Identification of a novel NY-ESO-1 promiscuous helper epitope presented by multiple MHC class II molecules found frequently in the Japanese population

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(Received February 14, 2007/Revised March 22, 2007/Accepted March 25, 2007/Online publication May 1, 2007)

NY-ESO-1 is a cancer-testis antigen that elicits strong cellular and humoral immune responses against NY-ESO-1-expressing tumors. Although CD4+ T cells play a critical role in inducing antitumor immunity, little is known about MHC class II-restricted helper epitopes of the NY-ESO-1 antigen compared with MHC class I-restricted epitopes. Here, we searched for new NY-ESO-1 helper epitopes presented by MHC class II molecules, especially those found frequently in the Japanese population. We established five NY-ESO-1-specific helper T-cell lines from healthy Japanese donors using NY-ESO-1 recombinant protein and peptide. Using MHC class II-specific antibodies and a panel of Epstein–Barr virus-transformed B-cell lines, it was demonstrated that four out of the five T-cell lines recognized a region within NY-ESO-$1_{119–143}$ in the context of HLA-DRB1*0802, DRB1*0901, DRB1*1502 or DRB1*0405/*0410. In addition, using a set of overlapping 15-mer synthetic peptides, we found that NY-ESO-$1_{122–138}$ was a promiscuous region that bound to four distinct HLA-DR molecules found in the Japanese population. These findings expand the usefulness of NY-ESO-1 as a tool for tumor vaccine therapy in eliciting NY-ESO-1-specific helper T-cell responses, especially in Japanese cancer patients. (*Cancer Sci* 2007; 98: 1092–1098)

The cancer-testis (CT) antigen family has been recognized as an attractive target for tumor immunotherapy. These antigens are expressed in a variety of malignant neoplasms but not in normal tissues, with the exception of the testis and the placenta.[1,2] Among these CT antigens, NY-ESO-1, which was first discovered by serological analysis of a recombinant cDNA expression library (SEREX) from an esophageal cancer,[3] was reported to be expressed in a variety of malignancies including melanomas and esophageal, breast, prostate, urinary tract, ovarian and lung cancers.[4–9] NY-ESO-1 was shown to elicit spontaneous humoral and cellular immune responses in a large proportion of patients with NY-ESO-1-positive tumors.[9,10]

Because $CD8^+$ T cells can exert direct cytotoxicity against tumor cells, cancer vaccine studies first focused on the activation of $CD8^+$ T cell-mediated antitumor immunity by MHC class I-restricted tumor peptides.[11–13] However, it appeared to be difficult to induce fully activated $CD8^+$ cytotoxic T cells (CTL) in cancer patients by tumor vaccine therapy using MHC class I-binding peptides because of the defect of helper arm or strong immunosuppression.[14,15] To overcome this problem, many investigators tried to improve the protocol for tumor vaccine therapy using adjuvants in addition to MHC class I-binding peptides.[16] Previously, we proposed that tumor-specific $CD4^+$ T cells, especially Th1 cells, play a critical role in inducing CTL-mediated antitumor immunity in tumor-bearing hosts.[17–20] Although CTL can act alone at the effector phase, $CD4^+$ T cells are required for the priming and maintenance of $CD8^+$ T cells.[21–27] $CD4^+$ T cells stimulate $CD8^+$ T cells through activating antigen-presenting cells, such as dendritic cells (DC), via the CD40–CD154 interaction.[28–32] $CD4^+$ T cells are also reported to stimulate $CD8^+$ T cells through $CD4^+$–$CD8^+$ T-cell communication by cytokines or cell-to-cell interaction.[23,24,33] The high titer of NY-ESO-1 antibodies found in a large proportion of patients suggested that $CD4^+$ T cells efficiently respond to NY-ESO-1 antigen. Recently, a number of MHC class II-restricted NY-ESO-1 epitopes recognized by $CD4^+$ T cells were identified.[34–38] Nevertheless, information on MHC class II-restricted epitopes of the NY-ESO-1 antigen is limited compared with information on the epitopes presented on MHC class I molecules. Moreover, most of the epitopes previously reported were restricted to the human leukocyte antigen (HLA) alleles found mainly among Caucasians (such as DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*1101 and DRB1*1501) and not among Japanese people (such as DRB1*0405, DRB1*0901 and DRB1*1502).[39–41]

In the present paper, we identified four novel NY-ESO-1 epitopes recognized by NY-ESO-1-specific $CD4^+$ T-cell lines established from healthy Japanese donors. These novel NY-ESO-1 helper epitopes were located on the NY-ESO-$1_{122–138}$ promiscuous region and presented by HLA-DRB1*0802, DRB1*0901, DRB1*1502 or DRB1*0405/*0410, whose HLA alleles are found frequently in the Japanese population. Thus, our defined NY-ESO-1 helper epitopes will become a useful tool for developing an efficient strategy for $CD4^+$ T cell-mediated tumor vaccine therapy in Japanese cancer patients.

Materials and Methods

Cell lines and culture medium. Epstein–Barr virus (EBV)-transformed B cells (EBV-B) were generated from peripheral blood mononuclear cells (PBMC) of healthy volunteers by culturing with culture supernatant from the EBV-producing B95-8 cell line after obtaining written informed consent and approval by the medical ethics committees of Hokkaido University Graduate School of Medicine. These cells were maintained in RPMI-1640 (Sigma, St Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Invitrogen, Carlsbad, CA, USA) with 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol (Sigma), 10 mM HEPES, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate. DC and T cells were derived from PBMC of healthy volunteers after obtaining written informed consent.

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doi: 10.1111/j.1349-7006.2007.00501.x

Table 1. HLA-DR genotypes of donors

Donor	HLA-DR genotype	
1	DRB1*0101	DRB1*0802
2	DRB1*0405	DRB1*0901
3	DRB1*0802	DRB1*1502
4	DRB1*0410	DRB1*1201

HLA, human leukocyte antigen.

HLA-DR haplotypes of all donors were typed and are listed in Table 1. DC were cultured in AIM-V medium (Life Technologies, Invitrogen) without serum. T cells were cultured in AIM-V medium containing 5% heat-inactivated pooled human AB serum (kindly donated by Hokkaido Red Cross Blood Center) or 10% heat-inactivated fetal bovine serum.

Peptides and recombinant protein. The peptide NY-ESO-$1_{119–143}$ (PGVLLKEFTVSGNILTIRLTAADHR), which was reported to bind multiple HLA-DR alleles (DRB1*0101, DRB1*0301, DRB1*0701, DRB1*1501, DRB3, DRB4 and DRB5),[34] was synthesized using a solid-phase strategy followed by purification with reverse-phase high-performance liquid chromatography (RP-HPLC), with purity >98% as assessed by RP-HPLC, mass spectrometry and amino acid analysis. Synthetic 15-mer peptides spanning the NY-ESO-$1_{119–143}$ region were synthesized with purity >98%. NY-ESO-1 overlapping peptides were purchased from Sigma Genosys (Hokkaido, Japan). Recombinant NY-ESO-1 protein was expressed in *Escherichia coli* as full-length protein.

***In vitro* generation of DC.** PBMC from healthy volunteers were isolated by Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden) gradient centrifugation. PBMC were incubated for 2 h at 37°C in a six-well plate (Nalge Nunc International, Roskilde, Denmark) in AIM-V medium without serum. Non-adherent cells were removed, and adherent cells were cultured in the presence of 30 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (KIRIN, Tokyo, Japan) and 30 ng/mL interleukin (IL)-3 (KIRIN) in AIM-V without serum. Half of the medium was exchanged for fresh medium containing GM-CSF and IL-3 on days 3 and 5. On day 7, the adherent DC were harvested with trypsine (Sigma) and used as antigen-presenting cell (APC). CD11c expression was >99% for these DC (data not shown).

***In vitro* induction of antigen-specific helper T cells.** $CD4^+$ T cells were purified from PBMC by positive selection with anti-CD4 monoclonal antibody coupled with magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA). *In vitro*-generated DC were pulsed with 50 μg/mL rNY-ESO-1 or 10 μg/mL synthetic peptide for 2 h at 37°C. The antigen-pulsed DC (1×10^4 or 1×10^5) were then treated with mitomycin C (MMC) and mixed with autologous $CD4^+$ T cells (3×10^4 or 1×10^6) in AIM-V supplemented with 5% human AB serum in 96-well round-bottomed or 24-well culture plates (BD Bioscience, San Jose, CA, USA). After 7 days, half of the medium was removed from each culture, and then the culture was added fresh medium containing MMC-treated autologous PBMC pulsed with relevant antigen (50 μg/mL protein or 10 μg/mL peptide) for 2 h. Two days after the second stimulation with antigen, human recombinant IL-2 (kindly supplied by Shionogi Pharmaceutical Institute, Osaka, Japan) was added to each well at a final concentration of 10 IU/mL. One week later, each well was tested for cytokine production in response to peptide-pulsed and MMC-treated autologous PBMC. The well showing a specific response to the relevant peptide was transferred to a 24-well plate (BD Bioscience) and restimulated at weekly intervals with MMC-treated autologous PBMC (1×10^6/well) pulsed with the peptides (10 μg/mL) in medium containing 20 IU/mL recombinant human IL-2.

Antigen-specific cytokine production of T cells. T cells (3×10^4/well) were mixed with MMC-treated autologous PBMC (3×10^4/well) or EBV-B cells (1×10^4/well) in the presence of relevant antigen in a 96-well round-bottomed culture plate (BD Bioscience) and incubated at 37°C in a 5% CO_2 incubator. After 20 h, culture supernatants were collected and the level of cytokines (interferon [IFN]-γ or IL-4) was measured using an enzyme-linked immunosorbent assay (ELISA) kit (BD Bioscience). MHC restriction of T cells was determined by adding anti-HLA-DR monoclonal antibody L243 (BD Bioscience) and anti-HLA-DP monoclonal antibody BRAFB6 (Serotech, Raleigh, NC, USA). All antibodies were used at a final concentration of 5 μg/mL throughout the 20-h incubation period.

Cytotoxicity assay. The cytotoxicity mediated by NY-ESO-1-specific $CD4^+$ T cells was measured using a 6 h ^{51}Cr-release assay, as described previously.[42] Tumor-specific cytotoxicity was determined using peptide-pulsed EBV-B cells as target cells. Unpulsed EBV-B cells were used as control target cells. The percentage cytotoxicity was calculated as described previously.[42]

Results

Generation of NY-ESO-1-specific $CD4^+$ T cells with recombinant protein. For the induction of NY-ESO-1-specific $CD4^+$ T cells, $CD4^+$ T cells from three different healthy donors, each of whom expressed various HLA-DR subtypes, were stimulated *in vitro* with autologous DC loaded with recombinant NY-ESO-1 protein (rNY-ESO-1). Seven days later, the cultured T cells were re-stimulated with autologous DC pulsed with rNY-ESO-1. At days 14 and 21, each of the divided T cells was restimulated with autologous PBMC pulsed with six mixes of overlapping peptides covering the entire NY-ESO-1 protein sequence. At day 28 the T cells were tested for their specific IFN-γ production against overlapping peptides in ELISA. As shown in Fig. 1, the T cells of donor 1 recognized autologous PBMC pulsed with NY-ESO-1 peptide N14, included in peptide mix (MIX) 4, whereas the N20 peptide in MIX5 stimulated the T cells of donors 2 and 3. The other peptide mixes did not stimulate the T cells from three of the donors. We designated these three T cell lines derived from donors 1, 2 and 3 as PreN4, NaN5 and OKN5, respectively. The peptide sequences included in MIX4 and MIX5 are listed in Table 2.

Identification of HLA restriction of NY-ESO-1-specific T-cell lines. For further analysis of the recognized NY-ESO-1 epitope, three specific T-cell lines were restimulated with cognate peptide weekly. To elucidate HLA restriction of the T cells, we first examined the blocking effect of monoclonal antibodies against HLA-DP or HLA-DR in the NY-ESO-1-specific responses of these T cells. The IFN-γ production of all T cells against the cognate NY-ESO-1 peptide was significantly reduced when monoclonal antibodies against HLA-DR were added, whereas monoclonal antibodies against HLA-DP had no effect (Fig. 2a–c). To further analyze the HLA restriction of these T-cell lines, we tested the reactivity of the T cells against a range of peptide-pulsed allogeneic EBV-B cell lines with known HLA-DR alleles. PreN4 T-cell lines recognized only HLA-DRB1*0101-expressing EBV-B cell lines (Fig. 2d). NaN5 T-cell lines released IFN-γ against peptide-pulsed allogeneic EBV-B cell lines with HLA-DRB1*0901, and OKN5 T cell lines recognized peptide-pulsed HLA-DRB1*0802-EBV-B cell lines (Fig. 2e,f). The N14 peptide sequence is LEFYLAMPFATPME (88–101), and the epitope recognized by PreN4 T-cell lines in the context of HLA-DR1 was reported previously by Mandic *et al*.[36] However, the N20 peptide (124–138) recognized by NaN5 lines and OKN5 lines is contained within a peptide sequence reported previously by Zarour *et al*. as a promiscuous peptide.[34] We newly identified that this region binds to HLA-DRB1*0802 and DRB1*0901, so we focused on this region in further experiments.

Induction of NY-ESO-1-specific $CD4^+$ T cells with synthetic peptide NY-ESO-$1_{119–143}$ and identification of its HLA restriction. To test whether

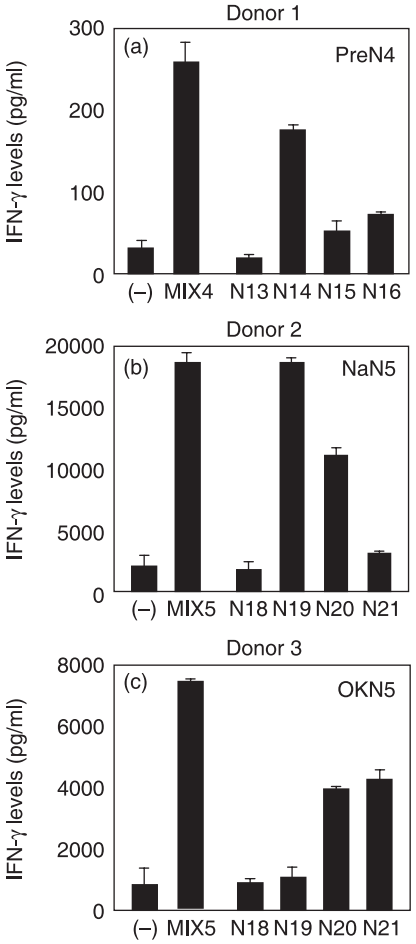

Fig. 1. Induction of NY-ESO-1-specific CD4⁺ T cells by dendritic cells (DC) pulsed with a recombinant NY-ESO-1 protein. (a–c) CD4⁺ T-cell cultures of healthy donors 1, 2 and 3 stimulated with autologous DC pulsed twice with a recombinant NY-ESO-1 protein. The cultures were then restimulated with autologous peripheral blood mononuclear cells (PBMC) pulsed twice with peptide mixtures containing some NY-ESO-1 overlapping peptides. At day 28 they were tested for their reactivity against autologous PBMC alone, cognate peptide mixture, or pulsed with 10 μg/mL of the overlapping peptide mixture. The T cells were incubated with the mitomycin C-treated target cells in duplicate for 20 h, and the culture supernatants were then assessed for interferon-γ levels by enzyme-linked immunosorbent assay. These experiments were carried out independently at least three times.

peptides containing this region induce peptide-specific T cells from freshly isolated CD4⁺ T cells, we prepared synthetic NY-ESO-$1_{119–143}$ peptide (PGVLLKEFTVSGNILTIRLTAADHR). Purified CD4⁺ T cells from two healthy donors, donors 3 and 4, were stimulated with their autologous DC pulsed with the NY-ESO-$1_{119–143}$ peptide. HLA-DR alleles of donor 4 are also listed in Table 1. CD4⁺ T cells were restimulated with autologous PBMC pulsed with NY-ESO-$1_{119–143}$ peptide at day 7, and tested for their reactivity against NY-ESO-1 at day 14. As shown in Fig. 3a,b. T cells derived from both of the healthy donors produced significant IFN-γ against autologous PBMC pulsed NY-ESO-$1_{119–143}$ peptide, but did not respond when autologous PBMC were not pulsed with any peptides. Moreover, these T cells also produced IFN-γ when cultured with their autologous PBMC pulsed with rNY-ESO-1 protein, suggesting that they recognized naturally processed NY-ESO-1 epitopes. These CD4⁺ T cells were further expanded by weekly stimulation. We designated these T-cell lines derived from donors 3 and 4 as

Table 2. Sequences of the 15-mer peptides comprising the overlapping peptide mixtures

Mix	No.	Position	Sequence
MIX4	N13	80–105	ARGPESRLLEFYLAMP
	N14	88–101	LEFYLAMPFATPME
	N15	93–107	AMPFATPMEAELARR
	N16	99–113	PMEAELARRSLAQDA
	N17†	105–119	ARRSLAQDAPPLPVP
MIX5	N18	111–126	QDAPPLPVPGVLLKEF
	N19	118–134	VPGVLLKEFTVSGNILT
	N20	124–138	KEFTVSGNILTIRLT
	N21	128–142	VSGNILTIRLTAADH

†N17 peptide was not used.

OKR26 and WAK3, respectively. The IFN-γ production of these T-cell lines against PBMC pulsed with a cognate NY-ESO-1 peptide was inhibited by monoclonal antibodies against HLA-DR but not against HLA-DP (Fig. 4a,b). Furthermore, OKR26 T-cell lines from donor 3 significantly produced IFN-γ in response to allogeneic HLA-DRB1*1502-expressing EBV-B cell lines pulsed with the peptide, suggested that OKR26 T-cell lines are restricted with DRB1*1502 (Fig. 4c). WAK3 T-cell lines from donor 4 responded to allogeneic HLA-DRB1*0405-expressing EBV-B cell lines in the presence of the peptide (Fig. 4d), but not against the allogeneic HLA-DRB1*1201-expressing cell line despite the fact that HLA-DRB1*1201 was expressed in autologous cells. Although we did not have allogeneic EBV-B cell lines with HLA-DR*0410, these data suggest that WAK3 T-cell lines are restricted with HLA-DR*0410 and cross-reacted with HLA-DRB1*0405 in response to the NY-ESO-$1_{119–143}$ peptide.

Identification of NY-ESO-1 minimal epitopes recognized by the CD4⁺ T-cell lines. To identify NY-ESO-1 minimal epitopes recognized by these CD4⁺ T-cell lines, a series of 15-mer overlapping peptides derived from NY-ESO-$1_{119–143}$ were synthesized and tested for their ability to stimulate IFN-γ release of these T cells. Because we failed to expand OKN5 T-cell lines, which are restricted to HLA-DRB1*0802, we were not able to carry out further experiments with this T-cell line. As shown in Fig. 5a, NaN5 T-cell lines generated by rNY-ESO-1 protein recognized autologous PBMC pulsed with p3, p4, p5 and p6 peptides, suggesting that this minimal region was NY-ESO-$1_{124–135}$ (KEFTVSGNILTI). As shown in Fig. 5b, OKR26 T-cell lines recognized autologous PBMC pulsed with p3, p4, p5, p6, p7 and p8 peptides, suggesting that this minimal region of NY-ESO-1 was NY-ESO-$1_{126–135}$ (FTVSGNILTI). As shown in Fig. 5c, WAK3 T-cell lines were stimulated by p1, p2, p3 and p4 peptides, suggesting that the minimal region existed within the region NY-ESO-$1_{122–133}$ (LLKEFTVSGNIL). These minimal regions of three T-cell lines are listed in Fig. 5d.

NY-ESO-1-specific CD4⁺ T-cell lines show both cytokine production and cytotoxicity. We assessed whether three CD4⁺ T-cell lines (NaN5, OKR26 and WAK3) show both Th1-cytokine production and cytotoxicity in response to cognate peptide-pulsed target cell lines in the context of the restricted HLA. As shown in Fig. 6, NaN5 T-cell lines efficiently lysed an allogeneic DRB1*0901-expressing EBV-B cell line in the presence of NY-ESO-$1_{119–143}$ peptide, but not in the absence of this peptide (Fig. 6a). Both OKR26 lines and WAK3 lines also displayed cytotoxicity against a peptide-pulsed EBV-B cell line in the context of their restricted HLA (Fig. 6b,c). It was also demonstrated that all CD4⁺ T-cell lines produce large amounts of IFN-γ but not IL-4 in response to relevant antigens (Fig. 6d–f).

doi: 10.1111/j.1349-7006.2007.00501.x

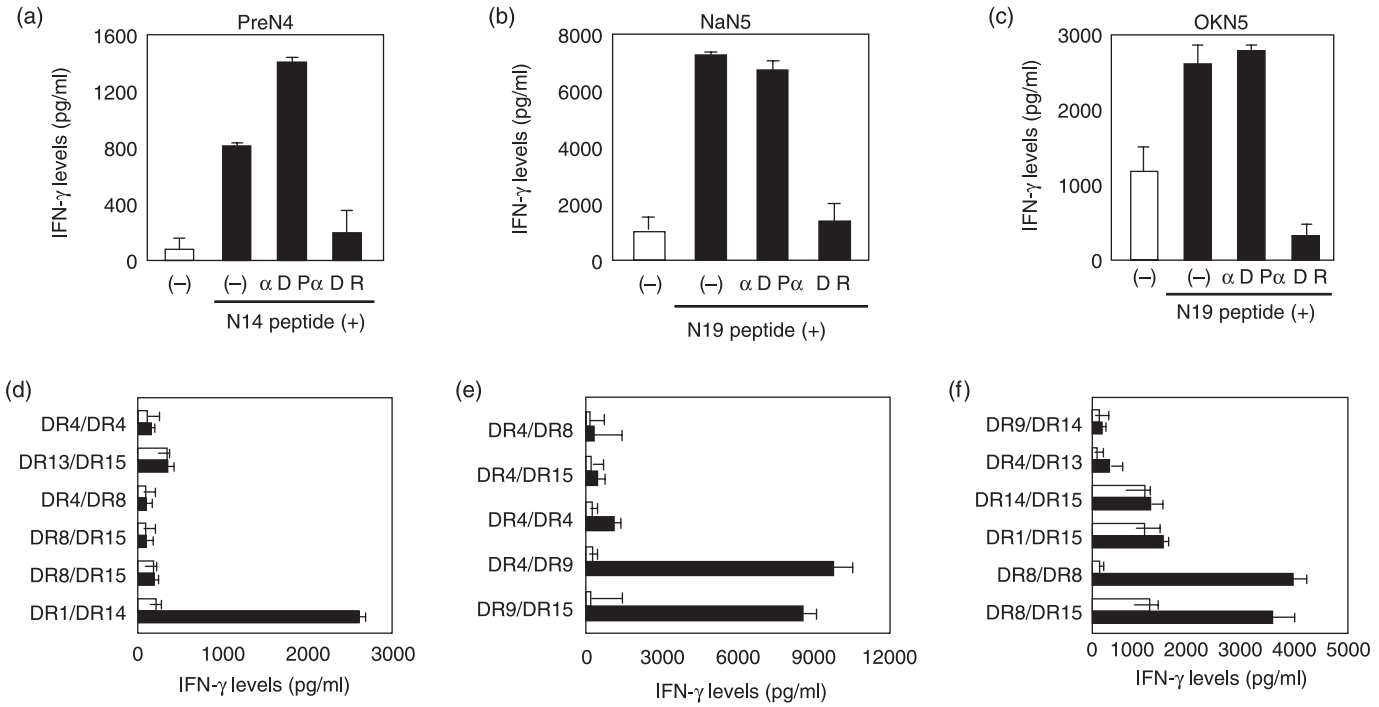

Fig. 2. MHC restriction analysis of the NY-ESO-1-specific T-cell lines generated by a recombinant NY-ESO-1 protein. (a,c,e) Mitomycin C (MMC)-treated autologous peripheral blood mononuclear cells pulsed with or without 10 μg/mL cognate NY-ESO-1 peptide were incubated together with PreN4 lines, NaN5 lines or OKN5 lines in the presence of monoclonal antibodies against HLA-DP or HLA-DR. (b,d,f) MMC-treated allogeneic Epstein–Barr virus-B-cell lines with known HLA-DR haplotypes pulsed with 10 μg/mL cognate NY-ESO-1 peptide were cultured with PreN4 lines, NaN5 lines or OKN5 lines. The culture supernatants were then assessed for interferon-γ levels by enzyme-linked immunosorbent assay. These experiments were carried out independently at least three times.

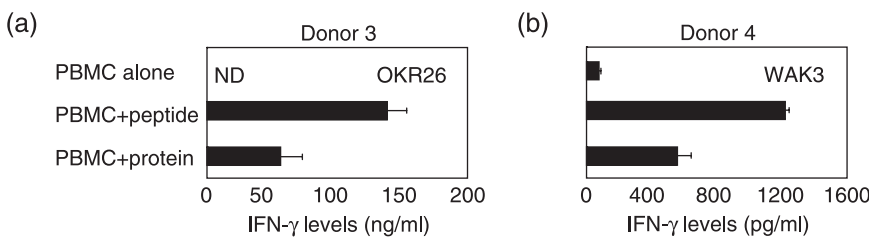

Fig. 3. Induction of the NY-ESO-1-specific T cells by dendritic cells pulsed with a promiscuous NY-ESO-$1_{119–143}$ peptide. (a,b) $CD4^+$ T-cell cultures of healthy donors 3 and 4 stimulated with promiscuous NY-ESO-$1_{119–143}$ peptide were tested for their reactivity at day 21 against autologous peripheral blood mononuclear cells alone, pulsed with 10 μg/mL relevant peptide, or pulsed with 50 μg/mL recombinant NY-ESO-1 protein. T cells (3×10^4/well) were incubated with the mitomycin C-treated target cells (3×10^4/well) in duplicate for 20 h, and the culture supernatants were then assessed for interferon-γ levels by enzyme-linked immunosorbent assay. These experiments were carried out independently at least three times. ND, not detected.

Thus, these $CD4^+$ T-cell lines were demonstrated to be NY-ESO-1-specific Th1 cells with cytotoxicity.

Discussion

The CT antigen, NY-ESO-1, is considered an important target for immunotherapeutic interventions because it has been shown to induce spontaneous humoral and cellular immune responses in a large proportion of patients with NY-ESO-1-expressing tumors.[35,43] A number of class I-restricted NY-ESO-1 epitopes recognized by $CD8^+$ CTL have been defined to develop an efficient tumor peptide vaccine therapy.[11] However, recent studies have demonstrated an important role for $CD4^+$ T cells in maintaining and enhancing antitumor immunity by $CD8^+$ CTL.[21–27] In particular, high titers of NY-ESO-1-reactive antibodies were detected frequently in patients with NY-ESO-1-expressing tumors,[11,44] whereas the production of high titers of antibodies against the melanoma antigen (MAGE) or differentiation antigens, such as tyrosinase, gp100, TRP-1 or TRP-2, have not been reported very often.[44] These studies suggest that NY-ESO-1-reactive $CD4^+$ T cells play a critical role in antitumor immunity in patients with NY-ESO-1-expressing tumors, presumably in both humoral and cellular immunity. Therefore, recent studies have focused on the identification of MHC class II-restricted helper epitopes recognized by $CD4^+$ T cells specific to tumor-associated antigens, including NY-ESO-1.[34–38] However, MHC restriction elements for the reported helper epitopes of NY-ESO-1 have been mostly limited to MHC class II alleles

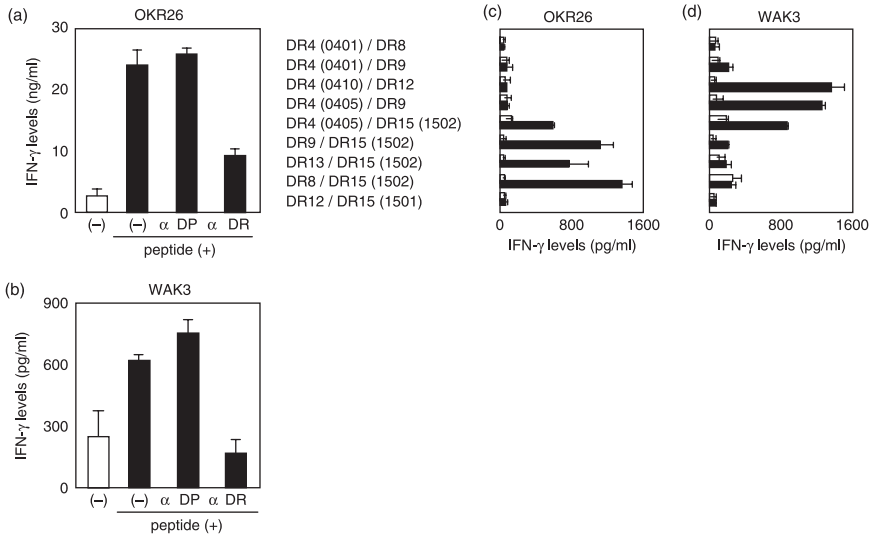

Fig. 4. MHC-restriction analysis of the NY-ESO-1-specific T-cell lines generated by a promiscuous NY-ESO-$1_{119–143}$ peptide. (a,b) Mitomycin C (MMC)-treated autologous peripheral blood mononuclear cells pulsed with or without 10 μg/mL NY-ESO-$1_{119–143}$ and (c,d) MMC-treated allogeneic Epstein–Barr virus-B-cell lines with known HLA-DR haplotypes pulsed with 10 μg/mL NY-ESO-$1_{119–143}$ were incubated together with OKR26 lines or WAK3 lines in the presence of monoclonal antibodies against HLA-DP or HLA-DR. The culture supernatants were then assessed for interferon-γ levels by enzyme-linked immunosorbent assay. These experiments were carried out independently at least three times.

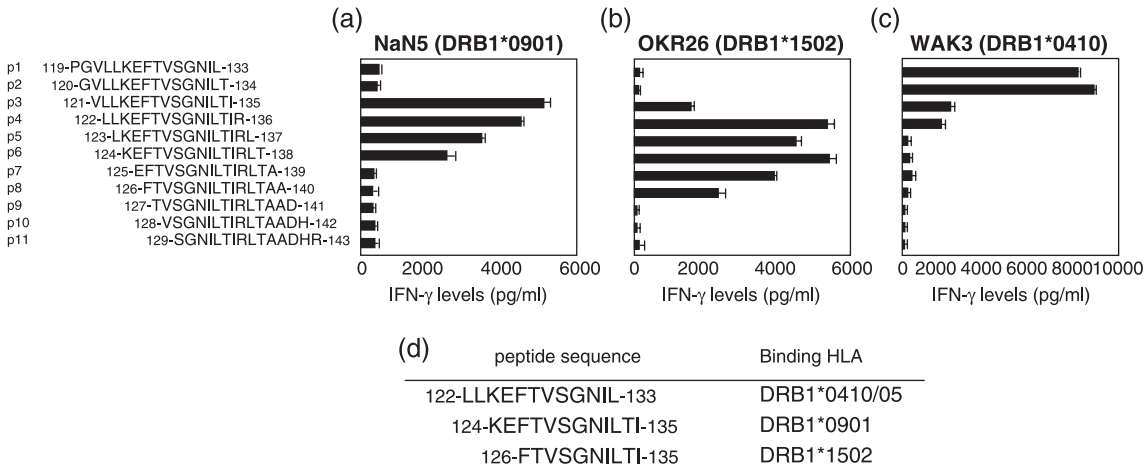

Fig. 5. Recognition of different epitopes within NY-ESO-$1_{119–143}$ by three $CD4^+$ T-cell lines (NaN5, OKR26 and WAK3). (a–c) NaN5, OKR26 and WAK3 were cultured with autologous peripheral blood mononuclear cells pulsed with a series of 15-mer overlapping peptides encompassing NY-ESO-$1_{119–143}$ in duplicate for 20 h. The culture supernatants were then assessed for interferon-γ levels by enzyme-linked immunosorbent assay. (d) Each of the recognition regions is listed. These experiments were carried out independently at least three times.

such as HLA-DRB1*0101, DRB1*0301, DRB1*0401 and DRB1*0701,[34–36] which are expressed frequently in Caucasian but not Japanese populations.

To expand the panel of MHC class II-restricted NY-ESO-1 epitopes that can be used to monitor spontaneous and vaccine-induced NY-ESO-1-specific immune responses, or to elicit effective immune responses in cancer patients, we aimed to identify helper epitopes presented by HLA alleles that occur frequently throughout the Japanese population, such as DRB1*0405, DRB1*0901 and DRB1*1502, occurring in 29, 23 and 16% of Japanese people, respectively. By stimulating $CD4^+$ T cells derived from healthy donors with NY-ESO-1 recombinant protein and peptide,[34] we generated five NY-ESO-1-specific $CD4^+$ T-cell lines (Figs 1,3). Four of these T-cell lines appeared to recognize novel NY-ESO-1-specific epitopes in the context of HLA-DRB1*0802, DRB1*0901, DRB1*1502 and DRB1*0405/*0410 (Figs 2,4). To our knowledge, this is the first report of a T-cell epitope from NY-ESO-1 presented by HLA-DRB1*0802, DRB1*0901 and DRB1*0405/*0410. Recently, another T-cell epitope from NY-ESO-1 presented by HLA-DRB1*1502 was reported by Hasegawa *et al*.[45] The DRB1*1502-restricted OKR26 T-cell lines failed to respond to DRB1*1501-expressing cell lines in the presence of the cognate peptide. Moreover, WAK3 T-cell lines responded not only to DRB1*0410 but also to DRB1*0405-expressing allogeneic cell lines, but not DRB1*0401 (Fig. 4d). This may indicate that the anchor position of DRB1*0410 is shared with DRB1*0405 but not with DRB1*0401. We confirmed that DRB1*0401-restricted T cell lines failed to recognize DRB1*0405-expressing cell lines (data not shown). Therefore, HLA restriction should be defined to the level of HLA alleles, but not to the serotype level.

NY-ESO-1 is known as one of the most immunogenic tumor antigens. This might be related to its ability to be presented by MHC molecules on APC. Indeed, it is reported that NY-ESO-1 has some promiscuous regions that bind to various HLA alleles.[34,36] However, previous reports focused only on HLA alleles

doi: 10.1111/j.1349-7006.2007.00501.x

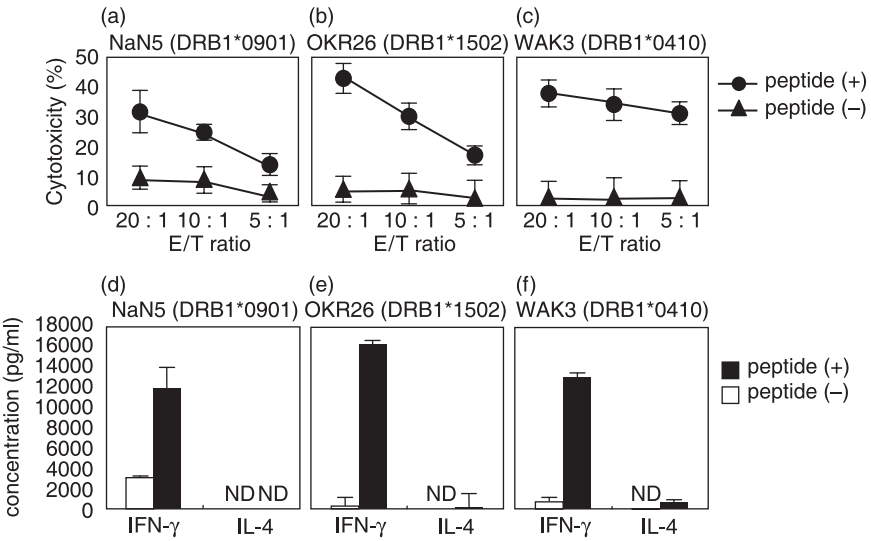

Fig. 6. Cytotoxicity and Th1-cytokine production of three CD4+ T-cell lines (NaN5, OKR26 and WAK3) in response to the cognate peptide-pulsed Epstein–Barr virus-B-cell lines (EBV-B). (a–c) The peptide-specific cytotoxicities of NaN5, OKR26 and WAK3 were tested using a 4 h,[51] chrominium-release assay against the human leukocyte antigen (HLA)-matched EBV-B pulsed with NY-ESO-$1_{119–143}$ peptide or unpulsed. (d–f) The HLA-matched EBV-B pulsed with 10 μg/mL NY-ESO-$1_{119–143}$ were cultured with NaN5, OKR26 or WAK3, and the culture supernatants were then assessed for interferon-γ and interleukin-4 levels by enzyme-linked immunosorbent assay. These experiments were carried out independently at least three times. ND, not detected.

found throughout the Caucasian population. Therefore, our work described here is the first report that focuses on HLA alleles found frequently throughout the Japanese population. In our protocol using a whole protein, we accidentally identified multiple epitopes within the peptide that were previously known to bind other alleles. Thus, our finding expands the usefulness of already known promiscuous helper regions, making it a powerful tool applicable to cancer patients throughout the world.

Both the OKN5 and NaN5 lines were generated using a recombinant NY-ESO-1 protein, so the epitopes of these T-cell lines were processed and presented naturally. OKR26 lines and WAK3 lines recognized autologous PBMC pulsed with not only the cognate peptide but also the recombinant NY-ESO-1 protein, suggesting that these epitopes were also processed and presented naturally (Fig. 3). Several peptide-induced CTL and helper T-cells lines that failed to recognize antigen-expressing cells have been reported.[46,47] Moreover, Gal *et al*. demonstrated distinct T-cell receptor (TCR) repertoires in naturally occurring versus vaccine-induced CTL responses to NY-ESO-1 antigen.[48] For future applications in the active immunization of cancer patients with epitope peptides, it is important to develop a method to induce antigen-specific T cells that can react to antigen-expressing cells. To identify new helper epitope peptides that are presented efficiently by APC, the NY-ESO-1 protein was first chosen as a stimulatory antigen. Simultaneous (four times) stimulation with protein alone failed to generate NY-ESO-1-specific CD4+ T cells but, rather, expanded non-specific CD4+ T cells. Therefore we improved the stimulation protocol to a combination stimulation consisting of two times protein and two times peptide stimulation. Using this stimulation protocol, we successfully expanded CD4+ T cells that could produce IFN-γ and IL-2 in response to APC pulsed with NY-ESO-1 protein antigen. The reasons why the peptide-induced T cells we generated could recognize protein-pulsed PBMC are unclear at present; analysis of the mechanisms underlying the efficient induction of T cells capable of recognizing naturally processed antigen is required in future studies. Moreover, NaN5, OKR26 and WAK3 lines showed both cytotoxic activity and Th1-cytokine production against cognate peptide-pulsed HLA-matched EBV-B cells (Fig. 6).

Several vaccination strategies utilizing NY-ESO-1 peptides, protein, DNA and recombinant viral vectors combined with different adjuvants or drug delivery systems have been evaluated for their efficiency in inducing antitumor immune responses, including specific CD4+ and CD8+ T-cell responses.[36,49,50] The monitoring of comprehensive immune responses and functional analysis of vaccinated cancer patients will help our understanding of vaccine-induced immune responses, which will be used to obtain favorable clinical responses. Therefore, an expanded panel of epitopes for CD4+ T cells is indispensable for developing efficient vaccine strategies that will benefit cancer patients with NY-ESO-1-expressing tumors.

Previous reports have shown that CD4+ T-cell responses to NY-ESO-1 are only detectable in cancer patients with antibodies against NY-ESO-1. In contrast, induction of NY-ESO-1-specific CD4+ T cells from healthy donors is limited by the removal of CD4+ CD25+ regulatory T cells from the culture.[51] In our study, however, NY-ESO-1-specific CD4+ T cells were induced from whole CD4+ T cells of healthy donors. It remains unclear why such a discrepancy occurred between previous results and our results. However, one possible explanation may be a different protocol used for inducing NY-ESO-1-specific CD4+ T cells. In a previous study, adherent cells from PBMC were used as APC. The adherent cells were cultured overnight without any cytokines to use as APC. In contrast, our study used adherent cell-derived DC generated by culture with cytokines for 1 week. Such DC are known as professional APC, which can efficiently present antigens and stimulate T cells. Therefore, our protocol may be superior for inducing NY-ESO-1-specific CD4+ T cells from healthy donors without removal of regulatory T cells. Using another tumor antigen such as MAGE-A4, antigen-specific CD4+ T cells were also induced from healthy donors using our protocol (unpublished data).

In conclusion, we identified a novel NY-ESO-$1_{122–138}$ promiscuous epitope recognized by CD4+ T cells in the context of HLA-DRB1*0802, DRB1*0901, DRB1*1502 and DRB1*0405/0410 that found frequently throughout the Japanese population. This finding expands the panel of epitopes applicable to the monitoring of NY-ESO-1-specific immune responses and to the active immunization of patients with NY-ESO-1-expressing tumors, and may help in the development of an efficient immunotherapy for cancer patients.

Acknowledgments

This study was supported by a Grant-in Aid from the Ministry of Education Culture, Sports, Science, and Technology, a Grant-in-Aid for Scientific Research on priority Areas, a Grant-in-Aid for Immunological Surveillance and its Regulation, and a Grant-in-Aid from the Ministry of Education, Culture Sports, Science and Technology Cancer Translational Research Project. We would like to thank Hokkaido Red Cross Blood Center for kind donations of pooled human AB serum. We would like to thank Takuko Sawada (Shionogi Pharmaceutical Institute, Osaka, Japan) for kind donations of recombinant IL-2.

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doi: 10.1111/j.1349-7006.2007.00501.x