EBNA1-specific $CD4^+$ T-cell responses have been shown to be mainly T helper type 1 in nature (Bickham *et al.*, 2001), with direct recognition of EBV-transformed cells (Khanna *et al.*, 1997; Munz *et al.*, 2000). Several MHC class II-restricted EBNA1 epitopes have been identified (Khanna *et al.*, 1995; Kruger *et al.*, 2003; Leen *et al.*, 2001; Paludan *et al.*, 2002; Voo *et al.*, 2002), implying that EBNA1-specific $CD4^+$ T cells may play a role in controlling tumour growth *in vivo*. Surprisingly, recent studies demonstrated that EBNA1-specific $CD8^+$ CTLs moderately lyse EBV-transformed lymphoblastoid cell lines (LCLs) and suppress LCL outgrowth *in vitro* (Lee *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004). Defective ribosomal products of EBNA1 were shown to be the sources of EBNA1 CTL epitopes presented on the cell surfaces.

In this study, we induced $CD8^+$ EBNA1-specific CTL clones from peripheral blood mononuclear cells (PBMCs) by using GAr-containing EBNA1 mRNA-transfected dendritic cells (DCs) as antigen-presenting cells (APCs). Antigen-transduced DCs have been demonstrated to prime antigen-specific CTLs efficiently *in vitro* (Grunebach *et al.*, 2003; Heiser *et al.*, 2000; Muller *et al.*, 2004; Nair *et al.*, 1998) and *in vivo* (Heiser *et al.*, 2002; Nair *et al.*, 2000; Su *et al.*, 2003; Zeis *et al.*, 2003). Furthermore, we identified a novel human leukocyte antigen (HLA)-C-restricted CTL epitope and confirmed EBNA1 recognition by CTL clones using tetrameric MHC–peptide complexes (tetramer). Finally, we estimated frequencies of EBNA1-specific CTL precursors (CTLp) in PBMCs of healthy EBV-seropositive donors and assessed effects of the CTL clones on EBNA1-expressing cells *in vitro*.

METHODS

Donors and cell lines. The study design and purpose, approved by the institutional review board of Aichi Cancer Center, Nagoya, Japan, were explained fully to all blood donors and informed consent was obtained. CD40-activated B (CD40-B) cells were generated from PBMCs of blood donors as described previously (Kondo *et al.*, 2002; Schultze *et al.*, 1997). Briefly, PBMCs were cultured with irradiated human CD40L-transfected NIH3T3 cells (referred as to t-CD40L; kindly provided by Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA, USA), recombinant interleukin-4 (IL-4) (Genzyme) and cyclosporine A (Sandoz) in the culture medium. Expanding CD40-B cells were stimulated twice a week. LCLs were prepared by transforming PBMCs with B95-8 cell-culture supernatant as described previously (Kuzushima *et al.*, 1999) and cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 U penicillin ml^{-1}, 50 μg streptomycin ml^{-1} and 50 μg kanamycin ml^{-1} (referred to as complete culture medium). HLA-Cw*0303-expressing gastric carcinoma cells [MKN45 (referred to as MKN45-Cw0303) cells] were generated from MKN45 cells by retroviral transduction using Phoenix GALV cells (kind gifts from Dr Kiem, Fred Hutchinson Cancer Research Center, Seattle, WA, USA, and Dr Nolan, Stanford University, Stanford, CA, USA). Retroviral transduction of HLA genes was performed as described previously (Akatsuka *et al.*, 2002; Kondo *et al.*, 2002). EBNA1 (without GAr)-expressing MKN45-Cw0303 cells (referred to as MKN45-Cw0303-ΔGA-EBNA1) were then generated from MKN45-Cw0303 cells by lentivirus transduction (Bai *et al.*, 2003). For this, an EBNA1-coding sequence without GAr (EBNA1 codons 92–323) was inserted into the lentivirus self-inactivating vector (CSIICMV-MCS; kindly provided by Dr Hiroyuki Miyoshi, RIKEN BioResource Center, Tsukuba, Japan) (Bai *et al.*, 2003). MKN45-Cw0303 cells expressing full-length EBNA1 (referred to as MKN45-Cw0303-full-EBNA1) were also generated from MKN45-Cw0303 cells by mRNA transduction. Transduction of *in vitro*-transcribed full-length EBNA1 mRNA was performed as described above. MKN45-Cw0303 and MKN45-Cw0303-ΔGA-EBNA1 cells were cultured in complete culture medium with 1.0 μg puromycin ml^{-1}.

Preparation of DCs. DCs were prepared as described previously (Dauer *et al.*, 2003; Romani *et al.*, 1994; Sallusto & Lanzavecchia, 1994). Briefly, $CD8^+$ T cells were isolated from PBMCs by using CD8 MicroBeads (Miltenyi Biotec) and stored at −135 °C. The CD8-depleted PBMCs were suspended in 4 ml RPMI 1640 medium supplemented with 5% human serum (MP Biomedicals), 2 mM L-glutamine, 50 U penicillin ml^{-1}, 50 μg streptomycin ml^{-1} and 50 μg kanamycin ml^{-1} (referred to as DC medium) and incubated for 2 h in six-well plates at 37 °C. Non-adherent cells were removed by gentle pipetting and adherent cells were cultured in DC medium in the presence of 50 ng granulocyte–macrophage colony-stimulating factor ml^{-1} (GM-CSF; Osteogenetics) and 10 ng IL-4 ml^{-1} (Osteogenetics). On days 2 and 4, half of the medium was replaced with fresh DC medium containing GM-CSF and IL-4. On day 6, DCs were collected and electroporated for mRNA transduction.

Production and transduction of *in vitro*-transcribed mRNA. To generate *in vitro*-transcribed EBNA1 mRNA, a pcDNA/EBNA1 vector was constructed. The coding sequence for EBNA1 was obtained by extraction of total RNA from B95-8-transformed LCLs using an RNeasy kit (Qiagen) and, after reverse transcription, EBNA1 cDNA was amplified by PCR with specific primers as follows: EBNA1 forward primer, 5′-AAGCTTGCCACCATGTCTGACGAGGGGCCAGGTACAG; reverse primer, 5′-GAATTCTCACTCCTGCCCTTCCTCACCCTC. The full-length EBNA1 fragment was then ligated into pcDNA3.1(+) (Invitrogen) using its *Hin*dIII and *Eco*RI sites (pcDNA/EBNA1). Clones were sequenced to verify their identity. Resulting plasmid DNA was linearized and transcribed *in vitro* by using an mMESSAGE and mMACHINE kit (Ambion) according to the manufacturer's instructions. A 3′-poly(A) tail was added by using poly(A) polymerase (Ambion) followed by purification with an RNeasy kit. The resulting mRNA was visualized by using the Reliant RNA gel system (Cambrex). DCs and CD40-B cells were transfected with mRNA by electroporation. First, they were washed twice with serum-free RPMI 1640 medium and suspended at a final concentration of 2.5×10^7 cells ml^{-1}. After mixing with 20 μg mRNA in 40 μl RPMI 1640 medium, they were then electroporated in a 2 mm cuvette by using an Electro Square Porator ECM 830 (Harvard Apparatus), under conditions of 450 V and 500 μS for DCs and 350 V and 350 μS for CD40-B cells. DCs were subsequently cultured in DC medium supplemented with GM-CSF and IL-4 for 3 h, followed with tumour necrosis factor alpha (PeproTech), IL-1β (PeproTech) and prostaglandin E_2 (Cayman Chemical) for maturation. CD40-B cells were seeded immediately onto irradiated t-CD40L cells and, after 36–48 h, these cells were used as APCs.

EBNA1 staining. EBNA1 mRNA-transfected CD40-B cells were collected and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, cells were permeabilized with PBS containing 0.5% Tween 20 and reacted with anti-EBNA1 rabbit polyclonal antibodies (kindly provided by Dr Tatsuya Tsurumi, Aichi Cancer Center Research Institute, Nagoya, Japan) for 30 min at 4 °C. After washing with PBS, cells were stained with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (Beckman Coulter) for 30 min at 4 °C. The stained cells were analysed by FACSCalibur (BD Biosciences) using CELLQUEST software (BD Biosciences).

CTL induction. The stored $CD8^+$ T cells were thawed, washed and co-cultured with irradiated (33 Gy) autologous EBNA1 mRNA-transfected DCs in 200 µl RPMI 1640 medium supplemented with 10 % human serum, 2 mM L-glutamine, 50 U penicillin ml^{-1}, 50 µg streptomycin ml^{-1} and 50 µg kanamycin ml^{-1} (referred to as CTL medium) in the presence of 5 ng IL-7 ml^{-1} (R&D Systems) and 5 ng IL-12 ml^{-1} (R&D systems) at 5 % CO_2 in a humidified incubator. On days 8, 16 and 23, T cells were restimulated with EBNA1 mRNA-transfected and irradiated DCs. One day after each restimulation, IL-2 (Shionogi) was added to a final concentration of 20 U ml^{-1}. To establish T-cell clones, limiting dilution of polyclonal CTLs was performed (Kuzushima *et al.*, 2001). In brief, polyclonal $CD8^+$ T cells were seeded at 1 cell per well in round-bottomed 96-well plates containing CTL medium with a monoclonal antibody (mAb) specific to CD3 (30 ng ml^{-1}; Ortho Biotech), 1×10^5 irradiated (33 Gy) PBMCs and 2×10^4 irradiated (55 Gy) LCLs. The next day, IL-2 was added to each well (50 U ml^{-1}). After 2 weeks culture, growing wells were split into two replicates and used as effectors in ELISPOT assays against either autologous EBNA1 mRNA-transfected CD40-B cells or autologous LCLs. Positive wells were transferred into flasks and expanded with anti-CD3 mAb, irradiated feeder cells and IL-2.

ELISPOT assays. ELISPOT assays were performed as described previously (Kuzushima *et al.*, 2003). Briefly, $CD8^+$ T cells were co-cultured with various stimulators in wells of Multiscreen-HA plates (Millipore) coated with anti-human gamma interferon (IFN-γ) mAb (Pierce Biotechnology). As stimulators, (i) autologous EBNA1 mRNA-transfected CD40-B cells or non-transfected CD40-B cells and (ii) autologous or allogeneic LCLs (1×10^5 cells per well) were seeded into each well. For peptide-titration assays and overlapping-peptide assay, serial concentrations of synthetic peptides were pulsed to autologous CD40-B cells for 1 h at room temperature. After probing with anti-human IFN-γ rabbit polyclonal antibodies (Pierce Biotechnology) followed by exposure to horseradish peroxidase-labelled anti-rabbit IgG antibody (Genzyme) and substrate, the plates were washed and dried. IFN-γ spots were enumerated under a dissecting microscope. The numbers of spots were confirmed by three investigators.

Peptides. Peptides were purchased from Bio-Synthesis Inc. The whole EBNA1 protein, excluding the GAr, deduced from the prototype B95-8 DNA sequence (GenBank accession no. V01555) was covered by 20 aa long synthetic peptides overlapping by 13 aa (total of 56 peptides). In addition, potential epitope peptides VYGGSKTSL (509–517), FVYGGSKTSL (508–517) and VFVYGGSKTSL (507–517) were synthesized, as predicted by the program SYFPEITHI (http://www.syfpeithi.de/) (Rammensee *et al.*, 1999). The known EBNA1 epitope peptide HPVGEADYFEY (Blake *et al.*, 1997) was also synthesized.

Tetramer production and staining. HLA-Cw*0303 and -Cw*0304 cDNA clones were used as templates to amplify sequences encoding the extracellular domains of HLA-Cw*0303 and -Cw*0304 heavy chains with primers C03F (5′-AACCATGGGCAGCCATTCTATGCGCTATTTTTACACCGCTGTGTCCCGGCC-3′) and C03R (5′-AAGGATCCTGGCTCCCATCTCAGGGTGAGG-3′). C03F contains several base changes designed to optimize protein expression in *Escherichia coli* BL21 (DE3) pLysS. The PCR product was digested with *Nco*I and *Bam*HI and cloned into a vector containing a BirA biotinylation site in frame with the 3′ end of the HLA sequence. Recombinant HLA-B*3501 protein was produced by using pGMT7-B35 (a kind gift of Dr McMichael, Weatherall Institute of Molecular Medicine, Oxford, UK). Recombinant MHC molecules were folded *in vitro* with β2-microglobulin and epitope peptides FVYGGSKTSL or VFVYGGSKTSL. Soluble complexes, purified by gel filtration, were biotinylated by using the BirA enzyme (Avidity LCC). Phycoerythrin (PE)-labelled tetramers were produced by mixing these biotinylated complexes with PE-labelled streptavidin (Molecular Probes). Tetramer staining was performed as follows. CTL clones (2×10^5) were incubated with tetramers at a concentration of 0.1 mg ml^{-1} and FITC–anti-CD8 mAb (Caltag) at 4 °C for 15 min. After washing twice, stained cells were fixed in 0.5 % paraformaldehyde and analysed by flow cytometry.

Mixed lymphocyte–peptide culture. Mixed lymphocyte–peptide culture was performed as described by Coulie *et al.* (2001) with modifications. PBMCs were isolated by density-gradient centrifugation and suspended in CTL medium. An aliquot was used for enumeration of $CD8^+$ cells. The cells were then distributed at 2×10^5, 1×10^5 or 5×10^4 cells per well in 96-well round-bottomed plates in 200 µl medium in the presence of epitope peptide (1 µg ml^{-1}) and IL-2 (20 U ml^{-1}). Thirty-two cultures of each PBMC density were set up. On day 7, half of the medium was replaced by fresh medium containing the peptide and IL-2. Tetramer staining was performed on day 14. We counted the tetramer-positive microcultures and estimated the anti-EBNA1 CTLp frequency by limiting-dilution evaluation using the L-Calc program (Stem Cell Technologies).

Outgrowth-inhibition assay. Outgrowth-inhibition assays were performed as described by Lee *et al.* (2004) with modifications. Briefly, target LCLs were seeded into round-bottomed 96-well plates at 2×10^4 cells per well in triplicate. EBNA1-specific CTL clones (1×10^4 cells per well) or CTL medium alone (as a control) were added to target-cell cultures. All cultures were maintained weekly by changing half of the medium. After 4 weeks culture, the number of LCLs in the culture at each setting was counted. Cell growth (percentage of control) was calculated as [no. LCLs from the culture with CTLs (clone B5 or clone C6)]/[no. LCLs from the culture without CTLs (medium)] × 100. B-cell identity of grown cells was confirmed by staining with PE–cyanin 5-labelled anti-CD19 and PE-labelled anti-CD8 mAbs (Beckman Coulter) and analysis by flow cytometry.

Detection of IFN-γ-producing $CD8^+$ T cells by flow cytometry. EBNA1-specific CTL recognition was measured as described previously (Kuzushima *et al.*, 2001) with slight modifications. Briefly, 5×10^5 B5 CTLs were incubated with 2×10^6 MKN45-Cw0303, MKN45-Cw0303-ΔGA-EBNA1 or MKN45-Cw0303-full-EBNA1 cells in 1 ml complete culture medium in a culture tube at 37 °C for 6 h, in the presence of brefeldin A (Sigma) during the last 5 h. After the incubation, the cell suspensions were fixed with 4 % paraformaldehyde in PBS. After washing with PBS, cells were permeabilized with IC Perm (BioSource International) and stained with PE–cyanin-5.1-labelled anti-CD8 (Beckman Coulter), PE-labelled anti-CD69 (Immunotech Coulter) and FITC-labelled anti-human IFN-γ (BD Biosciences) mAbs. Stained cells were analysed by flow cytometry. Live gating of the $CD8^+$ subset was performed and 50 000 events were acquired for each analysis.

RESULTS

EBNA1 expression in full-length EBNA1 mRNA-transfected CD40-B cells

To generate EBNA1-expressing APCs, we first produced full-length EBNA1 mRNA with a poly(A) tail from the pcDNA/EBNA1 plasmid by using an *in vitro* transcription system. The yield of capped mRNA was low, probably due to the presence of GAr (comprising GC-rich sequences), and this was not overcome fully by the change of reaction temperature or by adding single-stranded DNA-binding protein to the reaction mixture (data not shown). However,

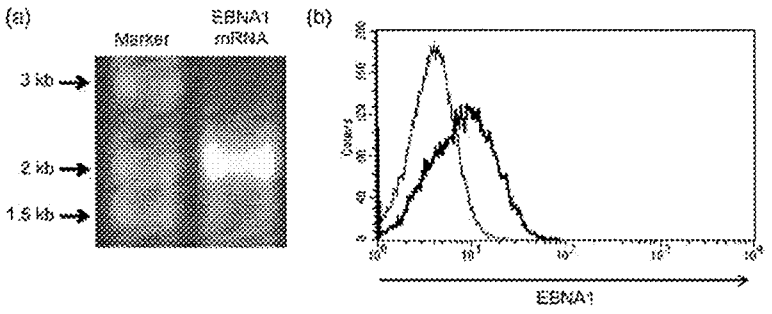

Fig. 1. EBNA1 expression in cells transfected with *in vitro*-transcribed full-length mRNA. (a) *In vitro*-transcribed full-length EBNA1 mRNA produced successfully from an EBNA1-cDNA plasmid. The quality of the EBNA1 mRNA was assessed by gel electrophoresis followed by staining with ethidium bromide. (b) EBNA1 protein expression in EBNA1 mRNA-transfected CD40-B cells. CD40-B cells were transfected with full-length EBNA1 mRNA by electroporation and intracellular staining of EBNA1 protein was performed and analysed by flow cytometry.

the amount of mRNA was sufficient and seen as a single band on the gel (Fig. 1a). Transfection was then performed by electroporation and EBNA1 expression was detected in most CD40-B cells, although the mean fluorescent intensity appeared to be low (Fig. 1b).

Induction of EBNA1-specific CTL lines and clones by using EBNA1 mRNA-transfected APCs

To explore the capacity for T-cell stimulation, autologous $CD8^+$ T lymphocytes were co-cultured with CD40-B cells transfected with full-length EBNA1 mRNA, and IFN-γ-producing cells were enumerated by ELISPOT assay. As shown in Fig. 2(a), $CD8^+$ T lymphocytes of donor Y01 produced IFN-γ spots without *in vitro* stimulation. As $CD8^+$ T lymphocytes of other donors did not produce significant spots on *ex vivo* analysis, the T cells were stimulated weekly with irradiated CD40-B cells that had been transfected with the full-length EBNA1 mRNA. After two rounds of stimulation, $CD8^+$ T lymphocytes of another donor (K04) produced IFN-γ spots upon contact with autologous CD40-B cells transduced with EBNA1 mRNA in the ELISPOT assay (Fig. 2a). These data indicate that the full-length EBNA1 mRNA was translated and that $CD8^+$ T-lymphocyte epitopes are processed and presented on APCs.

Next, EBNA1-specific CTL clones were established by using donor Y01 monocyte-derived DCs transfected with full-length EBNA1 mRNA. The transduced DCs were distributed in 96-well plates and used to stimulate autologous $CD8^+$ T lymphocytes in the presence of IL-7 and IL-12. After three rounds of stimulation, aliquots of each microculture were tested for their ability to secrete IFN-γ specifically upon contact with autologous CD40-B cells transduced with EBNA1 mRNA in the ELISPOT assay. Thirty-two microcultures out of 36 wells were scored as EBNA1-specific (data not shown), and lymphocytes from two well-growing microcultures were cloned by limiting dilution. CTL clones B5 and C6 were thus established, recognizing EBNA1 mRNA-transfected autologous CD40-B cells and autologous LCLs, but not mock-transfected autologous CD40-B cells or HLA-mismatched allogeneic LCLs (Fig. 2).

Identification of the presenting HLA molecules

The donor was typed genetically as HLA-A*2402, -A*3101, -B*1507, -B*3501 and -Cw*0303. To identify the antigen-presenting HLA molecule, a panel of partially HLA-matched LCLs was used to stimulate clones B5 or C6 to produce IFN-γ. In addition to autologous LCLs, allogeneic LCLs expressing HLA-B*3501 were recognized by CTL clone C6 (Fig. 3a), and one LCL with HLA-Cw*0303 and one with -Cw*0304 were recognized by clone B5 (Fig. 3b), demonstrating that HLA-B*3501 is the putative restriction element for clone C6 recognition, whilst both HLA-Cw*0303 and -Cw*0304 act for clone B5.

Identification of EBNA1 antigenic peptides

To identify the epitope region, clones B5 and C6 were stimulated with autologous CD40-B cells incubated with sets of peptides of 20 aa length, overlapping by 13 aa and covering the complete EBNA1 protein sequence without GAr. Because the primary structure of GAr is not likely to be contained in MHC class I epitopes, we did not include this part as an epitope source. Peptide 24 was recognized by clone C6 (Fig. 4). Regarding the HLA-B*3501-restricted epitope, HPVGEADYFEY has been reported previously (Blake *et al.*, 1997). As this epitope sequence is located in the centre of peptide 24 (aa 402–421) (Fig. 5a), we tested whether clone C6 might recognize HPVGEADYFEY-pulsed autologous CD40-B cells and confirmed a response to HPVGEADYFEY-pulsed stimulation (data not shown).

In the case of clone B5, two overlapping peptides, 38 (aa 500–519) and 39 (aa 507–526), were recognized (Fig. 4), sharing the 13 aa sequence VFVYGGSKTSLYN [underlined in Fig. 5(b)]. To predict the optimal epitope binding to HLA-Cw*0303, the program SYFPEITHI was applied. Because the anchor leucine at the C terminus and the auxiliary anchors valine and tyrosine at the third position of epitopes were predicted by the program, we examined the 11mer (VFVYGGSKTSL) and the 10mer (FVYGGSKTSL) (Fig. 5b). Half-maximal recognition of the peptide-pulsed target cells was obtained with 5–10 nM of the 10mer peptide and 1–5 nM of the 11mer (Fig. 5c), suggesting that these two peptides may be the optimal epitopes. The 9mer,

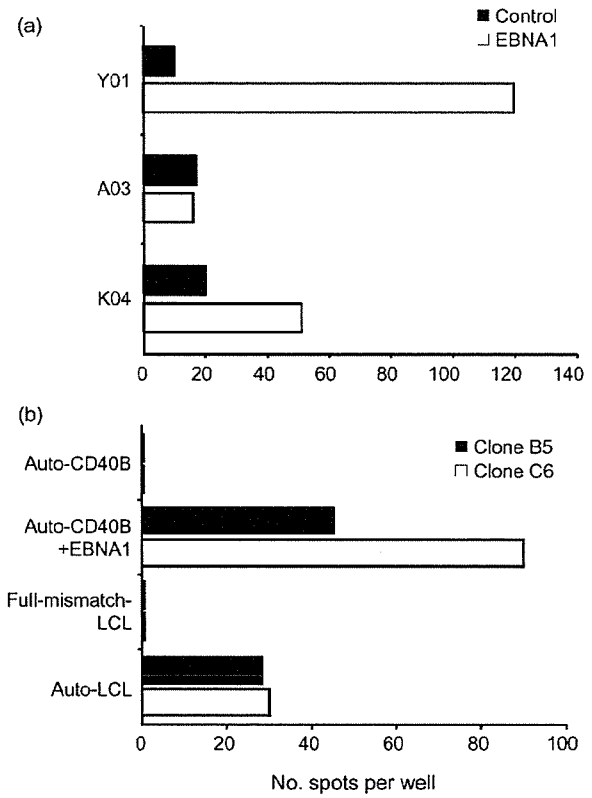

Fig. 2. Presence of anti-EBNA1 T cells in cultures primed with EBNA1 mRNA-transfected APCs. (a) Numbers of IFN-γ-producing cells from $CD8^+$ T lymphocytes without *in vitro* stimulation (Y01) or after two rounds of stimulation (A03 and K04) in ELISPOT assays. Aliquots of 100 000 cells were cultured in single wells with autologous CD40-B cells transduced with full-length EBNA1 mRNA for 20 h. Data from one representative experiment of two are shown. (b) $CD8^+$ T cells from one selected donor were stimulated with autologous DCs transfected with *in vitro*-transcribed EBNA1 mRNA. After three stimulations at weekly intervals, polyclonal $CD8^+$ T cells from two positive cultures were cloned by limiting dilution. Established clones B5 and C6 were then tested for recognition of EBNA1 mRNA-transfected autologous CD40-B cells and autologous LCLs by ELISPOT assay. Five thousand CTLs were seeded in each well. Data from one representative experiment of two are shown.

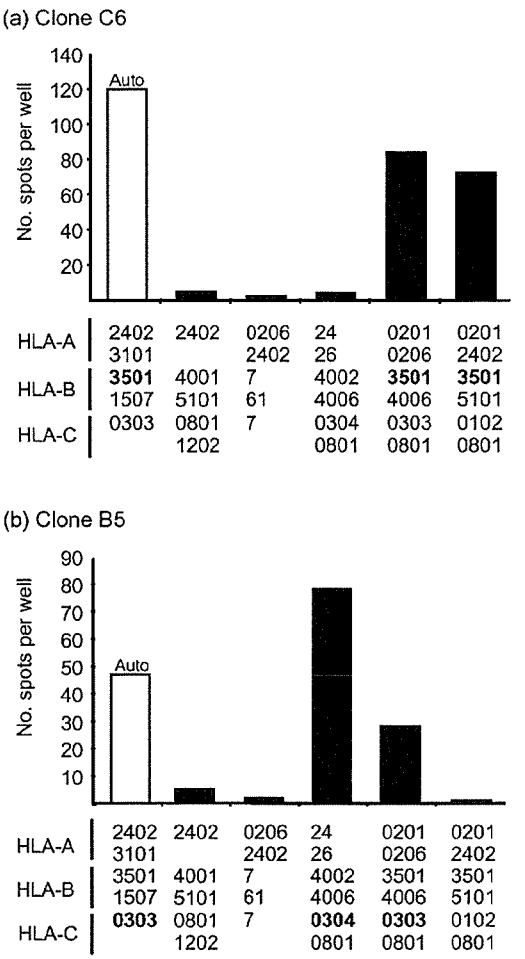

Fig. 3. Identification of presenting HLA molecules for EBNA1-specific CTL clones. (a) HLA-B*3501 molecules function as restriction elements for CTL clone C6. (b) HLA-Cw*0303 and -Cw*0304 molecules function as restriction elements for CTL clone B5. Autologous and allogeneic LCLs were used to stimulate clones B5 or C6 to produce IFN-γ spots. Single allele-matched LCLs were included and cultured with the CTLs (5×10^3) for 20 h. Each bar represents the mean number of spots in duplicate wells.

VYGGSKTSL, was not recognized, even at much higher concentrations.

Tetramers bind to the EBNA1-specific clone B5

As the peptide-dilution assay provided two optimal epitope candidates, we made fluorescently labelled tetramers incorporating the 10mer peptide FVYGGSKTSL or the 11mer VFVYGGSKTSL for further experiments. As shown in Fig. 5(d), these tetramers bound specifically to CTL clone B5. However, the tetramer incorporating the 10mer demonstrated higher avidity for the B5 clone than that incorporating the 11mer, suggesting the 10mer peptide FVYGGSKTSL to be the minimal and optimal epitope for the CTL. Moreover, clone B5 bound strongly to the HLA-Cw*0304 tetramer incorporating the 10mer, showing concordance with the results shown in Fig. 3(b). In addition, we characterized *in vitro*-expanded T cells from two donors by co-staining with MHC–peptide tetramer and CD62L. A proportion of 9.8% of HLA-Cw*0303-FVYGGSKTSL tetramer-positive lymphocytes were CD62-positive in one donor, and 6.5% in the other.

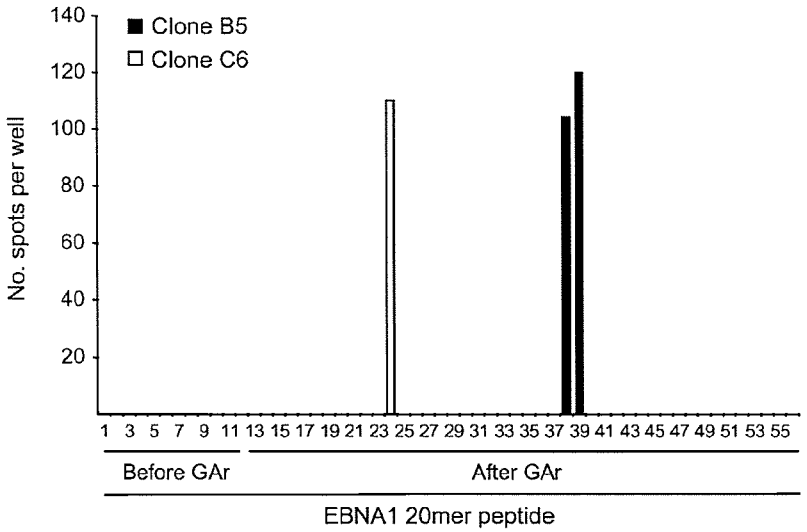

Fig. 4. Identification of overlapping peptides recognized by EBNA1-specific CTL clones. Autologous CD40-B cells (1×10^5 per well) were pulsed with 10 μg ml^{-1} of each of a set of 20mer overlapping peptides encompassing the EBNA1 protein, excluding GAr, and co-cultured with 5×10^2 CTL clone B5 or C6. Production of IFN-γ spots was then measured by ELISPOT assay.

Frequencies of EBNA1 epitope-specific $CD8^+$ T cells in PBMCs of healthy EBV-seropositive donors

We estimated frequencies of EBNA1 epitope-specific $CD8^+$ T cells in healthy EBV-seropositive donors by the mixed lymphocyte–peptide culture method. PBMCs from two donors with HLA-B*3501, one donor with HLA-Cw*0303 and three donors with HLA-Cw*0304 were tested. We could compare the anti-EBNA1 CTLp frequency in two donors with both HLA-B*3501 and HLA-Cw*0303 (HLA-Cw*0304). Representative tetramer staining of negative and positive microcultures from mixed lymphocyte–peptide culture wells is shown in Fig. 6. EBNA1-specific CTLp frequencies of HLA-B*3501-positive donors were 7.2×10^{-6} and 1.8×10^{-4}, and for HLA-Cw*0303 and -Cw*0304 were from 2.5×10^{-5} to $>2.1 \times 10^{-4}$. We did not find any hierarchy between the two EBNA1 epitopes in either of the donors with both HLA-B*3501 and HLA-Cw*0303 (Cw*0304) molecules (Table 1).

Effect of EBNA1-specific CTLs on EBV-infected B-cell growth

Clones B5 and C6 did not lyse autologous LCLs in the chromium-release assay (data not shown). Here, a final set of experiments was performed to ask whether these EBNA1-specific CTLs could affect the long-term growth and survival of EBNA1-expressing LCLs. Autologous and allogeneic LCLs with or without the restricting HLA molecules were seeded in 96-well plates in the presence or absence of responding CTLs. Cultures were then assayed for LCL outgrowth after 4 weeks. At the end, LCL outgrowth was assessed by microscopic inspection and confirmed by CD19 expression by flow cytometry. As shown in Fig. 7(a), both CTL clones clearly inhibited outgrowth of not only autologous LCLs, but also allogeneic LCLs with restricting HLA, suggesting that these CTL clones have the ability to inhibit outgrowth of EBV-positive cells with latency type III.

Recognition by EBNA1-specific CTL clone B5 of HLA-Cw*0303-transduced gastric carcinoma cells expressing EBNA1

Because naturally EBV-positive gastric cell lines are difficult to establish, we generated EBNA1-expressing gastric carcinoma cells designated MKN45-Cw0303-ΔGA-EBNA1 and MKN45-Cw0303-full-EBNA1 to verify that EBV-positive gastric cancer cell lines present the FVYGGSKTSL epitope. To investigate recognition by clone B5, we applied flow cytometry to detect EBNA1-specific CTLs producing IFN-γ. As shown in Fig. 7(b), 2.55 % of B5 clone cells produced IFN-γ when co-cultured with MKN45-Cw0303-ΔGA-EBNA1 cells, demonstrating specific recognition of the FVYGGSKTSL epitope on cells transduced with GAr-deleted EBNA1. Otherwise, B5 clone cells did not produce IFN-γ when co-cultured with MKN45-Cw0303-full-EBNA1 cells.

DISCUSSION

Recently, EBNA1-specific CTLs were shown to recognize and lyse HLA-matched LCLs (Lee *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004). In previous studies, such CTLs were generated from PBMCs of healthy donors after *in vitro* stimulation with autologous LCLs or EBNA1 peptide-pulsed PBMCs. As EBNA1 is expressed in all EBV-associated tumours and might be an important target for immunotherapy, we have explored the efficient induction of EBNA1-specific CTLs. Of the different methods used to obtain HLA class I-restricted epitopes on APCs for stimulation, we chose to employ full-length EBNA1 mRNA-transfected DCs. This strategy offers the following advantages: (i) the method is not dependent on knowledge of the HLA haplotype of each donor; (ii) there is complete deletion of antigenicity of the vector-backbone sequence; and (iii) the yield of mRNA *in vitro* transcription is highly reproducible and transduction is very efficient (Van

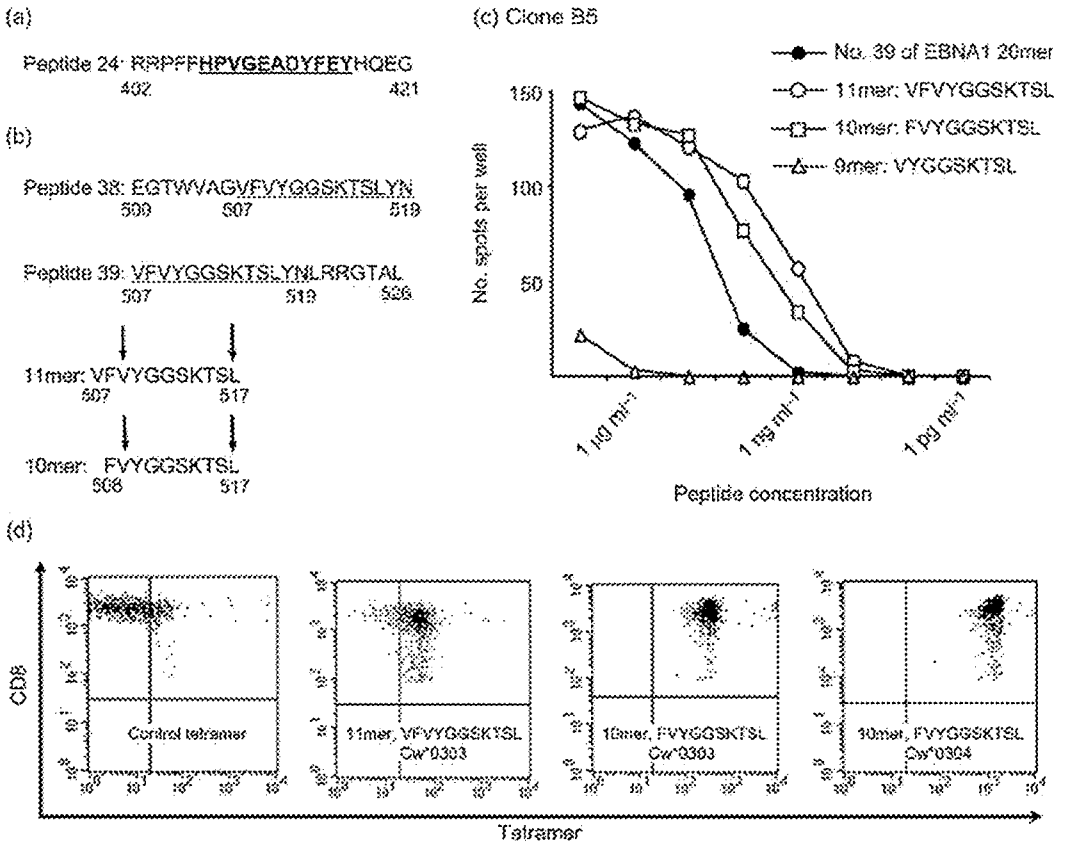

Fig. 5. Identification of optimal EBNA1 antigenic peptides recognized by EBNA1-specific CTL clones. (a) Amino acid sequence of the overlapping peptide recognized by the HLA-B*3501-restricted clone C6. The known epitope HPVGEADYFEY is indicated in bold and underlined. The numbers of the amino acid position in EBNA1 are shown. (b) Amino acid sequences of two consecutive overlapping peptides recognized by clone B5 and the potential optimal epitope sequences. The overlapping sequence between peptides 38 and 39 is underlined. Arrows indicate the primary and auxiliary anchors for HLA-Cw*0303 predicted by the program SYFPEITHI. The numbers of the amino acid positions in the EBNA1 protein are shown. (c) Titration of EBNA1-derived synthetic peptides. Autologous CD40-B cells were incubated for 1 h with 10-fold serial dilutions of synthetic peptides 507–526 (no. 39, 20mer), 507–517 (11mer), 508–17 (10mer) and 509–517 (9mer). CTL clone B5 (200 cells per well) was subsequently added and cultured for 20 h. Each symbol indicates the mean number of spots in duplicate wells. (d) The HLA-Cw*0303-restricted EBNA1-specific CTL clone B5 was stained with PE-conjugated HLA-Cw*0303–FVYGGSKTSL, HLA-Cw*0303–VFVYGGSKTSL or HLA-Cw*0304–FVYGGSKTSL tetrameric complexes and FITC-labelled anti-CD8 antibodies, and analysed by flow cytometry.

Tendeloo *et al.*, 2001). As an antigen, we used GAr-containing full-length EBNA1 instead of a GAr-deleted example to selectively activate CTL populations capable of reacting with epitopes that escape from the inhibitory mechanism governed by EBNA1 encoding GAr. Of note, even a low level of antigen delivery into DCs could induce antigen-specific CTL responses (Grunebach *et al.*, 2003), suggesting that this strategy has the potential to induce a CTL response even when a low density of EBNA1 epitopes is presented on DCs. In this study, we generated HLA-B- and -C-restricted EBNA1-specific CTLs successfully from a single donor, demonstrating that this method is a useful tool for generating EBNA1-specific CTLs, allowing investigation of the contribution of EBNA1 to cell-mediated immune responses in EBV-associated malignancies. Moreover, EBNA1 may have antigenicity when expressed on APCs, even if containing GAr *in vivo*. Finally, this induction method may be applicable for preparing EBNA1-specific CTLs for immunotherapy.

EBNA1 is generally immunologically invisible and only a small number of CTL epitopes have been identified (Blake *et al.*, 1997, 2000; Voo *et al.*, 2004). Of these, five epitopes are HLA-B-restricted and one is presented in the context of HLA-A. We detected two, one from HLA-B and another from HLA-C, from a single donor. To our knowledge, this is the first demonstration of an HLA-C-restricted EBNA1 epitope. To determine the minimal epitope, we compared

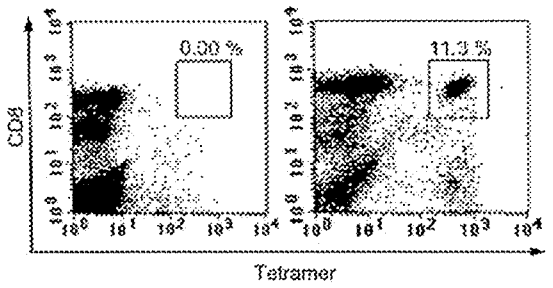

Fig. 6. Mixed lymphocyte–peptide culture analysis to estimate frequencies of C6 CTLp in $CD8^+$ lymphocytes of healthy EBV-seropositive donors. PBMCs from healthy EBV-seropositive donors with HLA-Cw*0303 were distributed at 2×10^5, 1×10^5 or 5×10^4 cells per well in 96-well round-bottomed plates in CTL medium with FVYGGSKTSL peptide (1 μg ml^{-1}) and IL-2 (20 U ml^{-1}). Half of the medium was replaced by fresh medium containing the relevant epitope peptide and IL-2 on day 7 and tetramer staining was performed on day 14. The plots show only data for $CD8^+$ lymphocytes, corresponding to 20–40 % of the cells in representative positive and negative cultures. The proportions of $CD8^+$ lymphocytes labelled specifically with the HLA-Cw*0303–FVYGGSKTSL tetramer are indicated.

the 11mer (VFVYGGSKTSL) and 10mer (FVYGGSKTSL) in a peptide-titration assay and found peptide concentration with half-maximal recognition of the target cells to be almost the same. However, clone C6 bound more strongly to tetramers incorporating the 10mer and we speculate that the N-terminal valine of the 11mer might be trimmed efficiently to yield 10mer in ELISPOT assay medium containing FCS. Moreover, the 10mer FVYGGSKTSL epitope was presented by HLA-Cw*0303 and -Cw*0304 molecules. As these two HLA-C alleles are possessed by >35 % of Japanese, >20 % of Northern Han Chinese (Hong *et al.*, 2005) and >25 % of Caucasians, this new epitope should enable us to analyse cellular immunity to EBNA1 in a broad population. Indeed, we estimated $CD8^+$ T-cell frequencies specific to either FVYGGSKTSL or HPVGEADYFEY in PBMCs of healthy EBV-seropositive donors by the mixed lymphocyte–peptide culture method followed by tetramer staining and found that EBNA1-specific CTLp frequencies of HLA-B*3501- or HLA-Cw*0303 (and -Cw*0304)-positive donors were between 1×10^{-5} and 1×10^{-4}. These data provide useful information for understanding cellular immunity to EBNA1. For determination of frequencies of EBNA1 epitope-specific CTLs, the *ex vivo* ELISPOT assay (Blake *et al.*, 2000) is simple and readily applicable, because frequencies can be predicted at the level of 1×10^{-4} $CD8^+$ lymphocytes.

Adoptive immunotherapy with CTLs has proved feasible for preventing and treating EBV-associated PTLD, HD and NPC (Bollard *et al.*, 2004; Gottschalk *et al.*, 2005; Straathof *et al.*, 2005). With respect to the targets for EBV-specific CTLs, EBNA3s and LMP2 are major EBV latent antigens; EBNA3s are immunodominant and LMP2 is recognized frequently, but is subdominant. In contrast, CTL responses to other antigens (EBNA2, EBNA-LP, LMP1 and EBNA1) seem to be less frequent (Rickinson & Moss, 1997), although EBNA1 can be immunodominant in some EBV-seropositive donors (Blake *et al.*, 2000). Indeed, LCL-activated EBV-specific CTL lines from NPC patients for adoptive immunotherapy demonstrate stronger responses to the immunodominant EBNA3s than against LMP1 and LMP2 (Straathof *et al.*, 2005). In addition, tetramer and functional

Table 1. Frequencies of anti-EBNA1 CTL precursors

Donor	HLA-B*3501				HLA-Cw*0303/0304			
	$CD8^+$ (%)	PBMCs per well	Positive/tested wells	Frequency (among $CD8^+$)	$CD8^+$ (%)	PBMCs per well	Positive/tested wells	Frequency (among $CD8^+$)
Y01*	38	2×10^5	32/32	$>1.8 \times 10^{-4}$	32	1×10^5	20/32	3.5×10^{-5}
		1×10^5	32/32			5×10^4	15/32	
		5×10^4	32/32			2.5×10^4	13/32	
T02†	32	2×10^5	10/32	7.2×10^{-6}	32	2×10^5	32/32	$>2.1 \times 10^{-4}$
		1×10^5	7/32			1×10^5	32/32	
		5×10^4	5/32			5×10^4	32/32	
A03†		NA‡			23	2×10^5	20/32	2.5×10^{-5}
						1×10^5	13/32	
						5×10^4	12/32	
K04†		NA			19	2×10^5	18/32	2.7×10^{-5}
						1×10^5	13/32	
						5×10^4	11/32	

*HLA-Cw*0303.
†HLA-Cw*0304.
‡NA, Not available.

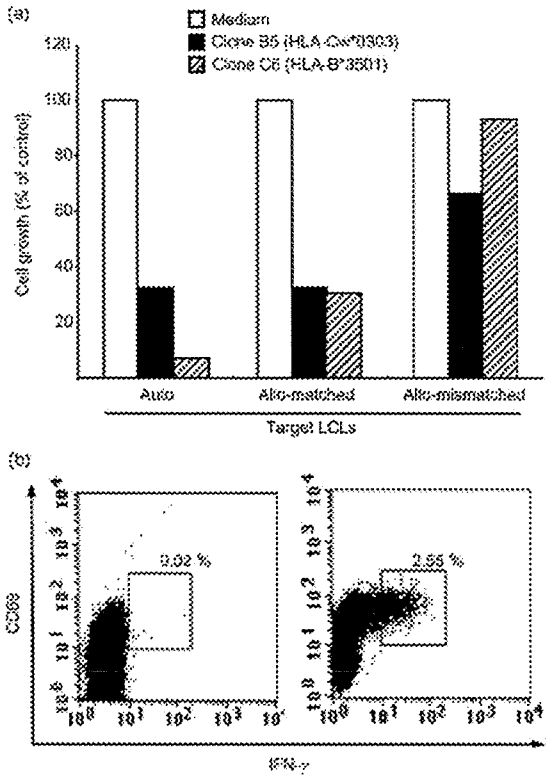

Fig. 7. Effects of EBNA1-specific CTLs on EBNA1-expressing cells. (a) EBNA1-specific CTL clones inhibit *in vitro* outgrowth of HLA-matched LCLs. Target LCLs (2×10^4) were cultured in triplicate wells of round-bottomed 96-well plates with EBNA1-specific CTL clones (1×10^4) or medium alone (control). After 4 weeks culture, the number of LCLs in the culture at each setting was counted. Cell growth (percentage of control) was calculated as [no. LCLs from the culture with CTLs (clone B5 or clone C6)]/[no. LCLs from the culture without CTLs (medium)] × 100. The B-cell (LCL) identity of the outgrowing cultures was confirmed by analysis of CD19 expression by flow cytometry. Data from one representative experiment of two are shown. (b) Detection of IFN-γ-producing anti-EBNA1 CTLs recognizing HLA-Cw*0303-positive cancer cells expressing EBNA1. B5 clones (5×10^5) were incubated with 2×10^6 MKN45-Cw0303 cells or MKN45-Cw0303-ΔGA-EBNA1 cells for 6 h, in the presence of brefeldin A for the last 5 h. After incubation, the cell suspensions were fixed with 4% paraformaldehyde in PBS and then permeabilized with IC Perm and stained with PE–cyanin-5.1-labelled anti-CD8, PE-labelled anti-CD69 or FITC-labelled anti-human IFN-γ mAbs. Stained cells were analysed by flow cytometry. Fifty thousand events were acquired for each analysis. The proportions of IFN-γ^+ CD69$^+$ CTLs among CD8$^+$ lymphocytes are indicated for one representative experiment of two performed.

analyses have shown that LMP2-specific CTLs are present in the infused CTLs used for adoptive immunotherapy and might have antiviral activity in patients with a good response to immunotherapy for HD (Bollard *et al.*, 2004). Interestingly, the CTL line from one NPC patient who attained a complete response was shown to contain a relatively large T-cell population for an EBNA1-derived CTL epitope (Straathof *et al.*, 2005). This suggests that increased attention should be focused on the contribution of EBNA1-specific CTLs to EBV cellular immunity. In this study, we showed two EBNA1-specific CTL clones to cause strong, specific inhibition of LCL outgrowth *in vitro*, which is consistent with recent observations with HLA-B8- and HLA-B*3501-restricted CTL clones (Tellam *et al.*, 2004; Voo *et al.*, 2004). C6 CTLs failed to respond to an HLA-Cw*0303-expressing gastric cancer cell line transduced with full-length EBNA1, although they produced IFN-γ when GAr-depleted EBNA1 was transduced (Fig. 7b). These data suggest differential antigen-processing machinery and presentation on class I molecules between LCLs and gastric cancer cells.

In conclusion, we have established EBNA1-specific CTL clones from PBMCs of a healthy donor by using EBNA1 mRNA-transfected DCs, and identified a novel CTL epitope of EBNA1 presented by HLA-Cw*0303 and -Cw*0304 molecules. The induction method adapted may be useful for generating EBNA1-specific CTLs and for investigating cellular immunity against EBNA1. Finally, the induced EBNA1-specific CTLs recognized EBNA1-expressing gastric carcinoma cells in the context of HLA-Cw*0303 *in vitro*, suggesting that EBNA1 is an important antigen for the further development of CTL therapy for EBV-associated malignancies.

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Identification of an HLA-A24-restricted cytotoxic T lymphocyte epitope from human papillomavirus type-16 E6: The combined effects of bortezomib and interferon-γ on the presentation of a cryptic epitope

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About 50% of cervical cancers are associated with human papillomavirus type 16 (HPV-16), and since the HPV-16 E6 and E7 oncoproteins are constitutively expressed in the tumor cells, they are attractive targets for cytotoxic T lymphocyte (CTL)-mediated immunotherapy. Nevertheless, only a limited number of HPV-16 E6 epitopes have been identified to date. Using reverse immunological methods, we have generated a CTL clone against the HPV-16 $E6_{49-57}$ epitope restricted by HLA-A*2402, which is the most common allele in Japan and relatively frequent worldwide, capable of lysing 293T cells transduced with HLA-A*2402 and HPV-16 E6. Although it was unable to recognize the SiHa cervical cancer cell line positive for HPV-16 and HLA-A*2402, the cells became susceptible to lysis when transduced with E6-E7 genes, which was unexpectedly offset by pretreatment with interferon (IFN)-γ alone. Interestingly, however, combined pretreatment with a proteasome inhibitor, bortezomib and IFN-γ fully restored CTL-mediated lysis of the original SiHa cells. Furthermore, such intervention of 2 of 4 other cervical cancer cell lines expressing HPV-16 E6 and HLA-A*2402 was found to induce IFN-γ production by specific CTLs. Tetramer analysis further revealed that induction of $E6_{49-57}$-specific T cells was possible in 5 of 7 patients with HPV-16-positive high grade cervical intraepithelial neoplasia or cervical cancer by *in vitro* stimulation with $E6_{49-57}$ peptide. Thus, these findings together indicate that $E6_{49-57}$ is a candidate epitope for immunotherapy and immunological monitoring of such patients.

Key words: cytotoxic T lymphocyte; tumor immunity; epitope, cervical neoplasm; bortezomib

Cervical cancer, the second most common cancer in women worldwide, with 250,000 new cases diagnosed each year,[1] is causally linked with human papillomavirus (HPV), the first proposed necessary cause of a human cancer as assessed by the World Health Organization.[2] High-risk HPV-16 is the most common type in all countries, with an overall prevalence of more than 50% in cervical cancers,[3] and 42.4% in Japanese patients.[4] Approximately 95% of HPV infections of the anogenital tract resolve spontaneously because of immune responses,[5] and the risk of cervical cancer is markedly increased in patients with immunodeficiency.[6–8] These findings suggest that the immune system plays a pivotal role in preventing development and progression of cervical cancer.

Two HPV oncoproteins, E6 and E7, which can inhibit the tumor suppressors, p53 and RB respectively, are constitutively expressed in cervical cancer cells and appear to be required to maintain their malignant growth.[9] These viral oncoproteins, which are not present in normal cells, have been considered to be attractive targets for specific immunotherapy against cervical cancer, and identification and characterization of cytotoxic T lymphocyte (CTL) epitopes for HPV have facilitated the development of peptide vaccines against cervical cancer. Most of the CTL epitopes identified to date for HPV-16 E6 and E7 are restricted by HLA-A*0201, the most frequent HLA allele in Caucasian populations. Identification of functional HPV-16 epitopes restricted by HLA-A*2402, which is very common in Japanese (phenotype frequency ~60%) and other Asian populations, should provide an opportunity of immunotherapy of HLA-A*2402^+ patients.

Proteasomes play a pivotal role in signal transduction, transcriptional regulation, response to stress, and control of receptor function by orderly degrading cellular proteins.[10] In addition, they make a critical contribution to the generation of antigenic peptides, displaying both exact N and C termini, or intermediates with exact C termini and N-terminal extensions with lengths ranging from 3–24 aa. $CD8^+$ T cells recognize 8–10 aa antigenic peptides presented on MHC class I molecules after transportation into endoplasmic reticulum and further N-terminus trimming. It is known that cells exposed to interferon (IFN)-γ up-regulate other catalytic components and can be equipped with "immunoproteasomes," which favor the generation of peptides suited for presentation by MHC I molecules. Thus, inhibition of proteasome functions by inhibitors can cause not only cellular apoptosis by affecting cellular regulatory proteins,[10] but also modulation of the generation of some antigenic peptides by proteasomes, leading to loss or gain of antigenicity.[11–13] Among various proteasome inhibitors, bortezomib, a boronic acid dipeptide, is the only one to have progressed to clinical trials in cancer patients, and has shown striking activity in patients with advanced multiple myeloma.[14] Bortezomib is a selective but reversible inhibitor of both standard proteasomes and immunoproteasomes.[15–18]

In the present study, using a reverse immunological approach, we identified an HLA-A*2402-restricted HPV-16 E6-derived nonameric epitope, $E6_{49-57}$. A $CD8^+$ T cell clone, specific for this epitope, initially failed to recognize untreated HPV-16^+ cervical

Abbreviations: B-LCL, B-lymphoblastoid cell line; BIMAS, bioinformatics and molecular analysis section; CD40-B, CD40-activated B; CIN, cervical intraepithelial neoplasia; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; HPV-16, human papillomavirus type 16; IFN, interferon; PBMCs, peripheral blood mononuclear cells.

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cancer cell lines; however, recognition was evident on bortezomib treatment, particularly with simultaneous IFN-γ treatment in combination. Furthermore, induction of $E6_{49-57}$-specific T cells detectable by HLA-A24/$E6_{49-57}$ tetramers was possible in 5 of 7 patients with HPV-16$^+$ high grade cervical intraepithelial neoplasia (CIN) or cervical cancer by *in vitro* stimulation with the $E6_{49-57}$ peptide. These results indicate $E6_{49-57}$ to be an endogenously processed HLA-A*2402-restricted CTL epitope, and the limited supply of HPV-16 E6 peptides observed in cervical cancer cells may be, at least in part, overcome by coadministration of IFN-γ and bortezomib.

Material and methods

Blood donors and cell lines

This study was approved by the Institutional Review Board of Aichi Cancer Center. Peripheral blood samples were obtained from patients with cervical cancer or cervical intraepithelial neoplasia (CIN) and healthy volunteer donors. All samples were collected after obtaining written informed consent according to the Declaration of Helsinki. HLA typing was carried out at the HLA Laboratory (Kyoto, Japan). Peripheral blood mononuclear cells (PBMCs) were isolated by a standard method, and CD8-positive and -negative fractions were isolated using CD8 microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and cryopreserved until use. B-lymphoblastoid cell lines (B-LCLs) were established by infecting an aliquot of PBMCs with B95-8 supernatant and maintained in RPMI1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and penicillin/streptomycin (referred below as complete medium).

CD40-activated B (CD40-B) cells were generated as previously described,[19] using a human CD40L-transfected NIH/3T3 cell line.[20] HPV-16-positive cervical cancer cell lines, CaSki, SiHa (ATCC, Manassa, VA), SKGIIIa and SKGIIIb, the latter 2 derived from a patient with a moderately differentiated epidermoid cervical cancer,[21] BOKU (Japanese Collection of Research Bioresources, Osaka, Japan) and 293T human embryonic kidney cells were cultured in IMDM, supplemented with 10% FCS, 2 mM L-glutamine and penicillin/streptomycin.

HPV genotyping

Cervical scrapes or tumor samples were evaluated for the presence of HPV DNA using a general primer (Gp5+/GP6+)-based PCR method as described previously.[22] Samples showing positive bands by ethidium bromide staining were further examined by PCR-based restriction fragment length polymorphism methods, using consensus primers for E6 ORF of HPV-16, -18 and -33, followed by digestion with *Rsa* I and *Sau* 3AI.[23] When the Gp5+/Gp6+ primer could not detect HPV DNA in cervical scrapes, total cellular DNA isolated from paraffin-embedded samples was explored as an alternative approach.

Construction of HPV-16 E6-E7 fusion gene expression plasmids

Plasmids encoding an in-frame HPV-16 E6-E7 fusion protein, devoid of 8 nt consisting of the E6 stop codon, the E7 start codon and 2 nt between them, were generated using a conventional overlapping PCR method. In brief, E6 and E7 genes were individually amplified using a template plasmid encoding genomic E6 and E7 (GenBank accession no. AF003015 and K02718) with the following primers: 5′ E6, 5′-ATAgtcgacTCACCATGTTTCAGGACCCACAGGA-3′; 3′ E6, 5′-TGTATCTCCATGCAGCTGGGTTTCTCTACGT-3′; 5′ E7, 5′-GAAACCCAGCTGCATGGAGATACACCTACAT-3′; 3′ E7, 5′-TATggatccTTATGGTTTCATAGAACAGATGG-3′.

The 3′ E6 and 5′ E7 primers contained overlapping sequences (underlined) for the targeted region. Restriction enzyme sites for *Sal*I and *Bam*HI are indicated with lower case letters. The 2 PCR products were conjugated by second PCR, using 5′ E6 and 3′ E7 primers and the final PCR product (E6-E7) was digested with *Sal*I and *Bam*HI and inserted into the pIRES2-EGFP vector (BD Biosciences, San Jose, CA). In addition, an E6-E7-IRES-EGFP fragment excised from pIRES2-EGFP/E6-E7 with *Xho*I and *Not*I was reinserted into the pLBPC retroviral vector and transfected into the Phoenix-GP-GALV cell line[24] (a gift from H.-P. Kiem, Fred Hutchinson Cancer Research Center, Seattle, WA, and G. Nolan, Stanford University, Stanford, CA) for retroviral gene transfer. HLA-A24-negative cell lines (BOKU and CaSki) were transduced retrovirally with HLA-A*2402 cDNA as previously described.[25] HLA-A*2402-transfected cell lines are referred to as "cell line name/A24." After 48 hr, HLA-A*2402-transduced cell lines were further infected with the retroviral vector pLBPC encoding E6-E7-IRES-EGFP. Individual transduced cells were selected with predetermined concentrations of puromycin.

Synthetic peptides and in vitro CTL induction

Peptides with the HLA-A*2402 binding motif within HPV-16 E6 and E7 were predicted by computer algorithms available at the BioInformatics & Molecular Analysis Section (BIMAS) web site (http://bimas.dcrt.nih.gov/molbio/hla_bind)[26] and synthesized using standard Fmoc chemistry.

Induction of CTLs was performed according to standard procedures as described previously.[19] Briefly, thawed CD8$^+$ cells (0.5×10^6/ml) were cocultured with irradiated (96 Gy) autologous peptide-pulsed (10 μM) CD40-B cells (0.5×10^6/ml) in 2 ml RPMI 1640, supplemented with 9% pooled human serum, 2 mM L-glutamine and penicillin/streptomycin (referred to as CTL medium) in the presence of recombinant human IL-7 (50 U/ml) and IL-12 (5 ng/ml; all from R&D Systems, Minneapolis, MN) in 48- or 24-well plates at 37°C in 5% CO_2. On days 7 and 14, the CD8$^+$ cells were restimulated as above. One and three days after each stimulation, recombinant human IL-2 (Chiron, Emeryville, CA) was added to the cultures to a final concentration of 20 IU/ml.

MHC stabilization assay

Binding of the synthesized peptides to HLA-A*2402 molecules was evaluated by an MHC stabilization assay, using T2-A24 cells, as described earlier.[27] Briefly, HLA-A24-transduced T2 cells (2×10^5) were incubated with 200 μL AIM-V medium (Invitrogen) and each of the peptides at a concentration of 10 μM at 27°C for 16 hr, followed by incubation at 37°C for 3 hr. Surface HLA-A24 molecules were then stained with the anti-HLA-A24 mAb (One Lambda, Canoga Park, CA) and FITC-conjugated anti-mouse mAb (BD Biosciences, San Jose, CA). Expression was measured with a FACS Calibur flow cytometer and CellQuest software (BD Biosciences), and mean fluorescence intensity (MFI) was recorded. Percentage MFI increase was calculated as follows: Percent MFI = (MFI with the given peptide − MFI without peptide)/(MFI without peptide) × 100.

Intracellular IFN-γ staining and T cell cloning

Intracellular IFN-γ was detected as previously described with modifications.[28] Briefly, a total of $0.3–0.5 \times 10^6$ cells from each T cell line were stimulated with autologous B-LCL (1×10^6) pulsed with each of 3 peptides (10 μM) in complete medium for 6 hr in the presence of 1 μg/ml brefeldin A and 20 IU/ml IL-2 at 37°C. After incubation, cells were stained with the aid of a Cytofix/Cytoperm Plus kit (BD Biosciences) according to the manufacturer's instructions. In brief, all cells were transferred into 5-ml round-bottomed tubes, washed and stained with PE-conjugated anti-CD3 (BD Biosciences) and PE-cyanine-5-conjugated anti-CD8 (Caltag, Burlingame, CA) for 15 min on ice. After membrane-staining, cells were washed and fixed with 250 μl of Cytofix (BD Biosciences) for 10 min on ice, stained intracellularly with FITC-conjugated anti-IFN-γ (BD Biosciences) for 30 min on ice and then analyzed as above.

T cells from CTL lines showing intracellular IFN-γ production were cloned by limiting dilution in 96-well round-bottomed plates with 5×10^4 PBMCs (33 Gy-irradiated) and 1×10^4 B-LCLs (66

TABLE I – CHARACTERS OF SYNTHETIC PEPTIDES AND RESULTS OF THE MHC STABILIZATION ASSAY

Peptide	Sequence	Score (BIMAS)	Score (SYFPEITHI)	% MFI increase
$E6_{49-57}$	VYDFAFRDL	240	23	54
$E6_{87-95}$	CYSVYGTTL	200	20	55
$E6_{98-106}$	QYNKPLCDL	300	21	58
CMV $pp65_{328-336}$	QYDPVAALF	168	24	325

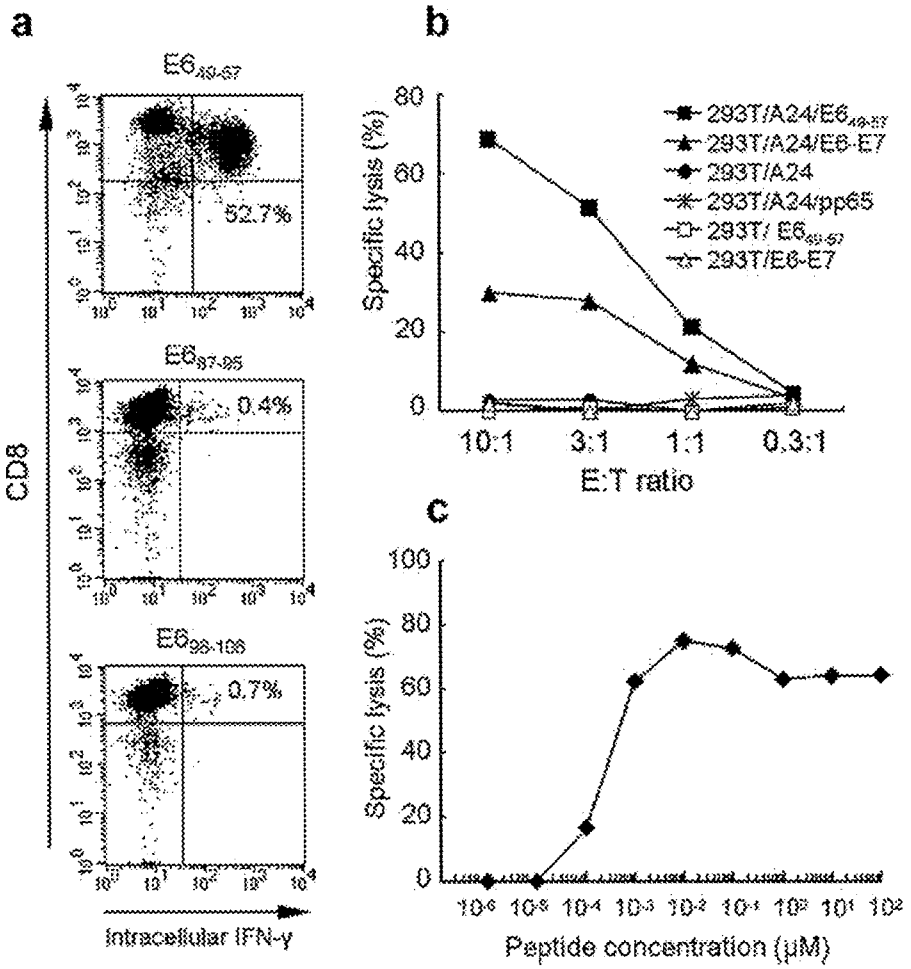

FIGURE 1 – Generation and characterization of HPV-16 E6-specific CTL lines and a clone. (*a*) Intracellular IFN-γ staining of CTL lines. $CD8^+$ cells were cocultured with irradiated autologous peptide-pulsed CD40-B cells in the presence of IL-7 and IL-12. On days 7 and 14, they were restimulated and 1 and 3 days after each stimulation, IL-2 was added to the cultures. Growing T cells were stimulated with autologous B-LCL pulsed with each of the 3 peptides indicated (Table I) for 6 hr in the presence of brefeldin A and IL-2. After incubation, cells were stained anti-CD3 and anti-CD8 followed by anti-IFN-γ, and then flow cytometric analysis was conducted. The percentage of cells gated for CD3 is shown. (*b*) Cytolytic activity of an HPV-16 $E6_{49-57}$-specific CTL clone. 2B2-CTL was isolated from the T cell line specific for the $E6_{49-57}$ peptide by limiting dilution, and cytotoxicity assays were performed by standard 4-hr ^{51}Cr release assay against various 293T target cells: 293T/A24, HLA-A*2402-transduced 293T cells; $E6_{49-57}$, $E6_{49-57}$-pulsed cells; E6-E7, E6-E7 fusion gene-transduced cells; pp65, HLA-A*2402-restricted cytomegalovirus pp65, control peptide-pulsed cells. (*c*) Evaluation of synthetic peptides for epitope reconstitution activity. Autologous B-LCLs were labeled with ^{51}Cr, then pulsed with serial dilutions of HPV-16 $E6_{49-57}$ peptide and used as targets for 2B2-CTL at an E:T ratio of 20:1.

Gy), 30 ng/ml anti-CD3 mAb (OKT3; Ortho Diagnostics, Raritan, NJ) and 50 IU/ml IL-2 in 200 μl CTL medium. Outgrowing wells were identified after 10–14 days and further expanded in 25-cm^2 flasks.

Cytotoxicity assay

Target cells were labeled with 0.1 mCi of $Na_2{}^{51}CrO_4$ overnight, and 1×10^3 target cells/well were mixed with CTL at the E:T ratios indicated in a standard 4-hr cytotoxicity assay in 96-well round-bottom plates. All assays were performed in triplicate. Peptide-pulsed target cells were incubated with peptide (1 μM) for 30 min after ^{51}Cr labeling, and washed twice before use. When indicated, target cells were treated with 100 U/ml IFN-γ (R&D Systems) for 48 hr, 10 μM epoxomicin (Peptide Institute, Osaka, Japan) for 5 hr or 10 μM bortezomib (Millennium Pharmaceuticals, Cambridge, MA) for 5 hr before cytotoxicity assay. Percent-

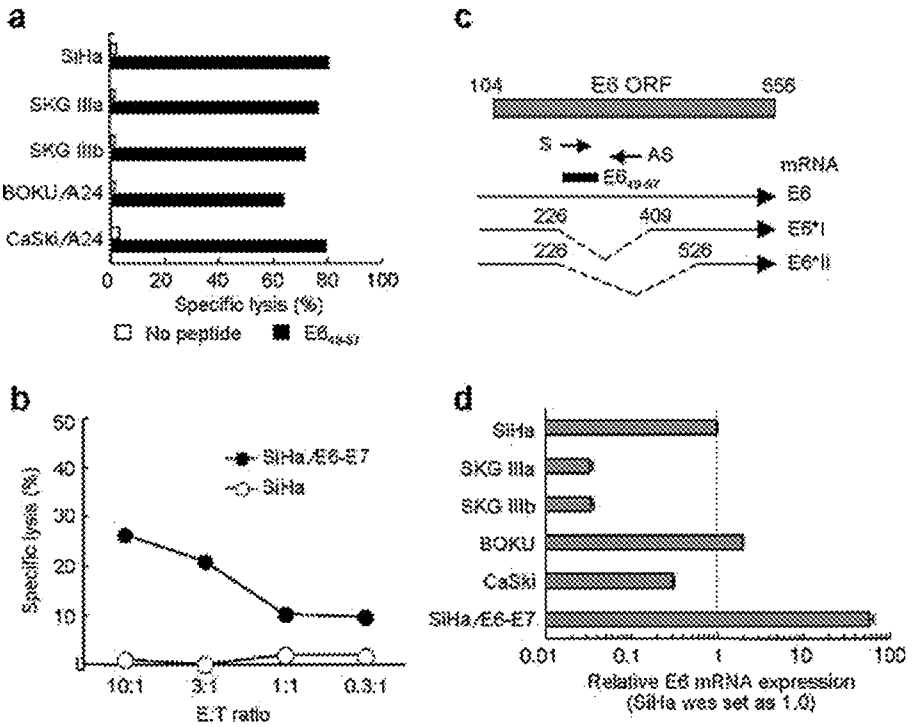

FIGURE 2 – Failure of 2B2-CTL lyse HLA-A*2402$^+$ HPV-16$^+$ cervical cancer cell lines and induction of susceptibility in SiHa cells by forced expression of the HPV-16 E6-E7 gene. (*a*) Cytolytic activity of 2B2-CTL against ^{51}Cr-labeled cervical cancer cell lines with or without the $E6_{49-57}$ peptide (1 μM) at an E:T ratio of 10:1. (*b*) Cytolytic activity of 2B2-CTL against ^{51}Cr-labeled original SiHa cells or those with E6-E7 fusion gene transduction (SiHa/E6-E7). (*c*) Schematic representation of the HPV-16 E6 ORF, mRNAs, locations of the $E6_{49-57}$ epitope and primers used to quantify HPV-16 E6 mRNA expression. (*d*) Real-time RT-PCR analysis of expression of the unspliced form of HPV-16 E6 mRNA in cervical cancer cell lines and SiHa/E6-E7. Expression of unspliced HPV-16 E6 transcripts in each sample was normalized to the internal GAPDH level, and values are expressed relative to the level found in SiHa cells as 1.0.

age specific lysis was calculated as follows: [(Experimental cpm − Spontaneous cpm)/(Maximum cpm − Spontaneous cpm)] × 100. For peptide reconstitution assays, ^{51}Cr-labeled HLA-A*2402$^+$ B-LCLs were incubated for 30 min in medium containing 10-fold serial dilutions of the peptides and then used as targets in standard cytotoxicity assays.

Enzyme-linked immunosorbent assay

Ten thousand CTLs per well were cocultured with the same number of target cells in complete medium in 96-well polypropylene plates. When necessary, target cells were pretreated with IFN-γ and individual proteasome inhibitor, (either) alone or in combination. After overnight incubation at 37°C, 50 μl of supernatant was collected and IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA).

Isolation of mRNA and quantitative RT-PCR for detection of E6 transcripts

Messenger RNA was isolated from ~8 × 10^6 cells of cervical cancer cell lines using a μMACS mRNA Isolation Kit (Miltenyi Biotec). One half microgram of mRNA was converted into cDNA in 30 μl of reaction mixture using an oligo(dT)$_{15}$ primer (Roche, Indianapolis, IN), and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitation of the HPV-16 E6 transcripts was performed using a fluorescence-based real-time detection method (ABI PRISM 7700 Sequence Detection System (TaqMan); Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The primer and probe sequences used to detect the unspliced form of E6 (GenBank Accession No. K02718) were as follows (Fig. 2*b*): sense, 5′-TGCATAGTATATAGAGATGG-GAATCCA-3′ (nt 254–280); antisense, 5′-ACGGTTTGTTGTAT-TGCTGTTCTAAT-3′ (nt 364–389); probe, 5′-(FAM)-TGCTG-TATGTGATAAATG-(MGB)-3′ (nt 282–300).

A primer and probe set for human GAPDH was used as an internal control. PCR was performed in a 1× TaqMan Universal PCR master mix containing 10 pmol of each sense and antisense primer, and 2 pmol of probe in a total volume of 25 μl (all from Applied Biosystems). The temperature profile was as follows: 50°C for 2 min, 95°C for 10 min and then 95°C for 15 sec and 60°C for 1 min for 40 cycles. Samples were quantified using relative standard curves for each experiment. All results were normalized with respect to the internal control, and are expressed relative to the levels indicated.

Tetramer construction and flow cytometric analysis

HLA-peptide tetramers were produced as described previously.[29] For staining, cells were incubated with the tetramer at a concentration of 20 μg/ml at room temperature for 15 min followed by FITC-conjugated anti-CD3 (BD Biosciences) and Tricolor anti-CD8 mAb (Caltag) on ice for 15 min. Cervical cancer cell lines were stained with FITC-labeled anti-HLA class I (Beckman Coulter, Fullerton, CA) and analyzed as above.

Results

HPV-16 $E6_{49-57}$ peptide-specific CTLs can be generated and lyse E6-transduced 293T cells

The primary amino acid sequence of HPV-16 E6 and E7 was first analyzed for consensus motifs for novel nonameric peptides capable of binding to HLA-A*2402, the most frequent allele in the Japanese population, and the top 3 candidate peptides with scores of >50 using the BIMAS computer algorithm were chosen (Table I). All were from E6 sequences, as E7 did not contain peptide motifs suitable for HLA-A*2402-binding. The MHC stabilization assay revealed that these peptides similarly increased HLA-A24 expression on the cells, although the increase was inferior to that with a strong A24-binder peptide, $CMVpp65_{328-336}$.[27] These results indicate that the peptides indeed bound and stabilized the HLA complex on cell surfaces.

HLA-A*2402$^+$ PBMCs collected from one CIN patient and 3 healthy volunteer donors were tested for *in vitro* induction of CTLs specific for these peptides. After the third stimulation, cytolytic activity of the CTL lines was assessed. A CTL line that could

lyse peptide-pulsed autologous B-LCL was successfully generated from the CIN patient but not from healthy volunteer donors. Intracellular cytokine staining demonstrated that $E6_{49-57}$ peptide-pulsed autologous B-LCL induced IFN-γ production in 52.7% of $CD3^+$ cells from the CTL line, whereas induction with the other 2 peptides was negligible (Fig. 1*a*). Therefore, T cells specific for the $E6_{49-57}$ peptide (VYDFARDL) were subsequently cloned. One of the representative T cell clones, designated 2B2-CTL, was further expanded and tested.

Cytotoxicity assays showed 2B2-CTL to lyse $E6_{49-57}$ peptide-pulsed HLA-A*2402-transduced 293T cells (referred as 293T/A24) and HPV-16 E6-E7-transduced 293T/A24 cells (Fig. 1*b*), indicating that 2B2-CTL is restricted by HLA-A*2402 and recognizes an endogenously processed E6-derived epitope in 293T cells. The $E6_{49-57}$ peptide was found to have high affinity to HLA-A*2402 molecules because half-maximal lysis of peptide-pulsed autologous B-LCL was achieved at a concentration of 300 pM (Fig. 1*c*), which is comparable to peptide concentrations required for reconstitution of other tumor-associated epitopes reported earlier.[30]

Failure to lyse HPV-16-positive cervical cancer cell lines by 2B2-CTL can be overcome by forced expression of full-length E6 cDNA by gene transfer

To examine the immunotherapeutic potential of HPV-16 $E6_{49-57}$-specific 2B2-CTL, cytotoxic activity against various HPV-16^+ cervical cancer cell lines was tested after transduction with HLA-A*2402 cDNA when necessary. Unfortunately, none of 5 cervical cancer cell lines was originally lysed, but all became susceptible to lysis when the $E6_{49-57}$ peptide was supplied exogenously (Fig. 2*a*), suggesting that E6 proteins are not sufficiently produced or processed by the cellular machinery in these cell lines. To determine whether forced expression of HPV-16 E6 would make such cell lines susceptible to 2B2-CTL, SiHa cells were transduced with E6-E7 cDNA (referred to as SiHa/E6-E7) and tested. As shown in Figure 2*b*, SiHa/E6-E7 cells became susceptible to lysis by 2B2-CTL, indicating that the lack of lysis of original cell lines was likely caused by an insufficient supply of E6 proteins, while the intracellular machinery necessary for processing the HPV-16 $E6_{49-57}$ epitope was retained.

Next, we performed real-time RT-PCR to quantify HPV-16 E6 mRNA. Oncogenic HPVs, such as HPV-16 and HPV-18, have been reported to potentially produce distinct E6 mRNAs including E6, E6*I, E6*II by alternative splicing (Fig. 2*c*), and these transcripts of HPV-16 have been found in cervical cancers and cell lines.[31,32] Since the $E6_{49-57}$ epitope is encoded by nucleotides 226–253, which are spliced out in E6*I and E6*II mRNAs, primers and the probe were designed to detect the unspliced form of E6 mRNA. All these cell lines expressed the unspliced E6 mRNA, but the level varied significantly (Fig. 2*d*). Indeed, the expression level in SiHa/E6-E7 cells was 60-fold higher than that in untransduced SiHa cells (Fig. 2*d*), and this large difference could account for susceptibility to lysis by 2B2-CTL.

Treatment of cervical cancer cell lines with proteasome inhibitors and IFN-γ results in recognition by 2B2-CTL

Although 2B2-CTL recognized the endogenously processed $E6_{49-57}$ epitope and lysed SiHa/E6-E7 cells, forced expression of E6 cDNA was still required to lyse SiHa cells. Since presentation of certain CTL epitopes of tumor and viral antigens is reported to be enhanced by treatment of target cells with proteasome inhibitors,[11–13] we first attempted to sensitize SiHa cells with epoxomicin or bortezomib. Epoxomicin is an irreversible proteasome inhibitor, which is about 100-fold more potent than lactacystin,[12] while bortezomib is a reversible proteasome inhibitor.[15] As shown in Figure 3*a*, treatment of SiHa cells with epoxomicin or bortezomib induced IFN-γ production from 2B2-CTL but not from an irrelevant CTL clone or SiHa cells themselves. This effect was more prominent with epoxomicin than bortezomib. Of note, addition of IFN-γ pretreatment to proteasome inhibitors induced more

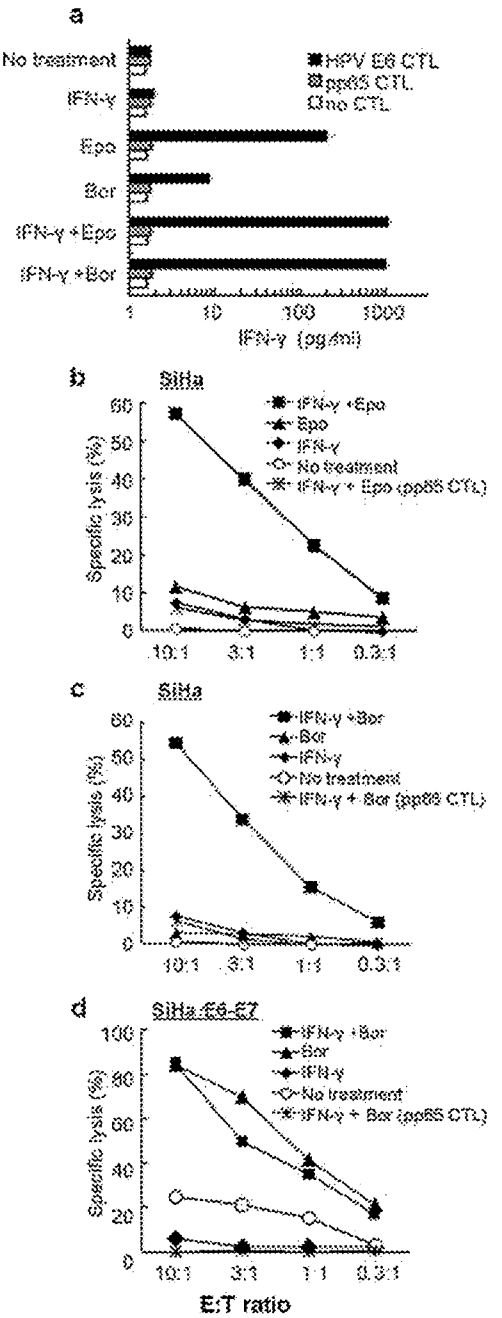

FIGURE 3 – Induction of susceptibility of SiHa cells to 2B2-CTL by treatment with proteasome inhibitors and IFN-γ. (*a*) IFN-γ production by 2B2-CTL after stimulation with pretreated SiHa cells. Supernatants were harvested and assayed by ELISA. Values are means ± SD obtained from triplicate samples in 1 of 2 independent experiments. The proteasome inhibitors used in this assay were epoxomicin (Epo) and bortezomib (Bor). pp65 CTL, with specificity for HLA-A24-restricted CMV pp65 epitope, was used as an irrelevant control CTL. (*b*) Cytolytic activity of 2B2-CTL against SiHa cells treated with epoxomicin (Epo) and IFN-γ was assessed with a standard 4-h ^{51}Cr release assay. (*c*) Cytolytic activity of 2B2-CTL against SiHa cells treated with bortezomib (Bor) and IFN-γ, assessed as in panel (*b*). (*d*) Cytolytic activity of 2B2-CTL against SiHa/E6-E7 cells treated with bortezomib (Bor) and IFN-γ assessed as in panel (*c*).

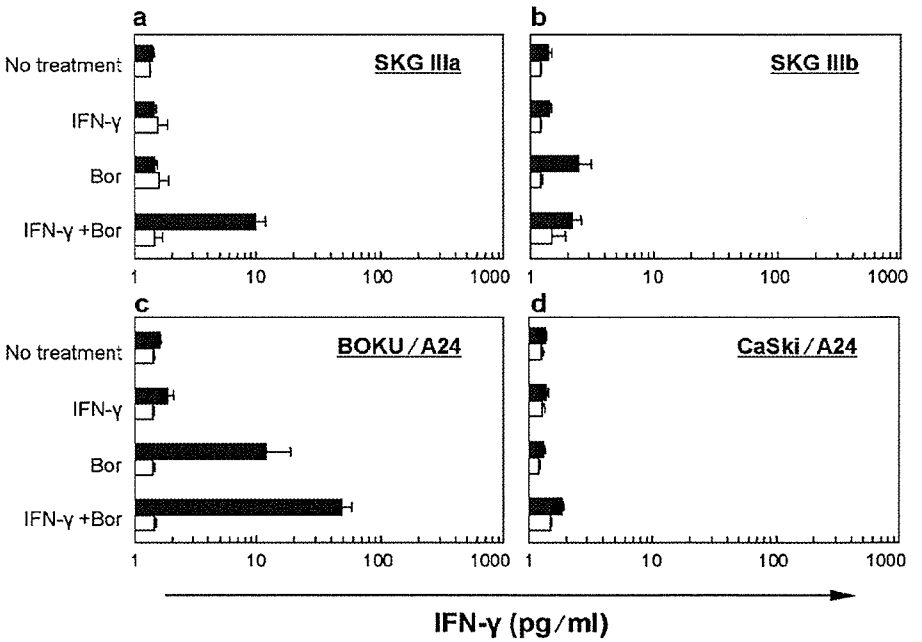

FIGURE 4 – IFN-γ production from 2B2-CTL cocultured with cervical cancer cell lines. After pretreatment of SKG IIIa (*a*), SKG IIIb (*b*), BOKU/A24 (*c*) and CaSki/A24 (*d*) cells with bortezomib (Bor) and IFN-γ, 2B2-CTL (solid bars) or no CTL (open bars) were added. Supernatants were harvested and assayed by ELISA. Values are means ± SD obtained from triplicate samples in 1 of 2 independent experiments.

IFN-γ production by 2B2-CTL, while IFN-γ pretreatment alone was not effective (Fig. 3*a*). The synergistic effect was clearly reflected by lytic activity of 2B2-CTL; SiHa cells pretreated with both IFN-γ and epoxomicin (Fig. 3*b*) or bortezomib (Fig. 3*c*) showed enhanced susceptibility to cytolysis by 2B2-CTL. Surprisingly, SiHa/E6-E7 cells, which were moderately lysed by 2B2-CTL (Fig. 2*d*), underwent no lysis when treated with IFN-γ alone (see Discussion), whereas pretreatment with bortezomib alone gave robust lysis (Fig. 3*d*).

The encouraging results of the efficacy of the combined treatment prompted us to test other cervical cancer cell lines by focusing on clinically available bortezomib.[14] Pretreatment of 4 cervical cancer cell lines (SKG IIIa, SKG IIIb, BOKU/A24 and CaSki/A24) with bortezomib and IFN-γ led to the production of IFN-γ by 2B2-CTL in BOKU/A24 and SKGIIIa, although the level of IFN-γ production was 20- and 100-fold lower, respectively (Figs. 4*a* and 4*c*), when compared with the results in SiHa cells (Fig. 3*a*). Little or no IFN-γ production by 2B2-CTL was observed upon stimulation with SKG IIIb and CaSki/A24 cells (Figs. 4*b* and 4*d*). However, when further transduced with E6-E7 cDNA, CaSki/A24 cells stimulated IFN-γ production to levels comparable to BOKU/A24 in the presence of bortezomib (data not shown), suggesting an insufficient amount of E6 protein in CaSki cells, partly accounting for the lack of recognition (Fig. 4*d*). In addition, none of these 4 cell lines pretreated with epoxomicin and IFN-γ induced significant IFN-γ production (data not shown), unlike SiHa cells. Finally, restoration of IFN-γ production by 2B2-CTL upon stimulation with the pretreated BOKU/A24 and SKGIIIa was not accompanied by efficient lysis by the CTL (data not shown).

Treatment with bortezomib reduces E6 mRNA but its effect on the cell surface expression of HLA class I is marginal

There are many intracellular steps in the generation of cell surface peptide-MHC complexes, which can be targeted in immune evasion by tumors and viruses. To investigate how bortezomib in combination with IFN-γ restored immunogenicity of the cervical cancer cell lines tested, we first examined cell surface expression of HLA class I molecules (Fig. 5*a*). As expected, expression detected by anti-pan HLA class I mAb (clone W6/32) on all the cell lines was up-regulated by IFN-γ (left panels), while 5-hr treatment with bortezomib alone exerted little effect (middle panels). The simultaneous treatment with bortezomib and IFN-γ, however, caused mild reduction of HLA class I expression of all cell lines when compared with those treated with IFN-γ alone (left *vs.* right panels). Because endogenous peptide supply is critical to stabilize cell surface HLA expression, these data indicate that at least net peptide supply to HLA molecules was only slightly affected in IFN-γ-treated cell lines by 5-hr treatment with this proteasome inhibitor.

Next, expression levels of unspliced E6 mRNA were assessed by real-time RT-PCR analysis. The value for SiHa cells was slightly up-regulated by IFN-γ, as previously reported.[33] Unexpectedly, E6 mRNA was down-regulated after treatment with bortezomib alone in all cell lines, to values ranging from 4 to 60% of the untreated cell levels (Fig. 5*b*). In SKG IIIa and SKG IIIb cells, IFN-γ slightly down-regulated E6 mRNA, with further down-regulation on addition of bortezomib. In the case of BOKU cells, bortezomib down-regulated E6 mRNA was fully restored by IFN-γ. The quantitative results of real-time RT-PCR assays were confirmed by Western blot analysis (T. Kiyono, unpublished observations). Collectively, pretreatment of the cervical cancer cell lines with bortezomib was always associated down-regulation of E6 mRNA, and addition of IFN-γ showed mixed results. These findings altogether indicated that the restoration of 2B2-CTL recognition by pretreatment with bortezomib especially in combination with IFN-γ is not due to E6 mRNA up-regulation, but rather is likely caused by modulation of proteasomal function by bortezomib, as reported previously.[11–13]

*HLA-A*2402-restricted $E6_{49-57}$-specific CD8+ T cells can be induced from CIN and cervical cancer patients despite low precursor frequencies*

Identification of the immunogenic HLA-A*2402-restricted HPV-16 E6-derived epitope enabled us to synthesize the HLA-A24/$E6_{49-57}$ tetramer, which clearly stained 2B2-CTL (Fig. 6*a*, right panel) and detected a growing population of $E6_{49-57}$-specific T cells in bulk culture after stimulation from which 2B2-CTL was generated (Fig. 6*a*, middle panel). In a prospective cohort study consisting of twenty HLA-A*2402$^+$ patients with CIN or cervical

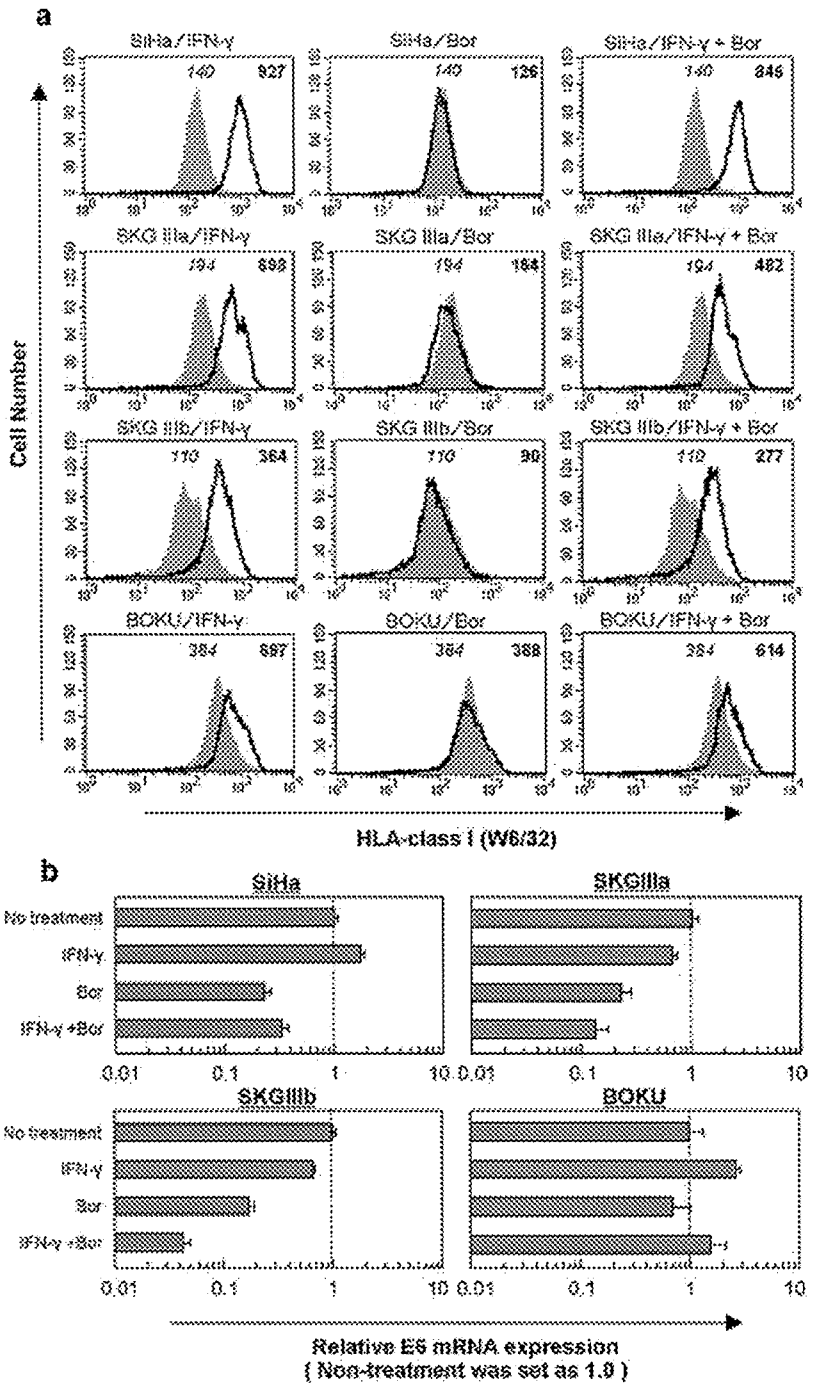

FIGURE 5 – Expression of cell surface HLA-class I molecules and unspliced form of E6 mRNA in cervical cancer cell lines after treatment with bortezomib (Bor) and IFN-γ. (*a*) Flow cytometric analysis of HLA-class I expression on SiHa, SKG IIIa, SKGIIIb and BOKU cells. Open areas represent fluorescence distributions of cervical cancer cells treated with bortezomib and IFN-γ, and filled areas represent those for untreated cervical cancer cells. The mean fluorescence intensity for each result after the indicated treatment (in bold) or no-treatment (in italics) is shown. The mean fluorescence intensity for negative controls (FITC isotype control) was always between 3 and 4. (*b*) Real-time RT-PCR analysis of unspliced HPV-16 E6 transcripts in the cell lines shown in panel (*a*), treated with bortezomib (Bor) and IFN-γ, compared with no-treatment controls. Expression of unspliced HPV-16 E6 transcripts in each sample was normalized to the internal GAPDH level, and values are expressed relative to the level found in the no-treatment control cells as 1.0.

cancer, we investigated if $E6_{49-57}$-specific $CD8^+$ T cells could be induced from their PBMCs by stimulation with peptide-pulsed autologous CD40B cells.[19] $CD8^+$ T cells were stimulated in multiple wells of 48-well plates depending on the yield from individual patients. All those undergoing conization or hysterectomy were found to be positive for certain HPV. Patient characteristics and a summary of the results are shown in Table II. Of the total, 7 were $HPV\text{-}16^+$, 6 were $HPV\text{-}18^+$ and the remaining 7 had unclassified HPV infections, which were at least not HPV-16. HLA-A24/$E6_{49-57}$ $tetramer^+$ T cells were successfully induced in 5 of 7 $HPV\text{-}16^+$ patients and 5 of 13 $HPV\text{-}16^-$ patients. Distinct populations of $CD8^+$ $tetramer^+$ cells were observed in these patients after 3 rounds of stimulation, at frequencies from 0.06 to 68.1% of the $CD8^+$ T cells (Figs. 6*b*, 6*c* and Table II). However, $tetramer^+$ cells were not detectable as a distinguishable cluster in the unstimulated $CD8^+$ fraction of any of the patients tested (data not shown), implying that the precursor frequency of HLA-A*2402-restricted $E6_{49-57}$-specific T cells in unstimulated PBMCs was less

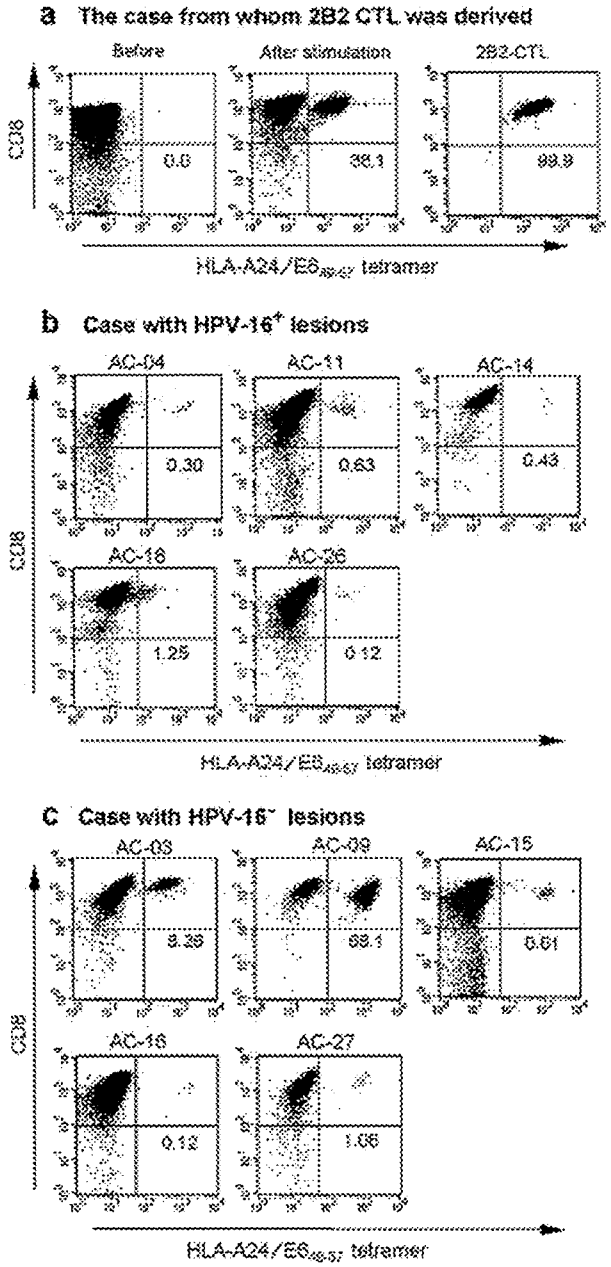

FIGURE 6 – Detection of HLA-A*2402-restricted $E6_{49-57}$-specific $CD8^+$ T cells using the HLA-A24/$E6_{49-57}$ tetramer. Unstimulated peripheral blood $CD8^+$ cells from patients with CIN or cervical cancer were stimulated with $E6_{49-57}$ peptide-pulsed CD40-B cells 3 times, as described in the Materials and Methods. The percentage of T cells binding to the tetramer among all $CD8^+$ is indicated in each panel. (*a*) Results of tetramer staining of unstimulated (left panel) and stimulated (middle panel) T cells from the patient from whom clone 2B2 was generated, and 2B2-CTL (right panel) are shown. (*b*) Representative positive staining results for T cell lines obtained from 5 of 7 patients with HPV-16-positive cervical lesions. (*c*) Representative positive staining results for T cell lines from 5 of 13 patients with HPV-16-negative cervical lesions.

than 10^{-4}. Finally, we further attempted to generate T cell lines from additional CIN patients by stimulating their PBMCs with 2 other candidate peptides (Table I), $E6_{87-95}$ and $E6_{98-106}$ in 2 and 8 patients, respectively, but no T cell lines specific for the peptides could be induced.

TABLE II – $E6_{49-57}$-SPECIFIC T CELL INDUCTION FROM PBMCS OF HLA-A*2402$^+$ PATIENTS DIAGNOSED WITH CIN OR CERVICAL CANCER

Patient group	Case	Age	Histological stage[1]	Induction efficiency[2]	% Tetramer$^+$ in $CD8^+$ cells[3]
HPV-16$^+$ patients	AC-04	24	CIN III	1/1	0.3
	AC-07	55	CIN III	0/3	–
	AC-10	58	CxCa	0/2	–
	AC-11	50	CIN III	1/4	0.63
	AC-14	40	CIN III	2/4	0.43, 0.38
	AC-18	27	CxCa	1/5	1.25
	AC-26	42	CxCa	1/2	0.12
HPV-16$^-$ patients	AC-01	58	CIN III	0/1	
	AC-03	55	CxCa	1/1	8.26
	AC-08	28	CIN III	0/2	–
	AC-09	36	CIN III	4/4	68.1, 38.9, 0.2, 0.06
	AC-15	34	CIN III	1/3	0.61
	AC-16	51	CIN III	2/3	0.12, 0.08
	AC-19	48	CIN II	0/2	–
	AC-20	52	CIN III	0/6	–
	AC-23	43	CIN III	0/5	–
	AC-24	53	CIN III	0/4	–
	AC-25	40	CIN III	0/4	–
	AC-27	36	CIN III	1/5	1.06
	AC-28	55	CIN III	0/2	–

[1]CIN, cervical intraepithelial neoplasia with histological grading; CxCa, cervical cancer.–[2]Number of wells containing A24/$E6_{49-57}$ tetramer$^+$ $CD8^+$ cells out of total wells set-up.–[3]Percentage of tetramer$^+$ cells among $CD8^+$ cells in the tetramer$^+$ wells.

Discussion

High-risk HPV E6 and E7 oncoproteins are indispensable to maintain the malignant growth of cervical cancer cells and are thus almost ubiquitously expressed in cervical lesions. It has been shown that 3 HLA-A*0201 restricted epitopes ($E7_{11-20}$, $E7_{82-90}$ and $E7_{86-93}$) induce CTL responses in HLA-A*0201 transgenic mice, and the generated CTLs lyse HLA-A*0201$^+$ HPV-16-positive CaSki cells.[34] As reported by Muderspach *et al.*,[35] increase of E7 epitope-specific reactivity in cytokine release and cytotoxicity assays was demonstrated in 10 of 16 HPV-16-positive HLA-A*0201$^+$ patients with high-grade cervical or vulvar CIN after vaccination with either $E7_{11-20}$ or $E7_{86-93}$ epitopes, and a proportion of these patients achieved partial clearance of virus infections and regression of lesions. Therefore, we sought to identify HLA-A*2402-restricted CTL epitopes from HPV-16 E6 and E7 oncoproteins by reverse immunology to facilitate immunotherapy for cervical cancer patients. The initial search for peptides carrying the HLA-A*2402 binding motif using a computer algorithm[26] gave only 3 candidate peptides within the E6 sequence. Two of these have been predicted and shown to bind to HLA-A*2402 molecules *in vitro* previously,[36] and we here demonstrated that a 2B2-CTL specific for one of these peptides, $E6_{49-57}$, could recognize an endogenously processed E6 epitope, although combined treatment of the cervical cancer cell lines with proteasome inhibitors and IFN-γ was necessary. Very recently, 2 other HLA-A*2402-restricted HPV-16 E6 CTL epitopes, $E6_{82-90}$ and $E6_{98-107}$, were reported by Hara *et al.*, whose BIMAS scores were similar to our candidate peptides (Table I), 200 and 360, respectively.[37] The reason why $E6_{82-90}$ was not included in our candidate peptides resulted from a nonsynonymous nucleotide difference corresponding to the E6 aa 82–90 region that disrupted the anchor motif for HLA-A*2402 in the HPV-16 DNA sequence we based on (GenBank accession no. AF003015). $E6_{98-107}$ was not considered because we paid little attention to decameric peptides, these being relatively rare for HLA-A*2402.[38] Comparative experiments are now underway to determine the immunogenecity among patients with CIN and cervical cancers.

The SiHa cells used in this study have been shown to express normal levels of TAP1, TAP2 and IFN-γ-inducible proteasome subunits of low molecular mass protein 2 and 7 molecules.[39] Since forced expression of E6 mRNA on transduction of E6-E7 cDNA rendered SiHa cells susceptible to lysis by 2B2-CTL and the recognition was augmented by bortezomib while it was abrogated by IFN-γ (Fig. 3*d*), and E6 mRNA was down-regulated by bortezomib while it was upregulated by IFN-γ (Fig. 5*b*, left upper panel), we speculate the presence of a complicated, but very intriguing balance among E6 protein supply, processing and destruction does exist, at least in SiHa cells. First, there seems no doubt that insufficient E6 oncoprotein supply is a major hurdle for tumor cell escape from CTL recognition. In this regard, it is of note that Evans *et al.* reported using CTL clones specific for an E6-derived epitope restricted by HLA-A*0201, $E6_{29-38}$, appropriate processing and presentation was observed in E6-transduced B-LCLs, but not in the HLA-A*0201 and HPV-16 expressing cell line, CaSki.[39] Although IFN-γ treatment upregulates immunoproteasomes and TAP proteins in CaSki cells, forced expression of E6 by a recombinant vaccinia virus encoding HPV-16 E6/E7 fusion protein was required to render them susceptible to CTL lysis.[39] In contrast, E7 has been shown to be more abundantly expressed than E6 in HPV-16-positive and HPV-18$^+$ cervical cancer cell lines[40,41] may explain why CTLs specific for 3 HLA-A*0201-restricted E7 epitopes ($E7_{11-20}$, $E7_{82-90}$ and $E7_{86-93}$)[36] could kill CaSki cells (HLA-A*0201$^+$).[34]

Second, IFN-γ gave a marginal effect on E6 mRNA expression, but its favorable effect on increased cell surface expression of HLA class I molecules was evident in most cell lines tested, which is critical for efficient recognition by CD8$^+$ T cells. Nevertheless, it is likely that IFN-γ treatment facilitated either active destruction of the $E6_{49-57}$ epitope or failure in epitope generation by immunoproteasome,[42] which is induced by IFN-γ as observed in IFN-γ-treated SiHa/E6-E7 cells. It remains to be determined in the future whether IFN-γ secreted from tumor-reactive CTLs might unexpectedly allow tumor cells to escape by further reducing the amount of epitopes possessing kinetics similar to $E6_{49-57}$. How such epitopes can sensitize cognate naive CTLs also warrants attention because professional antigen-presenting dendritic cells are equipped mainly with immunoproteasomes.

Third, bortezomib uniformly diminished mRNA expression levels probably as a result of general suppressive effects *via* NFκB inactivation, although the magnitude varied among cells (Fig. 5*b*). Nevertheless, treatment with bortezomib alone was sufficient to render SiHa/E6-E7 cells susceptible to lysis by 2B2-CTL. Because the restored IFN-γ secretion from 2B2-CTL was observed in all cervical cancer cell lines but the CaSki SKGIIIb cells, either with bortezomib alone or in combination with IFN-γ, it is of note that bortezomib functioned favorably to overcome the insufficient newly generation of HPV-16 $E6_{49-57}$ epitope for a very short time of 5 hr, probably by inhibiting proteasomal destruction of this particular epitope or another potential mechanism (see below). Immunoproteasomes are likely to have more catalytic activity against epitopes, but it is very important to note that this activity is also efficiently inhibited by bortezomib (Fig. 3*d*). Indeed, it has been reported that bortezomib can target both standard proteasomes and immunoproteasomes.[16–18] These findings encourage us to propose complementary roles of the 2 key drugs: bortezomib can facilitate cell lines to produce the $E6_{49-57}$ epitope, while IFN-γ harnesses the antigen-presenting machinery resulting in increased cell surface MHC expression. In addition, increased MHC biosynthesis due to IFN-γ may be favorable to quickly change peptide arrays presented on MHC molecules that include the $E6_{49-57}$ epitope. Finally, the possibility should be taken into consideration that target cancer cells could be prone to induction of apoptosis due to reduction of NF-κB activity by proteasome inhibitors,[43] although we did not observe increased spontaneous cell death in our ^{51}Cr-based cytotoxicity assays.

As described above, we chose proteasome inhibitors to make the limited amount of E6 protein efficiently available for antigen processing. However, several reports have demonstrated that the presentation of certain antigenic peptides is insensitive to proteasome inhibitors.[44,45] These reports provide another explanation for the enhancement of $E6_{49-57}$ epitope processing and presentation by proteasome inhibitors in our study. That is, the epitope might be generated by a nonproteasomal cytosolic protease or by a certain retained proteasome activity other than chymotrypsin-like activity, which is sensitive to epoxomicin or bortezomib treatment examined in our study. Epitopes produced in a proteasome-independent manner will have reduced competition for assembly with MHC class I molecules in the presence of proteasome inhibitors,[13] as the majority of MHC class I ligands are generated by proteasomes, resulting in rather efficient $E6_{49-57}$ peptide presentation. Finally, we surmise that enhanced CTL recognition by proteasome inhibitors is basically an epitope-specific phenomenon rather than a general one from the previous epitope-oriented reports. However, our preliminary studies showed that IFN-γ secretion from a CTL clone specific for HLA-A*0201-restricted HPV-16 $E7_{11-20}$ against SiHa/A2 (HLA-A*0201-transduced SiHa cells) was enhanced by exposure to bortezomib and IFN-γ (data not shown), suggesting that increase in epitope presentation by proteasome inhibitors may not be an exceptional phenomenon.

Results of $E6_{49-57}$ peptide-specific T cell induction from PBMCs of a prospective cohort of patients with CIN and cervical cancer demonstrated that the $E6_{49-57}$ peptide is indeed immunogenic and that most patients possess precursor T cells specific for this epitope. Youde *et al.*[46] have demonstrated HPV-16 $E7_{11-20}$ tetramer$^+$ CD8$^+$ cells in PBMCs to be rare in both CIN patients and healthy controls, but a CTL precursor population became detectable after peptide stimulation, as observed in our study. Thus, we surmise that immunity against E6 oncoprotein does exist in patients despite unsuccessful tumor eradication. There have been several conflicting results regarding CTL responses to HPV-16 E6 and E7 in cancer patients and healthy donors. Bontkes *et al.*[47] demonstrated that patients with persistent HPV-16 infection had specific memory CTL activity against E6 or E7, while no specific CTL activity was detected in HPV-16 negative patients or patients, who were cleared of HPV-16 infections. In contrast, Nakagawa *et al.*[48] demonstrated higher rates of HPV-16 E6-specific CTL responses in women, who had been cleared of HPV-16 infection compared with those who had not. In our study, HLA-A24/$E6_{49-57}$ tetramer$^+$ T cells were induced in both HPV-16$^+$ and HPV-16$^-$ patients at similar rates. We speculate that some of the latter group might have been successfully cleared of HPV-16 infection but the current cervical neoplasia was caused by a different type of HPV because superinfection with multiple types of HPVs has been shown to be common.[49] In fact, among 13 HPV-16$^-$ patients, 6 were found to have HPV-18$^+$ lesions, and 2 of them produced HLA-A24/$E6_{49-57}$ tetramer$^+$ T cells. In addition, the homology of E6 epitope of HPV-16 and HPV-18 is not very high (*i.e.* VYDFAFRDL *vs.* VFEFAFKDL; shared residues are underlined). These results suggest the possibility of past infection with HPV-16. The low CTL precursor frequency specific for HPV-16, possibly caused by poor natural immunogenicity of this virus as discussed above compared to more immunogenic viruses such as EBV, makes it difficult to address the question because serological tests do not detect previous infectious events with this virus.

In conclusion, our results demonstrate that $E6_{49-57}$ is a naturally-processed HPV-16 antigen, which can induce specific CTL in patients with cervical neoplasia. We also could demonstrate that the natural poor immunogenicity of HPV-associated cervical neoplasia may be in part overcome with a proteasome inhibitor, bortezomib, by facilitating generation of cryptic peptides in combination with IFN-γ. Since bortezomib and IFN-γ have been used clinically, they may provide patients suffering from advanced HPV-16 neoplasia with a new therapeutic option to be tested in future clinical studies.

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