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

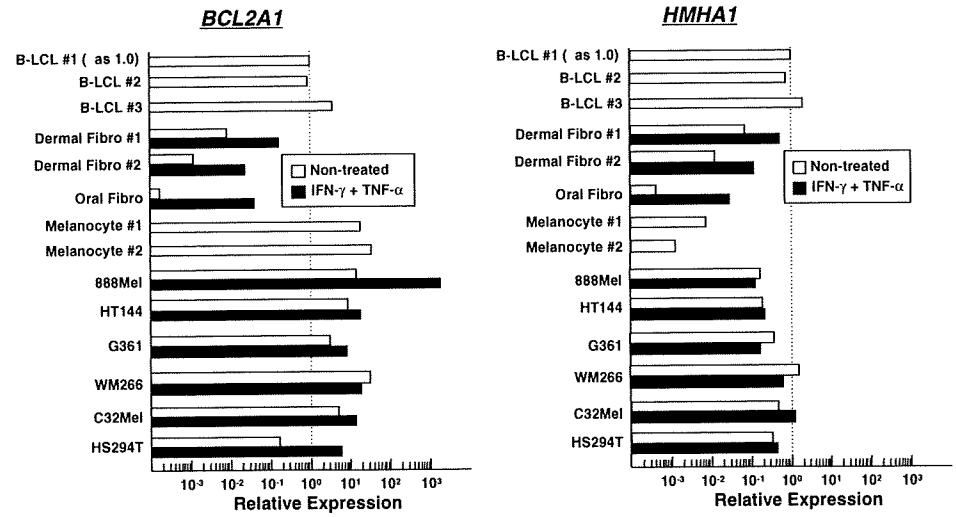

Fig. 1 Relative expression of *BCL2A1* (*left panel*) and *HMHA1* (*right panel*) in melanoma cell lines. Real-time reverse transcription PCR to quantify the mRNA expression of *BCL2A1* and *HMHA1* was performed using cDNA samples prepared from melanoma cell lines (kind gift from Dr. Kawakami, Keio University,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

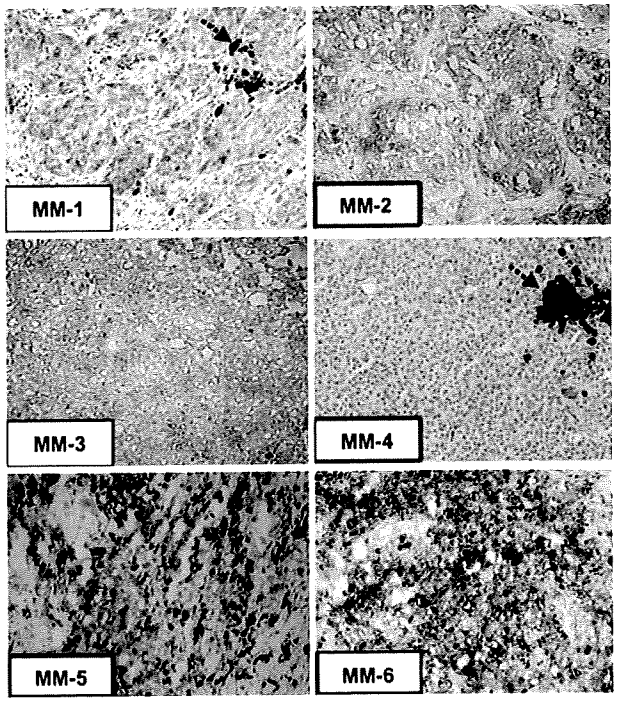

Fig. 2 Expression of BCL2A1 protein in primary metastatic melanoma (MM) cells. Frozen skin sections from six patients with metastatic melanoma were examined for BCL2A1 expression immunohistochemically by the standard avidin–biotin–peroxidase complex method using polyclonal antibodies against BCL2A1 (Santa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

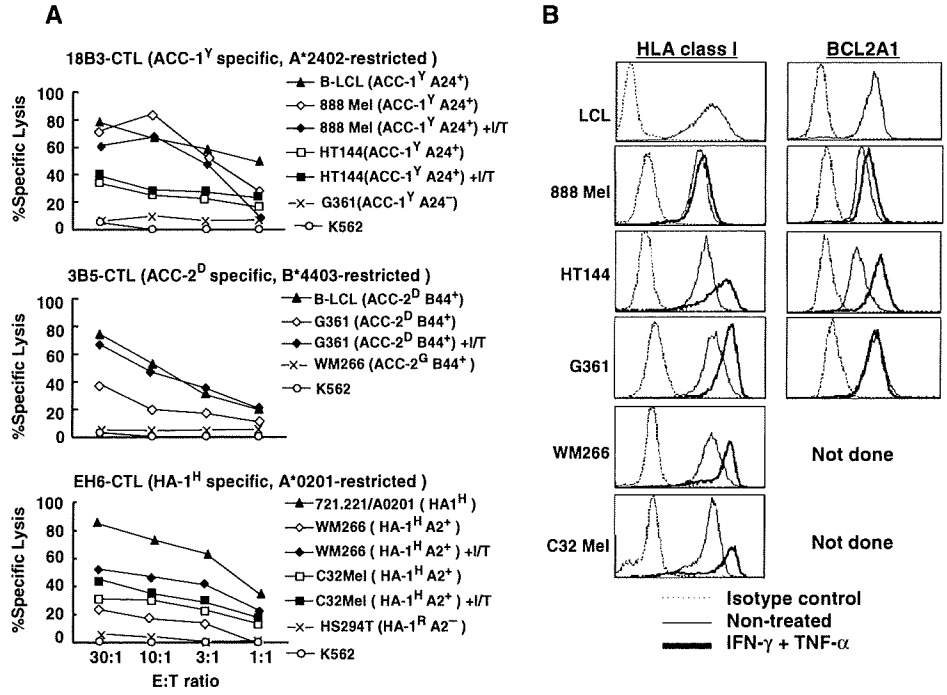

Fig. 3 Susceptibility of melanoma cell lines to mHag-specific CTL clones and the impact of cytokine treatment. **a** ^{51}Cr-release assay against melanoma cell lines. Standard 4-h ^{51}Cr-release assays were performed against various melanoma cell lines at the indicated E/T ratios and at least in duplicate. 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,

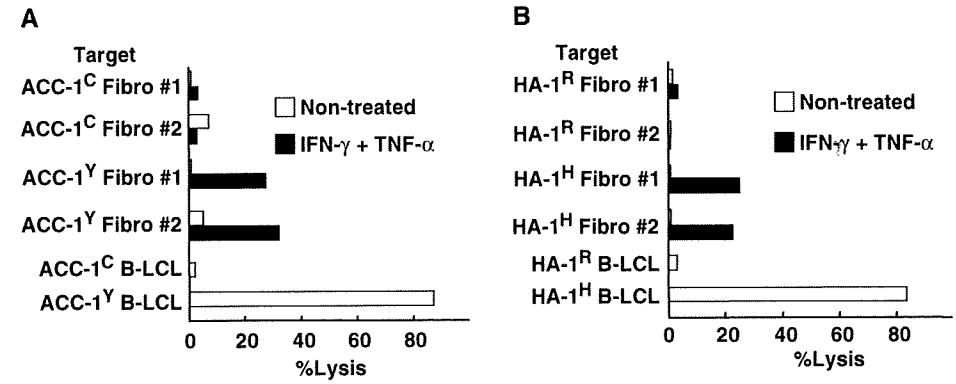

Fig. 4 Cytotoxicity of mHag-specific CTL clones against dermal fibroblasts. A standard 4-h ^{51}Cr-release assay was performed, as described above. **a** Cytotoxic activity of the HLA-A*2402-restricted, ACC-1^{Y} mHag-specific 1B3-CTL clone against HLA-A*2402-positive dermal fibroblasts (Fibro) and B-LCLs 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.

References

1. den Haan JM, Meadows LM, Wang W, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. Science. 1998;279:1054–7.
2. Kawase T, Akatsuka Y, Torikai H, et al. Alternative splicing due to an intronic SNP in HMSD generates a novel minor histocompatibility antigen. Blood. 2007;110:1055–63.
3. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. Nat Rev Cancer. 2004;4:371–80.
4. Atkins MB, Lotze MT, Dutcher JP, et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. J Clin Oncol. 1999;17:2105–16.
5. McDermott DF. Update on the application of interleukin-2 in the treatment of renal cell carcinoma. Clin Cancer Res. 2007;13:716s–20s.
6. Ravaud A, Dilhuydy MS. Interferon alpha for the treatment of advanced renal cancer. Expert Opin Biol Ther. 2005;5:749–62.
7. Bishop MR, Fowler DH, Marchigiani D, et al. Allogeneic lymphocytes induce tumor regression of advanced metastatic breast cancer. J Clin Oncol. 2004;22:3886–92.
8. Childs R, Chernoff A, Contentin N, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. N Engl J Med. 2000;343:750–8.
9. Klein CA, Wilke M, Pool J, et al. The hematopoietic system-specific minor histocompatibility antigen HA-1 shows aberrant expression in epithelial cancer cells. J Exp Med. 2002;196:359–68.
10. Fujii N, Hiraki A, Ikeda K, et al. Expression of minor histocompatibility antigen, HA-1, in solid tumor cells. Transplantation. 2002;73:1137–41.
11. Miyazaki M, Akatsuka Y, Nishida T, et al. Potential limitations in using minor histocompatibility antigen-specific cytotoxic T cells for targeting solid tumor cells. Clin Immunol. 2003;107:198–201.
12. Slager EH, Honders MW, van der Meijden ED, et al. Identification of the angiogenic endothelial-cell growth factor-1/thymidine phosphorylase as a potential target for immunotherapy of cancer. Blood. 2006;107:4954–60.
13. Akatsuka Y, Nishida T, Kondo E, et al. Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. J Exp Med. 2003;197:1489–500.
14. Childs R, Srinivasan R. Advances in allogeneic stem cell transplantation: directing graft-versus-leukemia at solid tumors. Cancer J. 2002;8:2–11.
15. Su AI, Cooke MP, Ching KA, et al. Large-scale analysis of the human and mouse transcriptomes. Proc Natl Acad Sci USA. 2002;99:4465–70.
16. Kloosterboer FM, van Luxemburg-Heijs SA, van Soest RA, van Egmond HM, Willemze R, Falkenburg JH. Up-regulated expression in nonhematopoietic tissues of the BCL2A1-derived minor histocompatibility antigens in response to inflammatory cytokines: relevance for allogeneic immunotherapy of leukemia. Blood. 2005;106:3955–7.
17. Khammari A, Nguyen JM, Pandolfino MC, et al. Long-term follow-up of patients treated by adoptive transfer of melanoma tumor-infiltrating lymphocytes as adjuvant therapy for stage III melanoma. Cancer Immunol Immunother. 2007;56:1853–60.
18. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. Human T cell responses against melanoma. Annu Rev Immunol. 2006;24:175–208.
19. Machiels JP, van Baren N, Marchand M. Peptide-based cancer vaccines. Semin Oncol. 2002;29:494–502.
20. Yee C, Thompson JA, Byrd D, et al. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci USA. 2002;99:16168–73.
21. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science. 2002;298:850–4.
22. Dudley ME, Wunderlich JR, Yang JC, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol. 2005;23:2346–57.
23. Kurokawa T, Fischer K, Bertz H, Hoegerle S, Finke J, Mackensen A. In vitro and in vivo characterization of graft-versus-tumor responses in melanoma patients after allogeneic peripheral blood stem cell transplantation. Int J Cancer. 2002;101:52–60.
24. Gottlieb DJ, Li YC, Lionello I, et al. Generation of tumour-specific cytotoxic T-cell clones from histocompatibility leucocyte antigen-identical siblings of patients with melanoma. Br J Cancer. 2006;95:181–8.
25. Akatsuka Y, Morishima Y, Kuzushima K, Kodera Y, Takahashi T. Minor histocompatibility antigens as targets for immunotherapy using allogeneic immune reactions. Cancer Sci. 2007;98:1139–46.
26. Vlaykova T, Talve L, Hahka-Kemppinen M, et al. Immunohistochemically detectable bcl-2 expression in metastatic melanoma: association with survival and treatment response. Oncology. 2002;62:259–68.
27. Goulmy E, Schipper R, Pool J, et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. N Engl J Med. 1996;334:281–5.
28. Tseng LH, Lin MT, Hansen JA, et al. Correlation between disparity for the minor histocompatibility antigen HA-1 and the development of acute graft-versus-host disease after allogeneic marrow transplantation. Blood. 1999;94:2911–4.
29. Murata M, Emi N, Hirabayashi N, et al. No significant association between HA-1 incompatibility and incidence of acute graft-versus-host disease after HLA-identical sibling bone marrow transplantation in Japanese patients. Int J Hematol. 2000;72:371–5.
30. Nishida T, Akatsuka Y, Morishima Y, et al. Clinical relevance of a newly identified HLA-A24-restricted minor histocompatibility antigen epitope derived from BCL2A1, ACC-1, in patients receiving HLA genotypically matched unrelated bone marrow transplant. Br J Haematol. 2004;124:629–35.
31. Dickinson AM, Wang XN, Sviland L, et al. In situ dissection of the graft-versus-host activities of cytotoxic T cells specific for minor histocompatibility antigens. Nat Med. 2002;8:410–4.

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ORIGINAL ARTICLE

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

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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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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

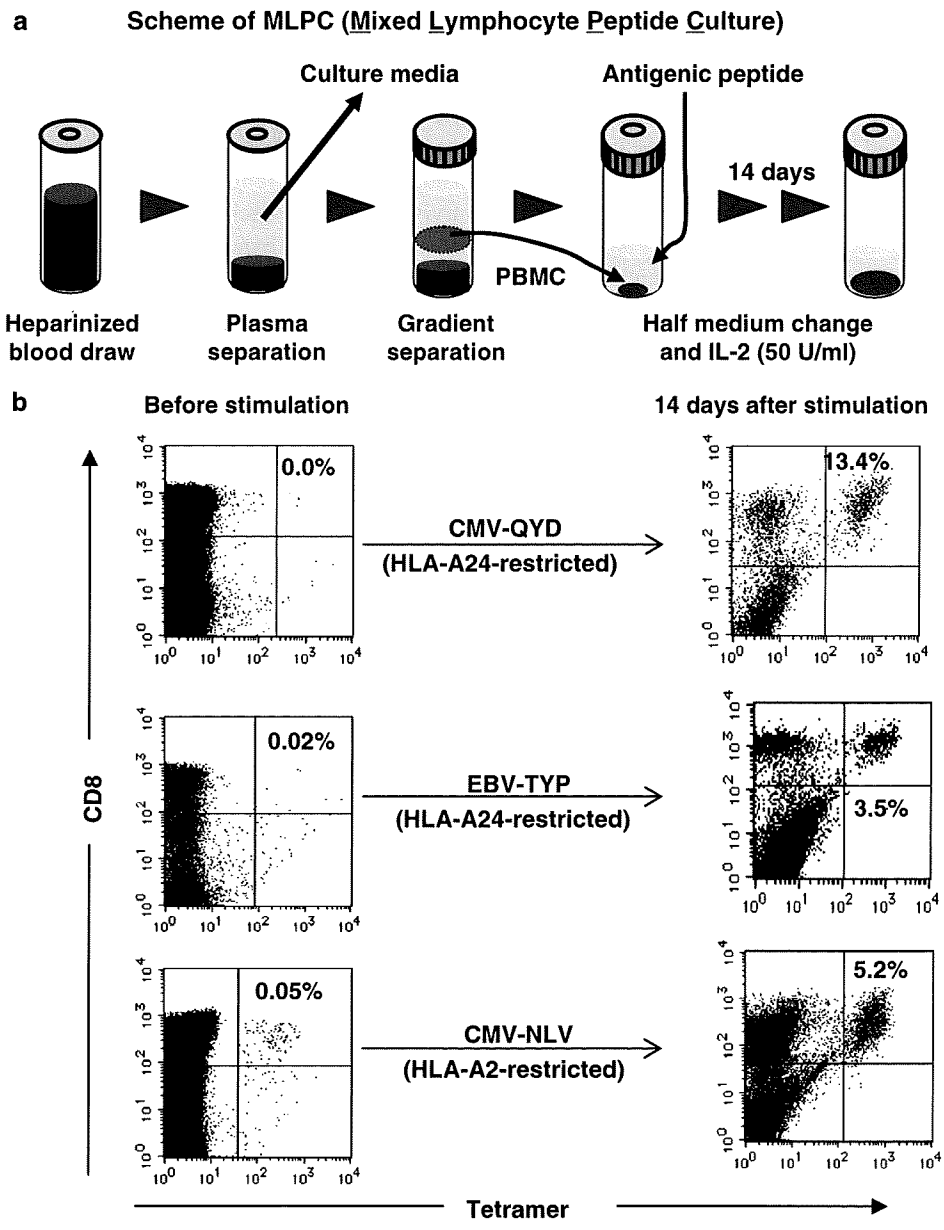

Fig. 1 **a** Schematic diagram of mixed lymphocyte peptide culture (MLPC). Heparinized whole blood was first centrifuged to obtain plasma for culture media preparation. Peripheral blood mononuclear cells (PBMCs) were then separated by density gradient centrifugation from the resuspended blood pellets 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

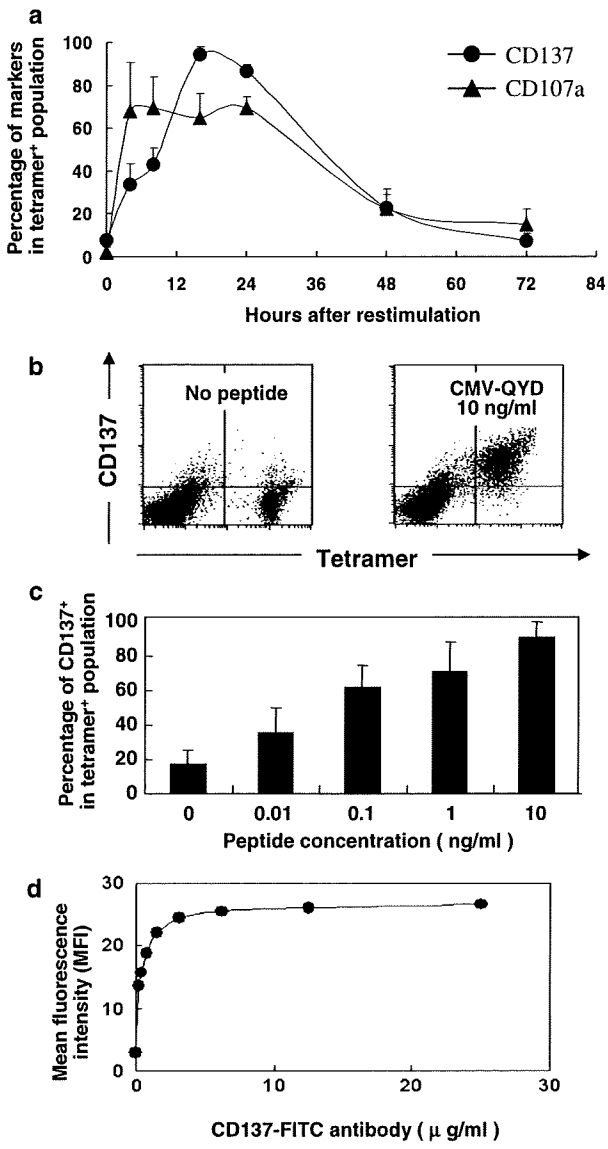

Fig. 2 Optimization of conditions for positive selection. **a** Expression kinetics of CD137 and CD107a on T cells generated by MLPC with the CMV-QYD peptide. The percentages of indicated marker (CD137 or CD107a)-positive cells among tetramer$^+$ T cell populations after stimulation with 10 ng/ml CMV-QYD 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

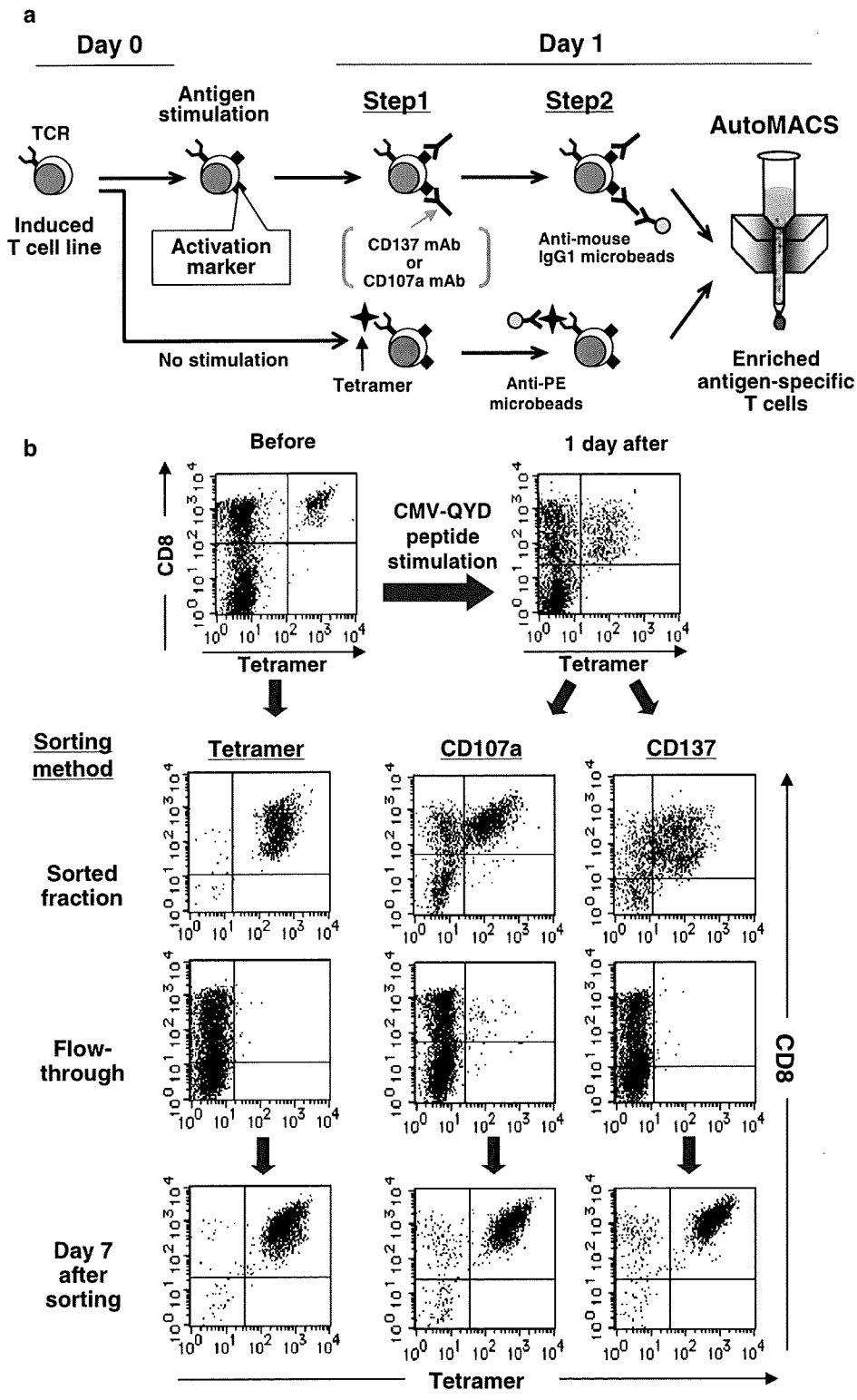

Fig. 3 Schematic illustration of positive selection using CD137, CD107a, or tetramer. **a** The MLPC-induced cell lines on days 14–16 after the initial stimulation were split and either restimulated with cognate antigenic peptide (2/3 part) or left without any stimulation (1/3 part) overnight. On the 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

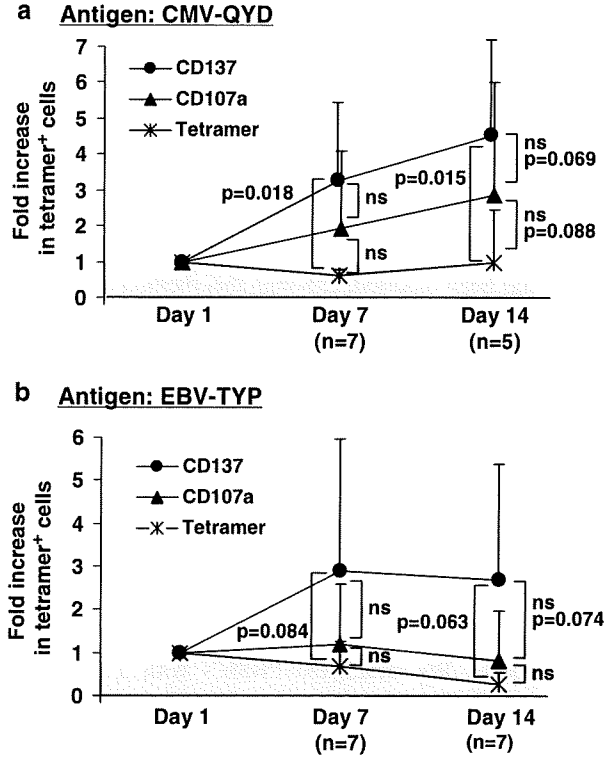

Fig. 4 Expansion of enriched cells after sorting with CD137, CD107a, or tetramers. AutoMACS-sorted fractions were cultured in a 24- or 96-well culture plate in ALyS505N-1000 media containing 1,000 U/ml IL-2 for the indicated period. Average fold increases of cognate tetramer$^+$ cells from seven individuals 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.

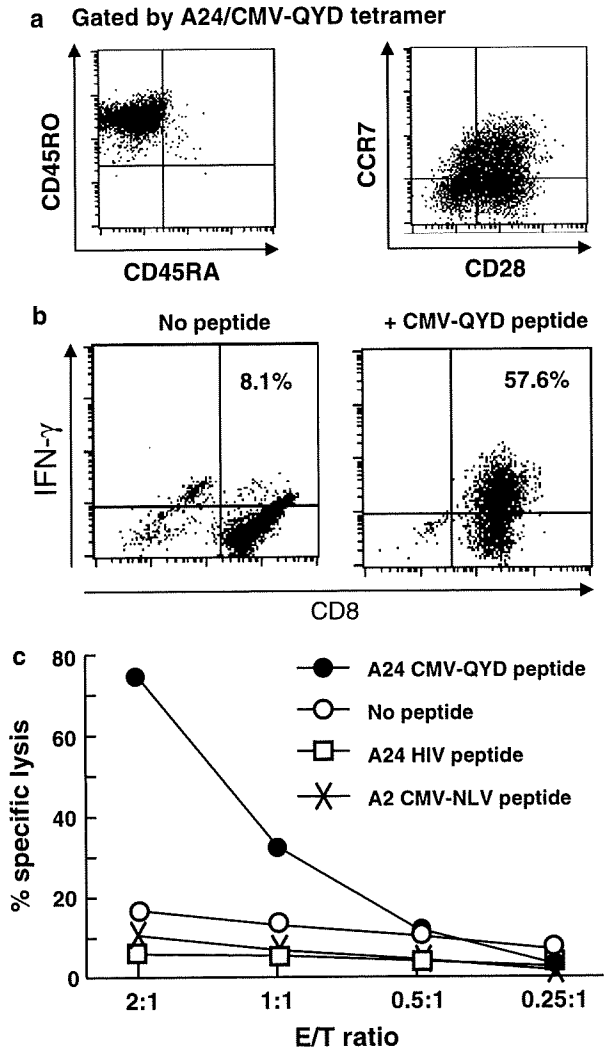

Fig. 5 Phenotypes and functions of CD137-sorted and 7-day cultured T cell lines. **a** Representative flow cytometry profile of CMV-QYD-specific T cell lines for differentiation markers. T cells gated for the cognate tetramer were analyzed with the indicated markers. **b** Capacity for IFN-γ production 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

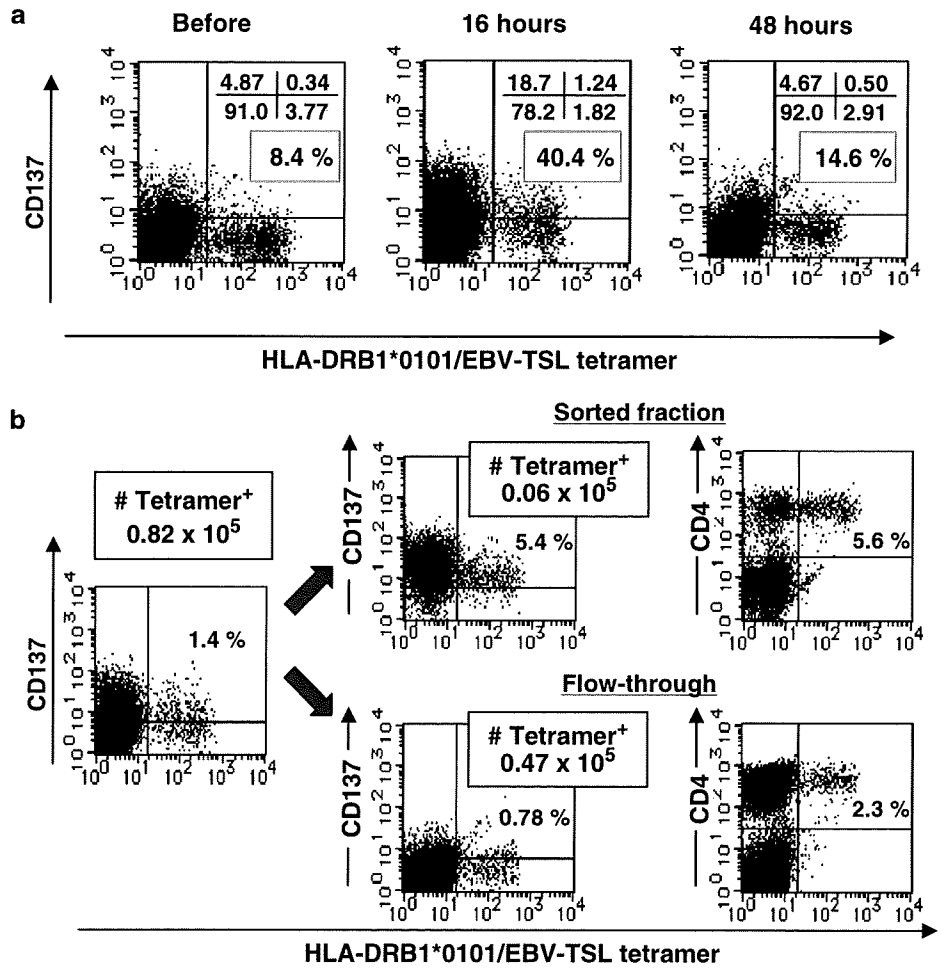

Fig. 6 Induction of CD137 expression on antigen-specific $CD4^+$ T-cells. **a** PBMCs were stimulated in MLPC with the HLA-DRB1*0101-restricted EBV-TSL peptide. On day 14 of culture, the T cells were stimulated with 10 ng/ml EBV-TSL peptide. The expression of CD137 was assessed along with HLA-DRB1*0101/EBV-TSL 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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References

1. Fishman JA, Emery V, Freeman R, et al. Cytomegalovirus in transplantation—challenging the status quo. Clin Transplant. 2007;21:149–58.
2. Ganne V, Siddiqi N, Kamaplath B, et al. Humanized anti-CD20 monoclonal antibody (Rituximab) treatment for post-transplant lymphoproliferative disorder. Clin Transplant. 2003;17:417–22.
3. Gottschalk S, Heslop HE, Rooney CM. Adoptive immunotherapy for EBV-associated malignancies. Leuk Lymphoma. 2005;46:1–10.
4. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med. 1995;333:1038–44.
5. Heslop HE, Ng CY, Li C, et al. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. Nat Med. 1996;2:551–5.
6. Savoldo B, Huls MH, Liu Z, et al. Autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for the treatment of persistent active EBV infection. Blood. 2002;100:4059–66.
7. Papadopoulos EB, Ladanyi M, Emanuel D, et al. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. N Engl J Med. 1994;330:1185–91.
8. Riddell SR, Bleakley M, Nishida T, Berger C, Warren EH. Adoptive transfer of allogeneic antigen-specific T cells. Biol Blood Marrow Transplant. 2006;12:9–12.
9. Rubio V, Stuge TB, Singh N, et al. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. Nat Med. 2003;9:1377–82.
10. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods. 2003; 281:65–78.
11. Brosterhus H, Brings S, Leyendeckers H, et al. Enrichment and detection of live antigen-specific CD4(+) and CD8(+) T cells based on cytokine secretion. Eur J Immunol. 1999;29:4053–9.
12. Wolfl M, Kuball J, Ho WY, et al. Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. Blood. 2007; 110:201–10.
13. Baumann S, Krueger A, Kirchhoff S, Krammer PH. Regulation of T cell apoptosis during the immune response. Curr Mol Med. 2002;2:257–72.
14. Watts TH. TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol. 2005;23:23–68.
15. Wen T, Bukczynski J, Watts TH. 4-1BB ligand-mediated costimulation of human T cells induces CD4 and CD8 T cell expansion, cytokine production, and the development of cytolytic effector function. J Immunol. 2002;168:4897–906.
16. Wehler TC, Nonn M, Brandt B, et al. Targeting the activation-induced antigen CD137 can selectively deplete alloreactive T cells from antileukemic and antitumor donor T-cell lines. Blood. 2007;109:365–73.
17. Frentsch M, Arbach O, Kirchhoff D, et al. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. Nat Med. 2005;11:1118–24.
18. Sabbagh L, Kaech SM, Bourbonniere M, et al. The selective increase in caspase-3 expression in effector but not memory T cells allows susceptibility to apoptosis. J Immunol. 2004; 173:5425–33.
19. Burrows SR, Suhrbier A, Khanna R, Moss DJ. Rapid visual assay of cytotoxic T-cell specificity utilizing synthetic peptide induced T-cell–T-cell killing. Immunology. 1992;76:174–5.
20. Gattinoni L, Klebanoff CA, Palmer DC, et al. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest. 2005;115:1616–26.
21. Diab A, Cohen AD, Alpdogan O, Perales MA. IL-15: targeting CD8+ T cells for immunotherapy. Cytotherapy. 2005;7:23–35.

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LETTER TO THE EDITOR

Consulting clinic for related family donors in hematopoietic stem cell transplantation

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When patients with hematologic or other intractable disorders need to undergo allogeneic HSCT (hematopoietic stem cell transplantation), related family members are most often considered as candidates for the hematopoietic stem cell (HSC) donation. According to a nationwide questionnaire survey in Japan, 98.0% of family donors are informed about the HSC donation procedure only by the clinicians involved in the care of the HSC transplant recipient ('recipient clinicians'). Moreover, when written informed consent for HSC donation was obtained, the recipient clinicians and the recipients themselves were present with the prospective donors in 89.3 and 70.7% of cases, respectively.[1] We questioned whether in such situations the donors were truly agreeing to donate of their own free will, and whether their safety in terms of physical eligibility was considered sufficiently. There have been many studies on the safety and ethical issues facing unrelated volunteer HSC donors[2,3] and especially on issues regarding PBSC collection.[4–6] However, surprisingly, few studies so far have addressed these issues with special reference to related donors.[7] Therefore, we started a unique donor consultation program, 'Consulting Clinic for Related HSC Donors (CRD),' in which two clinicians ('donor clinicians') who are not directly involved in the care of the prospective recipients play a central role in securing the rights and safety of related family donors.

We herein introduce the CRD process at our hospital (Figure 1). At the CRD visit, the donor candidate watches a video program to obtain general information about HSCT and completes a questionnaire to elicit a medical history. Then the donor clinician comprehensively explains what HSC donation is, especially focusing on the risks associated with BM and PBSC harvesting in addition to their potential alternatives. After the written informed consent for the donation is obtained, the donor candidate undergoes unified medical examinations to evaluate eligibility for the planned HSC donation; thereafter, the donor clinician and another physician who is also uninvolved in the care of the prospective recipient meet together and discuss whether the examination results satisfy our institutional eligibility criteria. Decisions are classified into three groups: (A) 'qualified', (B) 'reserved' (requesting more consideration) and (C) 'unqualified'. The patient is informed of the decision by the recipient clinician, who reports that the donor candidate is 'qualified' or 'unqualified' without disclosing the reasons, while the donor candidate is

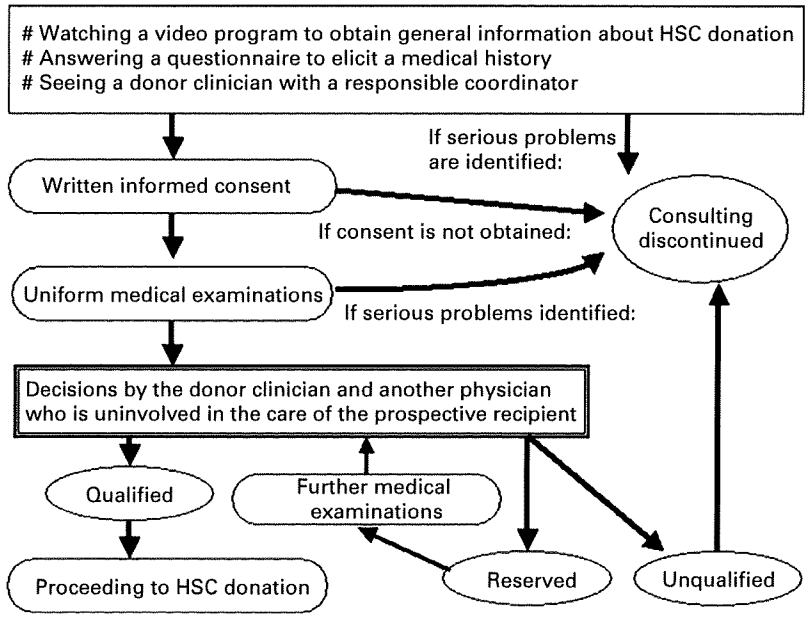

Figure 1 The diagram showing the process of our consulting clinic for related donors (CRD).

Table 1 Characteristics of donor candidates visiting our consulting clinic for related donors

Sex number (%)	
Male	38 (54)
Female	32 (46)
Median age, years (range, years)	48 (13–69)
Age group number (%)	
11–20 year	2 (3)
21–30 year	19 (27)
31–40 year	7 (10)
41–50 year	11 (16)
51–60 year	21 (30)
61–69 year	10 (14)
Relationship to the recipient number (%)	
Sibling	48 (69)
Son	8 (11)
Daughter	4 (6)
Father	3 (4)
Mother	7 (10)
Type of requested stem cell source number (%)	
Bone marrow	52 (74)
Peripheral blood	13 (19)
Bone marrow or peripheral blood	5 (7)

informed of the decision by the donor clinician. It is most important through this process for the donor clinician to avoid coercing the donation and to inform the donor candidates that they can cancel the donation at any time, even after providing written consent.

Between April 2003 and March 2007, a total of 70 donor candidates visited the CRD (Table 1). Two candidates were judged as 'unqualified' at the first medical interview. Sixty-eight qualification meetings by two clinicians were held to judge the eligibility of the candidates to undergo BM or PBSC donation; the initial decisions were 'qualified' in 43 cases (63%), 'reserved' in 21 (33%) and 'unqualified' in 4 (6%). The reasons for reservation were abnormalities in blood or urine examinations in 17 cases, suspicion of cardiovascular disease in 3 and suspicion of a neurological disorder in 1. The reasons for disqualification were suspicion of malignant tumors in three cases and presence of cardiovascular disease in one. Twenty of twenty-one candidates (95%) in the 'reserved' group were eventually judged 'qualified' after existing diseases were controlled and/or the donor was re-evaluated by another consultant physician or anesthesiologist. Three of four 'unqualified' candidates at the first CRD were reclassified as 'qualified' at the second CRD when the requested source of HSC was changed from PBSC to BM. Twelve of sixty-six 'finally qualified' donors (18%) did not actually donate HSCs due to various reasons, including death of the recipient before undergoing HSCT. To date, we have not observed any severe adverse events in the remaining 54 donors who actually donated HSC, with a median follow-up of 33 months (range, 5–51 months).

It is still difficult to estimate the contribution of our CRD to the improvement of donor safety because our experience is limited. However, we believe that our CRD has a meaningful impact on the ethical issues facing related donors. The psychological condition of the donor, particularly the donor's motives for considering the HSC donation, should be carefully assessed prior to giving informed consent. It is reported that family donor candidates may occasionally be subjected to coercion or external pressure, partly because such donors' desire to help relatives may be different from the motivations of unrelated donors.[8,9] In a survey of Japanese HSCT centers, 39.4% of related donors felt that they could not refuse to donate, while only 20% of them felt that they had a chance to refuse to donate HSCs if they did not want to do so.[1] Especially, in case the donor candidate is a child and the prospective recipient is one of their parents, there is a clear conflict of interest since the parent usually signs the consent for the child, raising a strong need for a guardian of the child donors.

In our CRD, several donor candidates hesitated over whether to agree or to refuse to donate. In those cases, the donor clinicians informed the candidate that even if the donation is refused, they would never tell the patient why the donor candidate could not donate HSC. This system largely put the candidates at their ease, and all of these candidates finally agreed to donate. Switzer *et al.*[10] reported that unrelated donors who felt they were pressured, irrespective of whether they were encouraged or discouraged to donate, are less likely to have a positive donation experience. In this way, our CRD program functioned as a buffer zone for the relationship between the donor and recipient.

In conclusion, we believe that this type of CRD program, which supports and respects the donors' free will, should become more widely used to secure the rights and safety of related donors. Further studies on the role of the CRD in the management of related familial donors are warranted.

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References

1 Kato S. Studies on quality of life of donors and recipients in transplantation medicine (Japanese). *2004 Annual Report of Research in Human Genome, Tissue Engineering, and Food Biotechnology, Ministry of Health, Labour and Welfare of Japan, 200400072A*, (http://mhlw-grants.niph.go.jp/niph/search/NIDD00.do).
2 Butterworth VA, Simmons RG, Bartsch G, Randall B, Schimmel M, Stroncek DF. Psychosocial effects of unrelated bone marrow donation: experiences of the National Marrow Donor Program. *Blood* 1993; **81**: 1947–1959.
3 Gandini A, Roata C, Franchini M, Agostini E, Guizzardi E, Pontiero Giacometti P *et al.* Unrelated allogeneic bone marrow

donation: short- and long-term follow-up of 103 consecutive volunteer donors. *Bone Marrow Transplant* 2001; **28**: 369–374.
4 Ordemann R, Hölig K, Wagner K, Rautenberg U, Bornhäuser M, Kroschinsky F *et al.* Acceptance and feasibility of peripheral blood stem cell mobilisation compared to bone marrow collection from healthy unrelated donors. *Bone Marrow Transplant* 1998; **21**: S25–S28.
5 Murata M, Harada M, Kato S, Takahashi S, Ogawa H, Okamoto S *et al.* Peripheral blood stem cell mobilization and apheresis: analysis of adverse events in 94 normal donors. *Bone Marrow Transplant* 1999; **24**: 1065–1071.
6 Anderlini P, Rizzo JD, Nugent ML, Schmitz N, Champlin RE, Horowitz MM *et al.* Peripheral blood stem cell donation: an analysis from the International Bone Marrow Transplant Registry (IBMTR) and European Group for Blood and Marrow Transplant (EBMT) databases. *Bone Marrow Transplant* 2001; **27**: 689–692.
7 Gluckman E, Socié G, Guivärch C, Rabannes F, Meresse V, Henry-Amar M. The long time forgotten HLA identical bone marrow donor: result of a survey on 818 patients. *Blood* 1996; **88**: 612a.
8 Christopher KA. The experience of donating bone marrow to a relative. *Oncol Nurs Forum* 2000; **27**: 693–700.
9 Chang G, McGarigle C, Spitzer TR, McAfee SL, Harris F, Piercy K *et al.* A comparison of related and unrelated marrow donors. *Psychosom Med* 1998; **60**: 163–167.
10 Switzer GE, Dew MA, Butterworth VA, Simmons RG, Schimmel M. Understanding donors' motivations: a study of unrelated bone marrow donors. *Soc Sci Med* 1997; **45**: 137–147.

Impact of ABO mismatching on the outcomes of allogeneic related and unrelated blood and marrow stem cell transplantations for hematologic malignancies: IPD-based meta-analysis of cohort studies

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BACKGROUND: The impact of donor-recipient ABO matching on outcomes after allogeneic stem cell transplantation has been a matter of controversy.
STUDY DESIGN AND METHODS: Individual patient data–based meta-analysis was conducted with a pooled data set provided through six published and one unpublished cohorts. Outcomes in recipients of peripheral blood or bone marrow transplantation for hematologic malignancies were evaluated. A multivariate Cox model was used to adjust differences in outcomes of patients receiving ABO-matched grafts with those receiving major, minor, or bidirectional mismatched grafts. Considering multiple testing, p values of less than 0.05 and 0.001 were considered significant for the primary and secondary endpoints, respectively.
RESULTS: In all, 1208 cases, including 697 ABO-matched and 202 major, 228 minor, and 81 bidirectional mismatched transplants, were analyzed. Overall, adverse impact of ABO matching on overall survival (OS), as a primary endpoint, was not observed (adjusted hazard ratios [95% confidence intervals]: major, 1.03 [0.82-1.30], p = 0.81; minor, 1.19 [0.97-1.47], p = 0.10; bidirectional, 1.25 [0.91-1.72], p = 0.17). Among related stem cell recipients, ABO matching had no significant influence on OS, while the minor and bidirectional mismatched groups among unrelated stem cell recipients exhibited lower OS with marginal significance, especially in patients with acute leukemia, patients who received transplants after 1998, and patients who underwent transplants at Asian centers.
CONCLUSIONS: Our meta-analysis demonstrates no adverse association between any ABO mismatching and survival. However, marginally lower OS found in recipients of minor or bidirectional mismatched grafts from unrelated donors suggested the need for larger studies focusing on unrelated transplants.

ABO matching between donor and recipient in solid organ transplantation is generally thought to be essential for better outcomes.[1] In contrast, blood or marrow stem cell transplantation (SCT) from an ABO-mismatched donor is sufficiently

ABBREVIATIONS: AL = acute biphenotypic or unclassifiable leukemia; ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; CLL = chronic lymphocytic leukemia; CML = chronic myelogenous leukemia; HR(s) = hazard ratio(s); IPD = individual patient data; MDS = myelodysplastic syndrome; ML = malignant lymphoma; MM = multiple myeloma; OS = overall survival; SCT = stem cell transplantation; TRM = treatment-related mortality.

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