashige T, Gnjatic S, Ritter G, Chen YT, Stockert E, Nasu Y, Tsushima T, Kumon H, Old LJ, Nakayama E (2003) NY-ESO-1 mRNA expression and immunogenicity in advanced prostate cancer. Cancer Immunol 3: 10.

Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* **12**: 252–264.

Sasako M, Sakuramoto S, Katai H, Kinoshita T, Furukawa H, Yamaguchi T, Nashimoto A, Fujii M, Nakajima T, Ohashi Y (2011) Five-year outcomes of a randomized phase III trial comparing adjuvant chemotherapy with S-1 versus surgery alone in stage II or III gastric cancer. *J Clin Oncol* **29**: 4387–4393.

Seamonds B, Yang N, Anderson K, Whitaker B, Shaw LM, Bollinger JR (1986) Evaluation of prostate-specific antigen and prostatic acid phosphatase as prostate cancer markers. *Urology* **28**: 472–479.

Shimada H, Takeda A, Arima M, Okazumi S, Matsubara H, Nabeya Y, Funami Y, Hayashi H, Gunji Y, Suzuki T, Kobayashi S, Ochiai T (2000) Serum p53 antibody is a useful tumor marker in superficial esophageal squamous cell carcinoma. *Cancer* **89**: 1677–1683.

Stockert E, Jäger E, Chen YT, Scanlan MJ, Gout I, Karbach J, Arand M, Knuth A, Old LJ (1998) A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med* **187**: 1349–1354.

Takahashi Y, Mai M, Kusama S (1998) Factors influencing growth rate of recurrent stomach cancer as determined by analysis of serum carcinoembryonic antigen. *Cancer* **75**: 1497–1502.

Takahashi Y, Takeuchi T, Sakamoto J, Touge T, Mai M, Ohkura H, Kodaira S, Okajima K, Nakazato H (2003) The usefulness of CEA and/or CA19-9 in monitoring for recurrence in gastric cancer patients: a prospective clinical study. *Gastric Cancer* **6**: 142–145.

Türeci O, Mack U, Luxemburger U, Heinen H, Krummenauer F, Sester M, Sester U, Sybrecht GW, Sahin U (2006) Humoral immune responses of lung cancer patients against tumor antigen NY-ESO-1. *Cancer Lett* **236**: 64–71.

Uenaka A, Wada H, Isobe M, Saika T, Tsuji K, Sato E, Sato S, Noguchi Y, Kawabata R, Yasuda T, Doki Y, Kumon H, Iwatsuki K, Shiku H, Monden M, Jungbluth AA, Ritter G, Murphy R, Hoffman E, Old LJ, Nakayama E (2007) 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* **7**: 9.

Wada H, Sato E, Uenaka A, Isobe M, Kawabata R, Nakamura Y, Iwae S, Yonezawa K, Yamasaki M, Miyata H, Doki Y, Shiku H, Jungbluth AA, Ritter G, Murphy R, Hoffman EW, Old LJ, Monden M, Nakayama E (2008) Analysis of peripheral and local anti-tumor immune response in esophageal cancer patients after NY-ESO-1 protein vaccination. *Int J Cancer* **123**: 2362–2369.

Yamamoto M, Baba H, Kakeji Y, Endo K, Ikeda Y, Toh Y, Kohnoe S, Okamura T, Maehara Y (2004) Prognostic significance of tumor markers in peritoneal lavage in advanced gastric cancer. *Oncology* **67**: 19–26.

Yuan J, Adamow M, Ginsberg BA, Rasalan TS, Ritter E, Gallardo HF, Xu Y, Pogoriler E, Terzulli SL, Kuk D, Panageas KS, Ritter G, Sznol M, Halaban R, Jungbluth AA, Allison JP, Old LJ, Wolchok JD, Gnjatic S (2011) Integrated NY-ESO-1 antibody and CD8 + T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proc Natl Acad Sci USA* **108**: 16723–16728.

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Overcoming regulatory T-cell suppression by a lyophilized preparation of *Streptococcus pyogenes*

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Cancer vaccines have yet to yield clinical benefit, despite the measurable induction of humoral and cellular immune responses. As immunosuppression by $CD4^+CD25^+$ regulatory T (Treg) cells has been linked to the failure of cancer immunotherapy, blocking suppression is therefore critical for successful clinical strategies. Here, we addressed whether a lyophilized preparation of *Streptococcus pyogenes* (OK-432), which stimulates Toll-like receptors, could overcome Treg-cell suppression of $CD4^+$ T-cell responses in vitro and in vivo. OK-432 significantly enhanced in vitro proliferation of $CD4^+$ effector T cells by blocking Treg-cell suppression and this blocking effect depended on IL-12 derived from antigen-presenting cells. Direct administration of OK-432 into tumor-associated exudate fluids resulted in a reduction of the frequency and suppressive function of $CD4^+CD25^+Foxp3^+$ Treg cells. Furthermore, when OK-432 was used as an adjuvant of vaccination with HER2 and NY-ESO-1 for esophageal cancer patients, NY-ESO-1–specific $CD4^+$ T-cell precursors were activated, and NY-ESO-1–specific $CD4^+$ T cells were detected within the effector/memory T-cell population. $CD4^+$ T-cell clones from these patients had high-affinity TCRs and recognized naturally processed NY-ESO-1 protein presented by dendritic cells. OK-432 therefore inhibits Treg-cell function and contributes to the activation of high-avidity tumor antigen-specific naive T-cell precursors.

Keywords: Cancer · Treg cells · Tumor immunology · Vaccination

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Introduction

Many tumor-associated antigens recognized by the immune system are normal self-constituents, and tumor immunity is considered to be in part an autoimmune response [1–3]. Therefore, mechanisms for maintaining immunological self-tolerance hamper effective anticancer immunity. $CD4^+CD25^+$ Treg cells are one of the major components in maintaining immunological self-tolerance in hosts by suppressing a wide range of immune responses [4–7]. Indeed, depletion of Treg-cell populations enhances spontaneous and vaccine-induced antitumor immune responses [6, 8, 9], and the stimulation of $CD4^+CD25^+$ Treg cells by immunization with self-antigens induces enhanced chemically induced primary tumor development and increased numbers of pulmonary metastasis following injection of transplantable tumor cells [10–12]. In human cancers, the presence of high numbers of $CD4^+CD25^+$ Treg cells or low ratio of $CD8^+$ T cells to $CD4^+CD25^+$ Treg cells in tumors is correlated with unfavorable prognosis [13, 14]. In addition, the depletion of $CD4^+CD25^+$ Treg cells in patients receiving a DC vaccine enhances the stimulation of tumor-specific T-cell responses, indicating a crucial role for Treg cells in the regulation of antitumor immune responses in humans [15].

NY-ESO-1, a germ cell protein, was found by SEREX (serological identification of antigens by recombinant expression cloning) using the serum of an esophageal cancer patient [16, 17]. We have previously shown that NY-ESO-1–specific $CD4^+$ T cells are detectable in cancer patients with spontaneous NY-ESO-1 serum Ab responses [17, 18]. In addition, NY-ESO-1–specific $CD4^+$ T-cell precursors can expand and become detectable in healthy individuals after in vitro antigenic stimulation of peripheral $CD4^+$ T cells, but only following depletion of $CD4^+CD25^+$ T cells [19, 20]. These results suggested that NY-ESO-1–specific $CD4^+$ T-cell precursors are actually present at relatively high frequencies in healthy individuals, and that the activation/expansion of NY-ESO-1–specific naive $CD4^+$ T cells is suppressed by $CD4^+CD25^+$ Treg cells. In healthy donors and in cancer patients with NY-ESO-1–expressing tumors but without spontaneous anti-NY-ESO-1 Ab (seronegative), naturally arising NY-ESO-1–specific T-cell responses are susceptible to Treg-cell suppression and are exclusively detected from naive populations ($CD4^+CD25^-CD45RA^+$). In contrast, most NY-ESO-1–specific $CD4^+$ T cells in cancer patients with spontaneous anti-NY-ESO-1 Ab (seropositive) are derived from memory populations ($CD4^+CD25^-CD45RO^+$) and are detectable even in the presence of $CD4^+CD25^+$ Treg cells [20, 21]. After vaccination with HLA-DPB1*0401/0402-restricted NY-ESO-$1_{157-170}$ peptide in incomplete Freund's adjuvant, ovarian cancer patients develop NY-ESO-1–specific $CD4^+$ T cells with only low avidity to antigen and low sensitivity to Treg cells, even though they have an effector/memory phenotype ($CD4^+CD25^-CD45RO^+$) [21]. Still, high-avidity naive NY-ESO-1–specific T-cell precursors are present in the peripheral blood of vaccinated patients, but they are subjected to continuous $CD4^+CD25^+$ Treg-cell suppression throughout vaccination [21]. Thus, a strategy to overcome Treg-cell suppression

on preexisting high-avidity naive T-cell precursors is an essential component for effective cancer vaccines.

Accumulating data shed light on recognition of pathogen-associated molecular patterns through TLRs to break the suppressive environment in tumors [22]. It has been reported that TLR stimulants, such as lipopolysaccharide or CpG, block the suppressive activity of $CD4^+CD25^+$ Treg cells partially by an IL-6–dependent mechanism [23]. TLR2 signaling was reported to stimulate the proliferation of $CD4^+CD25^+$ Treg cells and to induce temporal loss of suppressive activity of $CD4^+CD25^+$ Treg cells [24]. TLR2 signaling has also been shown to increase IL-2 secretion by effector T cells, thereby rendering them resistant to $CD4^+CD25^+$ Treg-cell–mediated suppression [25]. We and others have recently reported that vaccination of tumor antigens by TLR stimulating viral or bacterial vectors was able to not only inhibit the suppressive function of $CD4^+CD25^+$ Treg cells but also break tolerance or hyporesponsiveness of effector T cells to tumor antigens even in the presence of Treg cells [26–28].

OK-432 is a lyophilized preparation of *Streptococcus pyogenes* that binds TLR-2, TLR-4, and/or TLR-9 and activates APCs, making it attractive for potential use as an adjuvant of cancer vaccine [29–33]. OK-432–matured DCs effectively prime antigen-specific T cells in vitro [29, 34]. Importantly, OK-432 has already been used for many years as a direct anticancer agent, particularly in Japan, and has a well-established clinical safety profile. However, while it is considered that OK-432 may inhibit Treg-cell suppressive activity by stimulating several TLR signaling pathways, its influence on Treg cells has not yet been shown. In this study, we addressed whether OK-432 inhibits Treg-cell suppressive function and could be a promising adjuvant of cancer vaccines.

Results

OK-432 inhibits the suppressive activity of $CD4^+CD25^+$ Treg cells

To address whether OK-432 inhibited $CD4^+CD25^+$ Treg-cell suppression, we employed the standard in vitro suppression system. $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells (highest 3% of $CD4^+CD25^+$ cells) were isolated from PBMCs of healthy individuals. $CD4^+CD25^-$ T cells were cultured with irradiated autologous APCs (CD4-depleted PBMCs) and anti-CD3 Ab in the presence or absence of $CD4^+CD25^{high}$ Treg cells. $CD4^+CD25^-$ T-cell proliferation was analyzed as described in the *Materials and methods*. In accordance with previous reports [7], $CD4^+CD25^{high}$ Treg cells markedly suppressed the proliferation of $CD4^+CD25^-$ T cells (Fig. 1A and B). In sharp contrast, when OK-432 was added in the culture, suppressive activity of $CD4^+CD25^{high}$ T cells was significantly inhibited (Fig. 1A and B). In addition, OK-432 did not induce death of $CD4^+CD25^{high}$ Treg cells as the frequency of Annexin V^+ and 7-AAD^+ cells was not significantly increased in the presence of OK-432 (data not shown). Instead, $CD4^+CD25^{high}$ Treg cells exhibited marginal proliferation in the presence of
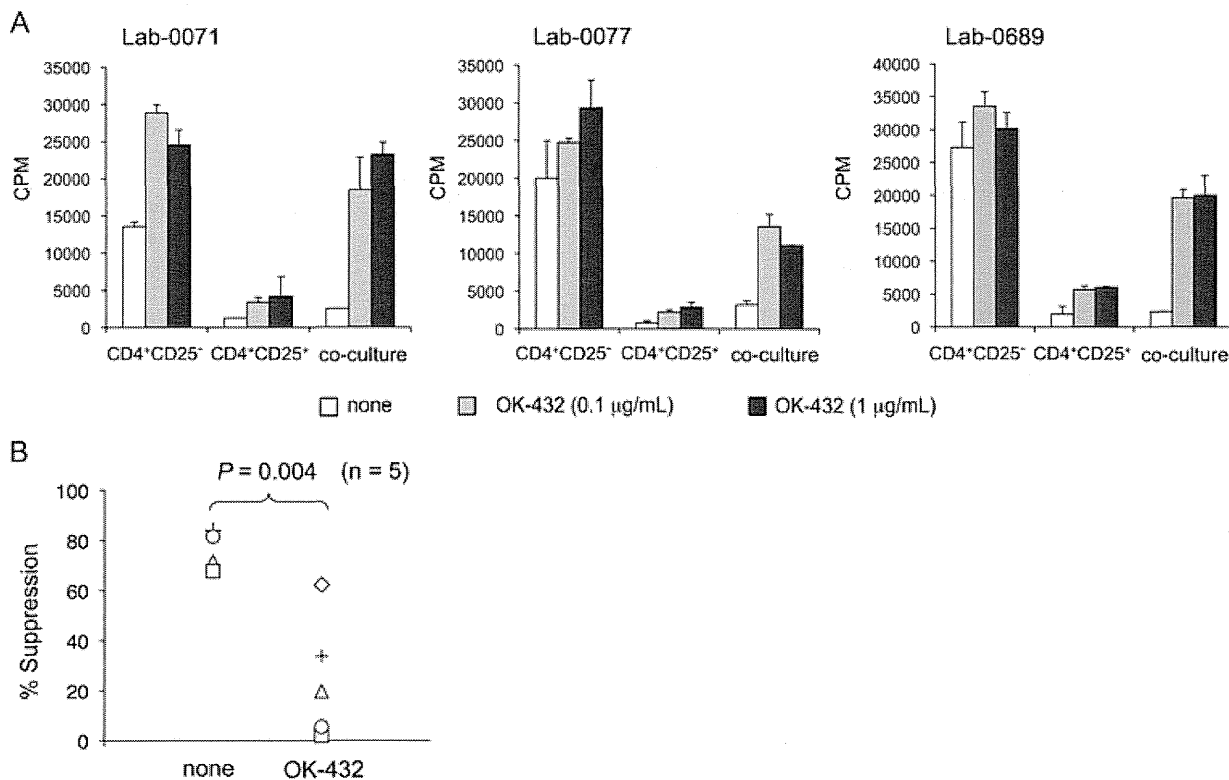
Figure 1. OK-432 overcomes Treg-cell suppression in vitro. (A, B) $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells were collected from PBMCs of healthy individuals as described in the *Materials and Methods*. 1×10^4 $CD4^+CD25^-$ T cells were cultured with irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 5×10^3 $CD4^+CD25^+$ Treg cells with/without OK-432. Proliferation was analyzed by ^{3}H-thymidine incorporation. Data of three donors are shown as mean + SD of two replicates/samples and are from one experiment representative of at least two performed. (B) Summary of percent suppression in five healthy individuals. Percent suppression was calculated as: (1 − (cpm in coculture)/(cpm in $CD4^+CD25^-$ T cells)) × 100. Data shown are from one experiment representative of at least two performed.

OK-432 (Fig. 1A). These data indicate that addition of OK-432 impairs the suppressive activity of $CD4^+CD25^{high}$ Treg cells and partially reverses anergy status of Treg cells.

Instead, $CD4^+CD25^{high}$ Treg cells slightly proliferated in the presence of OK-432 (Fig. 2B). These data suggest a critical role for IL-12 in the inhibition of Treg-cell suppression by OK-432.

Inhibition of the suppressive activity of $CD4^+CD25^+$ Treg cells by OK-432 is dependent on IL-12

Since OK-432 reportedly induces TLR-2, TLR-4, and/or TLR-9 activation and subsequent production of proinflammatory cytokines [29–33], we examined the involvement of cytokines in this inhibition of Treg-cell suppression. To this end, Abs against several candidate cytokines were added to cultures. Among cytokines tested, only blocking Ab against IL-12 significantly abrogated the inhibition of Treg-cell suppression by OK-432 (Fig. 2A).

To confirm the importance of IL-12, we next analyzed whether the addition of IL-12 could inhibit Treg-cell suppression as observed by OK-432. $CD4^+CD25^-$ T cells were cultured with $CD4^+CD25^{high}$ Treg cells, irradiated autologous APCs and anti-CD3 Ab in the presence of IL-12. Treg-cell suppressive activity was significantly inhibited by the addition of IL-12, but not IL-6 or IFN-γ (Fig. 2B). Again, IL-12 did not kill $CD4^+CD25^{high}$ Treg cells as the frequency of Annexin V^+ and 7-AAD$^+$ cells was not significantly increased in the presence of IL-12 (data not shown).

OK-432 induces higher amounts of IL-12 but not IL-10 from APCs compared with other stimuli

To gain insight into the cellular target(s) of OK-432, we explored the origin of IL-12 after OK-432 treatment based on the essential role of IL-12 in the inhibition of Treg-cell suppression by OK-432. We then analyzed whether OK-432 stimulation indeed induced IL-12 production from APCs, such as CD3-depleted PBMCs used in the standard Treg-cell suppression assays. CD3-depleted PBMCs from healthy donors were stimulated with OK-432, LPS, or TNF-α, and cytokine production was examined. OK-432 induced significantly higher amounts of IL-12 from CD3-depleted PBMCs than LPS or TNF-α (Fig. 3A). In addition, CD3-depleted PBMCs stimulated with OK-432 induced much less IL-10 production than LPS (Fig. 3A). Similar results, i.e. IL-12 rather than IL-10 was dominantly produced by CD3-depleted PBMCs stimulated with OK-432, were obtained from four esophageal cancer patients (Fig. 3B).
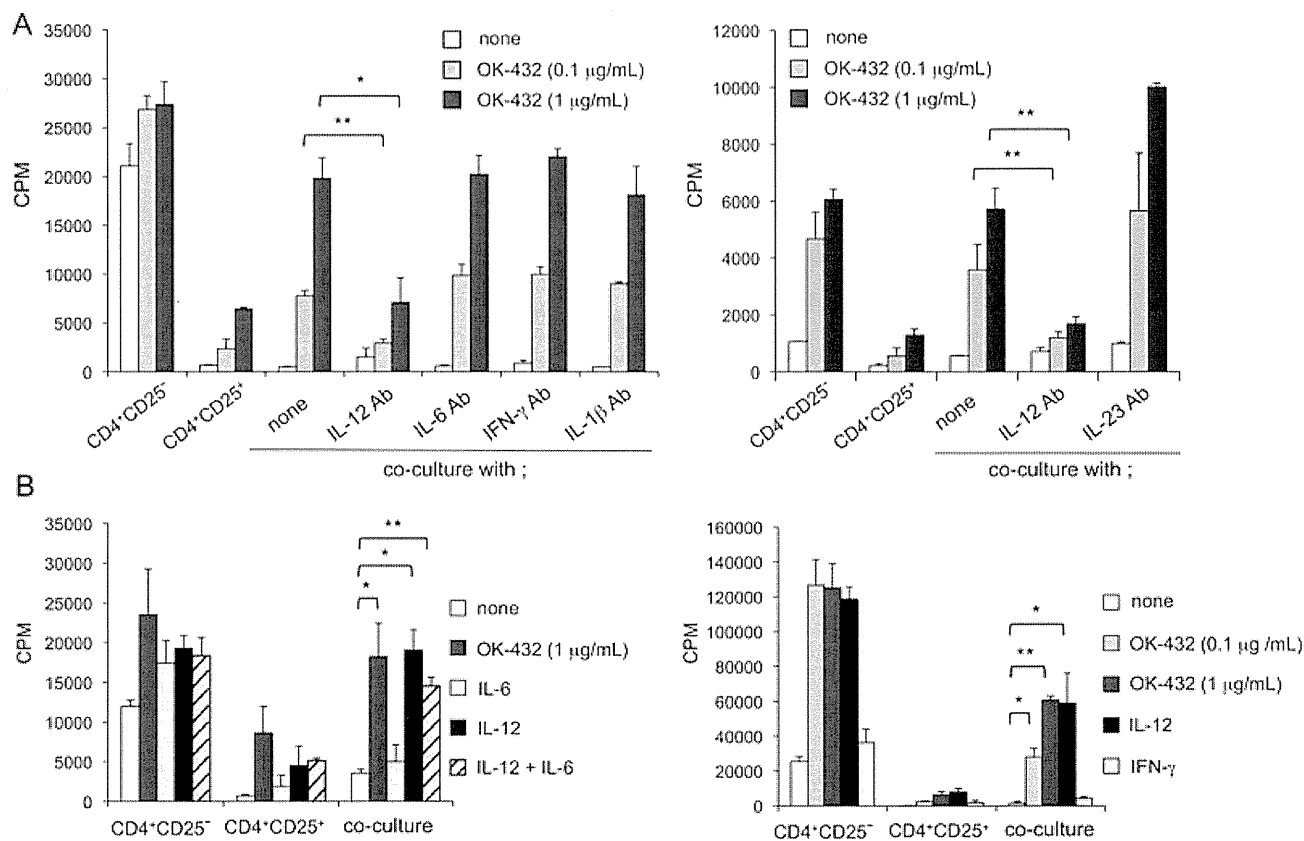
Figure 2. IL-12 is a critical cytokine to overcome Treg-cell suppression by OK-432. CD4$^+$CD25$^-$ T cells and CD4$^+$CD25^{high} Treg cells were collected from PBMCs of healthy individuals. 1×10^4 CD4$^+$CD25$^-$ T cells were cultured with 1×10^5 irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 2.5×10^3 CD4$^+$CD25^{high} Treg cells with/without OK-432. (A) Blocking Abs (10 μg/mL) against several cytokines as indicated were added in the culture and proliferation was measured. (B) Recombinant cytokines (IL-12; 5 ng/mL, IL-6; 5 ng/mL, IFN-γ; 100 U/mL) were added into the cultures and proliferation was analyzed. Data for one representative donor among three donors are expressed as mean + SD of two replicates/samples and are from one experiment representative of at least two performed. $^*p < 0.05$ and $^{**}p < 0.01$ as compared with control, Student's t-test.

We next examined which cell types in PBMCs produced IL-12 after OK-432 stimulation. The major sources of IL-12 in PBMCs after OK-432 stimulation were CD11c$^+$ and CD14$^+$ cells, and neither NK cells nor T cells produced IL-12 (Fig. 3C). Taken together, APCs, such as monocytes, macrophages, and DCs are considered to be the cellular targets of OK-432 to induce IL-12 which is a crucial component for the inhibition of Treg-cell suppression by OK-432.

OK-432 administration to tumor-associated exudates reduces local Treg-cell accumulation and function

As OK-432 is available as an anticancer agent in Japan and has been used for controlling tumor-associated exudate fluids by direct injection to the cavity, we next investigated its influence on Treg cells following in vivo treatment of OK-432. We analyzed the local Treg-cell accumulation and function of tumor-associated sites before and 2–3 days after local OK-432 administration. Cells were isolated from tumor-associated exudate fluids, such as pleu-

ral effusions and ascites. The frequency of Treg cells before and after treatment with OK-432 was examined by staining with Abs for CD4, CD25, and Foxp3. The Foxp3$^+$ T-cell population in CD4$^+$ T cells was markedly reduced (Fig. 4A). Furthermore, the proportion of Foxp3$^+$ T cells in CD4$^+$CD25$^+$ T cells was also significantly reduced after OK-432 administration (Fig. 4A and B), indicating that the balance of helper T cells to Treg cells had changed.

We next addressed the suppressive activity of CD4$^+$CD25^{high} T cells in tumor-associated exudate fluids. CD4$^+$CD25^{high} T cells (highest 3% gate of CD4$^+$CD25$^+$ cells defined with peripheral blood was applied) were isolated from tumor-associated exudate fluids and cultured with CD4$^+$CD25$^-$ T cells from PBMCs with irradiated autologous APCs and anti-CD3 Ab. After OK-432 administration, as the volume of tumor-associated exudate fluids decreased, sufficient amounts of CD4$^+$CD25^{high} T cells for proliferation assays were available only from two patients. CD4$^+$CD25$^-$ T-cell proliferation was analyzed as described in the Materials and Methods. There was a trend, albeit not significant, toward a decrease in Treg-cell function after OK-432 administration (Fig. 4C). In contrast, we did not observe any differences in frequency and function of Treg cells in PBMCs
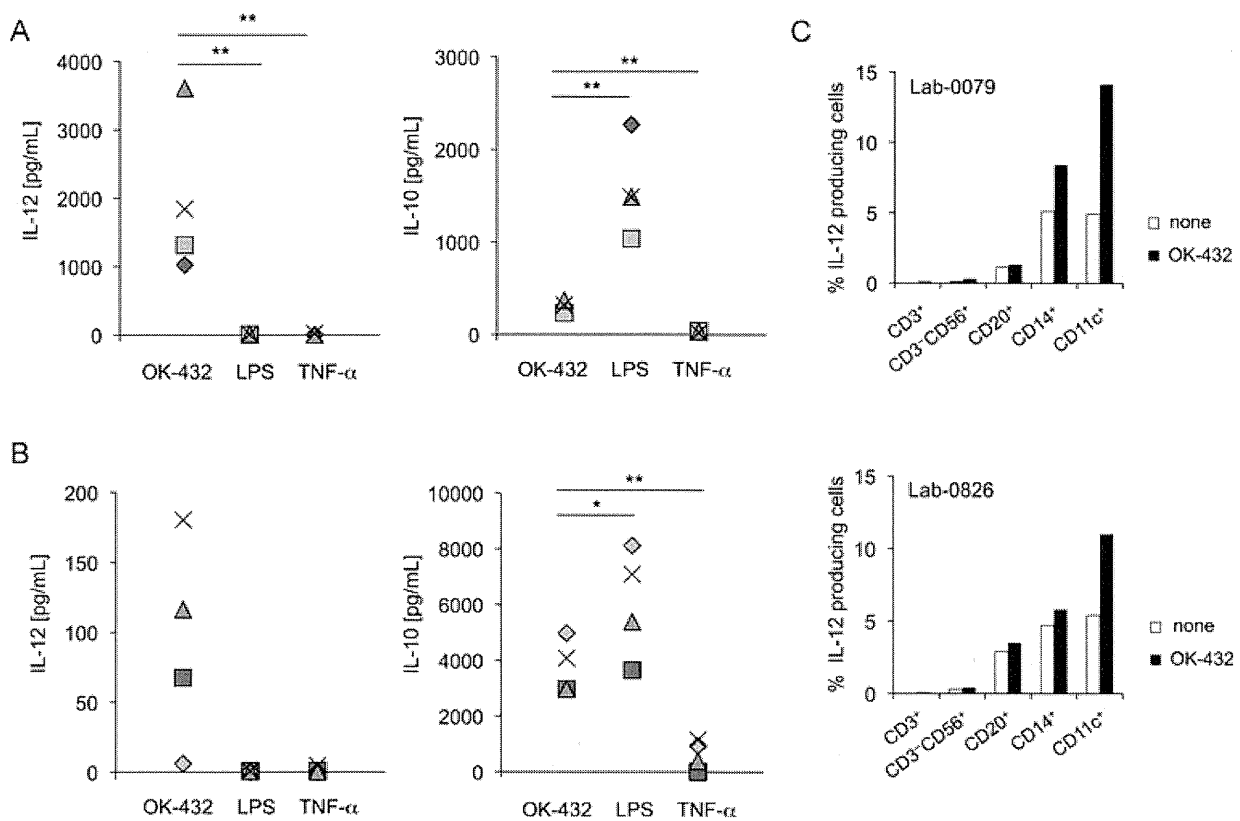
Figure 3. APCs stimulated with OK-432 produce significantly higher amounts of IL-12. (A) 1×10^5 CD3-depleted PBMCs from four healthy individuals were cultured with TNF-α (100 ng/mL), LPS (1 mg/mL), or OK-432 (1 μg/mL) and supernatant was collected 48 h later. IL-12p70 and IL-10 production was analyzed with ELISA. (B) 1×10^5 CD3-depleted PBMCs from four esophageal cancer patients were cultured as in (A) and supernatant was collected 48 h later. IL-12p70 and IL-10 production was analyzed with ELISA. (A, B) Each symbol represents an individual donor; data shown are from one experiment representative of at least two performed. (C) PBMCs from two healthy individuals were cultured with/without OK-432. Cells were subjected to staining with the indicated surface markers and then intracellular IL-12, and were analyzed by flow cytometry. Data of two donors from one experiment representative of at least two performed. $^*p < 0.05$ and $^{**}p < 0.01$ as compared with control, Student's t-test.

before and after OK-432 administration (data not shown). These data propose that in vivo injection of OK-432 decreases the local Treg-cell accumulation and function.

Origin of the repertoire of CD4$^+$ T-cell effectors elicited by vaccination with NY-ESO-1 and OK-432

To further explore the effect of OK-432 on the inhibition of in vivo Treg-cell activity, we also examined the potential of OK-432 as an adjuvant in a cancer vaccine. We have reported that high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors are present in naive CD45RA$^+$ populations and that their activation is rigorously suppressed by CD4$^+$CD25$^+$ Treg cells [20, 21]. We also found that synthetic peptide vaccination with incomplete Freund's adjuvant induces only peptide-specific CD4$^+$ T cells with low-avidity TCRs (recognition of >1 μM peptide but not naturally processed NY-ESO-1 protein), but not high-avidity CD4$^+$ T cells (recognition of naturally processed NY-ESO-1 protein or <0.1 μM peptide) that are susceptible to Treg-cell suppression [21]. Together, these data highlight the importance of blocking Treg-cell activity to allow activation/expansion of high-avidity

NY-ESO-1–specific CD4$^+$ T-cell precursors. For this reason, we investigated whether high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors were activated by NY-ESO-1 protein vaccination with OK-432 as an adjuvant and were present in memory CD45RO$^+$ populations.

Samples from two patients who received vaccination with cholesteryl hydrophobized pullulan (CHP)-HER2 and NY-ESO-1 with OK-432 (Supporting Information Fig. 1) were available for this analysis. Whole CD4$^+$ T cells or CD4$^+$CD25$^-$CD45RO$^+$ (effector/memory) T cells before and after vaccination were presensitized with NY-ESO-1–overlapping peptides covering the entire sequence of NY-ESO-1 and specific CD4$^+$ T-cell induction was analyzed with ELISPOT assays. As the sample size was not sufficient to analyze specific CD4$^+$ T-cell induction within CD4$^+$CD25$^-$CD45RA$^+$ (naive) T cells, we analyzed whether NY-ESO-1–specific high-avidity CD4$^+$ T cells were induced from the CD4$^+$CD25$^-$CD45RO$^+$ (effector/memory) T-cell population after vaccination in Pt #1 (HLA-DR 4, 12 and HLA -DQ 4, 8) and #2 (HLA-DR 9, 15 and HLA-DQ 6, 9). Pt #1 exhibited spontaneously induced CD4$^+$ T-cell responses against NY-ESO-1$_{91-110}$ before vaccination and the responses were maintained after extensive vaccination (Fig. 5A). These spontaneously