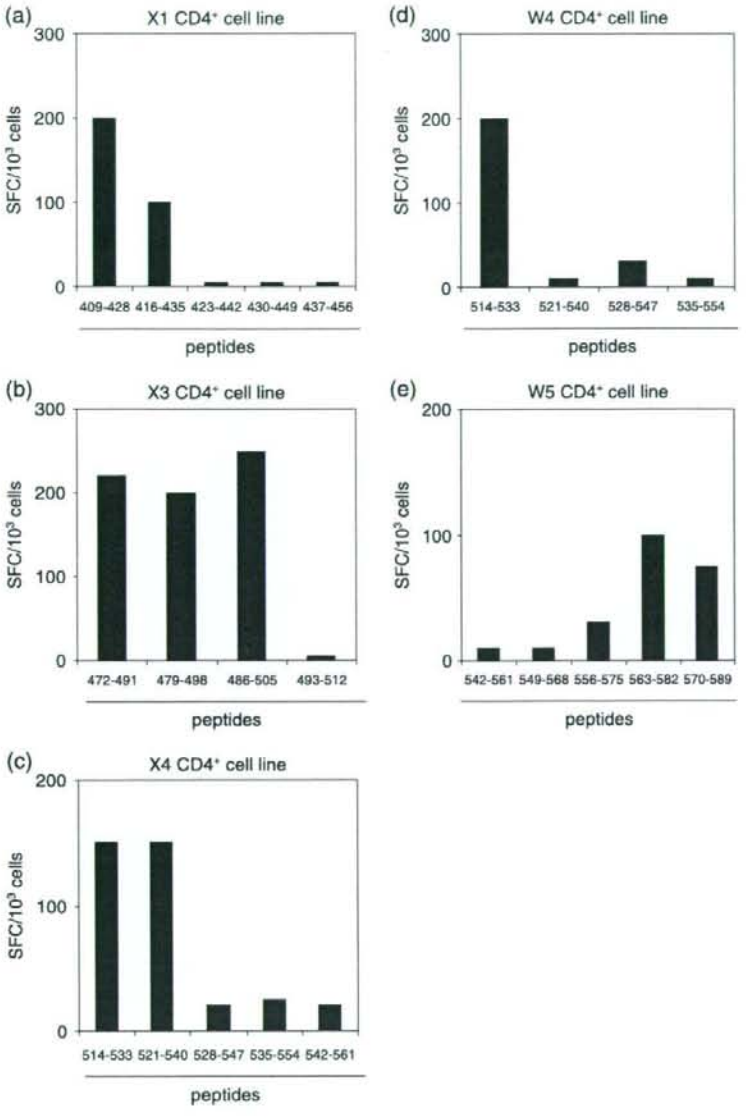

Fig. 2. Reactivity of CD4+ T-cell lines with individual peptides belonging to each pool. (a–e) Each CD4+ T-cell line was tested for its reactivity with individual peptides by enzyme-linked immunosorbent spot assay. Data are numbers of spots/1000 CD4+ T cells.

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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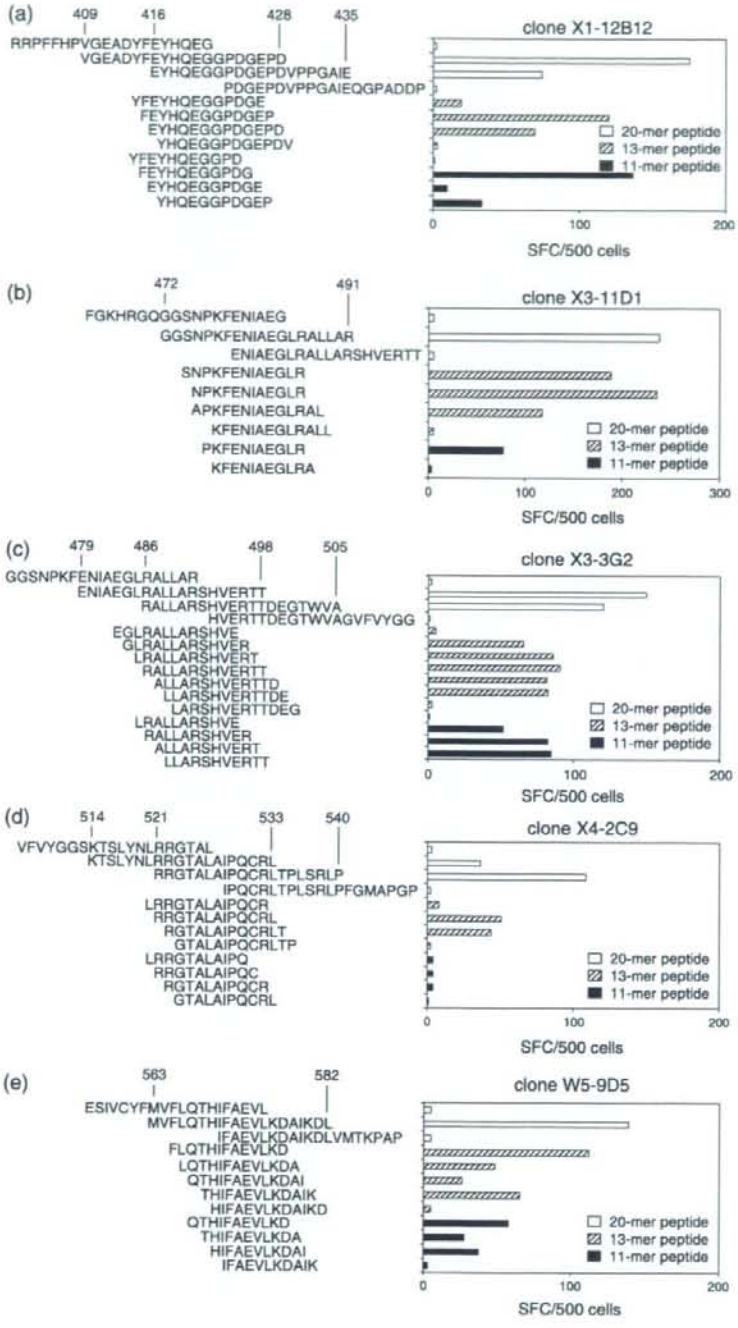

Fig. 3. Mapping of core regions recognized by CD4⁺ T-cell clones. (a–e) Peptides of 13- and 11-mer spanning overlapping regions were synthesized and tested for antigenicities in enzyme-linked immunosorbent spot assays. Numbers represent Epstein–Barr virus nuclear antigen 1 amino acid residues. All results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

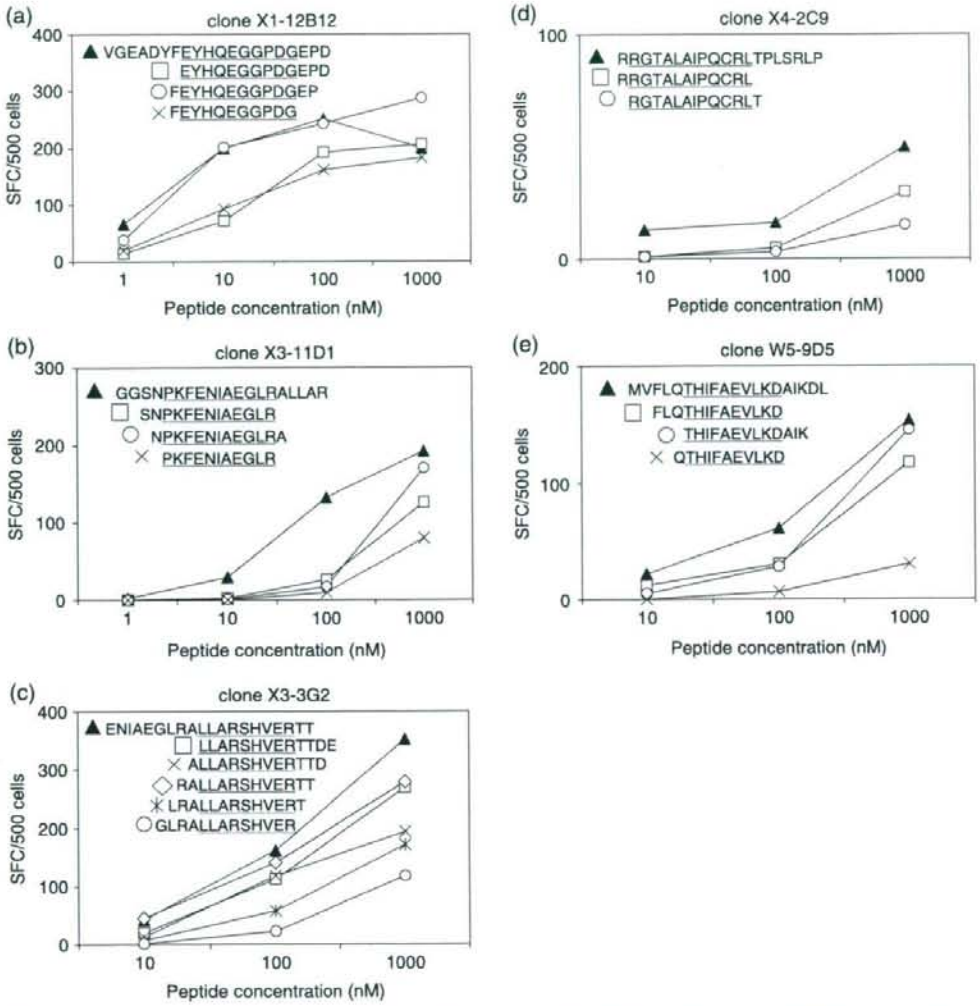

Fig. 4. Peptide dilution assay for CD4⁺ T-cell clones. (a–e) Each peptide at serial concentrations was tested in enzyme-linked immunosorbent spot assays. Data are numbers of spots/500 CD4⁺ T-cell clones. The core peptide sequences are underlined. All results are the mean of two duplicate values. SFC, 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[1]	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

[1]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.

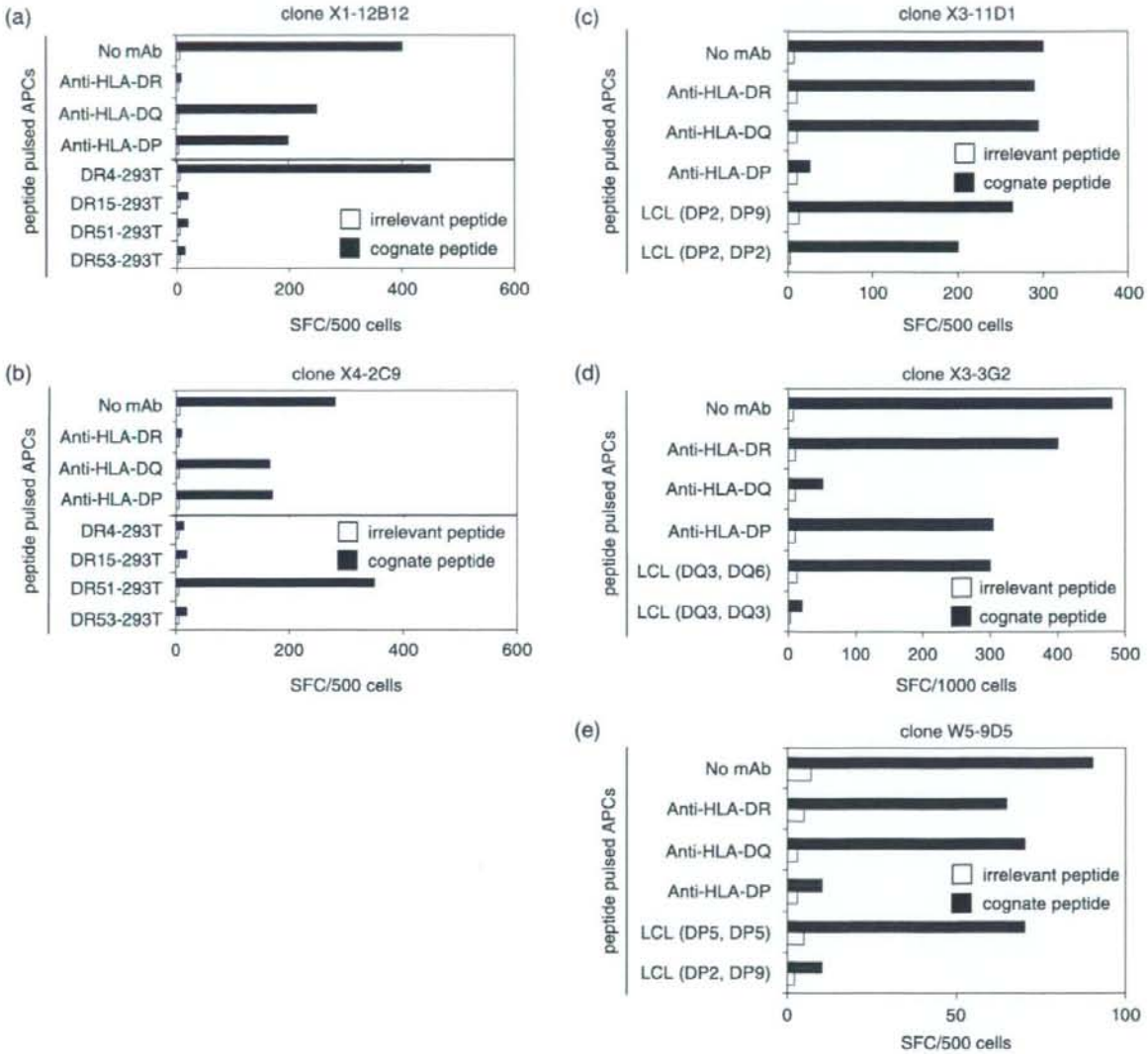

Fig. 5. Identification of human leukocyte antigen (HLA) class II restriction molecules. (a,b, top panel) Enzyme-linked immunosorbent spot (ELISPOT) assays were performed using autologous lymphoblastoid cell lines (LCL) pulsed with peptides in the presence of monoclonal antibody (mAb) against HLA-DR, 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.

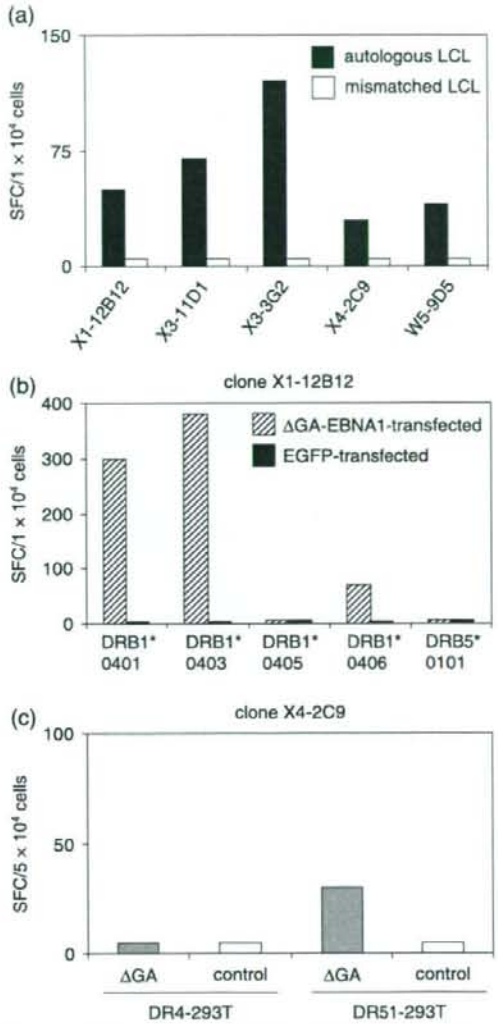

Fig. 6. Recognition of endogenously processed Epstein–Barr virus nuclear antigen (EBNA)1 by CD4⁺ T-cell clones. (a) γ-interferon spot production with CD4⁺ T-cell clones was estimated by enzyme-linked immunosorbent spot (ELISPOT) assays using lymphoblastoid cell lines (LCL)as antigen-presenting cells. 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.

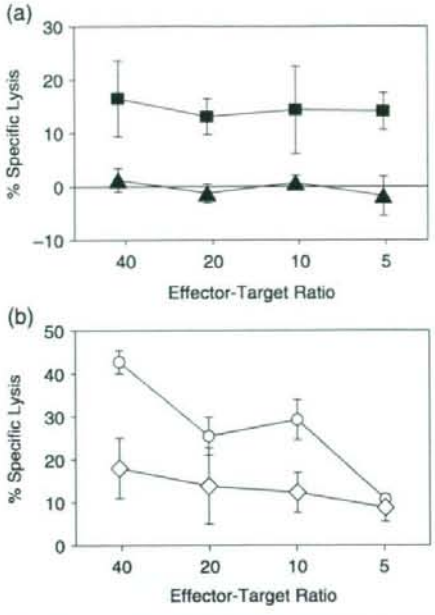

Fig. 7. Cytotoxic activity of CD4⁺ T cell clone X4-2C9 against EBV-carrying NK and T cell lines. (a) Fourteen-hour cytotoxic T lymphocytes assays were performed using Epstein–Barr virus-carrying T-cell lines as targets. Data for specific lysis of an human leukocyte antigen (HLA)-DR51-positive T-cell 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.

Acknowledgments

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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

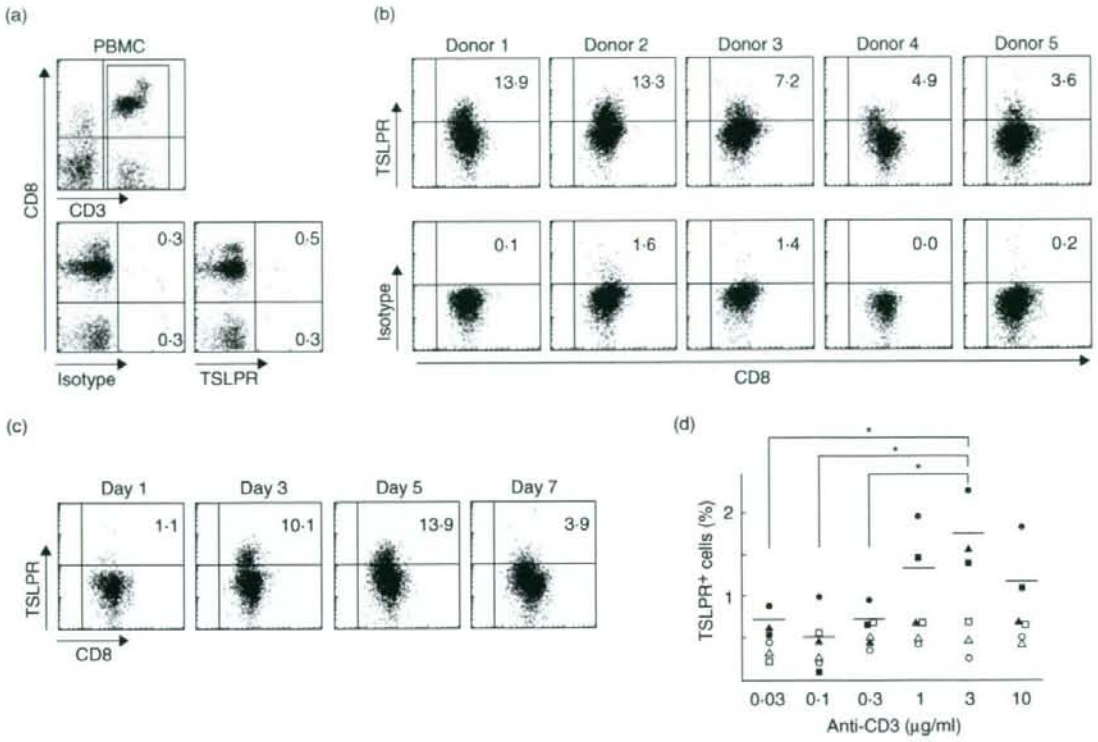

Fig. 1. TCR stimulation induces TSLPR expression on human CD8⁺ T cells. (a) PBMCs were stained with mAb to CD3, CD8 and TSLPR or isotype control. Cell surface marker phenotypes were determined by flow cytometry. Lower panels represent staining of CD3⁺ cells with TSLPR or isotype control. Numbers in quadrants 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.

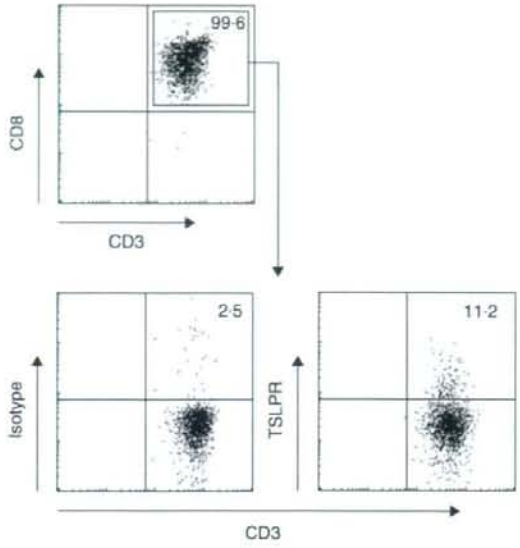

Fig. 2. CD8$^+$ T cells express TSLPR on their surface in mixed lymphocyte reaction. Purified human CD8$^+$ T cells were cultured with allogeneic MoDCs for 7 days at a DC : T cell ratio of 1:2. Cell surface marker phenotypes were determined by flow cytometry as in Fig. 1b. Data represent one of five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

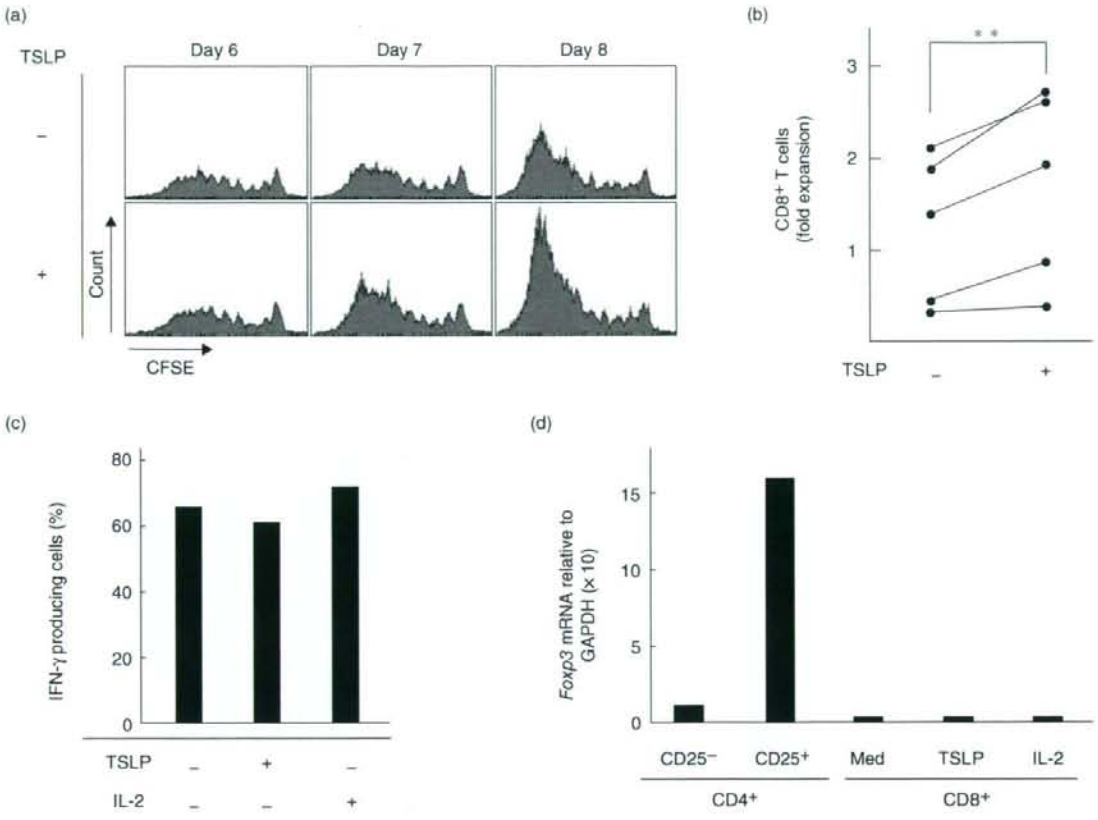

Fig. 3. TSLP directly enhances expansion of functional CD8+ T cells induced by TCR stimulation. (a) Cell divisions of CFSE-labelled purified human CD8+ T cells stimulated with anti-CD3 plus anti-CD28 with or without TSLP for indicated days. Filled histograms represent CFSE dilution in CD8+ T cells. Data 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

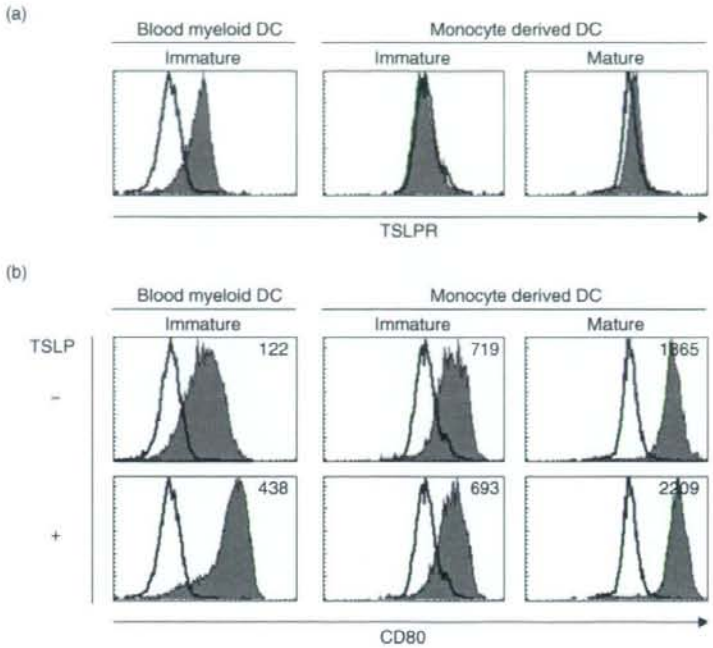

Fig. 4. TSLPR and CD80 expression on blood myeloid DCs and monocyte-derived DCs. (a) Purified immature CD11c$^+$ blood myeloid DCs and monocyte-derived DCs with or without maturation with IL-6, tumour necrosis factor-α, IL-1β and prostaglandin-E2, were stained with mAb to TSLPR or isotype control. Cell 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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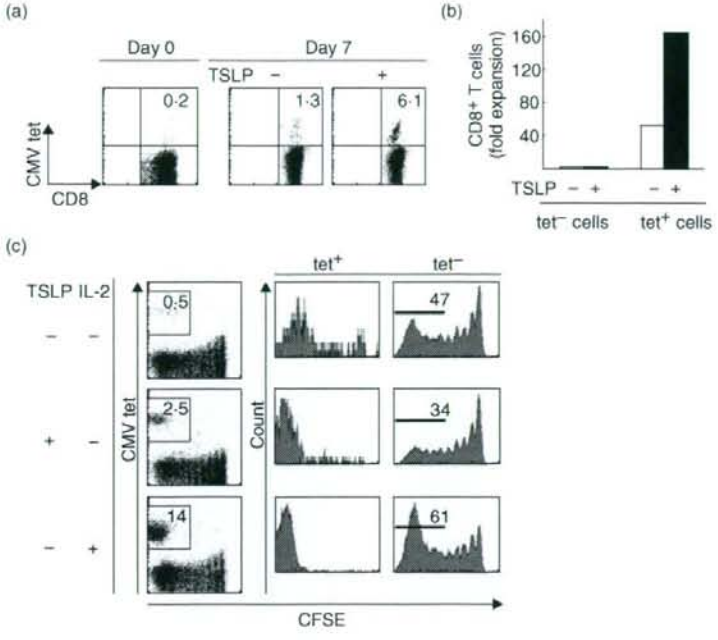

Fig. 5. Human TSLP induces predominant expansion of CMV-specific CD8⁺ T cells. (a) Purified human CD8⁺ T cells were stimulated with autologous MoDCs pulsed with HLA-A24-restricted CMVpp65 peptide for 7 days with or without TSLP at a DC : T cell ratio of 1:2. Cells were stained with mAb to cell surface 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

current setting. Thus, it is crucial to identify antigens that may serve as therapeutic targets for post-transplant vaccination or adoptive T-cell therapy to selectively augment the graft-versus-tumor (GVT) effects following allogeneic HSCT with modification to reduce fatal GVHD. Recently, we and others showed that the hematopoietic cell-restricted mHag HA-1^H, encoded by *HMHA1*, can be one of the potential targets for the GVT effect due to its aberrant expression in some solid tumors [9–11]. In addition, *ECGF1*-encoded mHag has been shown to be expressed in some solid tumors [12]. Thus, we sought to examine whether other mHags we had identified previously could also be expressed in any solid tumors and serve as potential targets for GVT effects.

The ACC-1 and ACC-2 mHags encoded by *BCL2A1* have been shown as hematopoietic cell lineage-restricted mHags [13]. Amino acid substitutions, ^{19}Cysteine→Tyrosine, and ^{82}Glycine→Aspartic acid, of BCL2A1 lead to donor-derived HLA-A*2402 and HLA-B*4403/4402-restricted $CD8^+$ CTL responses against the recipient's hematopoietic cells [13]. In this study, we demonstrate that *BCL2A1* is also highly expressed in melanoma cells and that they are effectively lysed by cognate CTLs. Although it has been reported that allogeneic HSCT was not promising against advanced melanoma in a small cohort of patients [14], our findings imply that targeting *BCL2A1*-encoded mHags may bring about beneficial GVT effects in a fraction of melanoma patients for whom these mHags are applicable.

2 Materials and methods

2.1 Cell lines and cell culture

$CD8^+$CTL clones recognizing $ACC1^Y$ (1B3-CTL) and ACC-2^D (3B5-CTL) were generated from post-HSCT recipients peripheral blood mononuclear cells (PBMCs) and HA-1^H (EH6-CTL) were from healthy volunteer's PBMCs, as previously described [11, 13]. These CTL clones were expanded using allogeneic PBMCs and Epstein Barr virus-transformed B cell lines (B-LCLs) as feeder cells and frozen until use.

Table 1 indicates the melanoma cell lines used in this study, kindly provided by Dr. Kawakami (Keio University, Tokyo, Japan). Their genotypes for the HLA class I and mHag allele were typed at the HLA Laboratory (Kyoto, Japan). All melanoma cell lines were cultured in Iscove modified Dulbecco medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and penicillin/streptomycin. B-LCLs established by infecting PBMCs with B95-8 (ATCC, Rockville, MD, USA) supernatant and HLA class I-deficient mutant 721.221 B-LCL were cultured in RPMI1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin/streptomycin. Primary melanocytes, NHE-Ma(L) and HEMa-LP, were purchased from KURABO (Osaka, Japan) and cultured in specified medium according to the manufacturer's protocol. All blood and tissue samples were collected after obtaining written informed consent, and the study was approved by the Institutional Review Board of Aichi Cancer Center.

Table 1 HLA and mHags typing of melanoma cell lines

Cell line	HLA		mHag [a]		
	A loci	B loci	ACC-1	ACC-2	HA-1
888Mel	0101/2402	5201/5501	Y/C	D/G	R/R
HT144	0101/2402	1501/5701	Y/C	D/G	R/R
G361	2301/2601	3801/4403	Y/C	D/G	R/R
WM266	0201/2902	1302/4403	C/C	G/G	H/R
C32Mel	0201/2501	1801/4402	C/C	G/G	H/H
HS294T	0101/2501	0702/0801	C/C	G/G	R/R

[a] The phenotypes of mHags of individual melanoma cell lines are shown using a single-lettered amino acid code. ACC-1 mHag is considered to be positive when carrying a Y (tyrosine) residue (referred to as $ACC\text{-}1^Y$) at its polymorphic site, while it is negative when carrying a C (cysteine) residue (referred to as $ACC\text{-}1^C$). Similarly, $ACC\text{-}2^D$ carrying D (asparaginic acid) is positive, while $ACC\text{-}2^G$ carry G (glycine) is negative; HA-1^H carrying H (histidine) is positive, while HA-1^R carrying R (arginine) is negative

2.2 Messenger RNA expression of BCL2A1 and HMHA1 in melanoma cell lines

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). Messenger RNA was magnetically isolated from total RNA using the μMACS mRNA Isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. Complementary DNA was synthesized in the presence of oligo $(dT)_{15}$ primer (Roche) and M-MLV-reverse transcriptase (Invitrogen) according to the manufacturers' instructions.

PCR amplification and real-time quantification analysis were performed using the TaqMan assay according to the manufacturer's instructions. The following sequences were used as primers with the TaqMan probe to detect the mRNA region of each gene:

BCL2A1-sense: 5′-TGAATAACACAGGAGAATGGATAAGG-3′,
BCL2A1-antisense: 5′-TTCAGGAGAGATAGCATTTCACAGAT-3′,
BCL2A1-probe: 5′-(FAM)-CTGGCTGGATGACTTT-(MGB)-3′
HMHA1-sense: 5′-GAGGGCCTTGAGAAACTTAAGGA-3′

HMHA1-antisense: 5′-CAGCGGGTACTTGGAGATG ATC-3′,
HMHA1-probe; 5′-(FAM)-CTGCGTGTCATGCAT-(MGB)-3′

For an internal control, a primer and probe set for human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) was used. 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 in the ABI PRISM 7900HT Sequence Detector System (Applied Biosystems). The temperature profile was: 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 60°C for 1 min for 40 cycles. Relative expressions were calculated by the $_{\Delta\Delta}C_T$ method after validation test described in the manufacturer's brochure (User Bulletin #2; Applied Biosystems 11 December 1997 (updated October 2001).

2.3 Immunohistochemical analysis of BCL2A1 expression in primary melanoma

To analyze the BCL2A1 protein expression in primary melanoma cells, we used frozen skin sections obtained from six patients with metastatic melanoma. The expression status was examined immunohistochemically with the standard avidin–biotin–peroxidase complex method using polyclonal antibodies against BCL2A1 [Santa Cruz, A1 (N-20): sc-6066].

2.4 Flow cytometric analysis of HLA Class I and BCL2A1 expression in melanoma cell lines

The cell surface HLA-class I expression of melanoma cell lines before and after treatment with interferon-γ (IFN-γ) and TNF-α was evaluated using W6/32 mAb (10 μg/mL) and FITC-conjugated anti-mouse IgG antibodies (Beckman Coulter). For the intracellular staining of BCL2A1 protein, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), washed once with PBS, and incubated with 40 μg/mL of goat polyclonal antibodies against BCL2A1 [Santa Cruz, A1 (N-20): sc-6066] for 15 min. After washing, bound antibodies were detected by incubation with FITC-conjugated donkey anti-goat IgG antibody (8 μg/mL, Santa Cruz, CA, USA) for 15 min. Cells were analyzed with a FACS Calibur flow cytometer and CellQuest software (Becton-Dickinson).

2.5 Cytotoxicity assay

Target cells were labeled with 0.1 mCi of ^{51}Cr for 2 h or overnight at 37°C, and 1 × 10^3 target cells/well were mixed with CTLs at an E/T ratio indicated in a standard 4-h cytotoxicity assay using 96 well, round-bottomed plates. All assays were performed at least in duplicate. Cells were treated either with IFN-γ (500 U/mL, R&D Systems) or TNF-α (10 ng/mL, Genzyme) for 48 h as indicated. Percent specific lysis was calculated as follows: ((Experimental cpm − Spontaneous cpm) / (Maximum cpm − Spontaneous cpm)) × 100.

3 Results

3.1 Melanoma cell lines express high levels of BCL2A1 gene

We previously demonstrated that *BCL2A1* is preferentially expressed in hematopoietic lineage cells but not other normal cells. By accessing a gene expression database, GNF (Genomic Institute of the Novartis Research Foundation, http://symatlas.gnf.org/SymAtlas/) [15], we found that *BCL2A1* is highly expressed in melanoma cell lines. Thus, we first tried to confirm the expression levels of *BCL2A1* in melanoma cell lines using real-time PCR. As shown in the lower part of Fig. 1a, most melanoma cell lines expressed the *BCL2A1* transcript at levels as high as B-LCLs, with the exception of the cell line HS294T, which eventually expressed a comparable level of the transcript after cytokine treatment (IFN-γ and TNF-α). Some cell lines also expressed *HMHA1* transcripts, but their levels were relatively low (Fig. 1b).

3.2 BCL2A1 expression in primary melanoma specimens

We subsequently tested whether primary melanoma cells expressed BCL2A1 protein. Skin sections from six patients (MM-1 to MM-6) with metastatic melanoma were stained with anti-BCL2A1 antibody (Fig. 2). Three specimens were positive for BCL2A1 (MM-1, 2, 3), while one was negative (MM-4). Another two samples were difficult to evaluate because of marked melanin pigmentation (MM-5, 6). Overall, 3/4 primary metastatic melanoma cells were positive for BCL2A1 protein. Along with the results of real-time PCR analysis, BCL2A1 was strongly and frequently expressed in melanoma cells.

3.3 Melanoma cell lines are susceptible to lysis by BCL2A1-specific CTL clones

To determine whether melanoma cell lines can indeed present BCL2A1-derived mHags on their cell surface HLA molecules, and are thus susceptible to lysis by CTLs specific for these mHags, we performed a standard

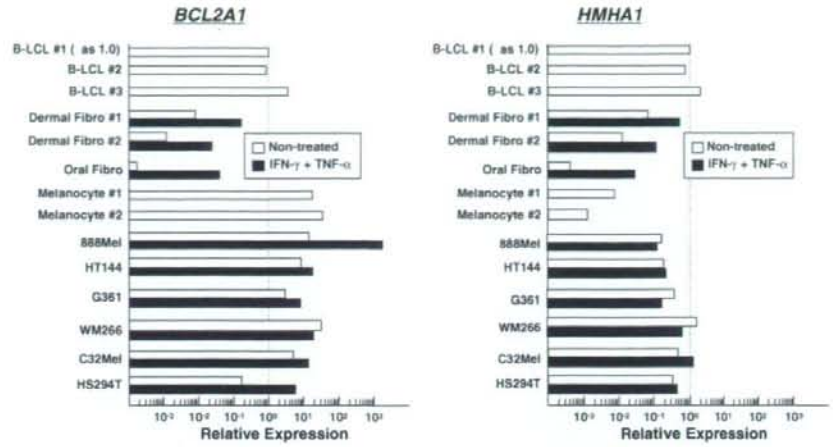

Fig. 1 Relative expression of *BCL2A1* (*left panel*) and *HMHA1* (*right panel*) in melanoma cell lines. Real-time reverse transcription PCR to quantify the mRNA expression of *BCL2A1* and *HMHA1* was performed using cDNA samples prepared from melanoma cell lines (kind gift from Dr. Kawakami, Keio University, Tokyo) and primary melanocytes, together with EBV-transformed B lymphoblasts (B-LCLs) and fibroblasts (Fibro) from skin and oral mucosa. IFN-γ + TNF-α (*solid bars*) denotes 48-h cytokine treatment with 500 U/mL of IFN-γ and 10 ng/mL of TNF-α. *GAPDH* was used as an internal control. mRNA expression in B-LCLs is set as 1.0

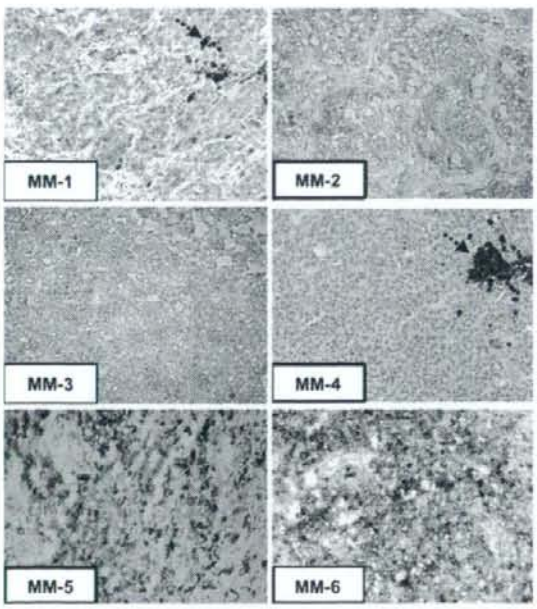

Fig. 2 Expression of BCL2A1 protein in primary metastatic melanoma (MM) cells. Frozen skin sections from six patients with metastatic melanoma were examined for BCL2A1 expression immunohistochemically by the standard avidin–biotin–peroxidase complex method using polyclonal antibodies against BCL2A1 (Santa Cruz, A1 (N-20): sc-6066). MM-1 to -3 were found to be positive; MM-4 negative; MM-5 and -6 showed marked melanin pigmentation. *Red arrows* in MM-1 and MM-4 indicate melanin spots, showing that the specimens were of melanoma origin

^{51}Cr-release assay. As shown in Fig. 3a, melanoma cell lines positive for respective mHags and restriction HLA alleles were lysed effectively by cognate CTL clones: 888Mel and HT144 by 18B3-CTL (HLA-A24-restricted, ACC-1^{Y}-specific), and G361 by 3B5-CTL (HLA-B44-restricted, ACC-2^{D}-specific). In contrast, HLA-class I-deficient K562 cell lines or melanoma cell lines lacking either the restriction HLA allele or cognate mHag allele that were used as control targets were not lysed at all, indicating that the observed cytotoxicity against melanoma cell lines by these CTL clones was antigen-specific. We also examined the expression of HLA-class I and intracellular BCL2A1 in these cell lines to evaluate the effect of cytokine treatment. All melanoma cell lines tested were positive for HLA-class I and BCL2A1, similarly to B-LCLs, with the one exception of HT144, whose BCL2A1 expression was 1-log lower than that of B-LCLs (Fig. 3B). Cytokine treatment upregulated HLA-class I expression in all melanoma cell lines, with one exception of 888 MEL, which might account for the increased lysis of G361 by 3B5-CTL and WM266 by EH6-CTL, respectively. The lower BCL2A1 expression in HT144 was also upregulated after treatment. However, cytokine treatment did not necessarily result in increased, specific lysis in cell lines other than G361 and WM266. Therefore, another mechanism might also be involved in the susceptibility to lysis of each cell line. In addition, two melanoma cell lines (WM266 and C32Mel) positive for the HLA-A*0201 and HA-1^{H} alleles could be recognized by EH6-CTL despite the relatively low expression of *HMHA1* compared to *BCL2A1*.