Epstein–Barr virus nuclear antigen 1-specific CD4+ T cells directly kill Epstein–Barr virus-carrying natural killer and T cells

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Epstein–Barr virus (EBV) nuclear antigen (EBNA)1 is expressed in every EBV-infected cell, regardless of the state of EBV infection. Although EBNA1 is thought to be a promising antigen for immunotherapy of all EBV-associated malignancies, it is less clear whether EBNA1-specific CD4+ T cells can act as direct effectors. Herein, we investigated the ability of CD4+ T-cell clones induced with overlapping peptides covering the C-terminal region of EBNA1, and identified minimal epitopes and their restricted major histocompatibility complex class II molecules. Of these, a novel epitope, EYHQEGGPD, was found to be presented by DRB1*0401, 0403 and 0406. Five CD4+ T-cell clones recognized endogenously processed and presented antigens on EBV-transformed lymphoblastoid cell lines (LCL) and one example proved capable of killing EBV-carrying natural killer (NK) and T-cell lines derived from patients with chronic active EBV infection (CAEBV). Identification of minimal epitopes facilitates design of peptide-based vaccines and our data suggest that EBNA1-specific CD4+ T cells may play roles as direct effectors for immunotherapy targeting EBV-carrying NK and T-cell malignancies. (*Cancer Sci* 2008; 99: 1633–1642)

The Epstein–Barr virus (EBV) is involved in development of many malignancies, including Burkitt's lymphoma (BL), Hodgkin's disease (HD) and the nasopharyngeal carcinoma, as well as post-transplant lymphoproliferative disorder.[1] It is also related to natural killer (NK) and T lymphomas and causes chronic active EBV infection (CAEBV).[1,2] Only EBV nuclear antigen (EBNA)1 is expressed in most BL, referred to as latency I. In addition to EBNA1, latent membrane protein (LMP)1 and/or 2 are expressed in HD, nasopharyngeal carcinomas, NK and T lymphomas, and CAEBV (latency II). All EBV latent antigens, EBNA1, 2, 3A, 3B, 3C and the leader protein, and LMP1 and 2 are expressed in post-transplant lymphoproliferative disorder (latency III). EBNA1 is expressed in common in all these diseases,[3] and may be present diffusely bound to mitotic chromosomes.[4] EBV has a *cis*-acting element, termed OriP, that enables the persistence of episomes in EBV-infected cells. EBNA1 also binds to OriP,[5] and is essential for EBV episome maintenance.[6] Thus, the existence of EBV DNA is tightly associated with EBNA1 expression in EBV-infected cells.

Evidence for the significance of EBV-specific T cells for control of EBV infection has been obtained from both *in vitro* and *in vivo* studies.[7,8] Human leukocyte antigen (HLA) class I-restricted CD8+ cytotoxic T lymphocytes (CTL) recognize latent and lytic EBV antigens,[8] and latent EBV antigen-specific CTL kill not only lymphoblastoid cell lines (LCL) expressing the full spectrum of latent viral proteins but also tumor cells with limited viral proteins.[9–11] EBV-specific CTL responses have been extensively studied, and CTL are thought to be the main effectors. EBNA1-specific CTL were long believed to be immunologically silent because EBNA1 contains an internal G-A repeat (GAr) domain which has an immune evasion function endowing resistance to proteasomal degradation in the antigen presentation pathway.[12,13] However, it has been reported that having GAr EBNA1 does not completely evade major histocompatibility complex (MHC) class I presentation.[10,14–16] There are thus some EBNA1 antigen epitopes, though the importance of EBNA1-specific CTL for EBV-positive tumors remains unclear.

Accumulating current evidence indicates that CD4+ T cells, as well as CTL, are required for effective antitumor immunity,[17–19] for example, playing an essential role in generation of CD8+ T memory cells.[20–22] In addition, there are reports of cytotoxic action of CD4+ T effector cells *in vitro*[23] and *in vivo*.[23,24] This has drawn attention to the possibility that EBV-specific CD4+ T cells are able to recognize EBV-infected cells. There is increasing interest in CD4+ T-cell responses to EBV as direct effectors. HLA class II-restricted CD4+ T cells specific for EBV latent and lytic antigens have been explored,[25–27] with the focus on EBV-infected B cells, mainly LCL, in which the HLA class II pathway of antigen presentation is active. Because immunoglobulin (Ig) isotype switching requires T-cell help, the presence of IgG antibodies to antigens implies that the latter are also targets of CD4+ T-cell responses.[28,29] Actually, healthy virus carriers are consistently positive for anti-EBNA1 IgG antibodies,[1] implying the existence of EBNA1-specific CD4+ T cells *in vivo*, because CD4+ T cells indeed recognize EBNA1 in healthy EBV carriers.[30,31] EBNA1 has been considered a promising antigen for T-helper (Th) cells. Moreover, it is clear that CD4+ T cells specific for EBNA1 can act as direct effectors for lysing BL cells and HD cells *in vitro*.[32] Furthermore, in a mouse model of BL, EBNA1-specific CD4+ T cells could suppress BL tumor growth *in vivo*.[33]

We report here the identification of five EBNA1-specific CD4+ T-cell clones recognizing LCL and their minimal epitopes. One HLA-DR4-restricted example is novel and the other four appear to be parts of longer peptides,[25,31] some of which are presented by other HLA class II molecules than those determined in this report. Of particular interest is a CD4+ T-cell clone killing EBV-infected T cells positive for DR51 and EBV-infected NK cells transduced with DRB5*0101. Because these EBV-infected NK and T cells express DR, DP and DQ, they could be potential targets of CD4+ T cells. These results imply that EBNA1-specific CD4+ T cells may also act as direct effectors *in vivo*.

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Table 1. Human leukocyte antigen (HLA) class II genotype of the donors and cell lines

Donors or cell lines	DRβ1	Other DR alleles	DPβ1	DQβ1
Donor X	*0401, *1501	DRβ4*0102, DRβ5*0101	*0201, *1401	*0301, *0602
Donor W	*0803, *1401	DRβ3*0202, NA	*0202, *0501	*0601, *0503
A1 LCL	*0101, *1401	DRβ3*0202, NA	*0402, *0501	*0501, *0502
A2 LCL	*0901, *0901	DRβ4*0103, DRβ4*0103	*0201, *0901	*0303, *0303
SNK10	*0901, *0901	DRβ4*0103, DRβ4*0103	*0201, *0402	*0303, *0303
SNT15	*0101, *0406	DRβ4*0103, NA	*0201, *0402	*0302, *0501
SNT16	*1201, *1502	DRβ3*0101, DRβ5*0102	*0501, *0901	*0303, *0601

NA, not assayed. LCL, lymphoblastoid cell lines.

Materials and Methods

Donors and cell lines. The study design and purpose, which had been approved by the institutional review board of Aichi Cancer Center, were fully explained and informed consent was obtained from all blood donors. HLA typing was carried out at the HLA Laboratory (Kyoto, Japan). The HLA class II genotype from donors X and W, and cell lines are shown in Table 1.

Epstein–Barr virus-transformed B-LCL were established as described previously,[34] and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin and 50 μg/mL kanamycin.

An EBV-carrying NK cell line, SNK10, and an EBV-carrying γδ T-cell line, SNT15, and an EBV-carrying αβ T-cell line, SNT16, were kindly provided by Dr Shimizu (Tokyo Medical and Dental University, Tokyo, Japan). All three were derived from different CAEBV patients and cultured as previously described,[35,36] along with HEK-293 T cells. CD40-activated B (CD40-B) cells were generated as detailed earlier,[37,38] using NIH/3T3-human CD40 ligand cells, kindly provided by Dr Freeman (Dana-Farber Cancer Institute, Boston, MA, USA).

Phoenix-GALV cells kindly provided by Dr Kiem (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) and Dr Nolan (Stanford University School of Medicine, Stanford, CA, USA) were cultured as previously described.[39] Retroviral transduction of HLA genes was performed as reported earlier.[37]

Plasmid construction. HLA-DRA cDNA were amplified by polymerase chain reaction (PCR) using a sense primer, 5′-ggatccgccaccATGGCCATAAGTGGAGTCCCTG-3′, and an antisense primer, 5′-gcggccgcTTACAGAGGCCCCCTGCGTTC-3′. The following primer pairs were used for PCR amplification of HLA-DRB1*0401 cDNA, HLA-DRB1*1501 cDNA, HLA-DRB4*0102 cDNA and HLA-DRB5*0101 cDNA: DRB1*0401 sense primer, 5′-ggatccgccaccATGGTGTGTCTGAAGTTCC-3′; DRB1*0401 antisense primer, 5′-gcggccgcTCAGCTCAGGAATCCTGTTG-3′. DRB1*0403, 0405 and 0406 cDNA were amplified by PCR with the DRB1*0401 sense primer and an antisense primer: DRB1*1501 sense primer, 5′-ggatccgccaccATGGTGTGTCTGAAGCTCC-3′ (fwd-1); DRB1*1501 antisense primer, 5′-gcggccgcTCAGCTCAGGAATCCTGTTG-3′; DRB4*0102 sense primer, fwd-1; DRB4*0102 antisense primer, 5′-gcggccgcTCAGCTCAAGAGTCCTGTTG-3′; DRB5*0101 sense primer, fwd-1; and DRB5*0101 antisense primer, 5′-gcggccgcTCAGCTCACGAGTCCTGTTG-3′. The resultant individual DNA fragments were cloned into pcDNA 3.1(+) using its *Bam*HI and *Not*I sites.

Full-length EBNA1 cDNA was cloned into pcDNA 3.1(+) (pcDNA/EBNA1).[16] EBNA1 without GAr (referred to as ΔGA-EBNA1) was constructed from pcDNA/EBNA1, as described previously.[40] The resulting construct was deleted from the GAr domain (EBNA1 codons 92–323).

Synthetic peptides. C-terminal EBNA1 polypeptides covering its 402–624 amino acids (a.a.) were deduced from the prototype B95-8 (National Center for Biotechnology Information accession no. V01555) DNA sequence. A total of thirty 20-mer peptides overlapping by 13 a.a. were designed, and purchased from Bio-Synthesis (Lewisville, TX, USA). A six-subpool was constructed from four to five peptides, excluding peptides including HLA class I-restricted epitopes. For example, HLA-B35-seropositive donors might be expected to respond to HPVGEADYFEY, and epitope-specific $CD8^+$ T cells might be expanded. Other known epitopes were excluded as well. To identify the core epitope sequence, 13- and 11-mer peptides were further designed and purchased from Bio-Synthesis. The one-letter a.a. code is used throughout the article.

Generation of $CD4^+$ T-cell lines and clones. Peripheral blood mononuclear cells (PBMC) from two donors were stimulated with individual peptide pools of 500 nM of each peptide in 2 mL RPMI-1640 medium supplemented with 10% human serum, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin and 50 μg/mL kanamycin at 5% CO_2 in a humidified incubator. On days 8 and 15, T cells were restimulated with peptide-pulsed γ-irradiated (33 Gy) autologous PBMC. One day after each restimulation, interleukin (IL)-2, kindly provided by Ms Sawada (Shionogi, Osaka, Japan), was added to a final concentration of 10 U/mL. After four rounds of stimulation, $CD4^+$ T cells were isolated with CD4 Microbeads (Miltenyi Biotec, Tokyo, Japan).

To establish T-cell clones, limiting dilution of isolated $CD4^+$ T cells was performed as previously described,[41] with slight modifications. Where necessary, EBNA1 peptide-reactive $CD4^+$ T cells were enriched from restimulated $CD4^+$ T cells using an γ-interferon (IFN-γ) Secretion Assay (Miltenyi Biotec), according to the manufacturer's instructions. Then, the purified $CD4^+$ T cells were seeded at 1 cell/well in round-bottomed 96-well plates in culture medium containing anti-CD3 monoclonal antibodies (mAb) (30 ng/mL), IL-2 (200 U/mL), γ-irradiated (33 Gy) 1×10^5 PBMC and γ-irradiated (55 Gy) 2×10^4 LCL. After 14–16 days of culture, the specificity of growing cells was examined with enzyme-linked immunosorbent spot (ELISPOT) assays of EBNA1 peptide-pulsed CD40 B cells and autologous LCL. Positive wells were transferred into flasks and expanded with anti-CD3 mAb, IL-2 (30 U/mL) and γ-irradiated feeders.

ELISPOT assays. ELISPOT assays were performed as previously described,[37,41] with minor modifications. Briefly, $CD4^+$ T cells were co-cultured with various stimulators for 20 h in AIM-V medium (Invitrogen, Carlsbad, CA, USA) in wells of Multiscreen-HA plates (MAHA S4510; Millipore, Billerica, MA, USA) coated with antihuman IFN-γ mAb (M700A; Pierce Biotechnology, Philadelphia, PA, USA). As stimulators, LCL (1×10^5 cells/well), HEK-293 T cells (5×10^4 cells/well) transfected with plasmids encoding HLA-DRA and DRB1 cDNA, and/or ΔGA-EBNA1 or EGFP,[37] cDNA with Lipofectamin 2000 (Invitrogen) 36 h earlier were seeded into each well. For peptide titration assays, serial concentrations of synthetic peptides were pulsed to HLA-DR-expressing HEK-293T (referred to as DR-293T) cells for 1 h at room temperature. In blocking assays, anti-HLA-DR mAb (L243; BD Bioscience, San Jose, CA, USA), anti-HLA-DQ mAb (TÜ169; BD Bioscience), and anti-HLA-DP mAb (BRA-FB6; MorphoSys,

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Kingston, NH, SUA) were added at the indicated concentrations, and incubated for 1 h prior to co-cultivation with T cells. After probing with polyclonal antirabbit IFN-γ antibody (P700; Pierce Biotechnology), and following exposure to horseradish peroxidase-labeled antirabbit IgG antibody and spot visualization, the plates were washed and dried. IFN-γ spots were enumerated using a dissecting microscope. In all experiments using $CD4^+$ T cell clones, results from ELISPOT assays are shown as the mean of two duplicate values.

CTL assays. Target cells were labeled with 1.85 MBq chromium (^{51}Cr) for 1.5 h at 37°C, washed and mixed with CTL at the indicated effector to target ratios in 96-well plates. After incubation for 14 h at 37°C, the radioactivity in the supernatants was counted in a γ-counter. The minimal release was less 30% of maximal release in all experiments. The percentage-specific ^{51}Cr release was calculated as follows: 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). All assays were done in triplicate wells. Standard deviations were calculated from each data.

Results

Induction of EBNA1-specific $CD4^+$ T cells. It has been reported that $CD4^+$ T-cell epitopes are concentrated within the C-terminal of the EBNA1 protein,[25,31,32] and we designed 30 peptides (20-mers overlapping by 13 residues) covering a.a. 402–624. To generate EBNA1-specific $CD4^+$ T cells, PBMC from two EBV-seropositive donors were stimulated with 6-peptide pools. After four rounds of stimulation, $CD4^+$ T cells were isolated using CD4 Microbeads and ELISPOT assays were performed to test the specificity of the $CD4^+$ T-cell lines. As shown in Fig. 1(a,b), X1, X3 and X4 $CD4^+$ T-cell lines and W4 and W5 $CD4^+$ T-cell lines specifically secreted IFN-γ in response to cognate peptide pool-pulsed autologous CD40-B cells, but not to irrelevant peptide pool-pulsed CD40-B cells.

To further assess IFN-γ secretion against each peptide, $CD4^+$ T-cell lines were tested against peptides belonging to each pool. As illustrated in Fig. 2(a), production of IFN-γ by the X1 $CD4^+$ T-cell line from donor X was raised by two peptides (residues 409–428 and 416–435). Interestingly, the X3 $CD4^+$ T-cell line recognized three peptides (residues 472–491, 479–498 and 486–505) (Fig. 2b). Fig. 2(c) shows that the X4 $CD4^+$ T-cell line responded to two peptides (residues 514–533 and 521–540). As shown in Fig. 2(d, e), the W4 $CD4^+$ T-cell line recognized one peptide (residues 514–533) and the W5 $CD4^+$ T-cell line recognized two (residues 563–582 and 570–589).

Identification of the EBNA1 epitope. We established five $CD4^+$ T-cell clones by limiting-dilution culture of $CD4^+$ T-cell lines. As expected from the specificity of the X3 $CD4^+$ T-cell line, the derived clones X3-11D1 and X3-3G2 responded to two distinct peptides. ELISPOT assays were performed to map the recognized epitope regions (Figs 3,4). Because clone X1-12B12 secreted IFN-γ in response to two overlapping peptides (residues 409–428 and 416–435, Fig. 2a), the epitope was speculated to be located around a.a. residues 416–428. We attempted to identify minimal epitopes despite the fact that they may not be as definitive as those recognized by $CD8^+$ T cells because MHC class II molecules can present peptides with various lengths.[42,43] To this end, 13- and 11-mer peptides spanning the overlapped regions were synthesized and tested in ELISPOT assays to explore the N- and C-terminal a.a. which are indispensable for recognition. As shown in Fig. 3(a), deletion of E at position 416 (referred to as E_{416}) from the 13-mer peptide abolished the IFN-γ production from clone X1-12B12. At the C-terminus, loss of G_{425} from the 11-mer peptide had the same effect. Accordingly we infer that the a.a. sequence, EYHQEGGPDG, is a putative minimal epitope for the clone recognition. Fig. 4(a) additionally demonstrates that a 13-mer peptide, (F)EYHQEGGPDG(EP), containing the minimal epitope

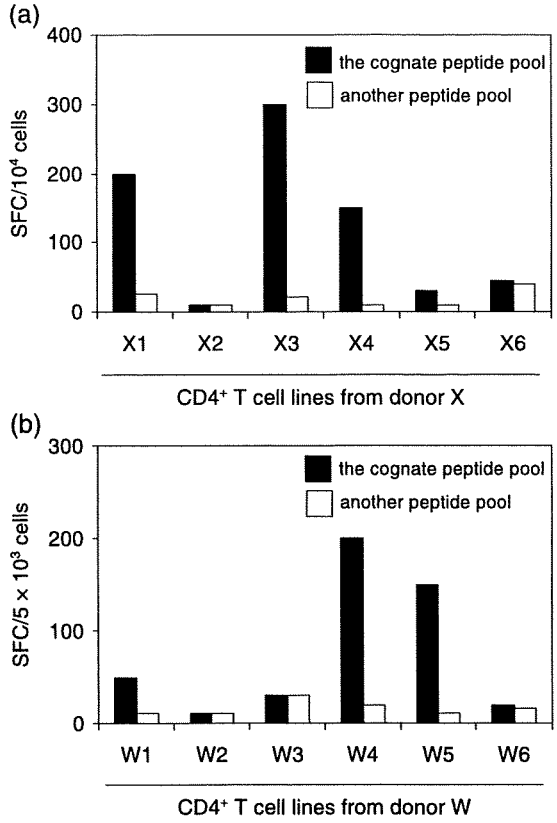

Fig. 1. Establishment of $CD4^+$ T-cell lines specific for Epstein–Barr virus nuclear antigen (EBNA)1 peptide pools. (a,b) Aliquots of peripheral blood mononuclear cells from donors X and W were stimulated *in vitro* with synthetic peptide pools four times. After CD4-positive selection, the responder cells were evaluated for their reactivity to the cognate peptide pools by enzyme-linked immunosorbent spot assays using autologous CD40-B cells as antigen-presenting cells. Another EBNA1 peptide pool that had not used in stimulation was tested for control. Data are numbers of spots per 10 000 $CD4^+$ T cells in (a) and 5000 $CD4^+$ T cells in (b). SFC, spot forming units.

sequence was better recognized than the 11-mer, (F)EYHQEGGPDG, and 13-mer, EYHQEGGPDG(EPD), peptides suggesting elongation of both N- and C-termini may augment the antigenicity.

As illustrated in Fig. 3(b), removal of P_{476} from 13- and 11-mer peptides completely abolished clone X3-11D1 recognition. The 13- and 11-mer peptides having R_{486} at the C-terminal stimulated the clone X3-11D1 to secrete IFN-γ. Thus, clone X3-11D1 responded to PKFENIAEGLR as the putative minimal epitope. Moreover, it recognized (SN)PKFENIAEGLR and (N)PKFENIAEGLR(A) more efficiently than the putative minimal epitope, PKFENIAEGLR (Fig. 4b).

As demonstrated in Fig. 3(c), deletion of L_{488} from the 13-mer peptide abolished the clone X3-3G2 recognition and IFN-γ secretion was also eliminated by deletion of R_{496} from the 13- and 11-mer peptides. This implies that LLARSHVER is the putative minimal epitope for the clone recognition. Furthermore, clone X3-3G2 could secrete IFN-γ in response to (RA)LLARSHVER(TT) and LLARSHVER(TTDE) as well as a 20-mer peptide (Fig. 4c).

As shown in Fig. 3(d), lack of R_{522} and L_{533} from 13-mer peptides abolished the clone X4-2C9 recognition. Accordingly, RGTALAIPQCRL is the putative minimal epitope required for IFN-γ secretion by clone X4-2C9, which did not respond to any 11-mer peptides. Moreover, clone X4-2C9 responded to 20-mer better than 13-mer peptides (Figs 3d, 4d).

As shown in Fig. 3(e), deletion of T_{568} from the 13-mer peptide abolished the IFN-γ production by clone W5-9D5, as did loss of

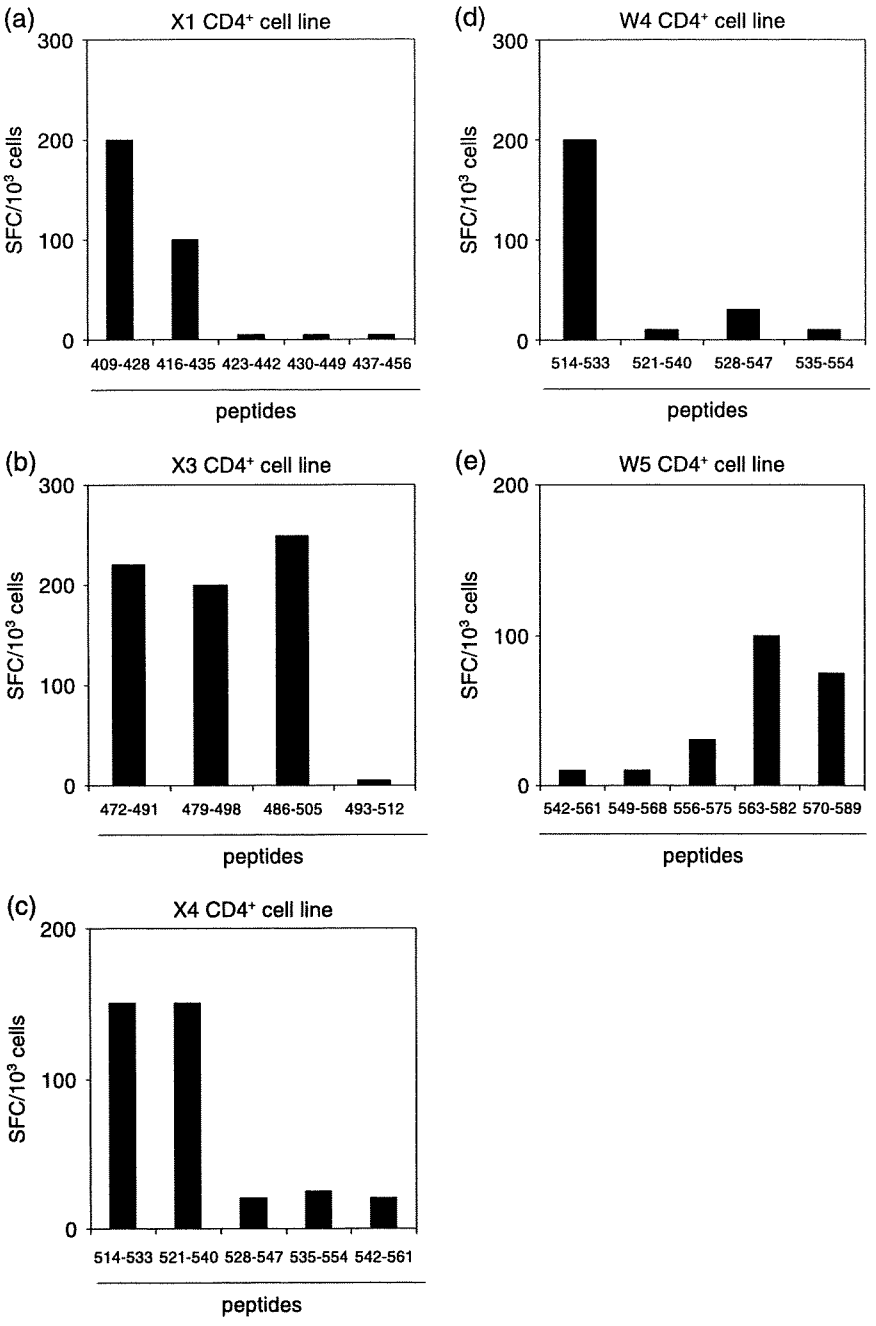

Fig. 2. Reactivity of CD4+ T-cell lines with individual peptides belonging to each pool. (a–e) Each CD4+ T-cell line was tested for its reactivity with individual peptides by enzyme-linked immunosorbent spot assay. Data are numbers of spots/1000 CD4+ T cells.

the C-terminal residues K_{576} and D_{577} from the 20-mer peptide. In consequence, THIFAEVLKD is the putative minimal epitope. Furthermore, removal of F_{565} from the 13- and 11-mer peptides resulted in decrease of IFN-γ secretion. As illustrated in Fig. 4(e), clone W5-9D5 could produce IFN-γ in response to (FLQ)THIFAEVLKD and THIFAEVLKD(AIK) as well as the 20-mer peptide. In conclusion, as listed in Table 2, the minimal peptide sequences recognized by CD4+ T-cell clones were identified. Four out of five established clones recognized minimal epitopes that are parts of epitope regions previously reported.[25,31]

HLA restriction of the EBNA1-specific clones. To identify the HLA molecule presenting EBNA1 to the CD4+ T-cell clones, ELISPOT assays were performed in the presence of antibodies against HLA-DR, DQ and DP molecules. Epitope-specific IFN-γ secretion by clones X1-12B12 and X4-2C9 was inhibited in the presence of the mAb against HLA-DR (Fig. 5a,b, top panel). The other mAb were without effect. To determine the restricting HLA-DR molecule, HEK-293 T cells expressing each donor HLA-DR allele were pulsed with their cognate peptides and used as stimulators in the ELISPOT assays. The cognate peptide was presented by HLA-DR4 for clone X1-12B12, and by HLA-DR51 for clone X4-2C9 (Fig. 5a,b bottom panel, respectively). As shown in Fig. 5(c,e), the HLA-DP-specific mAb blocked recognition by clones X3-11D1 and W5-9D5. No block was evident using mAb against HLA-DR and HLA-DQ. To verify the restriction molecule, ELISPOT assays were carried out using HLA-matched LCL pulsed with cognate or irrelevant peptides. Clones X3-11D1 and W5-9D5 recognized their cognate peptides in the context of HLA-DP2 and HLA-DP5 molecules, respectively. Accordingly, clone X3-11D1 could also recognize the epitope presented by HLA-DP5 (data not shown). As demonstrated in Fig. 5(d), clone X3-3G2 recognized its cognate peptide on the HLA-DQ6

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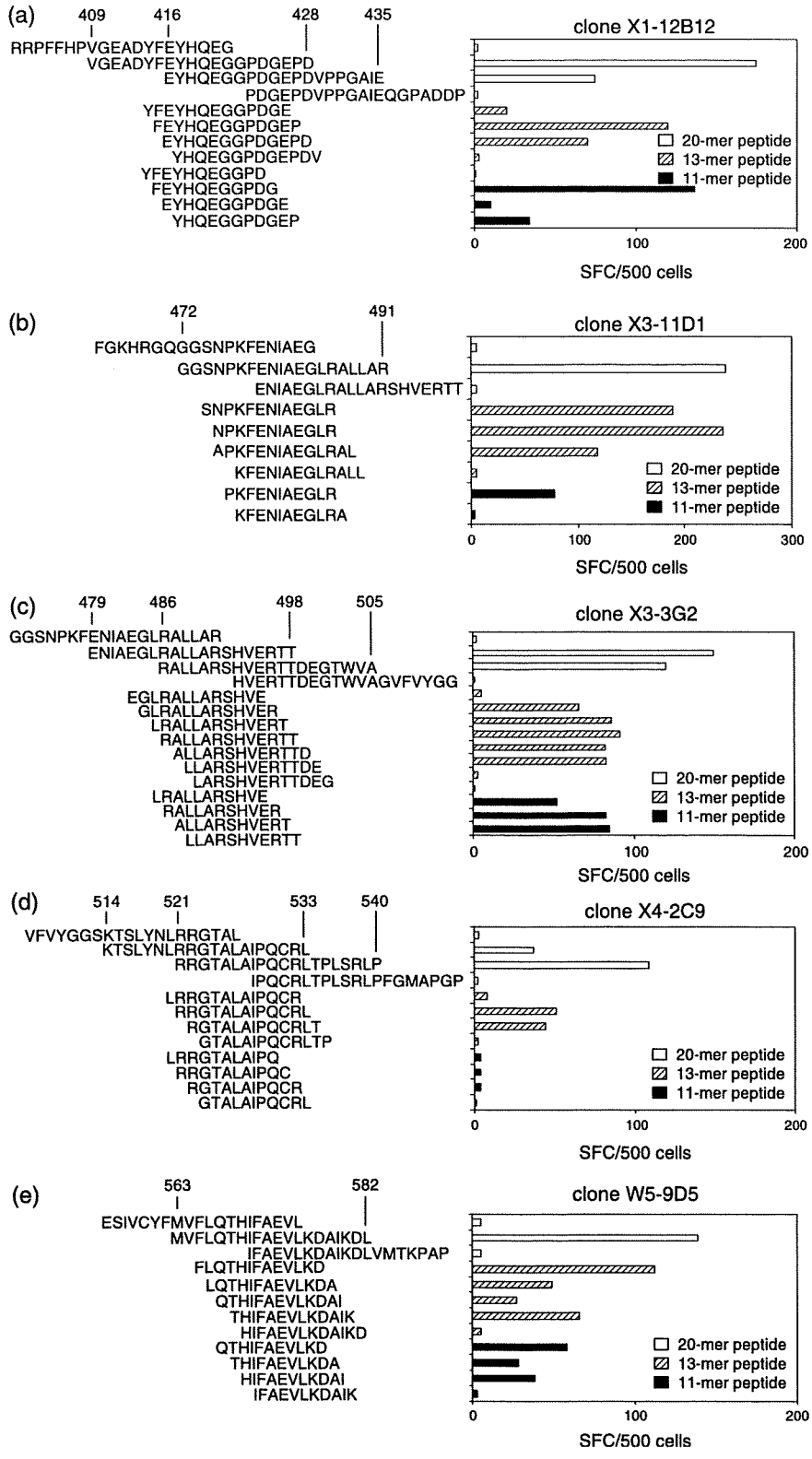

Fig. 3. Mapping of core regions recognized by CD4⁺ T-cell clones. (a–e) Peptides of 13- and 11-mer spanning overlapping regions were synthesized and tested for antigenicities in enzyme-linked immunosorbent spot assays. Numbers represent Epstein–Barr virus nuclear antigen 1 amino acid residues. All results are the mean of two duplicate values. SFC, spot forming units.

molecule. The identified HLA restriction alleles are listed in Table 2.

Recognition of LCL and DGA-transfected 293T by EBNA1-specific CD4⁺ T-cell clones. It has been reported that a CD4⁺ T-cell clone specific for the $EBNA1_{515-527}$ epitope fails to recognize LCL presenting naturally processed peptides on their surfaces.[44] To test whether established clones can recognize LCL without exogenous EBNA1 peptides, ELISPOT assays were performed. As shown in Fig. 6(a), all clones could secrete IFN-γ in response to autologous LCL. To identify their restricted molecules, HEK-293 T cells expressing the restricted HLA-DR alleles and ΔGA-EBNA1 protein were used in the ELISPOT assay (Fig. 6b,c).

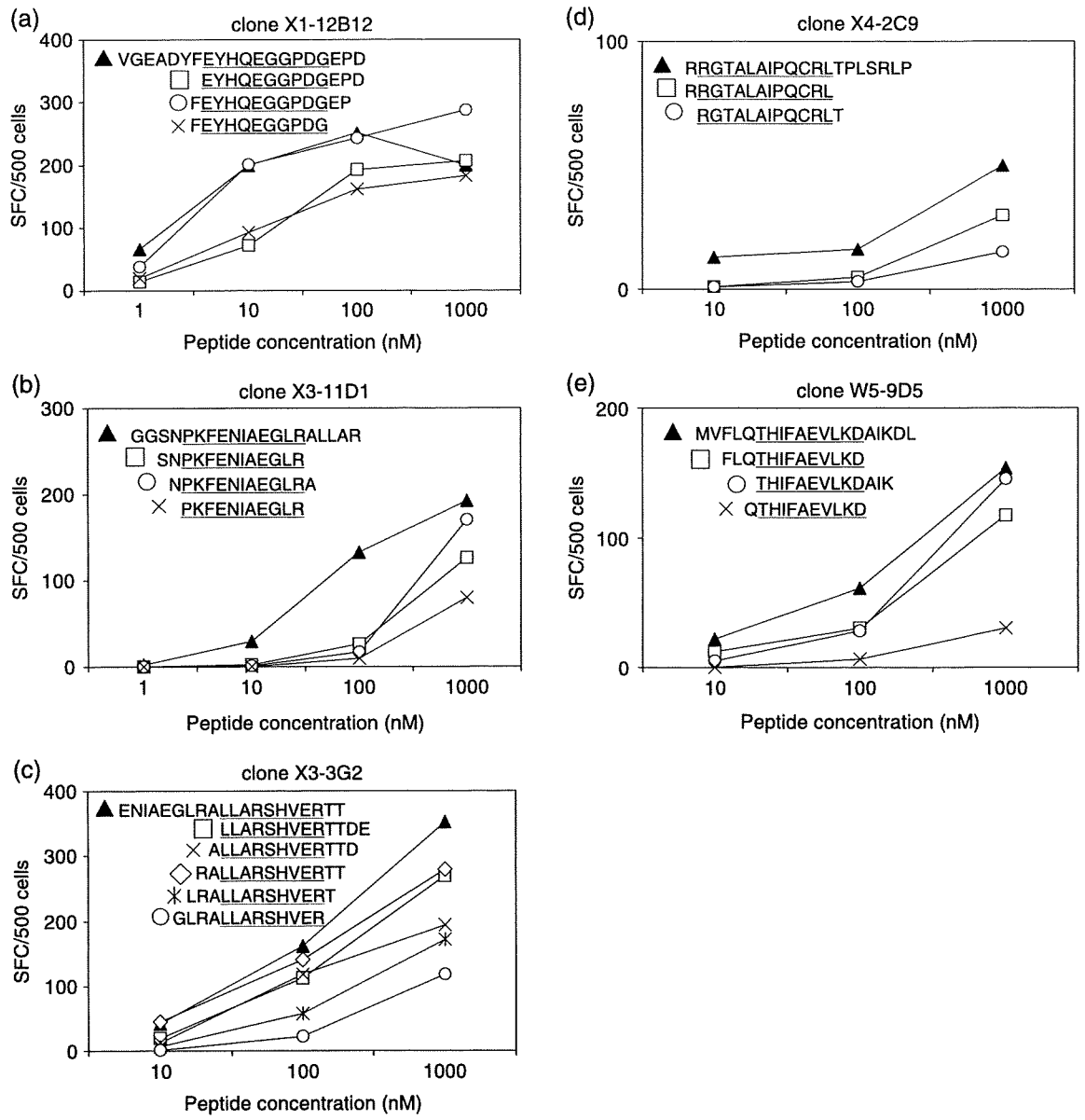

Fig. 4. Peptide dilution assay for CD4⁺ T-cell clones. (a–e) Each peptide at serial concentrations was tested in enzyme-linked immunosorbent spot assays. Data are numbers of spots/500 CD4⁺ T-cell clones. The core peptide sequences are underlined. All results are the mean of two duplicate values. SFC, spot forming units.

Clone X1-12B12 could recognize ΔGA-EBNA1-expressing HEK-293 T cells transfected with HLA-DRB1*0401, 0403 or 0406, but not DRB1*0405 (Fig. 6b). Clone X4-2C9 could recognize DR51-293 T cells transfected with ΔGA-EBNA1 (Fig. 6c).

Cytotoxic activity of EBNA1-specific CD4⁺ T-cell clones against EBV-positive NK and T-cell lines. It is reported that EBNA1-specific CD4⁺ T-cell clones can kill EBV-positive BL and HD cells. We tested the lytic activity of clones against EBV-carrying NK and T-cell lines established from patients with CAEBV as representative of EBV latency II malignancies and retaining characteristics of the original tumors, such as identical EBV clonality.[35] All clones with one exception (clone W5-9D5) had lytic activity against peptide-pulsed autologous CD40-B cells within 4-h incubation (data not shown). Clone X4-2C9, with the highest killing activity against peptide-pulsed targets, showed killing activity after 14-h incubation with HLA-matched EBV-carrying SNT16 cells (Fig. 7a) and HLA-DR51-transfected SNK10 cells (Fig. 7b).

Discussion

Previous studies revealed a DR4- or DQ3-restricted promiscuous epitope (NLRRGTALAIPQCRL), a DR4- or DR15-restricted promiscuous epitope (AEGLRALLARSHVER), a DR15-restricted epitope (MVFLQTHIFAEVLKD), a DP5-restricted (VFLQTHIFAEVLKDAIKDL) and a DP-restricted promiscuous epitope (NFKFENIAEGLRALL).[25,31,45] Our donors had HLA alleles that could present the above epitopes, but there were no T-cell responses to the HLA/peptide complexes with two exceptions (DP5- and DP-restricted epitopes). The DP5-restricted epitope (THIFAEVLKD) and the DP2- and DP5-restricted epitope (PKFENIAEGLR) may have strong immunogenicity. These results are in line with a report by Tsang *et al.*[31] who detected DP5-restricted epitope-specific CD4⁺ T cells in all donors positive for EBV and DP5. The HLA-DPB1*0501 allele is the most frequent HLA-DPB1 allele in the Japanese population and more than 60% of Japanese people are positive for this allele. It would

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Table 2. **Identification of epitopes recognized by five clones**

Clone	Minimal epitope†	Identified HLA-restriction	Known epitope sequence	Reported HLA-restriction	REF
X1-12B12	(F)EYHQEGGPDG(EP)	DR4			
X3-11D1	(SN)PKFENIAEGLR(A)	DP2, DP5	NPKFENIAEGLRALL	DP, DR11	25
X3-3G2	(RA)LLARSHVER(TTDE)	DQ6	LRALLARSHVERTTD	ND	25
X4-2C9	(R)RGTALAIPQCRL(T)	DR51	NLRRGTALAIPQCRL	DR4, DQ3	25
W5-9D5	(FLQ)THIFAEVLKD(AIK)	DP5	MVFLQTHIFAEVLKD	DR15	25
			VFLQTHIFAEVLKDLV	DP5	31

†The minimal epitope represents the shortest fragment where any further truncation may result in drastic reduction of antigenicity. Addition of amino acids in parentheses may augment the antigenicity. HLA, human leukocyte antigen; ND, not determined; REF, reference.

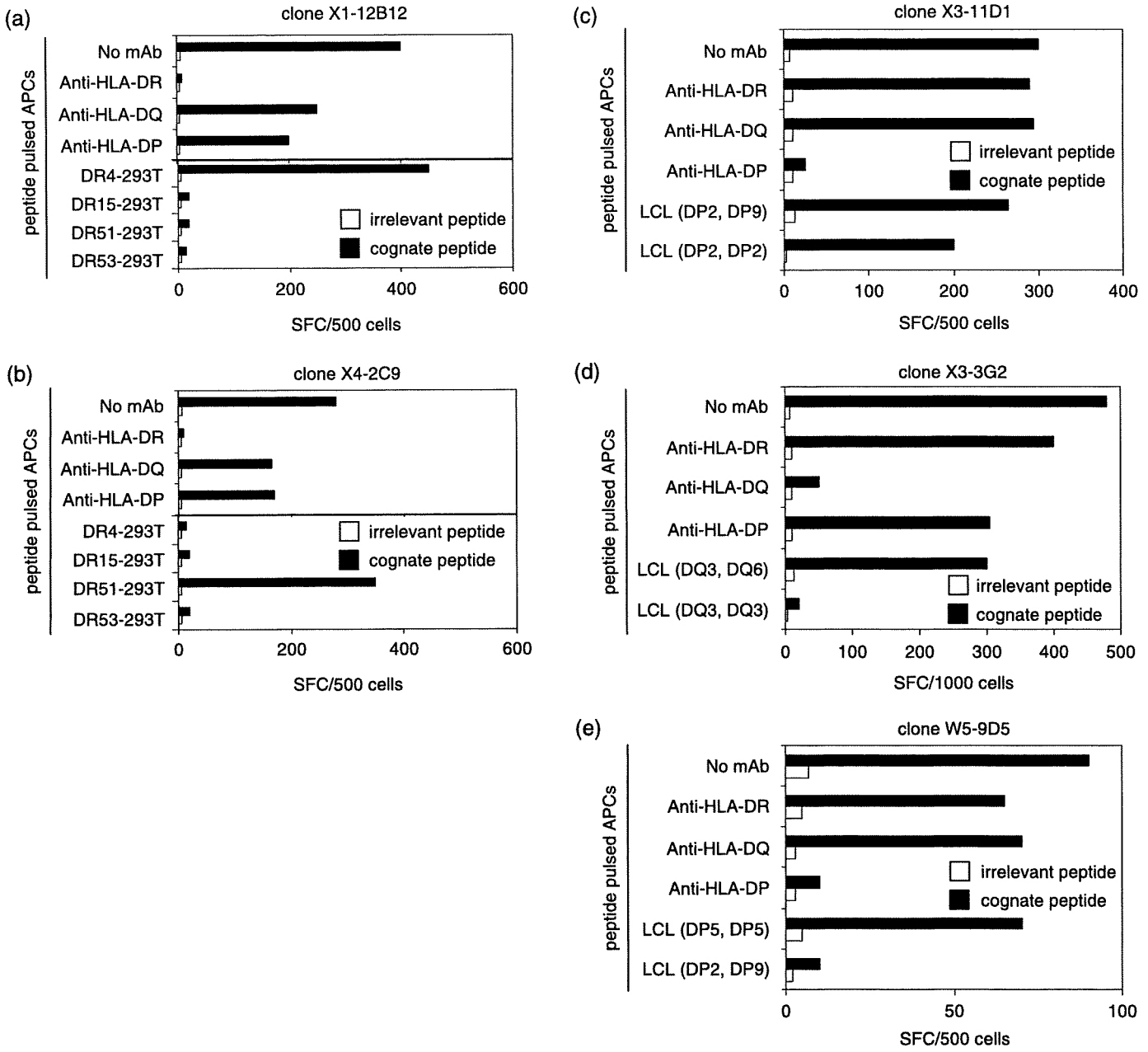

Fig. 5. Identification of human leukocyte antigen (HLA) class II restriction molecules. (a,b, top panel) Enzyme-linked immunosorbent spot (ELISPOT) assays were performed using autologous lymphoblastoid cell lines (LCL) pulsed with peptides in the presence of monoclonal antibody (mAb) against HLA-DR, DQ and DP. (bottom panel) $CD4^+$ T-cell clone recognition of HEK-293T cells transfected with each donor HLA-DR allele and pulsed with peptide was determined by ELISPOT assay. (c–e) ELISPOT assays were performed using autologous LCL pulsed with peptides in the presence of mAb against HLA-DR, DQ, DP or HLA-typed allogeneic LCL pulsed with peptides. All results are the mean of two duplicate values. SFC, spot forming units.

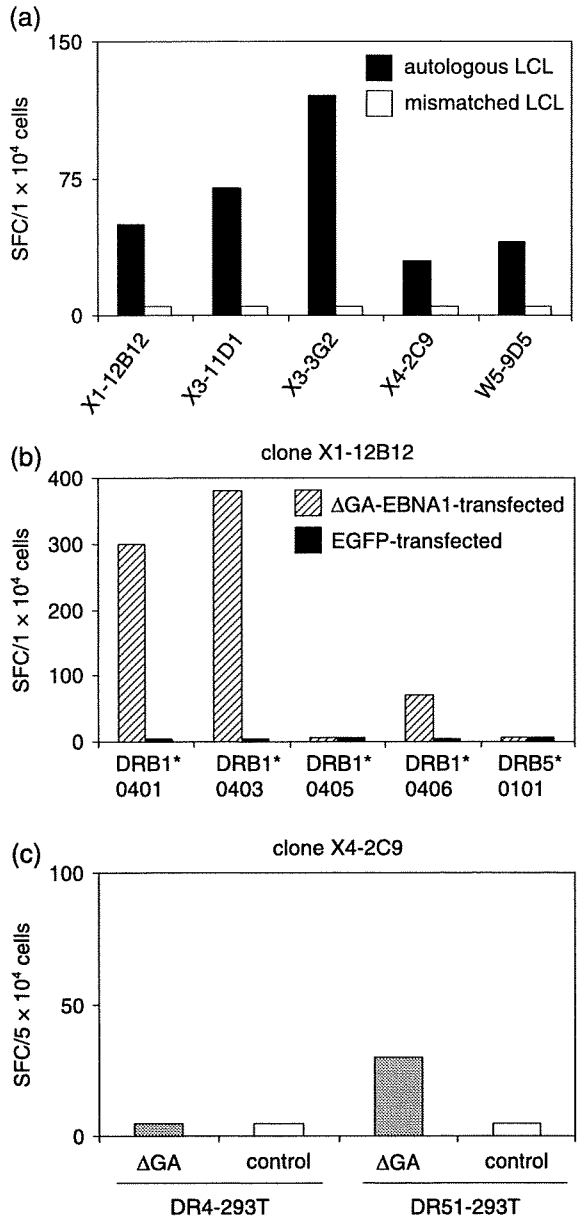

Fig. 6. Recognition of endogenously processed Epstein–Barr virus nuclear antigen (EBNA)1 by CD4⁺ T-cell clones. (a) γ-interferon spot production with CD4⁺ T-cell clones was estimated by enzyme-linked immunosorbent spot (ELISPOT) assays using lymphoblastoid cell lines (LCL)as antigen-presenting cells. Human leukocyte antigen (HLA)-mismatched LCL was A1 for clones from donor X and A2 for a clone from donor W. (b) Recognition by clone X1-12B12 of DR4- (DRB1*0401, 0403, 0405, or 0406) or DR51 (DB5*0101)-293T cells transfected with ΔGA-EBNA1 or a control construct was tested by ELISPOT assays. (c) Recognition by clone X4-2C9 of DR4- or DR51-293 T cells transfected with ΔGA-EBNA1 or control construct was tested by ELISPOT assays. All results are the mean of two duplicate values. EGFP, enhanced green fluorescent protein; SFC, spot forming units.

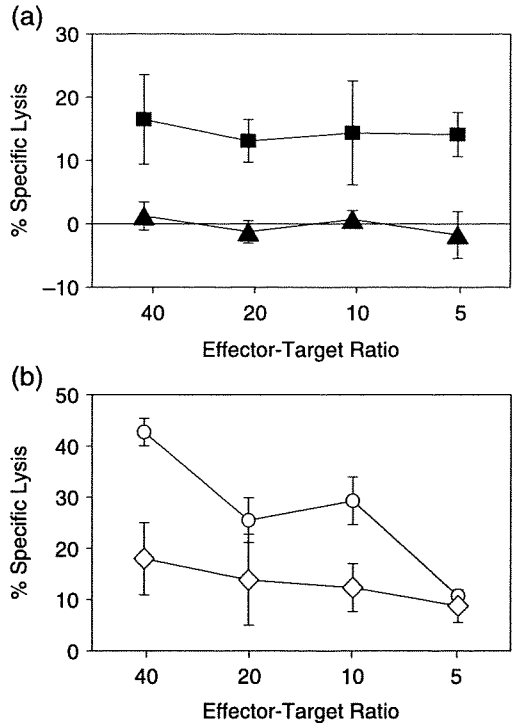

Fig. 7. Cytotoxic activity of CD4⁺ T cell clone X4-2C9 against EBV-carrying NK and T cell lines. (a) Fourteen-hour cytotoxic T lymphocytes assays were performed using Epstein–Barr virus-carrying T-cell lines as targets. Data for specific lysis of an human leukocyte antigen (HLA)-DR51-positive T-cell line (SNT16; ■) and an HLA-DR51-negative T-cell line (SNT15; ▲) are shown. (b) Cytolytic activity of the CD4⁺ T-cell clone X4-2C9 was assessed against HLA-DR51 (○) or HLA-DR1-retrovirally transduced (◇) SNK10 cells. All assays were done in triplicate wells. Bars indicate standard deviations calculated from each data. The minimal release was less 30% of maximal release in all experiments.

be useful for vaccine development to further investigate which epitopes are immunodominant *in vivo*.

In the present study, all clones with one exception (clone W5-9D5) demonstrated killing activity (30–80%) against peptide-pulsed targets when assayed after 4 h. The clone X4-2C9 had the highest killing activity; although it showed less lytic activity against an EBV-carrying T-cell line in 4-h cytotoxic assay (data not shown), cytolysis was found with a DR51-carrying EBV-positive T-cell line and a DR51-transfecting EBV-positive NK cell line after 14-h incubation. The present data confirm that EBNA1-specific CD4⁺ T cells work as direct effectors *in vitro*, in line with the data reported by Pauldan *et al.*[32] They documented that EBNA1-specific CD4⁺ T cells recognize and kill BL cells and Hodgkin's lymphoma cells *in vitro*.

Long *et al.*[46] established CD4⁺ T-cell clones specific for a single epitope of $EBNA2_{276-295}$ presented by four different MHC. Among them, only the HLA-DR52b-restricted clone could kill autologous LCL, though all four clones secreted IFN-γ against LCL. Two groups reported CD4⁺ T-cell clones specific for another single epitope of $EBNA2_{280-290}$ presented by several MHC.[47,48] Similarly, DQ2-, DQ7- and DR52-restricted clones could kill LCL, while the others could only secrete IFN-γ against LCL. Long *et al.*[46] mentioned that it was important for immunotherapeutic applications to identify combinations of epitopes and restriction molecules that have the capability for more efficient antigen presentation. In our study, only the clone X4-2C9 showed killing activity against EBV-positive NK and T-cell lines (Fig. 7a,b), underscoring the importance of particular combinations of epitopes and restriction molecules.

We reported earlier that EBV-infected NK cells established from patients with lymphomas or CAEBV are susceptible to LMP1-specific CTL-mediated lysis.[11] For immunotherapy against EBV-associated malignancies, it might be efficient to combine CD4⁺ T cells and CTL as direct effectors, but further studies are needed to clarify their synergistic effects *in vitro* and *in vivo*. For the present, we can conclude that the CD4⁺ T-cell-mediated

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lysis of EBV-carrying NK and T-cell lines demonstrated here provides clues to immunotherapy targeting EBV-associated malignancies.

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Human TSLP directly enhances expansion of CD8$^+$ T cells

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Summary

Human thymic stromal lymphopoietin (TSLP) promotes CD4$^+$ T-cell proliferation both directly and indirectly through dendritic cell (DC) activation. Although human TSLP-activated DCs induce CD8$^+$ T-cell proliferation, it is not clear whether TSLP acts directly on CD8$^+$ T cells. In this study, we show that human CD8$^+$ T cells activated by T-cell receptor stimulation expressed TSLP receptor (TSLPR), and that TSLP directly enhanced proliferation of activated CD8$^+$ T cells. Although non-stimulated human CD8$^+$ T cells from peripheral blood did not express TSLPR, CD8$^+$ T cells activated by anti-CD3 plus anti-CD28 did express TSLPR. After T-cell receptor stimulation, TSLP directly enhanced the expansion of activated CD8$^+$ T cells. Interestingly, using monocyte-derived DCs pulsed with a cytomegalovirus (CMV)-specific pp65 peptide, we found that although interleukin-2 allowed expansion of both CMV-specific and non-specific CD8$^+$ T cells, TSLP induced expansion of only CMV-specific CD8$^+$ T cells. These results suggest that human TSLP directly enhances expansion of CD8$^+$ T cells and that the direct and indirect action of TSLP on expansion of target antigen-specific CD8$^+$ T cells may be beneficial to adoptive cell transfer immunotherapy.

Keywords: CD8$^+$ T cells, direct action, human, proliferation, TSLP

Introduction

Human thymic stromal lymphopoietin (TSLP), an interleukin (IL)-7-like cytokine, activates CD11c$^+$ blood myeloid dendritic cells (DCs); subsequently, the primed DCs induce strong T-cell proliferation [1–4]. Immature myeloid DCs in blood highly express the functional receptor for TSLP, IL-7Rα chain and TSLP receptor (TSLPR) [1]. TSLP strongly up-regulates surface expression of costimulatory molecules on DCs, prolongs DC survival, and enhances DC-T cell conjugate formation, resulting in the strong proliferation of not only CD4$^+$ but also CD8$^+$ T cells [1–5]. Although TSLP-activated DCs strongly induce the proliferation of T cells, these DCs do not produce detectable amounts of proinflammatory cytokines, such as IL-1β, IL-6, IL-12p70, and tumour necrosis factor-α [2,3]. In physiological conditions, human TSLP is preferentially expressed by epithelial cells within the thymus and the mucosal surface in lymphoid tissues [3,6], suggesting that TSLP is involved in the proliferation and differentiation of T cells through DC activation *in vivo* [7,8].

In addition to indirect action through DC activation, the direct action of TSLP on CD4$^+$ T cell proliferation has recently been found in humans [9]. Freshly isolated peripheral blood CD4$^+$ T cells do not express the functional receptor for TSLP and do not respond to TSLP. However, CD4$^+$ T cells activated by anti-CD3 plus anti-CD28 express the functional receptor for TSLP, and directly respond to TSLP, resulting in enhanced proliferation [9]. Although human TSLP promotes CD4$^+$ T-cell proliferation both directly and indirectly through DC activation, whether it can directly act on CD8$^+$ T cells has yet to be clarified.

In this study, we show that human TSLP directly influenced activation of CD8$^+$ T cells. Non-stimulated human CD8$^+$ T cells from peripheral blood did not express TSLPR. However, stimulation of anti-CD3 plus anti-CD28 induced TSLPR expression in CD8$^+$ T cells. After T-cell receptor (TCR) stimulation, TSLP directly enhanced the expansion of activated CD8$^+$ T cells.

Materials and methods

CD8$^+$ T cell isolation and culture

This study was approved by the Institutional Review Board for Human Research in the Graduate School of Medicine,

Kyoto University. Peripheral blood mononuclear cells (PBMCs) were obtained from adult buffy coat of healthy donors (kindly provided by Kyoto Red Cross Blood Center, Kyoto, Japan), were frozen in a cell freezing medium without serum, Cellbanker™ (Nippon Zenyaku, Fukushima, Japan), and were kept in liquid N_2 until use. $CD8^+$ T cells were purified from PBMCs by positive selection using anti-CD8-coated magnetic beads (Miltenyi Biotec, Gladbach, Germany) to reach >99% purity. Isolated cells were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal calf serum (FCS) or 5% AB human serum (Sigma, St Louis, MO, USA), penicillin G and streptomycin (Gibco), 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Gibco), and 20 ng/ml IL-7 (R&D Systems, Minneapolis, MN, USA) (referred to as complete medium). The cells were seeded at a density of $2{\cdot}5 \times 10^5$ cells/ml in flat-bottomed 96-well plates in triplicate.

T cell proliferation and expansion assay

Purified $CD8^+$ T cells were cultured with FCS supplemented complete medium and stimulated with 5 μg/ml plate-bound anti-CD3 (UCHT1, eBioscience, San Diego, CA, USA) and soluble 1 μg/ml anti-CD28 (CD28·1, eBioscience). For some experiments, 3×10^{-2}–10 μg/ml plate-bound anti-CD3 were used, and 20 ng/ml recombinant human TSLP (R&D systems) was added on day 5 of culture. Viable cells were counted by trypan blue exclusion of dead cells. Purified $CD8^+$ T cells were labelled with carboxyfluorescein diacetate succinimidyl diester (CFSE, Molecular Probes, Invitrogen, Carlsbad, CA, USA) as described [3,6].

Flow cytometry

The following monoclonal antibodies (mAbs) were used for surface staining: fluorescein isothiocyanate (FITC)-conjugated anti-CD3, FITC and phycoerythrin-conjugated anti-CD8, all purchased from BD Bioscience (San Jose, CA, USA), and FITC-conjugated anti-CD80, phycoerythrin cyanine chrome 5-conjugated anti-CD8, biotinylated mouse IgG1 isotype control Ab, and allophycocyanin-conjugated streptavidin all from eBioscience. For detection of TSLPR, cells were stained with biotinylated anti-human TSLPR mAb as described [9]. Stained cells were analysed with a FACS Calibur™ (BD Bioscience).

T cell cytokine production

After 7 days of culture of $CD8^+$ T cells, intracellular cytokine production was assessed. Cultured $CD8^+$ T cells were collected, washed twice, and restimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) plus 2 μg/ml ionomycin (Sigma) in flat-bottomed 96-well plates at a concentration of 1×10^6 cell/ml. After 3·5 h, brefeldin A (Sigma) was added at 10 μg/ml. After 2·5 h, cells were collected and stained with phycoerythrin cyanine chrome 5-conjugated anti-CD8. Cells were fixed and permeabilized using Fix & Perm Cell Permeabilization Kit (Caltag Laboratories, An Der Grub, Austria), and stained with FITC-conjugated anti-interferon (IFN)-γ (eBioscience). Stained cells were analyzed with a FACS Calibur™.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

$CD4^+CD25^-$ T cells and $CD4^+CD25^+$ regulatory T (Treg) cells were isolated from PBMCs by cell sorting. Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA) and treated with DNase I (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed with SuperScript™ II (Invitrogen). Real-time quantitative reactions were performed with a LightCycler™ 480 Instrument (Roche Diagnostics Gmbh, Mannheim, Germany) according to the manufacturer's instructions. Values are expressed as arbitrary units relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers were used as previously described [6].

Monocyte-derived DC (MoDC) generation and blood myeloid DC purification

MoDCs were generated as described [10–12]. Briefly, $CD14^+$ monocytes were purified from PBMCs by positive selection with anti-CD14-coated magnetic beads (Miltenyi Biotec) to reach >97% purity and cultured in 12-well plates in FCS supplemented complete medium in the presence of 10 ng/ml IL-4 (Pepro Tech, Rocky Hill, NJ, USA) and granulocyte-macrophage colony-stimulating factor (Pepro Tech) for 5 days. On day 5, immature MoDCs were washed and resuspended in the same medium with 10 ng/ml of IL-6, tumour necrosis factor-α, IL-1β (all from Pepro Tech), and 1 μg/ml of prostaglandin-E2 (Sigma), and cultured for 2 days. On day 7, matured MoDCs were harvested. Viable DCs were counted by trypan blue exclusion of dead cells. $CD11c^+$ blood myeloid DCs were isolated from PBMCs as described previously [3,6]. $CD11c^+lineage^-$ cells were isolated by a FACS Aria™ (BD Biosciences) to reach >99% purity. Blood myeloid DCs were cultured immediately after being sorted in FCS supplemented complete medium. In experiments as shown in Fig. 4, DCs were seeded at a density of 1×10^6 cells/ml in 96-well plates with or without 20 ng/ml of TSLP and cultured for 24 h.

Synthetic peptide and human leukocyte antigen (HLA)-A2402-cytomegalovirus (CMV)pp65 tetramer

HLA-A24-restricted CMVpp65 peptide (QYDPVAALF aa 341-349), which is reported to be HLA-A24-restricted CMV-specific $CD8^+$ T cell epitope [13], was purchased from

OPERON Biotechnologies (Tokyo, Japan). HLA-A2402-CMVpp65 peptide tetramer conjugated with phycoerythrin were used for CMV-specific $CD8^+$ T cell staining as previously described [13,14].

DC-T cell co-culture

In experiments of allogeneic MoDC-$CD8^+$ T cell co-culture, T cells ($2{\cdot}5 \times 10^5$ cells/ml) were cultured with DCs at 1:2 DC : T cell ratio in round-bottomed 96-well plates in FCS supplemented complete medium. For analysis of CMVpp65-specific $CD8^+$ T cells, cells were isolated from HLA-A2402-positive CMV-seropositive healthy donors and cultured with autologous MoDCs. MoDCs were cultured with 1 μM HLA-A24-restricted CMVpp65 peptide for the last 4 h and washed three times to remove any cytokines before co-culture. The cells were co-cultured at the same ratio and density as used in the allogeneic condition in human serum supplemented complete medium with either 20 ng/ml of IL-2 (R&D Systems) or TSLP. The number of viable cells was determined by trypan blue exclusion.

Statistical analysis

Statistical significance ($P < 0{\cdot}05$) between groups was determined by paired *t*-test.

Results

TCR stimulation induces TSLPR expression on activated human $CD8^+$ T cells

Freshly isolated human $CD4^+$ T cells express IL-7Rα chain but not TSLPR, while $CD4^+$ T cells stimulated by anti-CD3 plus anti-CD28 induce the cell surface expression of TSLPR [9]. To examine whether TCR stimulation induces the cell surface expression of TSLPR on activated human $CD8^+$ T cells, purified peripheral blood $CD8^+$ T cells were cultured with the stimulation of anti-CD3 plus anti-CD28. Before purification from PBMCs, human $CD8^+$ T cells expressed an IL-7Rα chain, but they did not show any detectable level of TSLPR expression, as described previously [1,9] (Fig. 1a and data not shown). After 5 days culture of purified human $CD8^+$ T cells with TCR stimulation, activated $CD8^+$ T cells showed TSLPR expression of various degrees (3·6–13·9% of total $CD8^+$ T cells, Fig. 1b).

TCR stimulation induces brief expression of TSLPR on $CD8^+$ T cells

Next, we monitored TSLPR-expressing cells in $CD8^+$ T cells from 1 day to 7 days of culture with anti-CD3 plus anti-CD28 by flow cytometry. The induction of TSLPR on $CD8^+$ T cell surface by TCR stimulation was detectable by 3 days and reached a maximal level at 3–5 days after TCR stimulation (Fig. 1c). However, the percentages of $TSLPR^+$ cells in $CD8^+$ T cells decreased after 7 days of culture, suggesting that TCR stimulation induces the brief expression of TSLPR on $CD8^+$ T cells.

TSLPR induction of $CD8^+$ T cells depends on the strength of TCR stimulation

To further analyse the characteristics of TSLPR induction of $CD8^+$ T cells, we examined TSLPR induction on $CD8^+$ T cells in different concentrations of immobilized anti-CD3 in the presence of anti-CD28. The induction of TSLPR on $CD8^+$ T cell surface by TCR stimulation was detectable when we used 1 μg/ml of immobilized anti-CD3; it reached a maximal level at 3 μg/ml of anti-CD3 and was reduced at 10 μg/ml (Fig. 1d). These data suggest that the induction of TSLPR on $CD8^+$ T cells depends on the strength of TCR stimulation and may occur only within a narrow 'window' of the strength of TCR stimulation.

$CD8^+$ T cells express TSLPR on their surface in mixed lymphocyte reaction (MLR)

We showed that human $CD8^+$ T cells expressed TSLPR on their surface when we used anti-CD3 and anti-CD28 for polyclonal TCR stimulation. Next, we assessed whether human $CD8^+$ T cells express TSLPR on their surface in MLR in which $CD8^+$ T cells were co-cultured with activated allogeneic MoDCs. Purified human $CD8^+$ T cells were stimulated for 7 days with allogeneic MoDCs at a DC : T cell ratio of 1:2. After 7 days of co-culture, 99·6% of remaining viable cells showed $CD3^+CD8^+$, and these $CD8^+$ T cells contained 11·2% of $TSLPR^+$ cells (Fig. 2). These data suggest that human $CD8^+$ T cells express TSLPR on their surface in MLR.

TSLP directly enhances $CD8^+$ T-cell expansion induced by TCR stimulation

Human TSLP directly enhances the expansion of activated $CD4^+$ T cells expressing TSLPR [9]. To examine whether human TSLP directly enhances the expansion of activated $CD8^+$ T cells, purified $CD8^+$ T cells stimulated with anti-CD3 plus anti-CD28 were traced for cell division by using the CFSE dilution method. Without TSLP, TCR stimulation resulted in cell division of input $CD8^+$ T cells after 6 days of culture; these divided T cells underwent further divisions in the following 2 days of culture (Fig. 3a, upper panels). In contrast, although addition of TSLP on day 5 did not affect the cell division of $CD8^+$ T cells on day 6, TSLP induced a larger fraction of dividing cells in the following 2 days of culture (Fig. 3a, lower panels). In addition, fold expansion of activated $CD8^+$ T cells cultured with TSLP was significantly greater than that without TSLP (Fig. 3b). These data indicate

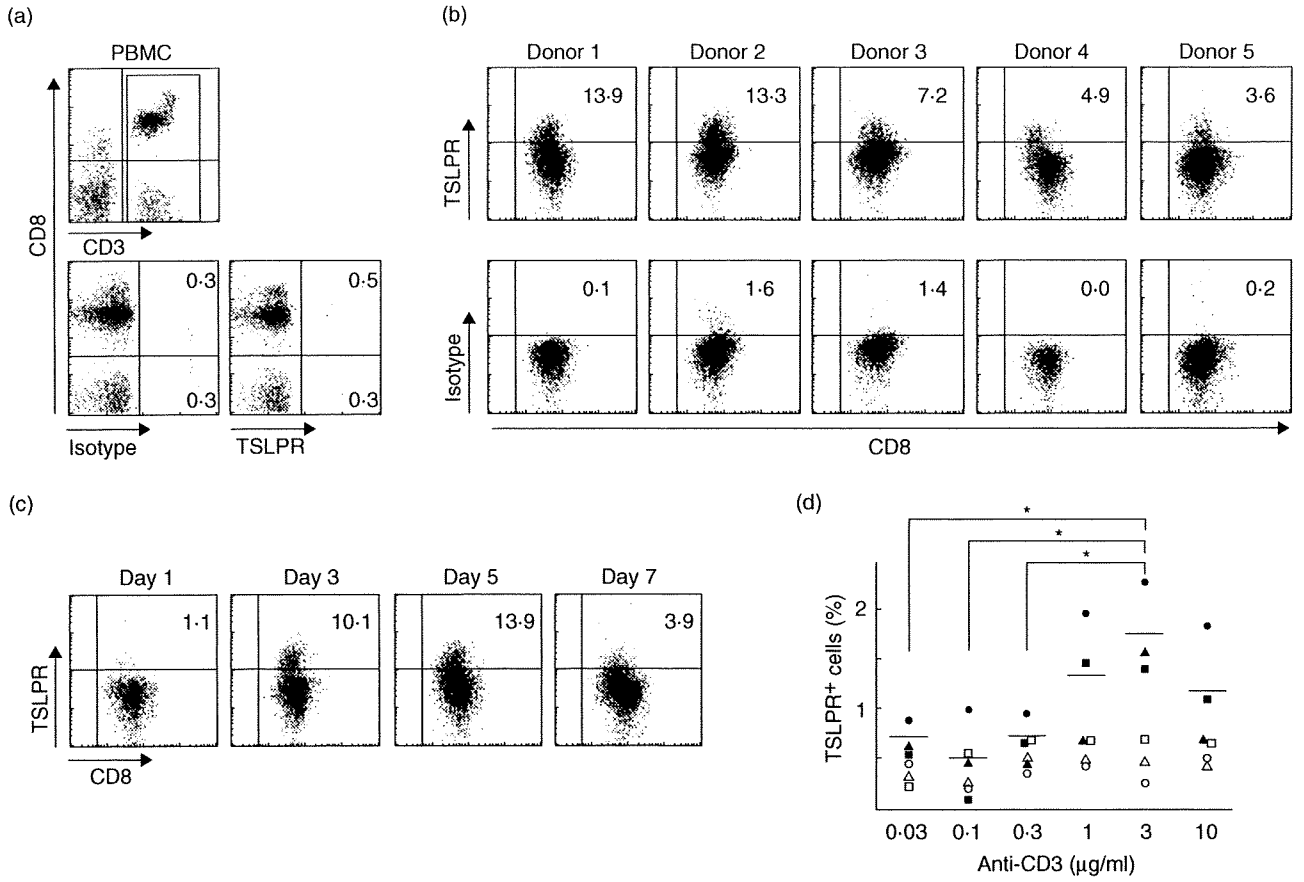

Fig. 1. TCR stimulation induces TSLPR expression on human CD8⁺ T cells. (a) PBMCs were stained with mAb to CD3, CD8 and TSLPR or isotype control. Cell surface marker phenotypes were determined by flow cytometry. Lower panels represent staining of CD3⁺ cells with TSLPR or isotype control. Numbers in quadrants indicate the percentages of cells for each given phenotype in CD3⁺ cells. Data represent one of five independent experiments. (b, c) Purified human CD8⁺ T cells were stimulated for 5 days (b) or indicated days (c) with immobilized anti-CD3 plus soluble anti-CD28, and numbers in quadrants indicate the percentages of cells for each given phenotype in CD3⁺CD8⁺ cells. Data shown from five individuals (b) and one of five independent experiments (c). (d) CD8⁺ T cells were stimulated with anti-CD28 and various concentrations of anti-CD3. Data shown are the percentages of TSLPR⁺ cells in CD3⁺CD8⁺ cells in three individual experiments (closed symbols). Horizontal bars indicate the mean of percentages of TSLPR⁺ cells and open symbols show isotype control from individuals. *P*-values as determined by the paired *t*-test. $^*P < 0.05$.

that human TSLP directly enhances the expansion of CD8⁺ T cells activated with TCR stimulation.

CD8⁺ T cells expanded by TCR plus TSLP stimulation produce IFN-γ, but do not express *forkhead box P3* (*Foxp3*) mRNA

Because of the indirect effect of TSLP on differentiation of CD4⁺Foxp3⁺ Treg cells in humans [6], we examined whether CD8⁺ T cells directly expanded by TCR plus TSLP stimulation are functional. We cultured CD8⁺ T cells under the stimulation of anti-CD3 plus anti-CD28 with or without TSLP or IL-2 and evaluated cytokine production capacity using intracellular cytokine staining of expanded T cells restimulated with PMA plus ionomycin and the expression level of *Foxp3* mRNA using real-time quantitative RT-PCR. Intracellular cytokine staining of CD8⁺ T cells demonstrated that the percentages of IFN-γ producing cells in CD8⁺ T cells cultured with TSLP is similar to those of CD8⁺ T cells cultured with IL-2 or medium alone (Fig. 3c). In addition, CD8⁺ T cells expanded by TSLP did not express *Foxp3* mRNA (Fig. 3d). These data suggest that CD8⁺ T cells expanded by TCR plus TSLP stimulation have cytotoxic activity, but not regulatory function.

TSLP enhances expansion of CMV-specific CD8⁺ T cells after TCR engagement

Both CD11c⁺ blood myeloid DCs and mature MoDCs expressed TSLPR and responded to TSLP, resulting in enhanced CD80 expression (Fig. 4). Although percentages of CD11c⁺ blood myeloid DCs in PBMCs are less than 1·0%, MoDCs are easily generated from PBMCs and widely used for clinical application of antigen (Ag)-specific T-cell expansion.

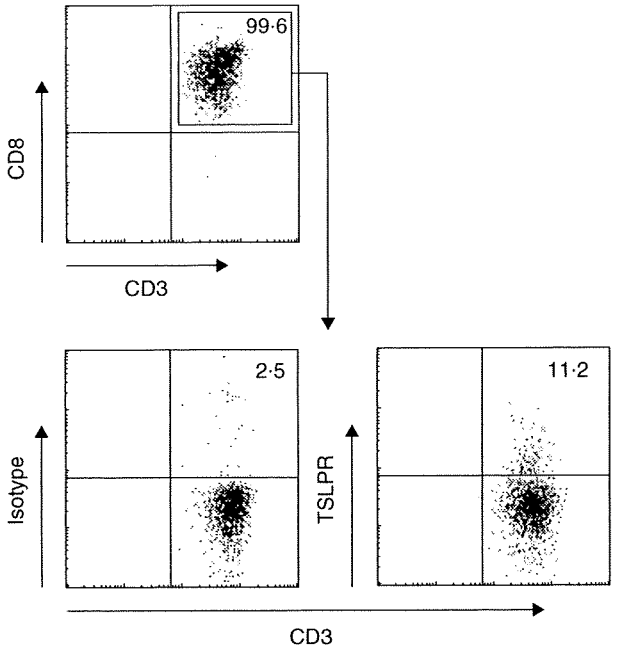

Fig. 2. CD8$^+$ T cells express TSLPR on their surface in mixed lymphocyte reaction. Purified human CD8$^+$ T cells were cultured with allogeneic MoDCs for 7 days at a DC : T cell ratio of 1:2. Cell surface marker phenotypes were determined by flow cytometry as in Fig. 1b. Data represent one of five independent experiments.

To assess whether human TSLP can enhance expansion of Ag-specific CD8$^+$ T cells after TCR engagement in the clinical setting, we purified CD8$^+$ T cells from HLA-A24-positive healthy donors and cultured these T cells with autologous MoDCs pulsed with HLA-A24-restricted CMVpp65 peptide in the presence or absence of TSLP. After 7 days of culture, tetramer positive CMV-specific CD8$^+$ T cells cultured with TSLP expanded more greatly than cells cultured without TSLP (Fig. 5a and b and Table 1). In contrast, TSLP did not affect expansion of tetramer negative non-specific CD8$^+$ T cells (Fig. 5b and Table 1). These data indicate that human TSLP enhances expansion of Ag-specific CD8$^+$ T cells in co-culture with MoDCs which are widely used for clinical application of Ag-specific T-cell expansion.

TSLP predominantly enhances expansion of Ag-stimulated CD8$^+$ T cells

To further test whether TSLP predominantly enhances the expansion of Ag-specific CD8$^+$ T cells after TCR engagement, we traced CD8$^+$ T cell division by using the CFSE dilution method for 7 days. TSLP enhanced cell division of tetramer positive CMV-specific CD8$^+$ T cells (Fig. 5c, left and middle panels), but reduced the population of divided tetramer negative non-specific CD8$^+$ T cells (Fig. 5c, right panels). In contrast, IL-2 enhanced cell division of both tetramer-positive and -negative CD8$^+$ T cells (Fig. 5c, lower panels). These data suggest that in comparison with IL-2, TSLP induces predominant expansion of Ag-specific CD8$^+$ T cells after TCR engagement.

Discussion

In the present study, we showed that human TSLP directly acts on activated CD8$^+$ T cells. TCR stimulation induced expression of the functional receptor for TSLP in CD8$^+$ T cells, and the activated CD8$^+$ T cells directly responded to TSLP, resulting in their enhanced expansion. The direct action of TSLP on CD4$^+$ T cell proliferation has recently been found in humans [9]. Moreover, TSLP-activated DCs induce the proliferation of both CD4$^+$ and CD8$^+$ T cells [1–5]. Therefore, human TSLP enhances proliferation of not only CD4$^+$ but also CD8$^+$ T cells, both directly and indirectly through DC activation. Although the species difference had appeared to exist in the effect of TSLP on DCs in humans and on DCs, T cells and mast cells in mice, the direct action of TSLP on T cells has become evident in humans, implying that human TSLP might additionally act on other unknown cells in the immune system.

IL-7, IL-15 and IL-2 bind to multimeric receptors that share the common γ chain (γc) and directly act on human CD8$^+$ T cells [15–17]. IL-7 binds to heterodimeric receptors composed of IL-7Rα and γc, whereas IL-15 binds to heterotrimeric receptors composed of IL-15Rα, IL-2/15Rβ and γc. All these subunits of receptors are expressed on resting human CD8$^+$ T cells. IL-7 and IL-15 can induce the proliferation of human CD8$^+$ T cells in the absence of TCR stimulation *in vitro* [17,18]. In contrast, IL-2Rα, which comprises the functional IL-2 receptor, is not expressed at significant levels in resting T cells; instead, expression of IL-2Rα is induced in T cells after TCR engagement. After activation by TCR engagement, IL-2 can efficiently enhance proliferation of human CD8$^+$ T cells *in vitro* [17,18]. In this study, we showed that although TSLPR was not expressed in resting human CD8$^+$ T cells, it was induced by TCR stimulation. Xu *et al.* reported that TSLPR is up-regulated by TSLP on MoDCs [19]. However, even in the presence of TSLP, IL-7 and IL-15, human CD8$^+$ T cells cultured without anti-CD3 plus anti-CD28 stimulation did not express TSLPR (data not shown). In addition, the stimulation of anti-CD3 plus anti-CD28 induced TSLPR, even in the absence of TSLP, IL-7 and IL-15; moreover, percentages of TSLPR$^+$CD8$^+$ T cells did not differ in the presence or absence of those cytokines (data not shown). These data suggest that TCR stimulation is sufficient to induce cell surface expression of TSLPR in human CD8$^+$ T cells.

Lee *et al.* reported that in mice, TSLP promotes the differentiation of Treg cells from CD4$^+$CD8$^-$CD25$^-$ single-positive thymocytes in a DC-independent manner [20]. In addition, we previously showed that the effect of human TSLP on Treg cell differentiation is restricted to the CD4$^+$CD8$^-$CD25$^-$ single-positive thymocyte stage in the human thymus [6]. However, CD8$^+$ T cells expanded with TSLP produced IFN-γ

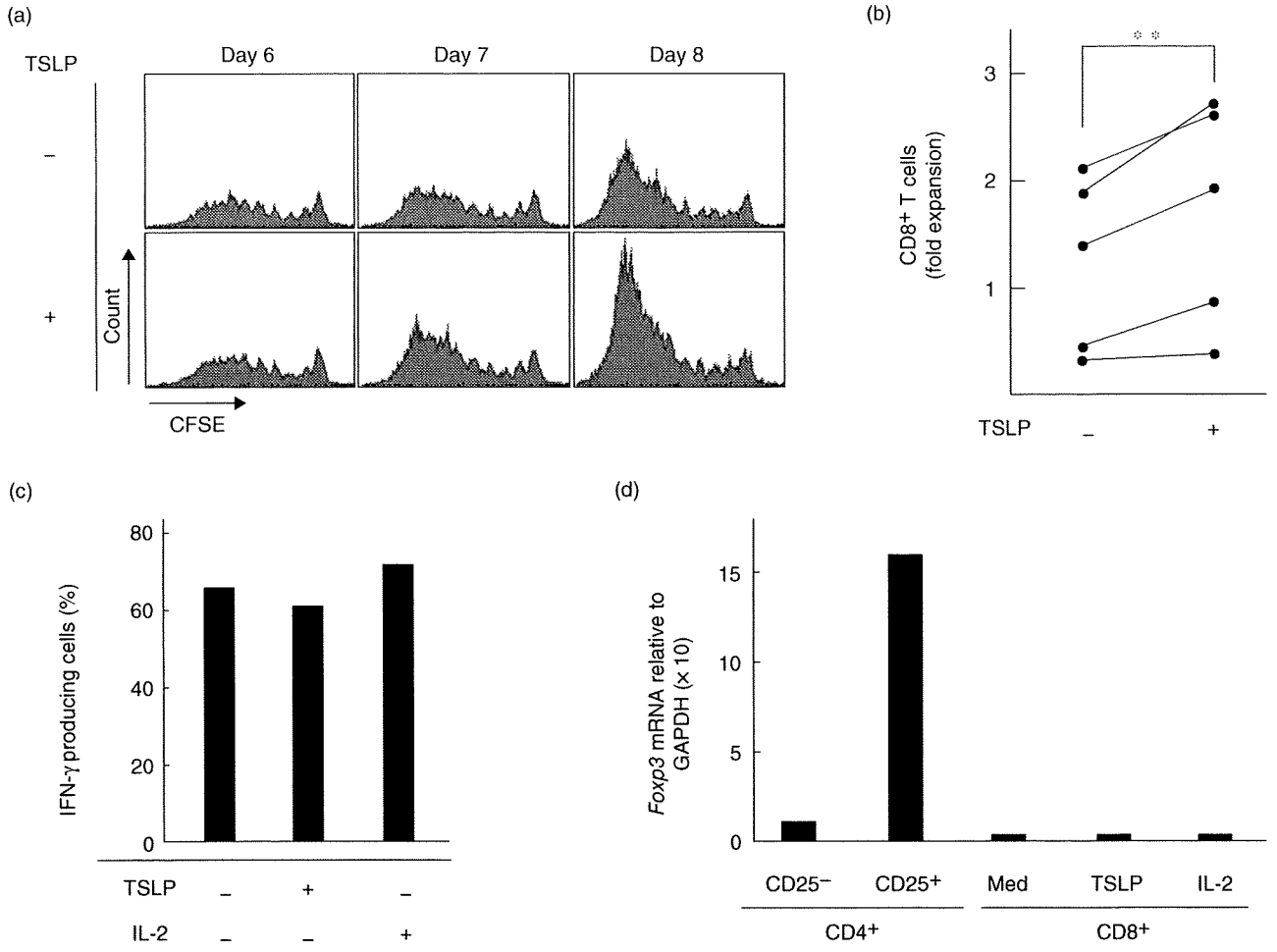

Fig. 3. TSLP directly enhances expansion of functional CD8+ T cells induced by TCR stimulation. (a) Cell divisions of CFSE-labelled purified human CD8+ T cells stimulated with anti-CD3 plus anti-CD28 with or without TSLP for indicated days. Filled histograms represent CFSE dilution in CD8+ T cells. Data represent one of five individual experiments. (b) Closed circles indicate fold expansion of CD8+ T cells stimulated with anti-CD3 plus anti-CD28 with or without TSLP compared with the initial CD8+ T-cell number in five individual experiments. The lines indicate CD8+ T cells from the same individual. (**$P < 0{\cdot}05$). (c) The percentages of IFN-γ producing cells in CD8+ T cells using intracellular cytokine staining. CD8+ T cells were stimulated with anti-CD3 plus anti-CD28 with or without TSLP or IL-2 and restimulated with PMA plus ionomycin. Data represent one of three independent experiments. (d) Expression levels of mRNA encoding *Foxp3* were measured using the real-time quantitative RT-PCR. CD8+ T cells were stimulated with anti-CD3 plus anti-CD28 with or without TSLP or IL-2. Data represent one of three independent experiments.

(Fig. 3c) and did not express *Foxp3* mRNA (Fig. 3d). Thus, it is not likely that the direct action of human TSLP on CD8+ T cells induces Treg cell differentiation.

In this study, we showed that in comparison to IL-2, TSLP induced exclusive expansion of CMV-specific CD8+ T cells after TCR engagement. One possibility is that indirect action of TSLP through MoDC activation affects the exclusive expansion of CMV-specific CD8+ T cells. There is also another possible explanation for our results. Although both IL-2Rα and TSLPR are induced after TCR engagement, the induction of TSLPR on CD8+ T cells may occur only within a narrow 'window' related to the strength of TCR stimulation (Fig. 1d). In addition, most naive CD8+ T cells down-regulate IL-7Rα after TCR activation, while memory and effector CD8+ T cells can selectively retain IL-7Rα expression [21,22]. Because tetramer positive CMV-specific CD8+ T cells show the effector function and memory phenotype [13,14,23,24], after TCR engagement, CMV-specific CD8+ T cells may retain IL-7Rα. Taken together, TCR engagement by CMVpp65-peptide–MHC complexes may induce efficient TSLPR expression and retain IL-7Rα in CMV-specific CD8+ T cells, resulting in TSLP mediated selective proliferation of these cells.

Recent studies have shown the efficacy of Ag-specific CD8+ T-cell transfer therapies for treatment of patients with selected metastatic cancers [25,26]. The procedure of adoptive cell transfer includes the isolation of Ag-specific CD8+ T cells, their *ex vivo* expansion and activation, and subsequent

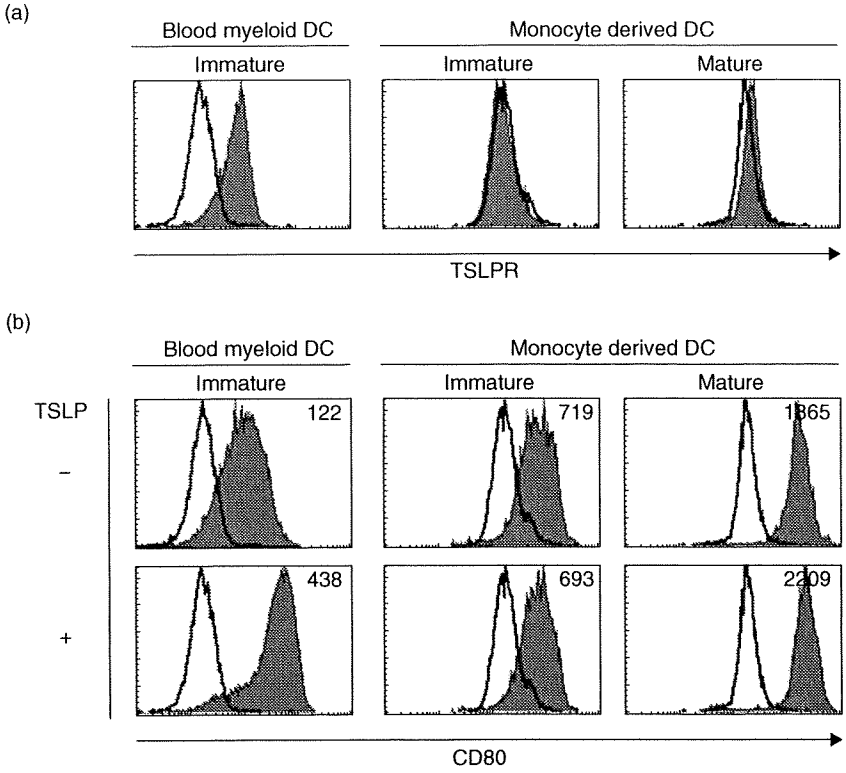

Fig. 4. TSLPR and CD80 expression on blood myeloid DCs and monocyte-derived DCs. (a) Purified immature CD11c$^+$ blood myeloid DCs and monocyte-derived DCs with or without maturation with IL-6, tumour necrosis factor-α, IL-1β and prostaglandin-E2, were stained with mAb to TSLPR or isotype control. Cell surface marker phenotypes were determined by flow cytometry. Filled histograms represent staining of DCs with TSLPR; open histograms represent isotype controls. Data represent one of three experiments. (b) Indicated DCs were cultured for 24 h with or without TSLP and stained with mAb to CD80 or isotype control. Filled histograms represent staining of DCs with CD80; open histograms represent isotype controls. Numbers in histograms indicate the mean fluorescence intensity. Data represent one of three experiments.

autologous administration. IL-2 is useful for *ex vivo* expansion and activation of Ag-specific CD8$^+$ T cells [25,26]. However, for therapeutic application in a variety of tumours, recent procedures using IL-2 appear not to be sufficient to generate large numbers of tumour-specific CD8$^+$ T cells [27–29]. In this study, we showed that in comparison to IL-2, TSLP induced predominant expansion of Ag-specific CD8$^+$ T cells after TCR engagement, suggesting that human TSLP may contribute to efficient expansion of tumour-specific CD8$^+$ T cells *ex vivo* for potential therapeutic application.

Table 1. The effect of TSLP on fold expansion of CMV-specific CD8$^+$ T cells.

CD8$^+$ T cells	TSLP stimulation	Fold expansion
Tetramer$^-$, $n = 12$	–	1·6 ± 0·8
	+	1·7 ± 0·9*
Tetramer$^+$, $n = 12$	–	60·6 ± 83·3
	+	101·5 ± 111·5**

Fold expansions of HLA-A2402-CMVpp65 tetramer$^-$ and tetramer$^+$CD8$^+$ T cells from 12 individuals after 7 days of culture with or without TSLP under the same conditions as Fig. 5a and b. The data shown are the mean ± SD. *P*-values *versus* fold expansion of the cells cultured without TSLP as determined by the paired *t*-test. $^*P = 0{\cdot}39$, $^{**}P < 0{\cdot}05$.

In conclusion, we demonstrated that TCR stimulation induced TSLPR expression in human CD8$^+$ T cells and that TSLP then directly enhanced proliferation of the activated CD8$^+$ T cells. Because TSLP is expressed in epithelial cells of mucosal lymphoid tissues in physiological conditions [3], the direct action of TSLP on TCR-stimulated CD8$^+$ T cells might contribute to enhancement of protective immune responses in the mucosa against invading microbes, leading to their eradication from the host. In addition, the direct and indirect action of human TSLP on expansion of target Ag-specific CD8$^+$ T cells after TCR engagement suggests that TSLP may be a useful tool for efficient adoptive cell transfer of immunotherapy.

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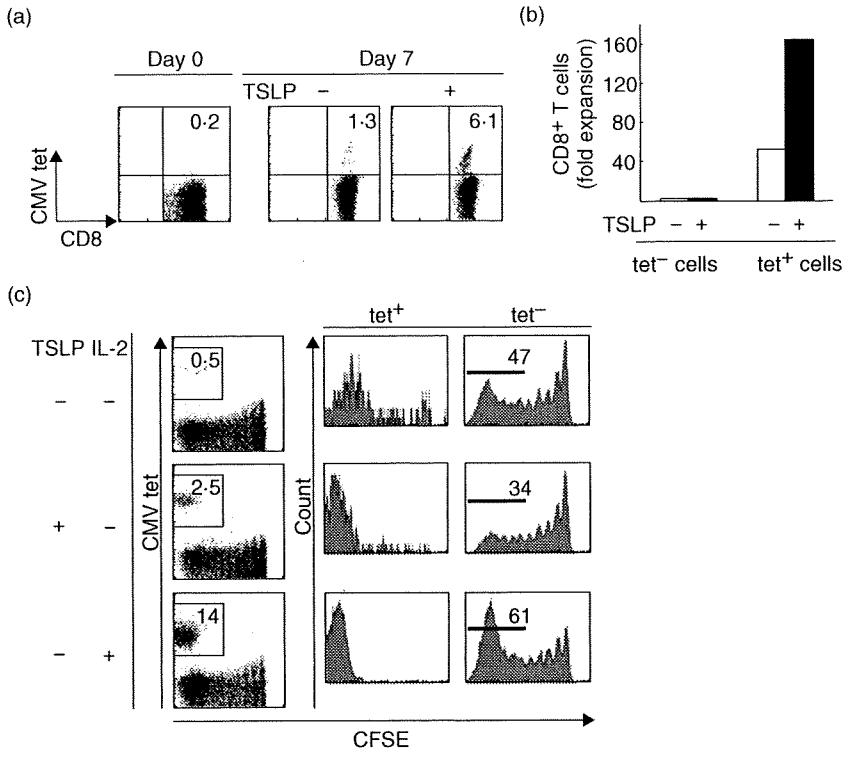

Fig. 5. Human TSLP induces predominant expansion of CMV-specific $CD8^+$ T cells. (a) Purified human $CD8^+$ T cells were stimulated with autologous MoDCs pulsed with HLA-A24-restricted CMVpp65 peptide for 7 days with or without TSLP at a DC : T cell ratio of 1:2. Cells were stained with mAb to cell surface marker phenotypes and HLA-A2402-CMVpp65 tetramer. (b) Fold expansion of $tetramer^-CD8^+$ T cells and $tetramer^+CD8^+$ T cells after 7 days of culture with (filled bars) and without (open bars) TSLP. Data represent one of 12 independent experiments. (c) Cell divisions of CFSE-labelled purified human $CD8^+$ T cells stimulated for 7 days with autologous MoDCs pulsed with CMVpp65 peptide (upper panels) or with either TSLP (middle panels) or IL-2 (lower panels). Cells were stained with anti-CD8 and CMVpp65 tetramer. Numbers indicate the percentages of proliferated $tetramer^+CD8^+$ T cells (left panels) and proliferated $tetramer^-CD8^+$ T cells (right panels) in $CD8^+$ cells. Data represent one of five independent experiments.

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ORIGINAL ARTICLE

Aberrant expression of BCL2A1-restricted minor histocompatibility antigens in melanoma cells: application for allogeneic transplantation

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Abstract It has been shown that allogeneic hematopoietic stem cell transplantation (HSCT) can be one of the therapeutic options for patients with metastatic solid tumors, such as renal cancer. However, the development of relatively severe GVHD seems to be necessary to achieve tumor regression in the current setting. Thus, it is crucial to identify minor histocompatibility antigens (mHags) only expressed in tumor cells but not GVHD target organs. In this study, we examined whether three mHags: ACC-1 and ACC-2 encoded by *BCL2A1*, and HA-1 encoded by *HMHA1*, could serve as such targets for melanoma. Real-time PCR and immunohistochemical analysis revealed that the expression of both *BCL2A1*and *HMHA1* in melanoma cell lines and primary melanoma cells was comparable to that of hematopoietic cells. Indeed, melanoma cell lines were efficiently lysed by cytotoxic T lymphocytes specific for ACC-1, ACC-2, and HA-1. Our data suggest that targeting mHags encoded not only by *HMHA1*, whose aberrant expression in solid tumors has been reported, but also *BCL2A1* may bring about beneficial selective graft-versus-tumor effects in a population of melanoma patients for whom these mHags are applicable.

Keywords Minor histocompatibility antigen · Allogeneic hematopoietic stem cell transplantation · Melanoma

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1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) can cure hematopoietic malignancies. The success of donor leukocyte infusion or a non-myeloablative conditioning regimen demonstrated that the therapeutic effects of allogeneic HSCT mostly rely on the allogeneic immune responses. In an HLA-matched setting, allogeneic immune responses are mediated by donor-derived cytotoxic T lymphocytes (CTLs) against minor histocompatibility antigens (mHags). Ubiquitously expressed mHags are responsible for both life-threatening graft-versus-host disease (GVHD) and the graft-versus-leukemia (GVL) effect, whereas hematopoietic cell-restricted mHags, such as HA-1 [1] or ACC-6 [2], may be optimal target antigens which can potentially separate the GVL effect from GVHD development [3].

In some solid tumors, such as melanoma or renal cell carcinoma, there has been accumulating evidence that immunological manipulation, e.g., IL-2 [4, 5] or interferon treatment [6], can lead to clinical responses in some patients with refractory disease, although responses have been limited. Since the late 1990s, it has been reported that nonmyeloablative allogeneic HSCT leads to apparent tumor regression in these immunogenic solid tumors [7, 8]. However, the development of relatively severe GVHD seems to be necessary to achieve tumor regression in the