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-1^Y) at its polymorphic site, while it is negative when carrying a C (cysteine) residue (referred to as ACC-1^C). Similarly, ACC-2^D carrying D (asparaginic acid) is positive, while ACC-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

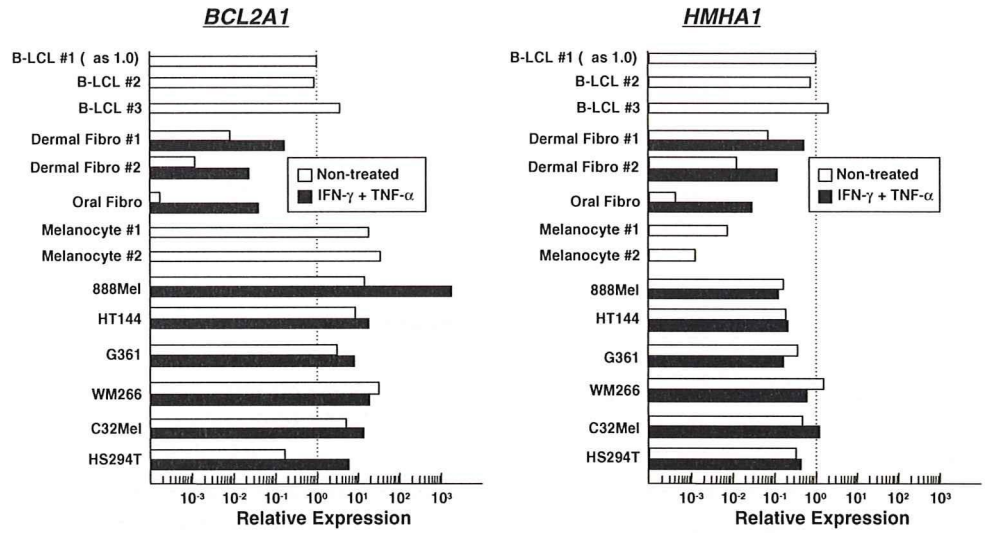

Fig. 1 Relative expression of *BCL2A1* (*left panel*) and *HMHA1* (*right panel*) in melanoma cell lines. Real-time reverse transcription PCR to quantify the mRNA expression of *BCL2A1* and *HMHA1* was performed using cDNA samples prepared from melanoma cell lines (kind gift from Dr. Kawakami, Keio University, 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

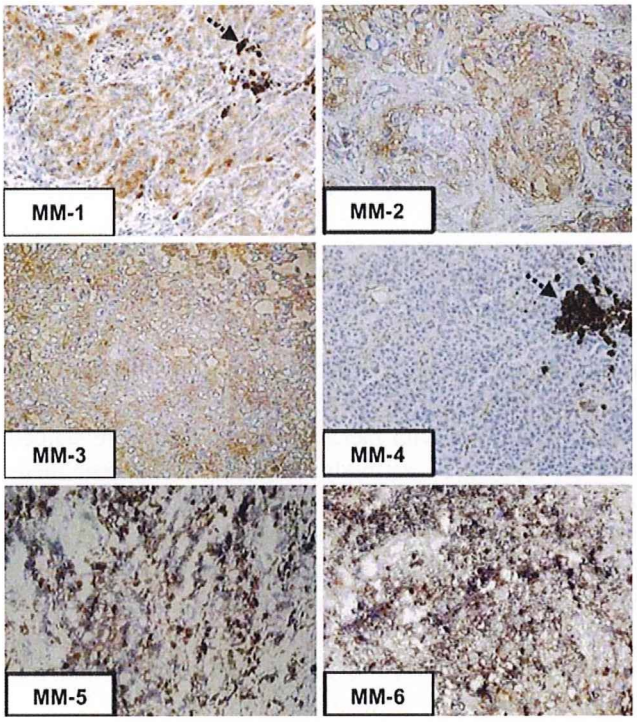

Fig. 2 Expression of BCL2A1 protein in primary metastatic melanoma (MM) cells. Frozen skin sections from six patients with metastatic melanoma were examined for BCL2A1 expression immunohistochemically by the standard avidin–biotin–peroxidase complex method using polyclonal antibodies against BCL2A1 (Santa 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*.

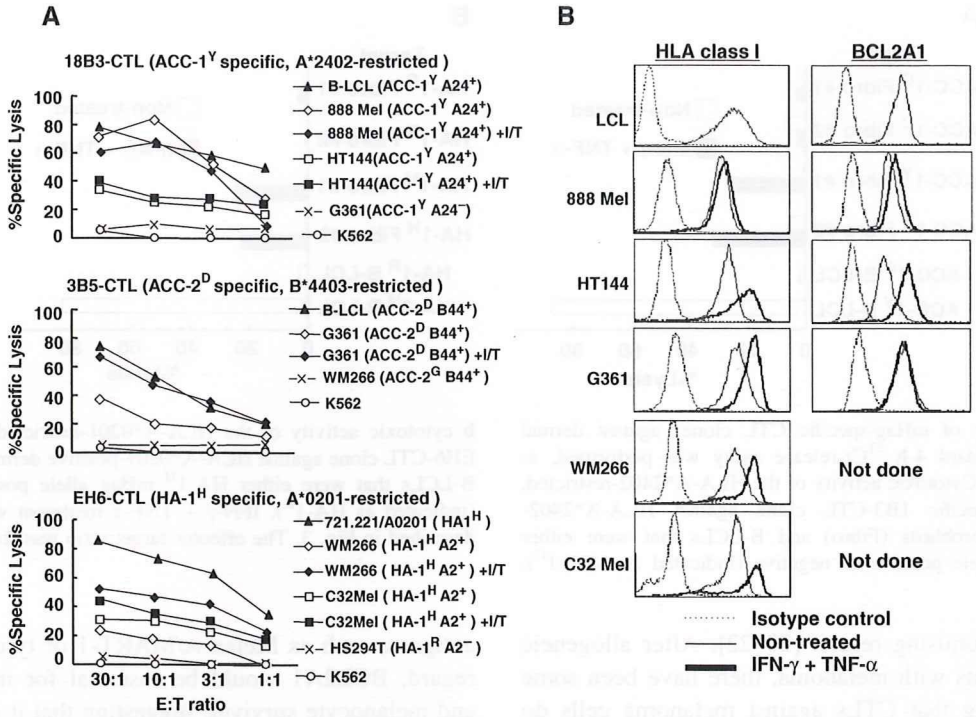

Fig. 3 Susceptibility of melanoma cell lines to mHag-specific CTL clones and the impact of cytokine treatment. **a** ^{51}Cr-release assay against melanoma cell lines. Standard 4-h ^{51}Cr-release assays were performed against various melanoma cell lines at the indicated E/T ratios and at least in duplicate. B-LCLs positive for the restriction HLA allele and mHag allele were used as positive controls for individual CTL clones. The 721.221 cell line comprised HLA-A*0201-transduced B-LCLs positive for the HA-1^{H} allele. Non-specific lysis of the individual CTL clones was examined and verified by testing against NK cell-sensitive K562 or melanoma cell lines that lacked either the cognate mHag or restriction HLA allele. I/T denotes the treatment of indicated cell lines with 500 U/mL of IFN-γ and 10 ng/mL of TNF-α for 48 hours prior to assays; **b** HLA class I and BCL2A1 expression of melanoma cell lines. Cell surface expression of HLA class I and intracellular staining of BCL2A1 was evaluated by flow cytometry before and after treatment with the above cytokines

3.4 HMHA1 in dermal fibroblasts is also upregulated by inflammatory cytokines

It has been reported that *HMHA1* encoding HA-1 mHag is not detected in normal nonhematopoietic cells such as dermal fibroblasts [9], while *BCL2A1* is upregulated in bone marrow-derived mesenchymal stem cells by inflammatory cytokines [16]. Thus, we examined whether dermal fibroblasts upregulated these mHag genes and became susceptible to cognate CTL clones. We found that the expression of both *BCL2A1* and *HMHA1* is upregulated in the dermal fibroblasts after cytokine treatment (Fig. 1a, b, upper part), indicating that these hematopoietic cell-specific mHags might be induced in a strong inflammatory cytokine milieu such as active GVHD after HSCT. Hematopoietic cell contamination was excluded by real-time PCR or flow cytometric analysis of the expression of CD45 in these fibroblasts (data not shown). Coincident with expression, the HLA-A*0201-restricted HA-1^{H}-specific CTL clone, EH6-CTL, and A*2402 restricted ACC-1^{Y} specific CTL clone, 18B3-CTL, could lyse these cytokine-treated mHag-positive dermal fibroblasts, although their level of lysis was relatively lower than that of hematopoietic cells (Fig. 4a, b).

4 Discussion

In this study, we demonstrated that HLA-A24-restricted ACC-1^{Y} and HLA-B44-restricted ACC-2^{D} mHAgs, whose expressions were shown to be limited to hematopoietic cells including leukemia cells, were also expressed in melanoma cell lines by real-time PCR and cytotoxicity assays. Melanoma is known as one of the representative immunogenic tumors. Previously, IL-2 administration [4] or the infusion of ex vivo expanded TILs [17] was tested, but resulted in a limited clinical response. In 1990s, many antigens of melanoma origin recognized by autologous T lymphocytes were identified [18], and these antigens were subsequently tested in clinical trials by peptide vaccination or adoptive CTL infusion. To date, peptide vaccination has resulted in a limited or marginal efficacy [19] while adoptive T lymphocyte infusion including Ag-specific CTL clones or TILs, especially after a lymphodepleted conditioning regimen,

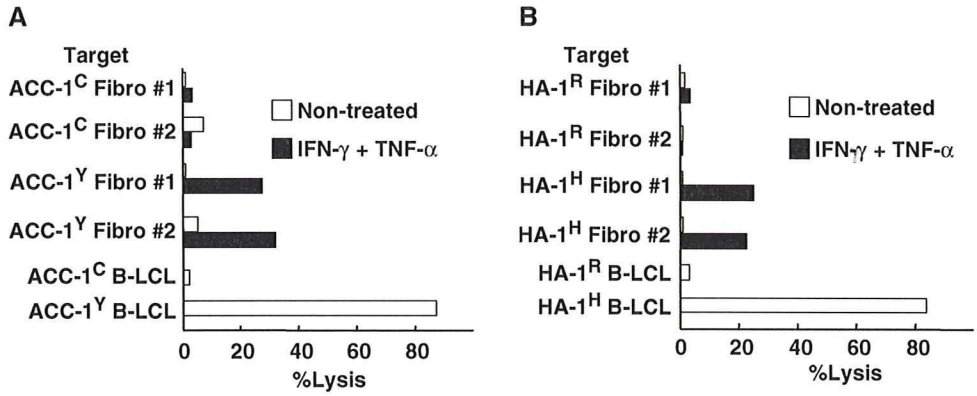

Fig. 4 Cytotoxicity of mHag-specific CTL clones against dermal fibroblasts. A standard 4-h ^{51}Cr-release assay was performed, as described above. **a** Cytotoxic activity of the HLA-A*2402-restricted, ACC-1^{Y} mHag-specific 1B3-CTL clone against HLA-A*2402-positive dermal fibroblasts (Fibro) and B-LCLs that were either ACC-1^{Y} mHag allele positive or negative (indicated as ACC-1^{C}); **b** cytotoxic activity of the HLA-A*0201-restricted, HA-1^{H}-specific EH6-CTL clone against HLA-A*0201-positive dermal fibroblasts and B-LCLs that were either HA-1^{H} mHag allele positive or negative (indicated as HA-1^{R}). IFN-γ + TNF-α treatment was performed as described in Fig. 3. The effector target ratio was fixed at 30:1

demonstrated promising results [20–22]. After allogeneic HSCT for patients with melanoma, there have been some reports indicating that CTLs against melanoma cells do exist and that these melanoma-reactive CTLs can be expanded in vitro [23, 24]. These observations suggest that allogeneic HSCT after a nonmyeloablative conditioning regimen might be a promising therapeutic strategy for patients with refractory metastatic melanoma.

Childs et al., however, reported relatively disappointing results in which 5 out of 11 metastatic melanoma patients receiving allogeneic HSCT died from rapid tumor growth, while the rest of the patients showed variable results [14]. As in the case of hematological malignancies, a high tumor burden should be one of the most unfavorable factors regarding treatment failure with allogeneic HSCT. Therefore, a treatment strategy combining the selection of patients with a lower tumor burden or slower growth kinetics and allogeneic HSCT may be explored for this poor-prognosis disease if the given donor and recipient are eligible for immunotherapy using ACC-1 and ACC-2 mHags, or other hematopoiesis-specific mHags are also highly expressed in melanoma cells. Since HSCT recipients eligible for ACC-1, ACC-2, and HA-1 mHags exist at a frequency of 11, 3, and 9%, respectively, in Japanese [25], it would be possible to apply these mHags to nearly a quarter of such patients.

BCL2A1 is a member of the B-cell lymphoma-2 (BCL2) family. *BCL2* is highly expressed in melanoma, which was shown to contribute to a chemoresistant phenotype [26]. The reduction of BCL2 by siRNA caused melanoma cells to become susceptible to chemotherapeutic agents. BCL2A1, although regulated differently from BCL2, also exerts antiapoptotic activity and is expressed even in normal melanocytes like other melanocyte differentiating antigens, such as Melan-A/MART-1 or tyrosinase. In this regard, BCL2A1 would be essential for melanoma cells and melanocyte survival, suggesting that it may be a good candidate antigen for immunotherapy against melanoma, although autoimmune depigmentation may also develop, as seen in adoptive immunotherapy targeting melanoma-associated antigens mentioned above [20, 21].

In addition, we unexpectedly found that, after cytokine treatment, dermal fibroblasts upregulated both *BCL2A1* and *HMHA1* expression and become susceptible to cognate CTL clones, respectively (Fig. 4). This suggests that, after allogeneic HSCT, they would also be upregulated under a "cytokine-storm", and may contribute in some way to the pathophysiology of skin GVHD. In the clinical setting, HA-1 was originally reported as an mHag associated with GVHD [27], and additional studies brought about mixed results, making it still too early to draw any conclusion [28, 29], while ACC-1^{Y} disparity did not seem to be associated with an increased incidence of acute GVHD [30]. In skin explant assays, it was shown that skin sections from HLA-A2^{+} HA-1^{+} individuals incubated with HA-1 CTLs developed only background grade I or low grade II GVH reactions, while male HLA-A2^{+} skin sections incubated with Y antigen-specific CTLs displayed severe GVH reactions of grade III–IV [31]. It is assumed that stronger GVH reactions might be observed if skin sections are pretreated with cytokines before incubation with HA-1-specific CTLs. IFN-γ, which is known to induce various transcription factors specific for hematopoiesis and immunity, might be a key in this upregulation of hematopoietic cell-restricted mHags in dermal fibroblasts. Since IFN-γ is strongly produced by CTLs and type 1 helper T cells, the IFN-γ secreted from mHag-specific CTLs could lead to the upregulation of target hematopoiesis-specific

mHags, resulting in GVHD or GVT effects in tumors sensitive to the IFN-γ-induced upregulation of such mHags. Therefore, it is crucial to develop a new treatment strategy to induce selective GVT effects while avoiding life-threatening GVHD using preconditioning and GVHD prophylaxis regimens to minimize GVHD, followed by selective immunotherapy targeting mHags mainly expressed in tumors and hematopoietic cells, such as ACC-1, -2, and HA-1, after the "cytokine storm" period is over.

In summary, *BCL2A1*-encoded mHags, ACC-1 and ACC-2, may be potential targets of immunological interventions for a fraction of patients with refractory, but not bulky melanoma following allogeneic HSCT.

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ORIGINAL ARTICLE

CD137-guided isolation and expansion of antigen-specific CD8 cells for potential use in adoptive immunotherapy

Kazue Watanabe · Susumu Suzuki · Michi Kamei · Shingo Toji · Takakazu Kawase · Toshitada Takahashi · Kiyotaka Kuzushima · Yoshiki Akatsuka

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Abstract The efficient isolation and ex vivo expansion of antigen-specific T cells are crucial for successful adoptive immunotherapy against uncontrollable infections and cancers. Several methods have been reported for this purpose, for example, employing MHC-multimeric complexes, interferon-gamma secretion, and antibodies specific for molecules expressed on T-cell surfaces, including CD25, CD69, CD107a, CD137, and CD154. Of the latter, CD137 has been shown to be one of the most promising targets since it is only expressed on $CD8^+$ T cells early after encountering antigen, while being almost undetectable on resting cells. However, detailed comparisons between CD137-based and other methods have not yet been conducted. In this study, we therefore compared three approaches (with CD137, CD107a, and tetramers) using HLA-A24-restricted CMV pp65 and EBV BRLF1 epitopes as model antigens. We found that the CD137-based isolation of antigen-stimulated $CD8^+$ T cells was comparable to tetramer-based sorting in terms of purity and superior to the other two methods in terms of subsequent cell expansion. The method was less applicable to $CD4^+$ T cells since their CD137 upregulation is not sufficiently high. Collectively, this approach is most likely to be optimal among the methods tested for the isolation and expansion of antigen-specific $CD8^+$ cells.

Keywords CD137 · Adoptive transfer · Cytotoxic T lymphocyte · Sorting

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K. Watanabe (✉) · S. Toji
Research Reagent Division, Medical Biological Laboratories Co., Ltd., 1063-103 Ohara, Terasawaoka, Ina, Nagano 396-0002, Japan
e-mail: watanabe.kazue@mbl.co.jp

K. Watanabe · M. Kamei · T. Kawase · T. Takahashi · K. Kuzushima · Y. Akatsuka (✉)
Division of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681, Japan
e-mail: yakatsuk@aichi-cc.jp

S. Suzuki
T Cell Technologies, Inc., 3-5-10 Marunouchi, Naka-ku, Nagoya 460-0002, Japan

M. Kamei
Department of Pediatrics and Neonatology, Nagoya City University, Graduate School of Medical Science, Nagoya, Japan

K. Kuzushima
Department of Cellular Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan

1 Introduction

Patients under severe immunosuppression after organ transplantation or chemotherapy, or due to congenital/acquired immunodeficiency, are vulnerable to infections with viruses such as cytomegalovirus (CMV) and Epstein–Barr virus (EBV), which are major causes of morbidity and mortality. Although the advent of new antiviral drugs for CMV [1] or anti-CD20 antibodies for EBV-associated B cell malignancies [2] has improved the survival of patients at risk, the adoptive transfer of T cells specific for these viruses still remains an attractive strategy, especially when the viruses or virus-associated tumors are resistant to such agents [3]. The powerful antiviral effects of infused T cells have been reported in various clinical settings [4–6]. There

are two ways to compensate for immunodeficiency in patients: (1) the infusion of ex vivo-expanded viral antigen-specific T cells; and (2) direct transfusion of peripheral blood T cells from healthy donors when the patients receive allogeneic hematopoietic cell transplantation. Although the latter method is feasible, there is a risk of graft versus host disease and it usually takes at least a few weeks before antiviral T cells have effectively expanded in vivo [7]. In contrast, although the former method is cumbersome and also time-consuming one at the ex vivo step, it is expected to be more effective and safer since only armed and selected viral antigen-specific T cells are infused [8].

Recently, several methods to detect and positively sort T cells specific for antigens of interest have been reported. These include the sorting of T cells stained with peptide/MHC multimers, with antibodies that react to cell surface-exposed CD107 (LAMP1) [9, 10], cell surface-captured interferon-gamma (IFN-γ), with the aid of a special biphasic antibody [11], and CD137 [12] as a more antigen-specific activation marker than CD25 or CD69. Except in tetramer or multimer cases, T cells activated with whole antigen without prior knowledge of the restriction HLA alleles or epitopes have been shown to be positively selected by flow-sorting or with magnetic beads using any of the above-mentioned methods. As these methods are based on the specific functions of individual cells, it is not easy to determine which method is most feasible for routine immunological studies and clinical application. In this report, we compared the results using three methods (using tetramers, CD107a, and CD137), all of which require a single staining step, employing CMV pp65 and EBV BRLF1 epitopes as model antigens, focusing on their merits and limitations.

2 Materials and methods

2.1 Cells and culture media

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll density gradient. All blood samples were collected after obtaining written informed consent, and the study was approved by the institutional review board of Aichi Cancer Center. Primary T cell lines were induced in RPMI 1640 (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 12.5 mM HEPES, 5% autologous plasma, penicillin/streptomycin, and 2 mM L-glutamine (referred to as T cell medium). Epstein–Barr virus-transformed B cells (B-LCL) were established by infecting an aliquot of PBMCs with B95-8 supernatant.

2.2 Antibodies, tetramers, and flow cytometric analysis

Antibodies used for sorting and phenotyping were as follows: anti-CD4-PC5, anti-CD8-PC5, anti-CD28-PE, anti-CD45RA-PE, anti-CD45RO-FITC (all from Beckman Coulter Inc., Miami, FL, USA) anti-CD137-FITC (MBL, Nagoya, Japan), anti-CD107a-FITC (Southern Biotech, Birmingham, AL, USA), anti-CD137-PE (BD Biosciences, San Diego, CA, USA), and anti-CCR7-FITC (R&D systems, Minneapolis, MN, USA). For intracellular interferon (IFN)-γ staining, anti-IFN-γ-FITC was from MBL (Nagoya, Japan). HLA-A*2402 CMVpp65, HLA-A*0201 CMV pp65, HLA-A*2402 EBV-BRLF1, and HLA-DRB1*0101 EBNA1 tetramers were purchased from MBL (Nagoya, Japan). Cells were first stained with tetramers for 15 min at room temperature, and then stained with appropriate combinations of antibodies for 15 min on ice. Flow cytometric analysis of the cells was performed using a FACSCalibur (BD Biosciences) with the aid of CellQuest software (BD Biosciences).

2.3 Peptides

The following peptides were synthesized by BioSynthesis (Lewisville, TX, USA): CMV/pp65(341–349) (QYDPVAALF, referred to as CMV-QYD hereafter), CMV/pp65(495–503) (NLVPMVATV, as CMV-NLV), EBV/BRLF1(320–328) (DYNFVKQLF, as EBV-DYN), and EBV/EBNA1(515–527) (TSLYNLRRGTALA, as EBV-TSL).

2.4 Induction of T cell lines by mixed lymphocyte-peptide cultures (MLPCs) (Fig. 1a)

The antigenic peptides listed above were directly added to PBMCs at 10 μg/ml suspended in 2 ml T cell medium in a 15-ml round-bottomed tube (BD Biosciences), and the cultures were maintained at 37°C and 5% CO_2. On day 2, recombinant human IL-2 (50 U/ml, Shionogi Pharmaceutical Institute Co., Osaka, Japan) was added. Starting on day 5, half-medium change and supplementation of IL-2 were performed every other day until day 14.

2.5 Restimulation and positive selection of antigen-specific T cells

Restimulation of MLPC T cell lines for the analysis of CD107a and CD137 expression followed by positive selection with MACS beads was performed 14 days after the primary stimulation. The optimal peptide concentration was predetermined for individual epitopes. Peptide was directly added to the aliquot of T cell lines without any antigen-presenting cells (APCs) and cytokines. For the determination of the optimal timing for positive selection either with anti-CD107a, or anti-CD137 antibody, the expression of CD107 and CD137 on antigen-specific T cells (identified by cognate tetramer) was assessed at

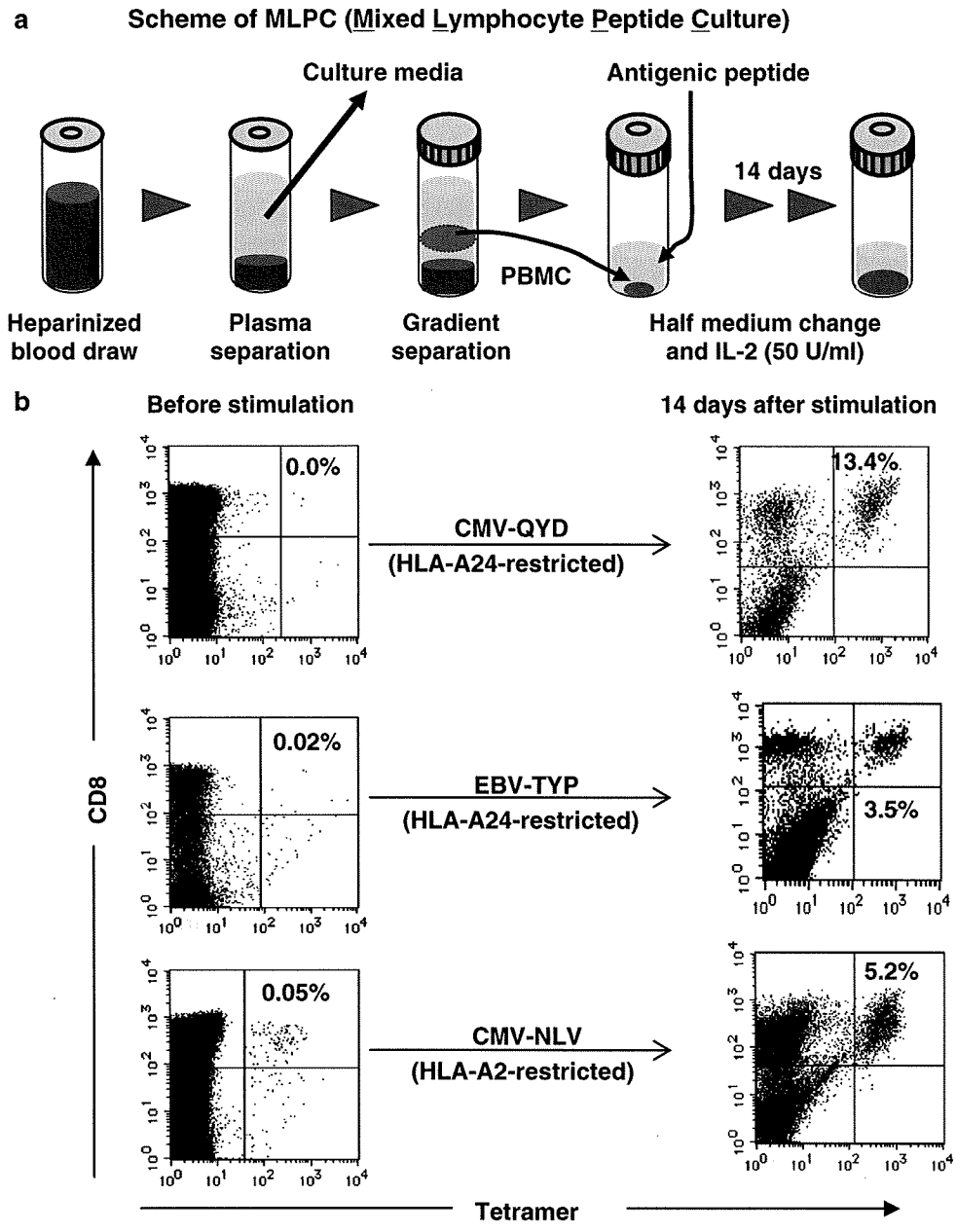

Fig. 1 **a** Schematic diagram of mixed lymphocyte peptide culture (MLPC). Heparinized whole blood was first centrifuged to obtain plasma for culture media preparation. Peripheral blood mononuclear cells (PBMCs) were then separated by density gradient centrifugation from the resuspended blood pellets and cultured in RPMI1640 medium supplemented with 5% autologous plasma in the presence of 10 μg/ml of antigenic peptide for 14 days. **b** Induction of viral antigen-specific T cell lines by MLPC. PBMCs were stained with the indicated tetramer before and after stimulation with the corresponding peptide. The percentages of tetramer$^+$ cells among CD3$^+$ populations are indicated. The data shown are representative of the following numbers of experiments: CMV-QYD, $n = 17$; EBV-TYP, $n = 10$; CMV-NLV, $n = 5$

various time points. After incubation for the predetermined time, T cell lines were washed and stained with either FITC-labeled CD107a, or CD137 antibody at 10 μg/ml in PBS containing 0.5% human serum albumin for 15 min at 4°C. After washing with MACS buffer (phosphate-buffered saline supplemented with 0.5% human serum albumin and 2 mM EDTA), the cells were incubated with anti-murine IgG1 MACS beads (Miltenyi Biotec, Auburn, CA, USA) for 15 min at 4°C. Cell separation was conducted using AutoMACS (Miltenyi Biotec). Antigen-specific T cells were also isolated without prior antigenic stimulation using cognate PE-conjugated tetramers followed by separation with anti-PE MACS beads and AutoMACS.

2.6 Expansion of sorted antigen-specific T cells

Sorted T cells were propagated in appropriately sized culture vessels in ALyS505N-1000 medium (Cell Science & Technology Institute, Inc., Sendai, Japan) originally containing 1000 U/ml of IL-2. Cultures were fed by changing half of the supernatant twice a week.

2.7 CFSE-based cytotoxicity assay

Target B-LCLs were labeled with 1 μM 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Wako Pure Chemical Industry, Osaka, Japan) for 10 min at 25°C.

After two washes, the CFSE-labeled target cells were cocultured with graded numbers of effector T cells for 5 h at 37°C and 5% CO_2 in the presence or absence of peptides in 96-well microtiter plates. The whole cells were harvested and stained with Annexin-V and Kusabira Orange (MBL) for 15 min at 25°C according to the manufacturer's instructions, and the absolute number of surviving cells was determined using a FACSCalibur with the aid of CellQuest software. The percentage lysis was calculated as follows:

$$[(ET - T0)/(100 - T0)] \times 100.$$

ET indicates percentage of $CFSE^+$ Annexin-V^+ target cells cocultured with effector cells, and T0 indicates the percentage of $CFSE^+$ Annexin-V^+ target cells without effector cells.

2.8 Statistical analysis

Data were expressed as the average ± SD of seven experiments. Samples were compared by paired Student's *t* test analyses using on-line software available at http://www.physics.csbsju.edu/stats/t-test.html.

3 Results

3.1 Induction of viral antigen-specific T cell lines by MLPC

We first sought to determine whether a simple MLPC could expand cognate antigen-specific T cells from healthy donors serologically positive for CMV and/or EBV (Fig. 1a). As shown in Fig. 1b, 3–15% of $CD8^+$ $tetramer^+$ populations among surviving cells with the cultured PBMCs were readily obtained after 14 days of culture, although the magnitude of responses varied depending on the epitope peptides and donors. The induction of T cells from seronegative donors was not attempted.

3.2 Kinetics of CD107a and CD137 expression following stimulation

It is important to determine when the activation markers are maximally upregulated for optimal sorting. Although CD137 expression kinetics have been reported elsewhere [12], we made a comparison with those of CD107a. As shown in Fig. 2a, CD137 expression among $tetramer^+$ cells exceeded 90% around 16 h following stimulation with the predetermined minimal concentration (10 ng/ml, see below) of CMV-QYD peptide. The expression started to decline after 24 h, and only 25% of the cells remained positive after 48 h. In the case of CD107a, upregulation was much quicker than with CD137, and a 70% level was maintained between 4 and 24 h, followed by a decline to 25% after 48 h. The maximal CD107a expression level was around 20% lower than that of CD137, and, unexpectedly, CD107a molecules exposed by the degranulation of CTLs remained on outer membranes for up to 24 h. Thus, we decided to perform the following positive selection experiments around 20 h after antigenic stimulation.

3.3 Optimization of peptide and primary antibody concentrations

Excessive antigenic stimulation is known to cause activation-induced cell death (AICD) in T cells [13]; thus, it is important to determine the minimal peptide concentration which results in sufficient CD137 expression. In the case of HLA-A24-restricted CMV-QYD peptide, the minimal concentration required to obtain more than 90% $CD137^+$ cells among the cognate $tetramer^+$ population was 10 ng/ml, and the use of 100 ng/ml resulted in a significant reduction of live cells, possibly due to AICD (Fig. 2b, c and data not shown). The optimal peptide concentrations differed among peptides; for example, 1 ng/ml was sufficient for the HLA-A24-restricted EBV-TYP peptide (data not shown), suggesting that the predetermination of optimal concentrations for individual peptides is necessary. A similar trend was observed when the extent of degranulation was assessed with CD107a antibody (data not shown).

The CD137 antibody (clone 4B4-1) itself does not induce AICD, but we also sought to determine sufficient concentrations by titration with measurement of the mean fluorescence intensity. In most cases, sufficient staining was obtained around 10 μg/ml (Fig. 2d and data now shown), which is a commonly employed concentration in most cell-staining procedures. Thus, we decided to use this concentration throughout the following experiments.

3.4 Comparison of three positive selection methods

Figure 3a shows the schematic procedures to positively select antigen-specific T cells by CD137, CD107a, or tetramer staining followed by MACS-based capture. In the case of tetramer-based sorting, peptide stimulation was not performed prior to sorting because it led to diminished tetramer staining, possibly due to the downregulation of T cell receptors (TCRs) on cognate T cells (upper right panel of Fig. 3b). The marked difference observed in sorted fractions just after positive selection was due to the fact that the tetramer-sorted fraction contained an average of 93% $CD8^+$ $tetramer^+$ cells (Table 1), while those obtained by CD107a and to lesser extent CD137 methods contained substantial numbers of $tetramer^-$ cells (Fig. 3b, second panel from the top). Since the $tetramer^-$ fractions were

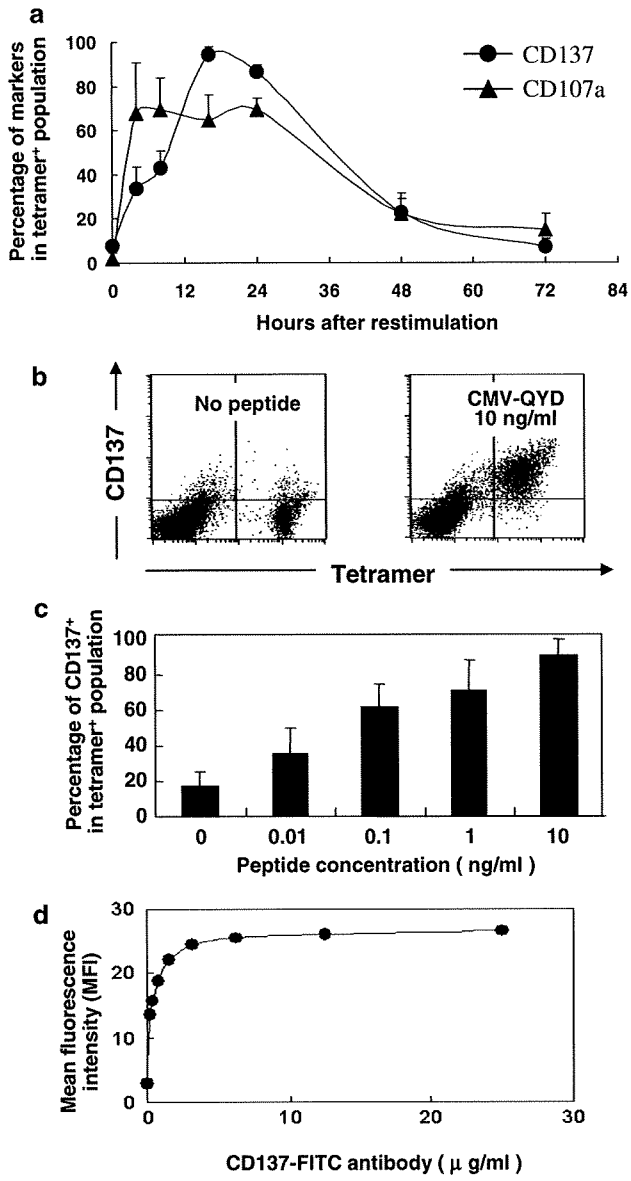

Fig. 2 Optimization of conditions for positive selection. **a** Expression kinetics of CD137 and CD107a on T cells generated by MLPC with the CMV-QYD peptide. The percentages of indicated marker (CD137 or CD107a)-positive cells among tetramer$^+$ T cell populations after stimulation with 10 ng/ml CMV-QYD peptide are longitudinally plotted. The data shown are mean and SD values from four independent experiments. **b** A representative profile of CD137 expression before and after stimulation with the CMV-QYD peptide. **c** Titration of the CMV-QYD peptide for the full upregulation of CD137. T cell lines generated by MLPC with CMV-QYD peptide were restimulated with the indicated concentrations of peptide, and the percentages of CD137$^+$ cells among the tetramer$^+$ population were plotted. The data shown are mean and SD values from five independent experiments. **d** Titration of CD137 antibodies. The mean fluorescence intensity (MFI) of CD137 staining with graded concentrations of FITC-conjugated CD137 antibodies is shown. T cell lines were the same as used in **c** and were stimulated with 10 ng/ml CMV-QYD peptide for CD137 upregulation

composed of both CD8$^+$ and CD4$^+$ cells, it is likely that these fractions came from T cells that expressed CD137 or CD107a molecules nonspecifically. Antigen-independent, spontaneous CD137 upregulation in tetramer$^-$ cells was indeed present (Fig. 2b), which might explain the recovery of tetramer$^-$ cells by CD137- and CD107a-based sorting. However, following culture for 7 days, these tetramer$^-$ cells showed a trend toward disappearance, suggesting either the loss of the growth of cells that had been expressing CD137/CD107a non-relevant to antigen stimulation, or relative outgrowth of antigen-specific cells after sorting (Fig. 3b, bottom panels and Table 1).

Data regarding the recovery of CMV-QYD-specific T cells with the three sorting methods are summarized in Table 1. Due to a consistently high percentage (average >93%) of tetramer$^+$ cells in the tetramer-sorted fraction, the total recovery of tetramer$^+$ cells was also constant (34–44.5%). In Experiment 3, however, the poor cell recovery, especially with CD107a-based sorting using the AutoMACS device, was most likely caused by unexpectedly low CD107a induction (11.3% among tetramer$^+$ cells). Nevertheless, in the other two experiments, both CD137- and CD107a-based methods resulted in a better recovery of tetramer$^+$ cells than the tetramer-based method.

We next sought to determine which method was most suitable for expanding enriched antigen-specific T cells after sorting. Fig. 4a and b shows the growth kinetics of sorted fractions cultured in the presence of IL-2, but without any feeder cells for T cell lines specific for CMV-QYD (Fig. 4a) and EBV-TYP (Fig. 4b) obtained from seven individuals. In the CMV-QYD group, T cell lines enriched with the CD137-based method readily showed significantly better growth than those enriched with tetramer (Fig. 4a). In the EBV-TYP group, T cell lines enriched with the CD137-based method showed a trend toward better growth than those enriched with tetramer ($P = 0.084$ for day 7 and $P = 0.063$ for day 14, Fig. 4b). In the case of T cell lines enriched with CD107a, those specific for CMV-QYD showed moderate growth (Fig. 4a), while those specific for EBV-TYP remained unchanged in number (Fig. 4b). The difference of growth kinetics did not reach significance for CD137-based versus CD107a-based methods; however, there was a constant trend toward an increased number of antigen-specific T cells among the CD137-based sorting group (Fig. 4a, b).

3.5 Phenotype and functional aspects of T cell lines sorted by CD137

Since CD137-based enrichment gave promising results, especially with expansion after sorting, we further

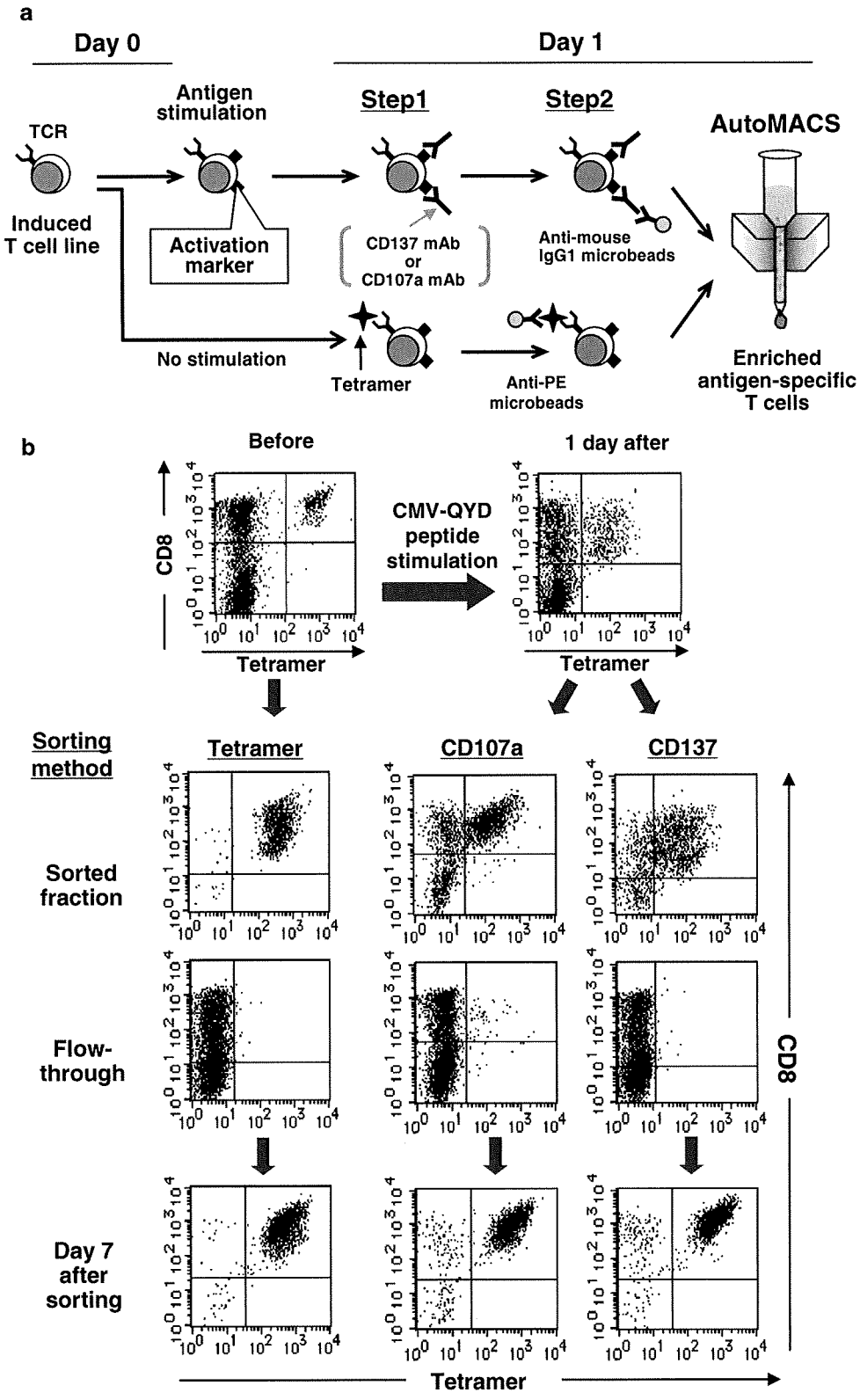

Fig. 3 Schematic illustration of positive selection using CD137, CD107a, or tetramer. **a** The MLPC-induced cell lines on days 14–16 after the initial stimulation were split and either restimulated with cognate antigenic peptide (2/3 part) or left without any stimulation (1/3 part) overnight. On the following day, T cells upregulating CD137 or CD107a by restimulation were first stained with individual antibodies and then incubated with anti-mouse IgG1 microbeads. T cells left untreated were first stained with cognate PE-conjugated tetramer and then incubated with anti-PE microbeads. T cells coated with the microbeads were then subjected to AutoMACS-based positive selection. **b** Representative flow cytometry data demonstrating the enrichment of CMV-QYD-specific T cells with the individual methods. The profiles of $CD8^+$ $tetramer^+$ cells in the AutoMACS-sorted, flow-through, and sorted fractions cultured for 7 days are shown

analyzed the phenotypes and functions of in vitro-expanded T cell lines obtained by the CD137 method. The CMV-QYD-specific T cell lines (gated by A24/CMV-QYD tetramer staining) were mostly $CD45RO^+$ and $CD45RA^-$, and more than a quarter of cells expressed both CCR7 and CD28, a hallmark for central memory

Table 1 Comparison of the recoveries of CMV/QYD-specific T cells among the three sorting methods

	% tetramer$^+$ cells (day 0)	Method	% CD137$^+$ or CD107a$^+$ among tetramer$^+$ cells	Number of tetramer$^+$ cells prior to sorting (day 1) ($\times 10^5$)[a]	Sorted fraction (day 1)		
					% tetramer$^+$	Number of tetramer$^+$ cells ($\times 10^5$)	% recovery of tetramer$^+$ cells
Experiment 1	8.3	CD137	95.6	6.4	66.3	3.7	58.0
		CD107a	95.0	6.4	58.4	3.3	51.1
		Tetramer	–	8.8	97.9	4.3	41.8
Experiment 2	12.4	CD137	98.3	1.74	80.8	1.29	74.1
		CD107a	87.3	1.74	75.2	0.98	56.3
		Tetramer	–	1.88	80.2	0.64	34.0
Experiment 3	22.5	CD137	99.2	23.8	96.0	2.88	12.1
		CD107a	11.3	23.8	32.1	0.29	1.2
		Tetramer	–	26.5	99.5	11.8	44.5

The experiment number corresponds to that shown in Fig. 4a

[a] Reduced number of tetramer$^+$ cells was caused mainly by activation-induced cell death during overnight stimulation with antigen

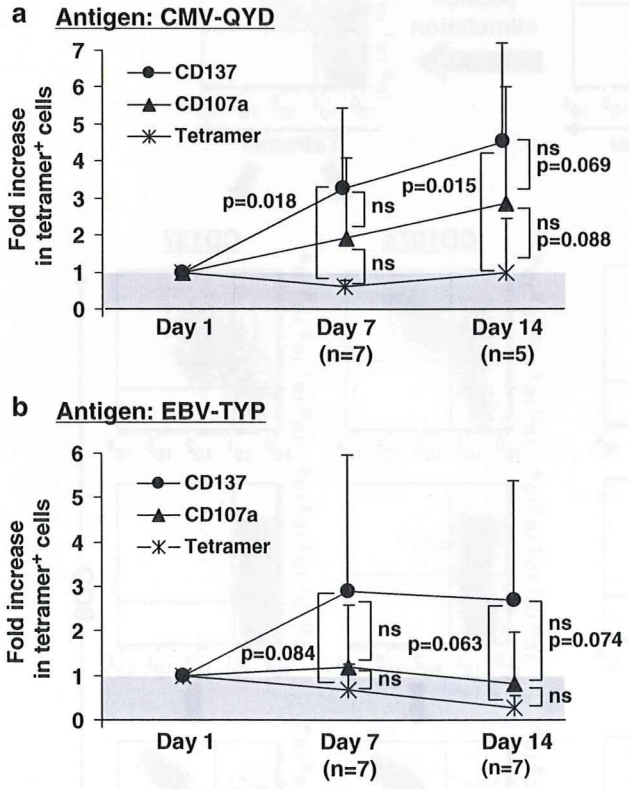

Fig. 4 Expansion of enriched cells after sorting with CD137, CD107a, or tetramers. AutoMACS-sorted fractions were cultured in a 24- or 96-well culture plate in ALyS505N-1000 media containing 1,000 U/ml IL-2 for the indicated period. Average fold increases of cognate tetramer$^+$ cells from seven individuals including three shown in Table 1 are shown. **a** Expansion of CMV-QYD-specific T cell lines. **b** Expansion of EBV-TYP-specific T cell lines. Statistical values were obtained using paired Student's *t* test. The *error bars* represent the mean SD of the seven experiments except one including five experiments for CMV-QYD on day 14. *ns* not significant

T cells (Fig. 5a). Upon stimulation with cognate peptide (CMV-QYD), nearly half of the T cells could produce IFN-γ (Fig. 5b). Finally, one of the T cell lines showed robust and specific lytic activity against CMV-QYD peptide-pulsed autologous B-LCLs (75% at an E/T ratio of 2, Fig. 5c).

3.6 Insufficient CD137 upregulation on antigen-stimulated CD4$^+$ T cells for positive selection

Since there is currently no feasible method to positively select antigen-specific CD4$^+$ cells, we examined whether CD137 might be sufficiently upregulated for MACS-based sorting. We first generated T cell lines by stimulating PBMC with an HLA-DRB1*0101-restricted EBV-TSL peptide. Figure 6a shows a representative kinetic profile of CD137 expression on a T cell line before and after restimulation with EBV-TSL peptide. Percentages of CD137$^+$ cells among (CD4$^+$) HLA-DRB1*0101/EBV-TSL tetramer$^+$ cells increased from 8.4 to 40.4% after 16 h of stimulation, and declined to 14.6% at 48 h. However, the (CD4$^+$) tetramer$^-$ fraction already showed upregulated CD137 expression before antigen stimulation, and its upregulation was more pronounced in terms of fluorescent intensity than that of the tetramer$^+$ fraction at 16 h, for unknown reasons (Fig. 6a, middle panel). As a result, although relatively more tetramer$^+$ CD137$^+$ cells were recovered in the sorted fraction (Fig. 6b, middle panels), the majority of tetramer$^+$ cells were eventually lost into the flow-through fraction, probably due to a weaker upregulation of CD137 insufficient for MACS-based sorting.

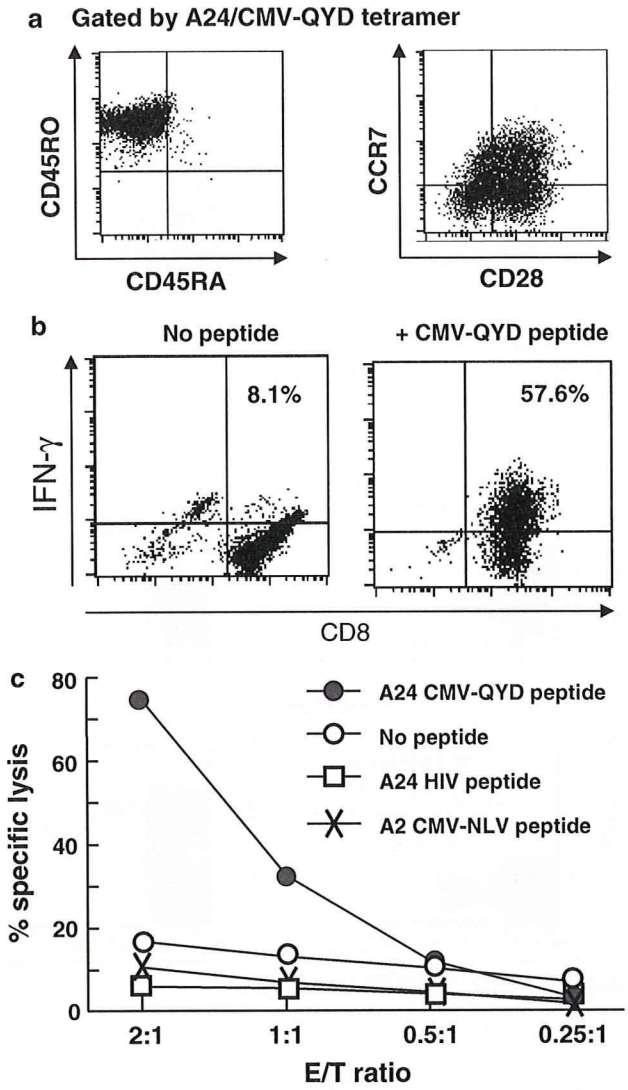

Fig. 5 Phenotypes and functions of CD137-sorted and 7-day cultured T cell lines. **a** Representative flow cytometry profile of CMV-QYD-specific T cell lines for differentiation markers. T cells gated for the cognate tetramer were analyzed with the indicated markers. **b** Capacity for IFN-γ production upon stimulation with autologous B-LCL pulsed with or without cognate peptide. **c** Cytotoxicity of T cell lines against peptide pulsed autologous B-LCL at the indicated effector:target (E:T) ratios. The data shown are representative of three independent experiments for **b** and **c**

4 Discussion

The enrichment of antigen-specific T cells is the first key step for successful adoptive immunotherapy, necessary to maximize efficacy and minimize unwanted reactivity to self-antigens that may result in autoimmunity. The present comparison of three methods (with CD137, CD107a, and HLA multimers) that can isolate T cells simply (i.e., by staining and separation with a MACS-based sorter) without any need for expensive flow cytometric cell sorters, showed a comparable recovery of antigen-specific $CD8^+$ cells assessed by cognate tetramer staining. However, the CD137-based method was superior when cell proliferation following enrichment was also taken into consideration (Fig. 4), although the difference between this and the CD107a-based method did not reach significance, possibly due to limited number ($n = 7$) of individuals tested and the inter-individual variation in the level of CD137 and CD107a upregulation after stimulation (data not shown). Nevertheless, the advantage of the CD137-based method is reasonable because CD137 has been shown to deliver a survival signal to activated T cells [14, 15]. In addition, CD137 was found to be upregulated in almost all (>90%) antigen-specific T cells, based on tetramer staining, when compared with CD107a (up to 70%), so that the former is likely to cover the full repertoire of antigen-specific T cells. Finally, we learned that CD137-based sorting is not suitable for antigen-specific $CD4^+$ T cells, at least with our current approach using simple "bulk" cultures, due to high background and bystander expression of CD137. However, CD137 was indeed upregulated upon antigen stimulation of cognate $CD4^+$ cells (Fig. 6a), as shown by others [16]. Because monocytes constitutively express CD137 (data not shown), the residual monocytes which were not killed by antigen-specific helper $CD4^+$ could contaminate the sorted fraction, likely resulting in the low-level purity of antigen-specific $CD4^+$ cells. To isolate antigen-specific $CD4^+$ helper T cells, the positive selection of CD154 or the CD40 ligand has been reported, although this method requires the addition of CD40-specific blocking antibodies to avoid the downregulation of CD154 induced by antigen stimulation [17]. We initially wished to isolate both antigen-specific $CD8^+$ and $CD4^+$ T cells with a single reagent, CD137, but our data demonstrated that it might be a suboptimal method at present, unless the IFN-γ secretion assay, which requires two more steps, is performed [11].

In the current study, to induce cell surface CD137 or CD107a expression with antigenic peptides, they were simply added directly to PBMC suspensions without antigen-presenting cells in order to minimize in vitro manipulation. We stimulated PBMCs with a commonly used concentration (i.e., 10 μg/ml) of antigenic peptides for simplicity because resting memory T cells in PBMCs are relatively resistant to AICD compared to activated effector T cells [18]. Restimulation of in vitro-activated T cells just before positive selection, however, did induce moderate reduction of cognate T cells (data not shown), possibly due to AICD [18] or T cell versus T cell killing [19], whereby antigen-specific T cells presenting the pulsed peptide are killed by other antigen-specific T cells. AICD could be avoided using more precisely titrated concentrations of peptides, but this might be difficult since the occurrence of AICD may also depend on other factors, including the T cell activation status, co-existing cytokines, and

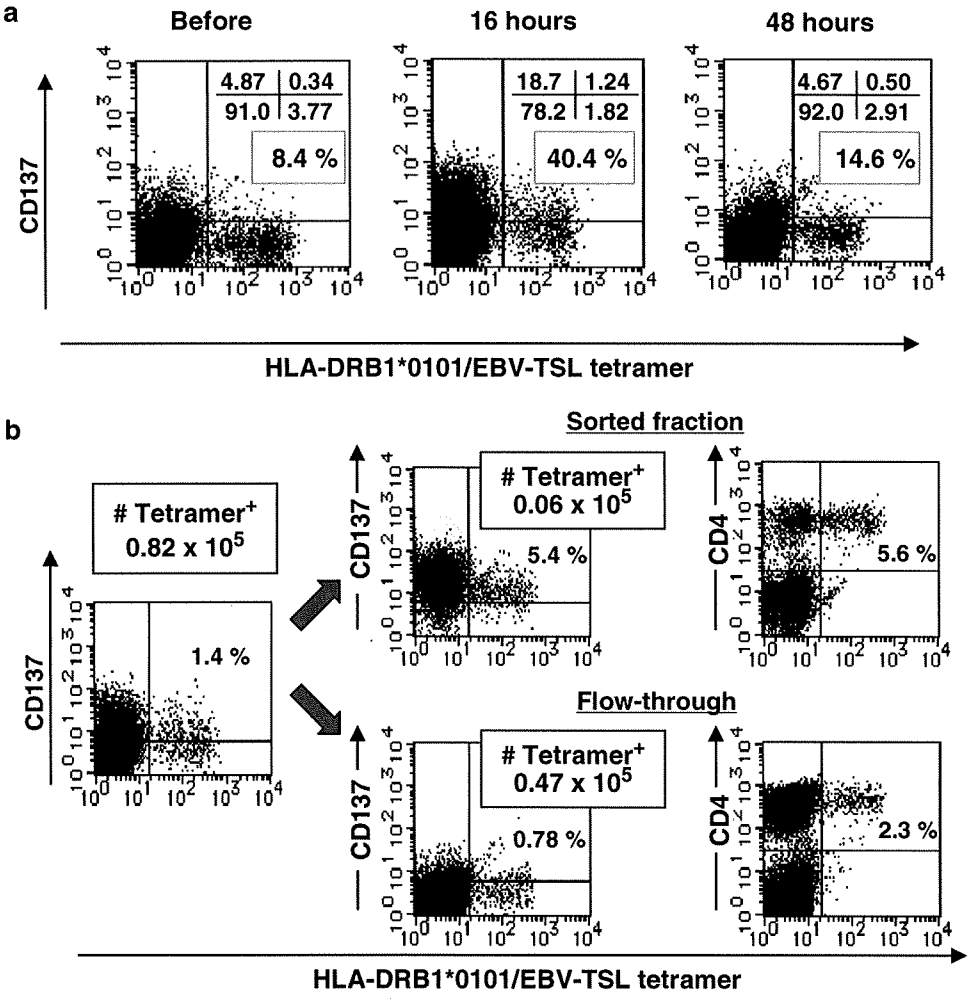

Fig. 6 Induction of CD137 expression on antigen-specific $CD4^+$ T-cells. **a** PBMCs were stimulated in MLPC with the HLA-DRB1*0101-restricted EBV-TSL peptide. On day 14 of culture, the T cells were stimulated with 10 ng/ml EBV-TSL peptide. The expression of CD137 was assessed along with HLA-DRB1*0101/EBV-TSL tetramer staining before and 16 and 48 h after stimulation. **b** Representative flow cytometry data demonstrating the enrichment of EBV-TSL-specific T cells with the CD137-based method. The profiles of $tetramer^+$ cells counterstained with either CD137 (*middle column*) or CD4 (*right column*) in the AutoMACS-sorted and flow-through fractions are shown. Numbers in *squares* represent the absolute numbers of $tetramer^+$ cells, indicating the loss of most antigen-specific T cells into the flow-through fraction

costimulatory molecules [13]. The latter "mutual" killing could be avoided using peptide-pulsed autologous antigen-presenting cells; however, any usage of cells, even autologous, requires multiple steps, including thawing, washing, peptide pulsing, and irradiation, with which the risk of bacterial contamination may increase. Thus, the optimization of simple and safe restimulation conditions for the maximal induction of CD137 or CD107a while minimizing the loss of antigen-specific T cells should be further explored.

As previously shown, CD137- and CD107a-based methods can be performed without prior knowledge of precise peptide sequences or HLA restriction, unlike the tetramer-based approach. Although we used predetermined CMV- and EBV-derived peptides as model antigens in this study, we also confirmed that T cell enrichment followed by the cloning of minor histocompatibility antigen-specific T cells are possible with CD107a- or CD137-based sorting after T cell lines are restimulated using endogenously antigen-expressing PBMCs or B-LCLs (our unpublished observations). This suggests that both methods are applicable for the positive selection of various T cell lines.

The long-term in vitro culture or expansion of T cells, especially after cloning, is known to be detrimental to T cell survival after returning to in vivo conditions due to progression to terminal differentiation [20]. Therefore, short-term induction culture, followed by enrichment and/or further short-term expansion are warranted. In our phenotypic and functional analyses, most T cells enriched with the CD137-based method and cultured for 7 days retained a central memory phenotype (Fig. 5a), IFN-γ production capacity, and cytolytic activity when challenged with cognate antigen-presenting cells (Fig. 5b, c). Thus, short-term culture for 7 days did not result in the loss of critical functions of T cells necessary for adoptive immunotherapy. It has been shown that an average ninefold expansion over 8 days is possible for CD137-enriched cells when cultured in the presence of IL-2, IL-7 and, IL-15 [12]. In our expansion study, only an average 2.6-fold expansion was obtained. The difference might be caused partly

because we did not use IL-7 and IL-15, especially the latter, which is known to deliver anti-apoptotic signals and augment the proliferation and homeostasis of memory $CD8^+$ T-cells [21]. The other reason could be that we sorted antigen-specific cells from memory T cell pools of CMV- or EBV-seropositive individuals while others have employed $CD45RA^+$ naïve cells as a source of antigen-specific T cells [12]. Collectively, our data demonstrate that CD137-based sorting is indeed superior to other "one step" sorting methods.

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Identification of a human leukocyte antigen-A24–restricted T-cell epitope derived from interleukin-13 receptor α2 chain, a glioma-associated antigen

Laboratory investigation

SHINJI SHIMATO, M.D., PH.D.,[1] ATSUSHI NATSUME, M.D., PH.D.,[1,2] TOSHIHIKO WAKABAYASHI, M.D., PH.D.,[1,2] KUNIO TSUJIMURA, M.D., PH.D.,[3] NORIMOTO NAKAHARA, M.D., PH.D.,[4] JUN ISHII, B.S.,[1] MOTOKAZU ITO, M.D.,[1] YOSHIKI AKATSUKA, M.D., PH.D.,[3] KIYOTAKA KUZUSHIMA, M.D., PH.D.,[3] AND JUN YOSHIDA, M.D., PH.D.[1,2]

[1]Department of Neurosurgery, Nagoya University School of Medicine; [2]Center for Genetic and Regenerative Medicine, Nagoya University Hospital; [3]Division of Immunology, Aichi Cancer Center Research Institute, Nagoya; and [4]Division of Neurological Surgery, Nagoya Central Hospital, Nagoya, Japan

Object. The human leukocyte antigen-A24 (HLA-A24) allele is highly expressed in Asians. This allele is expressed in 60% of the Japanese population and in a significant number of people of other ethnicities. The interleukin-13 type α2 receptor (IL-13Rα2) has been shown to be a glioma-specific antigen, and is abundantly expressed in a majority of high-grade astrocytomas. In this study, the authors first investigated the suitability of IL-13Rα2 as a target antigen of malignant glioma cells, and then identified a potential HLA-A24–restricted peptide derived from IL-13Rα2.

Methods. The expression of IL-13Rα2 in glioma tissues was examined by reverse transcription–polymerase chain reaction analysis. To identify the desired epitope, the authors selected 5 candidate peptides from IL-13Rα2 that were predicted to bind to HLA-A24. The lytic activity of cytotoxic T lymphocytes (CTLs) induced by peptide-pulsed dendritic cells was analyzed against various glioma cell lines and freshly isolated human glioma cells.

Results. In a series of glioma tissues obtained in 29 patients, the authors found that > 50% of high-grade gliomas expressed IL-13Rα2. Of the 5 peptides tested, P174 (WYEGLDHAL) was found to be the most useful for the induction of HLA-A24–restricted and IL-13Rα2–specific CTLs. A CTL line induced by P174 also showed antigen-specific cytotoxicity to surgically removed glioma cells depending on their level of expression of IL-13Rα2 and HLA-A24.

Conclusions. Interleukin-13Rα2 is a glioma-specific antigen, and the immunogenic peptide P174 may contribute to a peptide-based immunotherapy against malignant glioma cells expressing HLA-A24.
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KEY WORDS • glioma • human leukocyte antigen-A24 • interleukin-13 receptor α2 chain • T-cell epitope

MALIGNANT gliomas remain untreatable and lethal despite the extensive application of surgical excision and adjuvant radio- and/or chemotherapy. Therefore, various immunotherapy approaches are being explored and appear promising as new therapeutic methods.[21,24] Immunotherapy for tumors in humans has been proposed based on the finding that $CD8^+$ CTLs are capable of effective recognition and destruction of tumor cells.[20] Consequently, much attention has focused on the identification and characterization of glioma-associated antigens that elicit strong and highly glioma-specific immune reactions.

Recently, IL-13Rα2 has been shown to be abundantly expressed in a majority of high-grade astrocytomas.[5–7] Interleukin-13 is an immunoregulatory cytokine that shares a variety of functions with IL-4 through an IL-13/IL-4 receptor complex, which commonly contributes to normal physiological functions;[25,26] IL-13Rα1 is a component of this receptor complex.[14] However, IL-13Rα2 does not interact with IL-4.[16] Because IL-13Rα2 is expressed in tumor cells but not in most adult somatic tissues—with the exception of the testis—it may be considered a kind of cancer/testis antigen.[4] Mintz et al.[15] reported that mice treated with IL-13–based cytotoxins showed significant regression in IL-13Rα2–expressing tumors without any obvious complications. Interleukin-13Rα2 is therefore a promising target for glioma-specific immunotherapy.

Abbreviations used in this paper: BIMAS = BioInformatics and Molecular Analysis Section; CTL = cytotoxic T lymphocyte; DC = dendritic cell; FBS = fetal bovine serum; HLA = human leukocyte antigen; IL = interleukin; IL-13Rα2 = IL-13 type α2 receptor; MHC = major histocompatibility complex; PCR = polymerase chain reaction; rh = recombinant human; rhGM-CSF = rh granulocyte–macrophage colony-stimulating factor; RT-PCR = reverse transcription–PCR.

Vaccination with a tumor-specific antigen peptide is one of the most relevant strategies for tumor immunotherapy, and it is essential to identify the tumor antigen peptides that will elicit maximal immunological reactions in an MHC Class I/peptide complex–restricted manner.[2] The *HLA-A24* allele is highly expressed in Asians; in particular, 60% of the Japanese population has this allele.[3] A significant number of people of other ethnicities also express this antigen. Therefore, the identification of an HLA-A24–restricted CTL epitope would contribute to the development of immunotherapies for use in patients worldwide. In the present study, we investigated the suitability of IL-13Rα2 as a target antigen for malignant gliomas by examining its expression in normal tissues and gliomas. We then identified a potential HLA-A24–restricted peptide derived from IL-13Rα2 that can effectively induce HLA-A24–restricted and IL-13Rα2–specific CTLs, and demonstrated that the CTLs induced by this novel antigenic peptide can kill freshly isolated HLA-A24$^+$ IL-13Rα2$^+$ human-derived glioma cells.

Methods

Cells and Cultures

The glioma cell lines SKMG-1, KNS42, T98, U87MG, U251MG, and AO2 were grown in Eagle minimal essential medium (Nissui) containing 10% FBS, 5-mM L-glutamine, 2-mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. The HLAs in these cell lines were genetically typed by SRL, Inc.

The peptide transporter-negative BxT hybrid cell line 174×CEM (referred to as T2), was transfected with a plasmid encoding HLA-A2402. The transfected cell line was cloned by limiting dilution and designated T2-A24.[13] This cell line was grown in RPMI1640 supplemented with 10% FBS, 5-mM L-glutamine, 2-mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.8 mg/ml G418 (GIBCO).

Tumor tissues obtained in 3 patients who had undergone resection for high-grade gliomas at Nagoya University Hospital, Nagoya, Japan were placed in primary culture. Immediately after the extraction, the tissues were homogenized and digested with 1% DNase and 0.1% trypsin for 30 minutes at 37°C, and then centrifuged at 800 rpm for 5 minutes. The cells were seeded at a density of 2×10^6 cells per 100-mm dish and maintained in Dulbecco modified Eagle's medium (GIBCO) supplemented with 10% FBS, 5-mM L-glutamine, 2-mM nonessential amino acids, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were then incubated in a standard tissue culture incubator (at 100% relative humidity and 5% CO_2 in air). After achieving 80–90% confluence, the cells were subcultured onto a new 100-mm plate at a density of 2×10^6. The established cell lines were designated as NNS03, NNS04, and NNS08, and their HLAs were typed as HLA-A24/A33, A2, and A2/A26, respectively. All cells were confirmed to be glioma cells based on their expression of glial fibrilliary acidic protein.

Ribonucleic Acid Isolation and RT-PCR Analysis of IL-13Rα2 Expression

Reverse transcription was performed using total cellular RNA extracted by the guanidinium thiocyanate/cesium chloride method in the presence of random hexamers and SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The primers used for the amplification of human IL-13Rα2 were 5′-TGGTCAGAAGTGTGCCTGTC-3′ (sense) and 5′-TCTGCCCAGGAACTTTAAC-3′ (antisense). In the Takara Thermal Cycler, 25 amplification cycles, comprising denaturation for 30 seconds at 94°C, annealing for 30 seconds at 57°C, and extension for 1 minute at 72°C were conducted using AmpliTaq-gold DNA polymerase (Applied Biosystems). The PCR products were analyzed by electrophoresis on a 1.5% agarose gel, and stained with ethidium bromide. Band intensities were quantified by densitometric scanning using the National Institutes of Health IMAGE software. The results were evaluated semiquantitatively by comparison with the relative amounts of β-actin PCR products, and classified into groups of high (++: IL-13Rα2/β-actin > 0.5), moderate (+: < 0.5), or no expression (−: 0).

Peptide Synthesis

We used the binding-prediction software program BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind/) to identify potential HLA-A24–binding peptides within IL-13Rα2. All peptides were synthesized by Thermo Electron GmbH and purchased from Greiner Bio-One Japan. The purity of the peptides was shown to be $> 90\%$.

Major Histocompatibility Complex Stabilization Assay

The 5 synthesized peptides were used in an MHC stabilization assay by means of T2-A24 cells as described elsewhere.[13] Briefly, T2-A24 cells (3×10^5) were incubated with 200 μl of RPMI1640 containing 0.1% FBS and 10-μM peptides at 26°C for 16 hours, followed by incubation at 37°C for 3 hours. Surface HLA-A24 molecules were then indirectly stained with the anti–A24 monoclonal antibody (A11.1; One Lambda, Inc.) and fluorescein isothiocyanate-labeled anti–mouse immunoglobulin G. Expression was measured in a FACS Calibur (Becton Dickinson), and the mean fluorescence intensity was recorded.

Generation of DCs

All DCs derived from patients were found to be HLA-A24$^+$ cells on whole HLA typing conducted by SRL, Inc. The DCs were generated and matured as reported in other studies with minor modifications.[18] Briefly, peripheral blood mononuclear cells were isolated using Ficoll-Paque (Amersham Biosciences), and the monocyte fraction was enriched by plastic adherence. After a 2-hour incubation period at 37°C, the nonadherent cells were removed, and the adherent cells were cultured with 500 U/ml of rhGM-CSF and 500 U/ml of rhIL-4 (Strathmann Biotech AG) in AIM-V medium (GIBCO). On Day 6, 500 U/ml of rhGM-CSF, 500 U/ml of rhIL-4, 1000 U/ml of rhIL-6, 10 U/ml of human recombinant tumor necrosis factor–α, and 10 U/ml of rhIL-1β (Strathmann Biotech AG) were added to the wells. On Day 8, the matured DCs were harvested from the wells by vigorous washing.

Peptide Pulsing of DCs and Induction of IL-13Rα2–Specific CTL Lines

The mature DCs generated were resuspended in AIM-V at a density of 10^6 cells/ml and pulsed with 10 μg/ml of peptides for 4 hours at 37°C. The peptide-pulsed DCs were then treated with mitomycin C for 45 minutes, washed, and resuspended in AIM-V medium containing 10% human AB serum. The autologous $CD8^+$ T-cell population was magnetically isolated from peripheral blood mononuclear cells by using CD8 MicroBeads (Miltenyi Biotech). Next, 1 million $CD8^{++}$ T cells were cocultured in each well of a 24-well plate with 10^5 peptide-pulsed DCs in 2 ml of AIM-V medium supplemented with 10% human AB serum, 1000 U/ml rhIL-6, and 10 ng/ml rhIL-12 (Strathmann Biotech AG). On Day 7, the lymphocytes were restimulated with autologous peptide-pulsed DCs treated with mitomycin C in AIM-V medium supplemented with 10% human AB serum, 10 U/ml rhIL-2 (PeproTech EC), and 10 U/ml rhIL-7 (Strathmann Biotech AG). Restimulation of the lymphocytes by peptide-pulsed DCs was repeated once a week to establish CTL lines.

Cytotoxic T-Lymphocyte Assay

Target cells were labeled with $Na_2{}^{51}CrO_4$ (Perkin-Elmer), and the labeled cells (100 μl) were incubated with effector cells (100 μl) in a U-bottomed 96-well microtiter plate. After incubation for 4 hours at 37°C, the supernatants were collected and their radioactivity was measured with a gamma counter. The percentage specific lysis was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Monoclonal antibodies against HLA-ABC (W6/32) and HLA-DR (HDR-1) were kindly provided by Dr. K. Itoh, Kurume University, and were used for antibody-blocking tests.

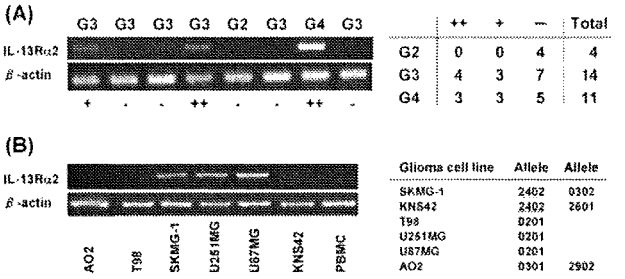
Statistical Analysis

The statistical significance of the difference between groups was determined by analysis of variance, using the Bonferroni correction for the multiple post-hoc analyses performed. The statistical analysis was conducted using commercially available software (Systat 9).

Results

Expression of IL-13Rα2 in Glioma Tissue Samples and Cell Lines

To assess the frequency of IL-13Rα2 expression in glioma cells, we performed RT-PCR on histopathologically confirmed glioma samples obtained in 29 patients. The gliomas examined in this analysis included 25 high-grade and 4 low-grade gliomas. A sample of nonglioma tissue was also examined for comparison. Interleukin-13Rα2 expression was observed in 14 (56%) of the high-grade gliomas (World Health Organization Grades 3 and 4), and high- and moderate-level expression was observed in 7 cases each. One low-grade glioma (Grade 2) and the nonglioma tissue sample did not express IL-13Rα2 (Fig. 1A, data not shown). Interleukin-13Rα2 was highly expressed in 3 of 6 glioma cell lines (Fig. 1B). We also examined the expression of IL-13Rα2 in other cancer cell lines such as medulloblastoma, colon cancer, melanoma, and pancreatic cancer, and found that only 1 of 2 medulloblastoma types expressed IL-13Rα2.



	++	+	−	Total
G2	0	0	4	4
G3	4	3	7	14
G4	3	3	5	11

Glioma cell line	Allele	Allele
SKMG-1	2402	0302
KNS42	2402	2601
T98	0201	
U251MG	0201	
U87MG	0201	
AO2	0301	2902

FIG. 1. Detection of IL-13Rα2 in glioma cells by RT-PCR on agarose gel electrophoresis. A: Representative results in a surgical specimen. The *table* represents the summarized data showing that IL-13Rα2 is expressed only in high-grade glioma cells. The expression intensity was semiquantitatively determined by comparison with the relative amounts of β-actin PCR products that were used as an internal control, and classified into groups of high (++), moderate (+), and no expression (−), based on the band intensities quantified by densitometric scanning using the National Institutes of Health IMAGE program. B: Results in human glioma cell lines. High expression of IL-13Rα2 was observed in 3 of 6 glioma cell lines. The *table* shows the results of the HLA typing of all glioma cell lines and shows that SKMG-1 and KNS42 have the A2402 allele *(underlined)*.

Selection of Potential HLA-A24–Binding Peptides Within the IL-13Rα2 Protein

Using computer-based algorithms, the following 5 peptide sequences with a high predictive score on BIMAS were selected for synthesis: WYEGLDHAL, LYLQWQPPL, VYYNWQYLL, TYPKMIPEF, and EYELKYRNI (Table 1). These sequences were designated P174, P49, P146, P368, and P68, respectively. Human leukocyte antigen stabilization assays were performed to test their affinity to the HLA-A2402 molecule by using T2-A24 cells. As shown in Fig. 2, P146 bound most strongly to the HLA-A24 molecule. Despite having lower affinities than P146, the other 4 peptides also showed positive values. Therefore, we used all the 5 peptides for the subsequent experiments.

Induction of HLA-A24–Restricted and IL-13Rα2–Specific CTL Lines

To determine which peptide could best induce HLA-A24–restricted and IL-13Rα2–specific CTLs, we examined the cytotoxic activities of CTL lines induced by repetitive stimulation with autologous DCs pulsed with the peptides listed in Table 1. After we performed the CTL assays using at least 3 separately obtained CTL lines for each peptide, we confirmed that 3 peptides (P174, P49, and P146) could induce CTL lines cytotoxic to the $HLA\text{-}A24^+$ $IL\text{-}13R\alpha2^+$ glioma cell line SKMG-1 (Fig. 3). Of these, the P174-induced CTL lines consistently showed the strongest cytotoxicity in several independent experiments in glioma tissues obtained in 2 patients.

To confirm whether the cytotoxicity of the CTL lines induced by IL-13Rα2–derived peptides was truly restrict-

TABLE 1

Characterization of IL-13Rα2–derived peptides used for CTL induction

Peptide Designation	Position*	Amino Acid Sequence	Predictive Score†
P174	174–182	WYEGLDHAL	360.000
P49	49–57	LYLQWQPPL	300.000
P146	146–154	VYYNWQYLL	200.000
P368	368–376	TYPKMIPEF	165.000
P68	68–76	EYELKYRNI	75.000

* Numbers indicate the position of the peptide in the amino acid sequence of IL-13Rα2.

† Estimated half-time of dissociation from HLA-A24 molecules (in minutes).

ed to both HLA-A24 and IL-13Rα2, we assessed their lytic activity against 6 glioma cell lines that differentially express IL-13Rα2 and HLA-A24. Representative results with a CTL line induced by the most immunogenic peptide (P174) are shown in Fig. 4A. Only the SKMG-1 cell line showed remarkable lysis by the P174-induced CTL line. The specificity of the lytic activity of this CTL line against the SKMG-1 cell line was statistically significant compared with its activity against all other 5 glioma cell lines. Furthermore, an antibody-blocking assay revealed that anti–HLA-Class I but not Class II antibodies could reduce the lytic activity of P174-induced CTLs in a dose-dependent manner. Taken together, these results clearly demonstrate that CTL with a desirable specificity, such as being HLA-A24–restricted and IL-13Rα2–specific, can be effectively induced by P174-pulsed DCs.

Cytotoxicity of P174-Induced CTLs Against Surgically Removed Glioma Cells

To assess the possibility of P174 use in patients with gliomas, we tested the cytotoxicity of P174-induced CTLs against glioma cells in primary cultures that had been freshly isolated from 3 patients. As shown in Fig. 5, only NNS03 cells expressing both HLA-A24 and IL-13Rα2 were significantly lysed by the P174-induced CTLs, suggesting that P174 can be applied in patients with glioma cells that express both HLA-A24 and IL-13Rα2.

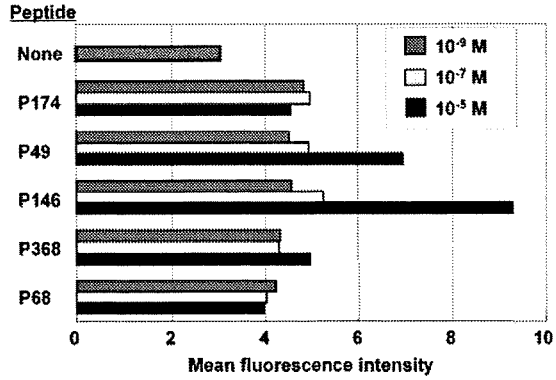


FIG. 2. Bar graph of MHC stabilization assay. The T2-A24 cells were incubated with the indicated peptides and HLA-A24 expression was analyzed by flow cytometry. The results are presented as the mean fluorescence intensity.

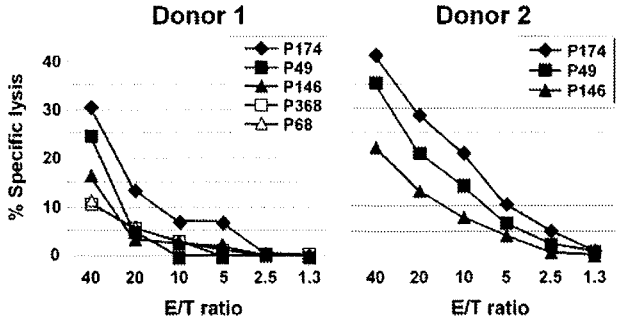


FIG. 3. Graphs comparing the cytotoxic activity of CTL lines induced by DCs pulsed with IL-13Rα2–derived peptides. The cytotoxic activity of the CTL lines was tested using the standard 4-hour ^{51}Cr release assay by using SKMG-1 cells (HLA-A24$^+$ and IL-13Rα2$^+$) as a target. E/T ratio = effector to target ratio.

Discussion

Debinski et al.[5–7] have reported that almost all human glioblastoma multiforme cells express IL-13R. They further demonstrated that IL-13Rα2, which binds IL-13 in an IL-4–independent manner, is the restricted binding site for IL-13 on malignant gliomas.[16] Another study by Joshi et al.[10] also has demonstrated high specificity of IL-13Rα2 for glioblastoma multiforme. In the present study, we first investigated the IL-13Rα2 expression in a series of resected glioma tissues and demonstrated that more than half of the high-grade gliomas expressed IL-13Rα2. This high expression in glioma cells was also confirmed on immunostaining with anti–IL-13Rα2 antibody. In contrast, none of the samples from Grade 2 gliomas expressed the antigen. These results suggest that IL-13Rα2 expression is restricted to malignant lesions and might be a prognostic marker in low-grade gliomas, as suggested previously.[7] Our data could render this protein a very promising target of glioma-specific immunotherapy. However, the frequency of IL-13Rα2 expression observed in the present study is not as high as that reported in previous studies. The difference might be related to the methods for examination or the backgrounds of the patients included in the studies. The availability of IL-13Rα2 as a target antigen should be confirmed by further investigations in a larger population. For reference, we conducted RT-PCR analysis of the expression of IL-13Rα2 in other cancer cell lines including colon cancers, melanomas, pancreatic cancers, and medulloblastomas, and found that only the medulloblastoma cell line expressed IL-13Rα2. However, ovarian or renal cancers have also been reported to express IL-13Rα2,[1,12] which may imply that IL-13Rα2 is associated with malignancy.

As a mainstay of this study, we investigated peptides derived from IL-13Rα2 that have the highest potential to elicit cellular immune responses against glioma cells expressing IL-13Rα2 and HLA-A24. First, 5 candidate peptides from the IL-13Rα2 protein were predicted using a bioinformatics approach. Second, we pursued the most promising peptide that can induce CTLs specific to IL-13Rα2 together with HLA-A24, and found P174 to be the most immunogenic. Finally, we tested the specificity of the P174-induced CTLs to IL-13Rα2 and HLA-A24, and their cytotoxicity against HLA-A24$^+$ IL-13Rα2$^+$ primary glioma cells as well as an established glioma cell line.