Int J Hematol (2008) 88:311–320
DOI 10.1007/s12185-008-0134-z

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

Received: 20 September 2007 / Revised: 7 May 2008 / Accepted: 9 June 2008 / Published online: 5 August 2008

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

K.W. and S.T. are employees of Medical Biological Laboratories Co., Ltd. S.S. is a representative executive of T Cell Technologies, Inc. Y.A. has received financial support through collaboration with Medical Biological Laboratories Co., Ltd.

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

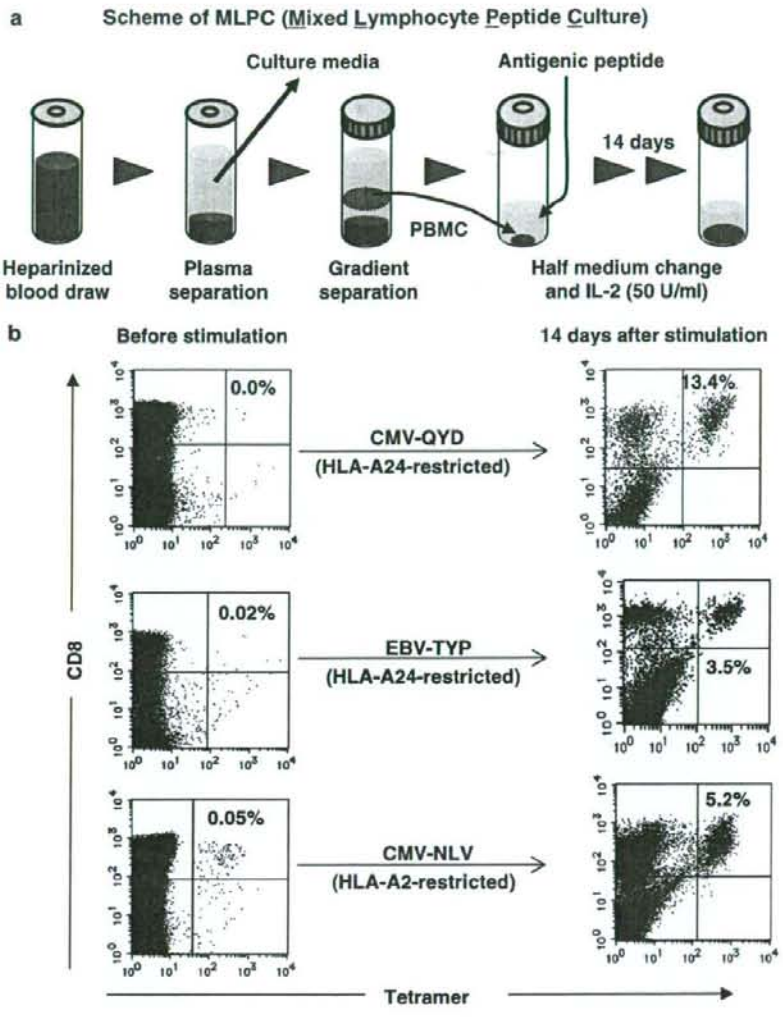

Fig. 1 **a** Schematic diagram of mixed lymphocyte peptide culture (MLPC). Heparinized whole blood was first centrifuged to obtain plasma for culture media preparation. Peripheral blood mononuclear cells (PBMCs) were then separated by density gradient centrifugation from the resuspended blood pellets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

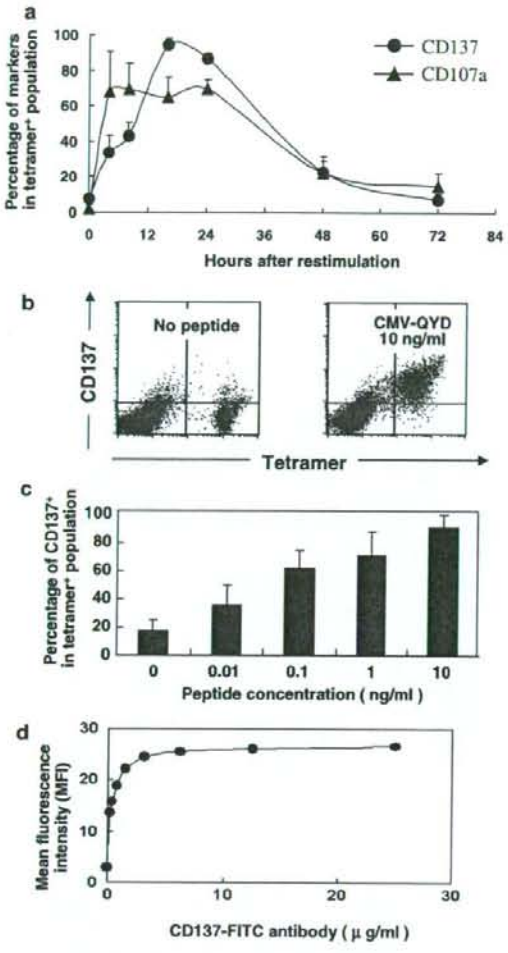

Fig. 2 Optimization of conditions for positive selection. **a** Expression kinetics of CD137 and CD107a on T cells generated by MLPC with the CMV-QYD peptide. The percentages of indicated marker (CD137 or CD107a)-positive cells among tetramer$^+$ T cell populations after stimulation with 10 ng/ml CMV-QYD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

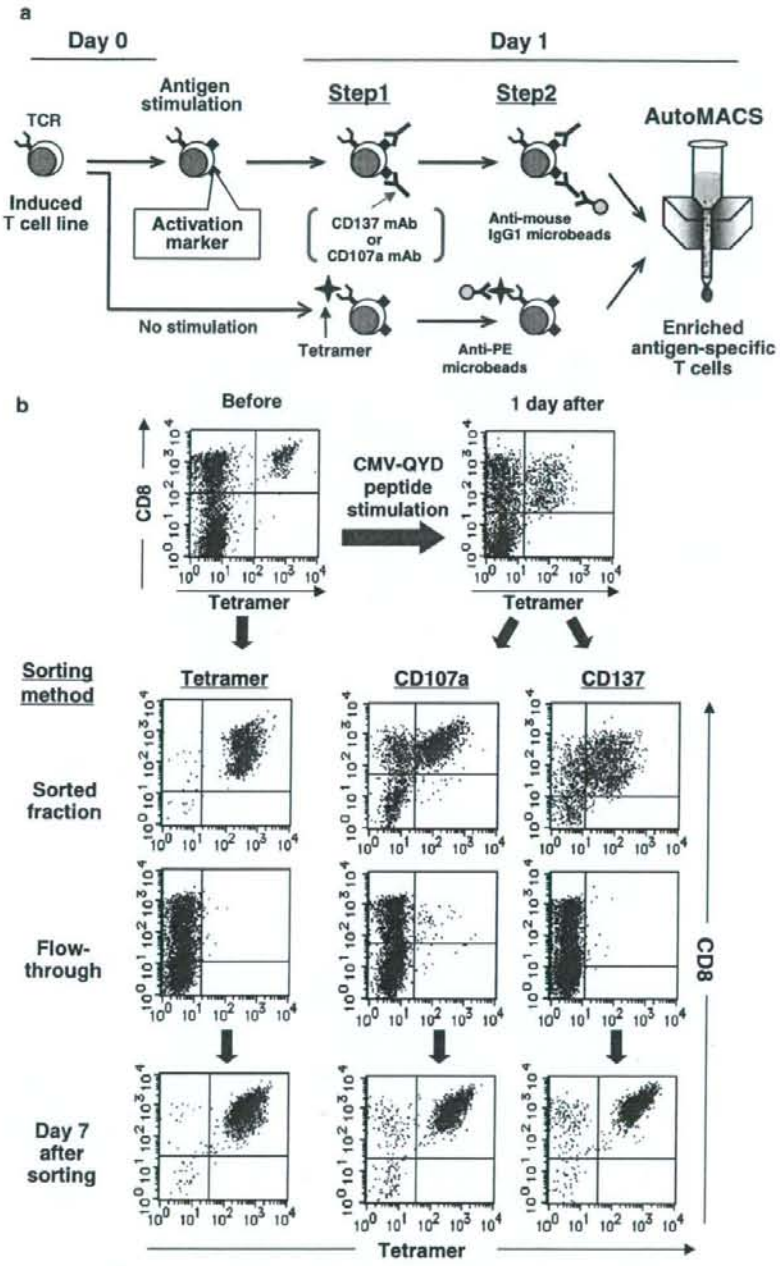

Fig. 3 Schematic illustration of positive selection using CD137, CD107a, or tetramer. **a** The MLPC-induced cell lines on days 14–16 after the initial stimulation were split and either restimulated with cognate antigenic peptide (2/3 part) or left without any stimulation (1/3 part) overnight. On the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

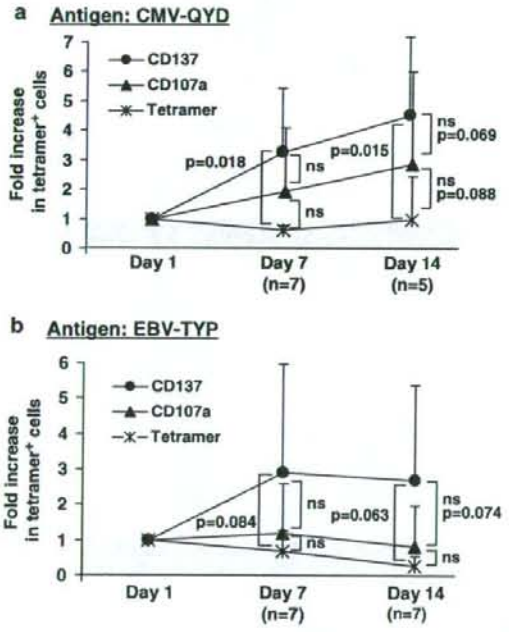

Fig. 4 Expansion of enriched cells after sorting with CD137, CD107a, or tetramers. AutoMACS-sorted fractions were cultured in a 24- or 96-well culture plate in ALyS505N-1000 media containing 1,000 U/ml IL-2 for the indicated period. Average fold increases of cognate tetramer$^+$ cells from seven individuals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

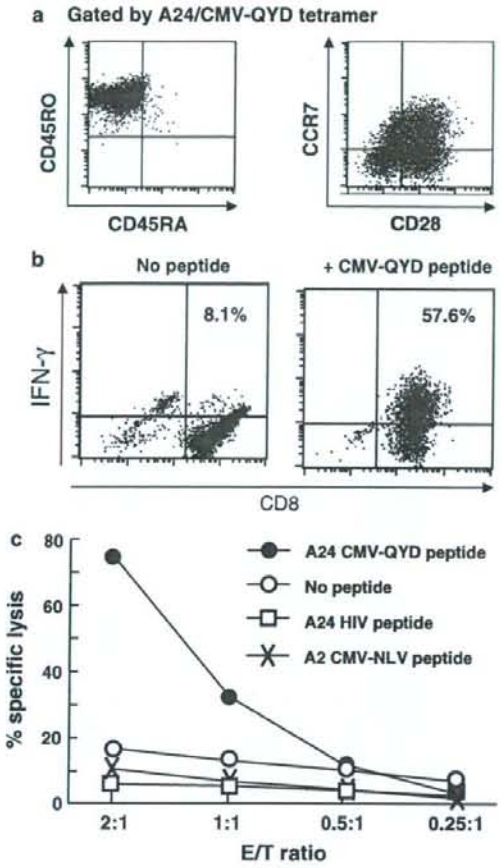

Fig. 5 Phenotypes and functions of CD137-sorted and 7-day cultured T cell lines. **a** Representative flow cytometry profile of CMV-QYD-specific T cell lines for differentiation markers. T cells gated for the cognate tetramer were analyzed with the indicated markers. **b** Capacity for IFN-γ production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

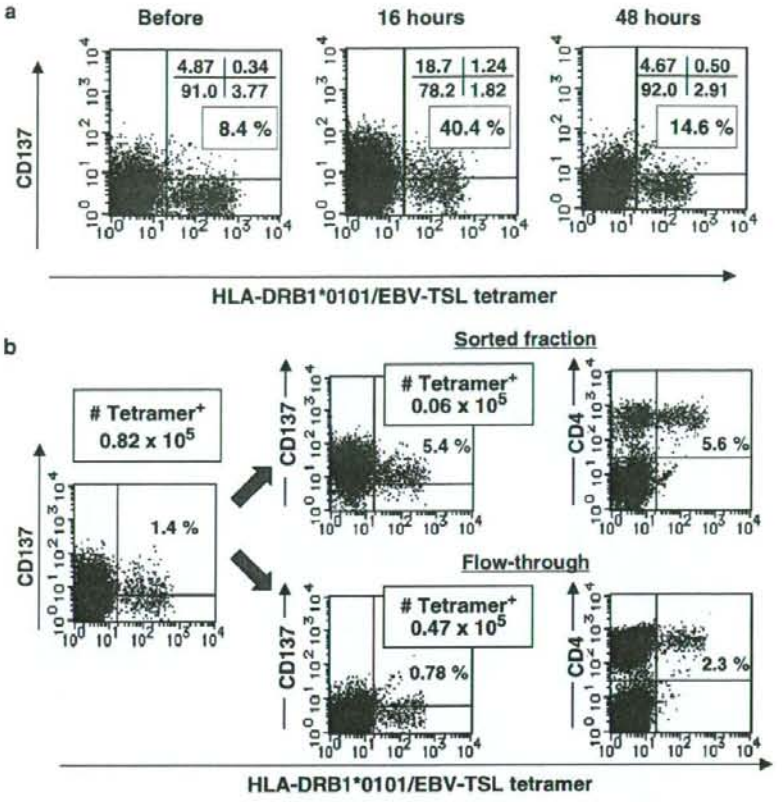

Fig. 6 Induction of CD137 expression on antigen-specific $CD4^+$ T-cells. **a** PBMCs were stimulated in MLPC with the HLA-DRB1*0101-restricted EBV-TSL peptide. On day 14 of culture, the T cells were stimulated with 10 ng/ml EBV-TSL peptide. The expression of CD137 was assessed along with HLA-DRB1*0101/EBV-TSL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

Acknowledgments The authors thank Dr. Hiroki Torikai, Dr. Satoko Morishima, Dr. Hidemasa Miyauchi, Dr. Ayako Demachi-Okamura, Ms. Yumi Nakao-Ohashi, Ms. Hiromi Tamaki, and Ms. Keiko Nishida for their expert technical assistance. This study was supported in part by Scientific Research on Priority Areas (B01) (no. 17016089), from the Ministry of Education, Culture, Science, Sports, and Technology, Japan; Research on Human Genome, Tissue Engineering Food Biotechnology and the Second and Third Team Comprehensive 10-year Strategy for Cancer Control (no. 26), from the Ministry of Health, Labour; and Collaboration with Medical Biological Laboratories Co., Ltd.

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.

blood

2008 111: 1054-1059
Prepublished online Nov 7, 2007;
doi:10.1182/blood-2007-08-099168

Prospective multicenter trial comparing repeated immunosuppressive therapy with stem-cell transplantation from an alternative donor as second-line treatment for children with severe and very severe aplastic anemia

Yoshiyuki Kosaka, Hiroshi Yagasaki, Kimihiko Sano, Ryoji Kobayashi, Hiroshi Ayukawa, Takashi Kaneko, Hiromasa Yabe, Masahiro Tsuchida, Hideo Mugishima, Akira Ohara, Akira Morimoto, Yoshitoshi Otsuka, Shouichi Ohga, Fumio Bessho, Tatsutoshi Nakahata, Ichiro Tsukimoto, Seiji Kojima and on behalf of the Japan Childhood Aplastic Anemia Study Group

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.

Prospective multicenter trial comparing repeated immunosuppressive therapy with stem-cell transplantation from an alternative donor as second-line treatment for children with severe and very severe aplastic anemia

Yoshiyuki Kosaka,[1] Hiroshi Yagasaki,[2] Kimihiko Sano,[3] Ryoji Kobayashi,[4] Hiroshi Ayukawa,[5] Takashi Kaneko,[6] Hiromasa Yabe,[7] Masahiro Tsuchida,[8] Hideo Mugishima,[9] Akira Ohara,[10] Akira Morimoto,[11] Yoshitoshi Otsuka,[12] Shouichi Ohga,[13] Fumio Bessho,[14] Tatsutoshi Nakahata,[15] Ichiro Tsukimoto,[16] and Seiji Kojima,[2] on behalf of the Japan Childhood Aplastic Anemia Study Group

[1]Department of Pediatrics, Hyogo Children Hospital, Hyogo; [2]Department of Pediatrics, Nagoya University Graduate School of Medicine, Aichi; [3]Department of Pediatrics, Kobe University Graduate School of Medicine, Hyogo; [4]Department of Pediatrics, Hokkaido University Graduate School of Medicine, Hokkaido; [5]Department of Pediatrics, Yamaguchi University School of Medicine, Yamaguchi; [6]Department of Pediatrics, Kiyose Children's Hospital, Tokyo; [7]Specialized Clinical Science, Pediatrics, Tokai University School of Medicine, Kanagawa; [8]Department of Pediatrics, Ibaraki Children's Hospital, Ibaraki; [9]Department of Pediatrics, Nihon University School of Medicine, Tokyo; [10]Department of Transfusion, Toho University School of Medicine, Tokyo; [11]Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto; [12]Department of Pediatrics, Hyogo College of Medicine, Hyogo; [13]Department of Pediatrics, Kyushu University Graduate School of Medicine, Fukuoka; [14]Department of Pediatrics, Kyorin University School of Medicine, Tokyo; [15]Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto; and [16]Department of Pediatrics, Toho University School of Medicine, Tokyo, Japan

We conducted a prospective multicenter study to compare the efficacy of repeated immunosuppressive therapy (IST) with stem-cell transplantation (SCT) from an alternative donor in children with acquired aplastic anemia (AA) who failed to respond to an initial course of IST. Patients with severe (n = 86) and very severe disease (n = 119) received initial IST consisting of antithymocyte globulin (ATG) and cyclosporine. Sixty patients failed to respond to IST after 6 months from the initial IST and were eligible for second-line treatment. Among them, 21 patients lacking suitable donors received a second course of IST. Three patients developed an anaphylactoid reaction to ATG and could not complete the second IST. A trilineage response was seen in only 2 of 18 (11%) evaluable patients after 6 months. Thirty-one patients received SCT from an alternative donor. At 5 years from the initiation of second-line therapy, the estimated failure-free survival (FFS), defined as survival with response, was 83.9% (± 16.1%, SD) in the SCT group compared with 9.5% (± 9.0%) in the IST group ($P = .001$). These results suggest that SCT from an alternative donor offers a better chance of FFS than a second IST in patients not responding to an initial IST. (Blood. 2008;111: 1054-1059)

Introduction

Acquired aplastic anemia (AA) is a heterogeneous disorder characterized by pancytopenia of peripheral blood and hypocellular marrow. Currently 2 effective treatments are available for this disorder: hematopoietic stem cell transplantation (SCT) and immunosuppressive therapy (IST). There are several reports comparing bone marrow transplantation (BMT) and IST as first-line treatment for AA.[1-4] These studies indicate that allogeneic BMT from an HLA-matched sibling donor is the treatment of choice for young patients. IST consisting of antithymocyte globulin (ATG) and cyclosporine (CyA) with or without granulocyte-colony stimulating factor (G-CSF) has been successfully used for patients with AA who lack an HLA-matched sibling donor or who are not eligible for SCT. Several reports indicate that 2- to 5-year survival following IST is between 60% and 90%.[5-7] We reported results of a multicenter trial of IST for children younger than 18 years with AA (AA-92 trial).[8] In the AA-92 trial, 119 children with newly diagnosed AA were enrolled, and the response rate at 6 months was 71%, with the probability of survival at 4 years greater than 90%. However, approximately 30% of the patients did not respond to an initial course of IST. Moreover, a significant proportion of patients subsequently relapsed and required second-line therapy.[9] The optimal treatment for such patients has not been established.

A repeated course of IST has been used for patients who fail to respond to, or who have relapsed after an initial course of, IST. Tichelli et al reported the results of a Basel study that consisted of repeated courses of IST, using ATG from the same species (horse) for nonresponders.[10] In their study, repeated IST was well tolerated and the response rate was 63%. An Italian group reported the results of repeated IST using ATG from different species (horse to rabbit), where the response rate was also high.[11] Investigators at the National Institutes of Health (NIH) recently reported the results of retreatment with rabbit ATG and CyA in 22 patients refractory to horse ATG and CyA. Contrary to the reports from Europe, the overall response rate was only 27% and no patients achieved complete response.[12]

SCT from an alternative donor has also been used as salvage therapy for patients not responding to IST because recent progress

Submitted August 7, 2007; accepted October 8, 2007. Prepublished online as *Blood* First Edition paper, November 7, 2007; DOI 10.1182/blood-2007-08-099168.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

in the management of patients who undergo SCT, and better selection of donors by DNA typing of HLA loci, has improved the outcome for these patients.[13,14] However, no prospective study has been performed to date comparing repeated IST versus SCT from an alternative donor as second-line therapy. Therefore, we conducted a prospective multicenter trial to compare these 2 treatment options for pediatric patients with severe and very severe AA who had failed to respond to initial IST.

Methods

Patients

This multicenter study was designed by the Japan Childhood Aplastic Anemia Study Group and involved 79 hospitals in Japan. The eligibility criteria were as follows: age younger than 18 years, diagnosis less than 180 days before registration, no specific prior treatment for AA, and severe to very severe disease. The definition of disease severity was determined according to currently used criteria.[15] The disease was considered severe if at least 2 of the following were noted: a neutrophil count less than 0.5×10^9/L, a platelet count less than 20×10^9/L, and a reticulocyte count less than 20×10^9/L with hypocellular bone marrow. AA was considered very severe if the criteria for severe disease were fulfilled and the neutrophil count was less than 0.2×10^9/L. Patients were excluded if they had congenital AA. Patients were screened for paroxysmal nocturnal hemoglobinuria (PNH) by flow cytometry using anti-CD55 and anti-CD59 antibodies. Bone marrow cytogenetic studies were performed in all patients. Allogeneic SCT was recommended for patients with severe or very severe disease who had an HLA-matched sibling: these patients were not included in AA-97 study.

Treatment protocol

Patients with very severe disease were treated with IST, which consisted of horse ATG (Lymphoglobulin; IMTIX-SANGSTAT, Lyon, France) 15 mg/kg per day on days 1 through 5; CyA 6 mg/kg per day from day 1 until at least day 180, with subsequent adjustment according to whole blood CyA concentration between 100 and 200 ng/mL; methylprednisolone (MePred) 2 mg/kg per day for 5 days, with subsequent halving of the dose every week until discontinuation on day 28 for prophylaxis of allergic reaction of ATG; and G-CSF (Filgrastim, Kirin, Tokyo, Japan) 400 $\mu g/m^2$ per day from day 1, with responding patients (neutrophil count $> 10^9$/L) receiving the same dose 3 times a week for 60 days (ATG/CyA/MePred/G-CSF). Patients with severe disease were given the same treatment regimen, with the exception that G-CSF was not given unless severe infection was documented (ATG/CyA/MePred).

The hematologic response was evaluated at 6 months after the initiation of therapy. A complete response (CR) was defined for all patients as a neutrophil count more than 1.5×10^9/L, a platelet count more than 100×10^9/L, and a hemoglobin level more than 11.0 g/dL.[8] A partial response (PR) was defined as a neutrophil count more than 0.5×10^9/L, a platelet count more than 20×10^9/L, a hemoglobin level more than 80 g/L (8.0 g/dL) and no requirement of blood transfusions. Patients with very severe or severe disease who failed to respond to initial IST underwent SCT if they had a serologically HLA-matched unrelated donor, HLA-one antigen mismatched family donor, or HLA-matched or HLA-one antigen mismatched unrelated cord blood donor at the time of evaluation. Those lacking a suitable donor received a second course of IST. A second course of IST consisted of the same regimen (horse ATG/CyA/MePred) used in the initial treatment of each patient. To reduce the risk of an anaphylactoid reaction to treatment with horse ATG, patients were initially given a 100-fold diluted dose of ATG as a test dose. An antihistamine was administered to all patients receiving a second course of IST to suppress allergic reactions.

The recommended conditioning regimen for SCT from an alternative donor consisted of cyclophosphamide (CY, 120 mg/kg), rabbit ATG (Thymoglobulin, IMTIX-SANGSTAT, 10 mg/kg), and total body irradiation (TBI, 10 Gy) or CY (3000 mg/m^2), rabbit ATG (10 mg/kg), fludarabine (100 mg/m^2), and local field irradiation (3 Gy).[16,17] Prophylaxis against graft versus host disease (GVHD) consisted of a combination of CyA (3mg/kg per day) or tacrolimus (0.02mg/kg per day) plus short-term methotrexate. CyA dose were adjusted to maintain whole blood concentration of 100 to 200 ng/mL and tacrolimus dose 5 to 10 ng/mL, respectively.

Informed written consent was obtained from all patients or their parents in accordance with the Declaration of Helsinki. The study was approved by the ethics committee of each participating hospital. The list of participating hospitals can be found in Document S1, (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Table 1. Pretreatment characteristics

	SAA	VSAA
Registered	86	119
Evaluable	84	117
Sex (M/F)	48/36	65/52
Median age, y (range)	8 (0-17)	9 (0-15)
Cause of AA		
Idiopathic	73	91
Hepatitis	8	24
Viral infection	1	2
Drug	2	0
Median days from diagnosis to treatment (range)	13 (1-94)	19 (1-179)

SAA indicates severe aplastic anemia; VSAA, very severe aplastic anemia

Statistical analysis

The primary end point of this study was failure-free survival (FFS) after second-line therapy, which was defined as survival with response. Death, no response by 6 months, disease progression requiring clinical intervention, or relapse were considered treatment failures.[18] Overall survival and FFS were analyzed using the Kaplan-Meier method. Differences between the 2 arms of the study were evaluated by the log-rank test. P less than .05 was considered statistically significant.

Results

Patient characteristics

From October 1997 to April 2004, 205 patients with newly diagnosed severe (n = 86) and very severe AA (n = 119) were enrolled in the AA-97 study (Table 1). An interim analysis was performed in April 2005. Four patients were excluded from further analysis for the following reasons: IST without ATG (2 patients) or stem cell transplantation within 4 months of diagnosis (2 patients). Two patients without any granulocytes were not treated with ATG because of severe infections; both of them died of fungal pneumonia within 2 months of diagnosis. Both patients who underwent SCT within 4 months of diagnosis died of graft rejection or cardiac toxicity to the preconditioning regimen. There were 2 further deaths within 6 months of patient registration: hemolysis of unknown cause and aspiration pneumonia. None of the patients was diagnosed with PNH at the time of registration. Severe and very severe AA were associated with hepatitis in 32 patients, with other viral infection in 3 patients, and with medication use in 2 patients. The median days (range) from diagnosis to treatment of severe and very severe AA were 13 (1-94) days and 19 (1-179) days, respectively (Table 1).

Trilineage hematologic response

At 3 months after the initiation of therapy, 49 patients (58%) with severe AA and 46 patients (39%) with very severe AA had

Table 2. Response to treatment after initial treatment

	SAA	VSAA
3 months		
Evaluable	84	117
CR	9*	6†
PR	40*	40†
NR	35	71
Alive	34	71
Dead	1	0
6 months		
Evaluable	83	115
CR	17‡	20§
PR	38‡	63§
NR	28	32
Alive	27	31
Dead	1	1

Data are numbers (%) of responders.
NR indicates no response.
*These classes combined were 58% to the total number.
†These classes combined were 39% to the total number.
‡These classes combined were 66% to the total number.
§These classes combined were 72% to the total number

responded to the initial course of IST (Table 2). By 6 months, 55 patients (66%) with severe AA and 83 patients (72%) with very severe AA had evidence of a trilineage response and had become transfusion-independent. Three patients died between 3 and 6 months. Overall, of 198 evaluable patients receiving an initial course of IST, 37 patients (19%) had a complete response and 101 patients (51%) showed a partial response, for an overall response rate of 70% after 6 months. Sixty patients (30%), 28 (34%) with severe AA and 32 (28%) with very severe AA, did not attain CR or PR status at 6 months, and were therefore eligible for second-line therapy (Fig 1).

Repeated IST versus SCT as second-line therapy

Figure 1 shows the outcome of 201 patients with treatment assigned. Twenty-one patients lacking a suitable donor at the time of evaluation were assigned to receive a second course of IST. Three of these patients developed an anaphylactoid reaction to ATG and thus could not complete their second course of treatment. Anaphylactoid reactions were not observed during the first course of IST in these 3 patients. These patients were

Table 3. Characteristics of 52 patients who underwent second-line therapy

	SAA	VSAA
Patients	21	31
Sex, M/F	14/7	14/17
Median age, y (range)	9 (2-17)	8 (0-17)
Cause of AA		
Idiopathic	17	29
Hepatitis	4	2
Severity of disease		
SAA	7	21
VSAA	14	10
Median months from diagnosis to second-line therapy (range)	7 (5-25)	8 (5-20)

Data are numbers except where indicated.

subsequently treated with corticosteroids, which rapidly resolved their symptoms. Among them, 1 patient died from complications of severe pancytopenia and 2 patients are alive with a late hematologic response.

Thirty-one patients received SCT from an alternative donor as follows: BMT from an HLA-matched unrelated donor (UBMT; n = 25), cord blood transplantation from an unrelated donor (UCBT; n = 2), and BMT from an HLA-mismatched family donor (n = 4). Twenty patients were conditioned with a CY, ATG, and TBI regimen and 4 received CY, Flu, ATG, and local field irradiation. Others received other types of conditioning regimen. Methotrexate and CyA were given for the prevention of GVHD in 5 patients. Tacrolimus was used instead of CyA in other patients. Five patients who had transformed to myelodysplastic syndrome (MDS) and 3 patients who were searching for an alternative donor still were excluded from the analysis.

In all, 52 patients were evaluated for response to second-line therapy. Characteristics of both groups are shown in Table 3. The median interval between the first course of IST and a second-line treatment was 7 months for the IST group and 8 months for the SCT group. At 6 months after the initiation of second IST, a trilineage response was seen in only 2 of 18 evaluable patients (11%). Among 16 nonresponders, 8 patients received UBMT as a third-line therapy and all of them are alive. They could not find a suitable donor at 6 months after initial therapy and received a second IST, but failed to respond.

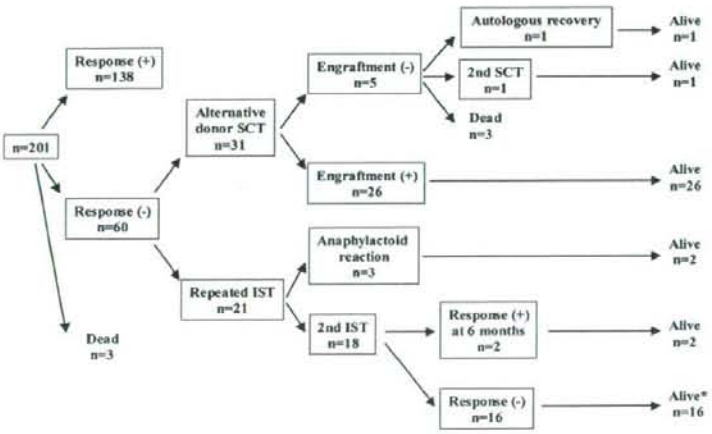

Figure 1. Overall outcome of 201 patients assigned to second-line therapy. *Among 16 patients who failed to respond to second IST, 8 patients received SCT and were alive. Four of the remaining 8 patients attained a late hematologic response and were alive. The other 4 patients were alive without response.

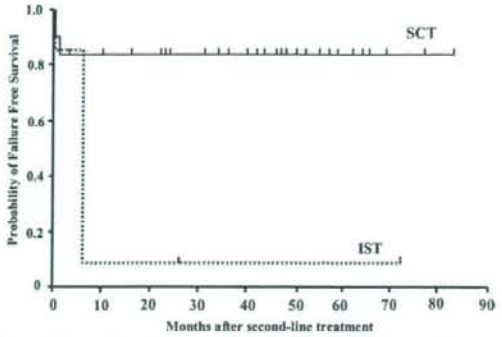

Figure 2. Actuarial probability of failure-free survival after second-line treatments with immunosuppressive therapy (n = 21) or stem-cell transplantation from an alternative donor (n = 31). FFS is defined as survival with response. Death, nonresponse by 6 months, disease progression requiring a second-line therapies, and relapse were considered as treatment failure.

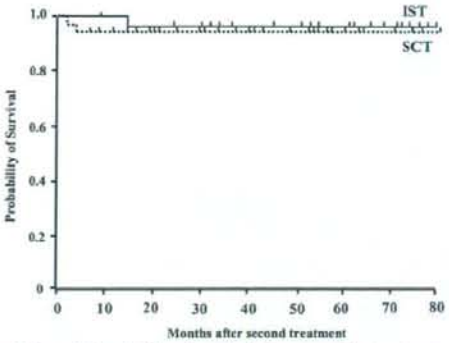

Figure 3. Actuarial probability of survival after second-line treatments with immunosuppressive therapy (n = 21) or stem cell transplantation from an alternative donor (n = 31).

Marrow donors included HLA-one antigen mismatched unrelated donor (n = 3) and HLA serologically 6/6 matched unrelated donor (n = 5). Four patients attained late response and another 4 patients are alive with regular blood transfusions. Overall, 20 of 21 patients are alive with a median follow-up period of 66 months from the start of second IST (range: 9-80 months).

In the SCT group, 5 patients did not engraft. Bacterial or fungal infections resulted in the death of 2 patients at an early phase of SCT. One patient who received UCBT had recovery of autologous bone marrow function and is alive 68 months after the transplant. One patient transplanted from an HLA-mismatched sibling had a successful second transplant from an unrelated donor. Another patient who failed to engraft after UBMT was rescued by second transplant from an HLA-2 antigen mismatched mother. Blood count normalized in the remaining 26 patients and they are all alive. Four evaluable patients developed grade II to IV acute GVHD, and chronic GVHD was observed in 4 patients. Twenty-nine of 31 patients are alive with a median follow up period of 35 months from the alternative donor transplantation (range: 4-83 months). The probability of FFS was calculated after excluding deaths and patients failing to respond to a second-line treatment by 6 months and requiring further treatment, that is, including only patients who were alive with hematologic response. The estimated FFS at 5 years from the beginning of second-line therapy was 83.9% (± 16.1% SD) in the SCT group compared with 9.5% (± 9.0%) in the IST group (P = .001) (Fig 2). The overall survival rate was not different between the IST group (95.2 ± 6.7%) and the SCT group (93.5 ± 4.2%) after second-line treatment (Fig 3).

Cytogenetic analysis and clonal disease

At the time of diagnosis, a clonal cytogenetic abnormality (monosomy 7, trisomy 8) was detected in 2 patients, who had morphologically typical AA. The disappearance of monosomy 7 was observed in 1 patient,[19] but trisomy 8 remained for 52 months after IST in another patient. New clonal cytogenetic abnormalities appeared in 10 patients after IST: monosomy 7 (5 patients), trisomy 8 (2 patients), trisomy 8 and del(7) (1 patient), monosomy X (1 patient), and t (3;3)(q21;q26) (1 patient). Eight patients underwent SCT from alternative donors and 6 of them are still alive.

Stem-cell transplantation

SCT was attempted in 52 patients in whom the initial IST failed (n = 31), the second IST failed (n = 8), who had relapse after initial response (n = 5), or who developed MDS and leukemia (n = 8). Alternative donors included unrelated bone marrow donors (n = 40), HLA-mismatched family donors (n = 6), and unrelated cord blood donors (n = 6). Five patients died: 3 received UCBT and 2 received UBMT. Causes of death were bacterial or fungal infections (n = 3), relapse of leukemia (n = 1), and venoocclussive disease (n = 1).

Survival

We analyzed the actuarial survival of 201 enrolled patients according to the severity of their disease. The actuarial survival of all enrolled patients was 94.5% (± 1.7%) with a median follow-up period of 48 months (range: 12-90 months). The actuarial survival was 92.6% (± 2.8%) in the 117 patients with very severe AA and 96.8% (± 2.1%) in the 84 patients with severe AA. There were 6 deaths in the very severe AA group and 3 in the severe AA group. The causes of death were SCT-related toxicities (n = 5), MDS/acute myelogenous leukemia (AML) (n = 1), bacteremia (n = 1), hemolysis of unknown causes (n = 1), and aspiration pneumonia (n = 1).

Discussion

The introduction of intensive IST with ATG and CyA has dramatically improved the outcome of patients with severe and very severe AA.[5-8] However, 30% to 40% of patients still fail to respond to IST and require second-line therapy. The treatment options for patients not responding to IST include further treatment with immunosuppressive agents or SCT from an alternative donor. At present, however, there is no consensus as to the best therapy for these patients. Recent studies have reported a high response rate and a favorable outcome after repeated ATG therapy in these patients, suggesting that SCT from an alternative donor should perhaps be considered third-line therapy.[10,11] However, the majority of patients in these studies were adults. Because the outcome after alternative donor transplantation is better in children than in adults,[20] the treatment choice may be different in children from in adults. Our prospective study showed that SCT from an alternative donor is superior to the repeated IST for FFS. At 6 months, the response rate to a second course of IST was only 11% (2/18), which increased to 33% (6/18) at

12 months, much lower than figures reported by others. The overall response was 63% in the Basel[10] and 77% in the Italian[11] studies. In addition, none of our patients achieved CR, whereas CR was achieved in 42% of patients in the Basel,[10] and 30% in the Italian study.[11] In the recent study from the National Institutes of Health, the overall response rate was 30%, although no one achieved a CR.[12] The reasons for the discrepancy in the response rates among these studies are not known. However, there are a number of differences between our study (AA-97) and the others. First, our study group consisted of only pediatric patients, whereas other studies included both pediatric and adult patients. The median age of the patients was 9, 15, 18, and 31 years, in the AA-97, Basel, Italian, and National Institutes of Health studies, respectively. Until now, there have been no reports of repeated IST restricted to children with AA.

In the majority of patients with acquired AA, bone marrow failure is believed to result from immunologically mediated destruction of the hematopoietic progenitor cells.[21] Whereas in some patients, a single course of ATG is not sufficient to achieve the degree of immunosuppression required to restore bone marrow function, necessitating further ATG therapy, the results of our study may indicate that pediatric patients are more susceptible than adult patients to the intensive IST currently used and that a single course of IST is adequate to discern their response to these immunosuppressive agents. Our results may also suggest that the efficacy of any immunosuppressive therapy for children with AA should be evaluated separately from adult patients.[22]

In the Italian study,[11] the assessment of response to the first course of IST was carried out at 120 days and some patients received a second course of treatment as early as 2 months after initiation of immunosuppressive therapy. In our previous study (AA-92), we observed no further patient response to an initial course of IST after 6 months, thus making the time of assessment at 6 months.[8] The timing of the evaluation of response to an initial course of IST is an important factor in determining the need for further treatment, making it difficult to compare the response rates to a second course of IST between our study and other studies.

In the AA-97 study, 31 severe and very severe patients who did not respond to immunosuppressive therapy received SCT from an alternative donor. Twenty-nine of these patients are alive with their bone marrow function restored. Importantly, all 26 engrafted patients are alive without failure. Of 2 patients who received UCBT, 1 died of fungal infection before engraftment, and the other reconstituted autologous bone marrow function. In a recent analysis of a large series of UCBT from the New York Blood Center, only 8 of 19 patients with severe AA engrafted after UCBT. The cohort of AA patients was among the group with the highest incidence of transplant-related mortality.[23] Because of discouraging results in the early period, we thereafter recommend that UCBT not be used as a second-line therapy. In contrast, in our study, results after BMT from an unrelated donor were excellent. Twenty-four of the 25 patients are alive and well. The National Marrow Donor Program in the United States reported on the results of UBMT for IST-resistant AA patients.[14] Fifty-one of 131 patients (39%) were alive at 11 to 94 months (median: 36 months) after transplantation. The major causes of death were graft failure and treatment-related events including GVHD and infections. Fifty-five patients were matched with donors using both serology and allele-level DRB1 typing; these patients had a survival rate of 56%. In a recent report from the Japan Marrow Donor Program, the overall survival rate for AA patients receiving HLA-matched unrelated BMT was 56%, with 81% survival in patients younger than 15 years and 32% survival in patients aged 16 and older.[20] Therefore, younger patients clearly have a survival advantage after UBMT. Similarly, in our AA-92 study, 13 of 15 patients who failed IST and who were subsequently treated with UBMT are alive and well, with a median follow-up of 36 months.[8] The duration of FFS of these pediatric patients with AA appeared to plateau at 2 years after SCT. Recently, 2 novel transplant regimens were reported: one from the United States and another from Europe.[24,25] The first tested de-escalating doses of radiation from 6 Gy to 2 Gy. The best results were achieved with 2 Gy TBI. The European group designed a radiation-free preparative regimen consisted of fludarabine, cyclophosphamide, and ATG. The Japan Marrow Donor Program is now performing high-resolution HLA typing at the DNA level at loci A and B as well as DRB1. It is expected that more precise HLA matching between patient and donor will further improve the outcome for UBMT recipients.

On the other hand, IST appears to be associated with an increased risk of evolution of clonal diseases such as MDS and PNH.[26,27] In our previous study, 11 of 50 children with AA that were treated with IST developed MDS/AML. None of the 48 patients who underwent SCT developed a clonal disorder.[28] In the Basel study, clonal disease developed in 53% of patients who received multiple courses of IST.[10] In the current study, there were 5 patients (8.3%) with MDS among 60 patients in whom initial IST was not effective, whereas no patient developed MDS/AML after SCT from an alternative donor treated as second-line therapy. This issue must be taken into consideration in the discussion of the appropriate second-line therapy for patients with AA.

Our study clearly demonstrates that SCT from an alternative donor provides a better chance of FFS than a second course of IST for children with AA who have failed to respond to an initial course of IST. Thus, we recommend SCT, and in particular BMT from an alternative donor, rather than a second course of IST as salvage therapy for these patients.

Authorship

Contribution: F.B., T.N., I.T., and S. K. designed the study. Y.K., H.Y., K.S., and S.K. analyzed results and wrote the manuscript. R.K., H.A., T.K., H.Y., M.T., H.M., A.O., A.M., Y. O., and S.O. enrolled the patients.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Seiji Kojima MD, PhD, Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan; e-mail: kojimas@med.nagoya-u.ac.jp.

References

1. Locasciulli A, van't Veer L, Bacigalupo A, et al. Treatment with marrow transplantation or immunosuppression of childhood acquired severe aplastic anemia: a report from the EBMT SAA Working Party. Bone Marrow Transplant. 1990;6: 211-217.
2. Doney K, Leisenring W, Storb R, et al. Primary treatment of acquired aplastic anemia: Outcomes with bone marrow transplantation and immunosuppressive therapy. Ann Intern Med. 1997;126: 107-115.
3. Bacigalupo A, Brand R, Oneto R, et al. Treatment of acquired severe aplastic anemia: bone marrow transplantation compared with immunosuppressive therapy. The European Group for Blood and Marrow Transplantation experience. Semin Hematol. 2000;37: 69-80.

4. Kojima S, Horibe K, Inaba J, et al. Long-term outcome of acquired aplastic anaemia in children: comparison between immunosuppressive therapy and bone marrow transplantation. Br J Haematol. 2000;111:321-328.
5. Frickhofen N, Kaltwasser JP, Schrezenmeier H, et al. Treatment of aplastic anemia with antithymocyte globulin and methylprednisolone with or without cyclosporine. N Engl J Med. 1991;324: 1297-1303.
6. Rosenfeld SE, Kimball J, Vining D, Young NS. Intensive immunosuppression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anemia. Blood. 1995;85:3058-3065.
7. Bacigalupo A, Bruno B, Saracco P, et al. Antilymphocyte globulin, cyclosporine, prednisolone, and granulocyte colony-stimulating factor for severe aplastic anemia: an update of the GITMO/EBMT study on 100 patients. European Group for Blood and Marrow Transplantation (EBMT) Working Party on Severe Aplastic Anemia and the Gruppo Italiano Trapianti di Midollo Osseo (GITMO). Blood. 2000;95:1931-1934.
8. Kojima S, Hibi S, Kosaka Y, et al. Immunosuppressive therapy using antithymocyte globulin, cyclosporine, and danazol with or without human granulocyte colony-stimulating factor in children with acquired aplastic anemia. Blood. 2000;96: 2049-2054.
9. Schrezenmeier H, Marin P, Raghavachar A, et al. Relapse of aplastic anemia after immunosuppressive treatment: A report from the European Bone Marrow Transplantation Group SAA Working Party. Br J Haematol. 1993;85:371-377.
10. Tichelli A, Passweg J, Nissen C, et al. Repeated treatment with horse antilymphocyte globulin for severe aplastic anaemia. Br J Haematol. 1998; 100:393-400.
11. Di Bona E, Rodeghiero F, Bruno B, et al. Rabbit antithymocyte globulin (r-ATG) plus cyclosporine and granulocyte colony stimulating factor is an effective treatment for aplastic anaemia patients unresponsive to a first course of intensive immunosuppressive therapy. Br J Haematol. 1999;107: 330-334.
12. Scheinberg P, Nunez O, Young NS. Retreatment with rabbit anti-thymocyte globulin and cyclosporine for patients with relapsed or refractory severe aplastic anemia. Br J Haematol. 2006;133:622-627.
13. Margolis D, Camitta B, Pietryga D, et al. Unrelated donor bone marrow transplantation to treat severe aplastic anaemia in children and young adults. Br J Haematol. 1996;94:65-72.
14. Deeg HJ, Seidel K, Casper J, et al. Marrow transplantation from unrelated donors for patients with severe aplastic anemia who have failed immunosuppressive therapy. Biol Blood Marrow Transplant. 1999;5:243-252.
15. Camitta BM, Thomas ED, Nathan DG, et al. A prospective study of androgens and bone marrow transplantation for treatment of severe aplastic anemia. Blood. 1979;53:504-514.
16. Kojima S, Inaba J, Kondo M, Kato K, Matsuyama T, Horikoshi Y & Mimaya J. Unrelated donor marrow transplantation for severe acquired aplastic anemia using cyclophosphamide, anti-thymocyte globulin, and total body irradiation. Br J Haematol. 2001;114:706-711.
17. Yabe H, Kato S, Yabe M, et al. Comparison of conditioning regimens with or without total body irradiation in bone marrow transplantation from an alternative donor. Blood. 2004;104 (Suppl. 2): 377b.
18. Marsh J, Schrezenmeier P, Marin O, et al. Prospective randomized multicenter study comparing cyclosporine alone versus the combination of antithymocyte globulin and cyclosporine for treatment of patients with nonsevere aplastic anemia: A report from the European Blood and Marrow Transplant (EBMT) Severe Aplastic Working Party. Blood. 1999;93:2191-2195.
19. Nagasawa M, Tomizawa D, Tsuji Y, et al. Pancytopenia presenting with monosomy 7 which disappeared after immunosuppressive therapy. Leuk Res. 2004;28:315-319.
20. Kojima S, Matsuyama T, Kato S, et al. Outcome pf 154 patients with severe aplastic anemia who received transplants from unrelated donors: the Japan Marrow Donor Program. Blood. 2002;100: 799-803.
21. Young NS. Hematopoietic cell destruction by immune mechanism in acquired aplastic anemia. Semin Hematol. 2000;37:3-14.
22. Reddy V, Khan S, Wingard JR. Treatment results in aplastic anemia trials need to be analyzed separately for pediatric and adult populations. Blood. 1999;94:1833-1834.
23. Rubinstein P, Carrier C, Scaradavou A, et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. N Engl J Med. 1998;339:1565-1577.
24. Deeg HJ, O'Donnell M, Tolar J, et al. Optimization of conditioning for marrow transplantation from unrelated donors for patients with aplastic anemia after failure of immunosuppressive therapy. Blood. 2006;108:1485-1491.
25. Bacigalupo A, Locatelli F, Lanino E, etal. Fludarabine, cyclophosphamide and anti-thymocyte globulin for alternative donor transplants in acquired severe aplastic anemia: a report from the EBMT-SAA Working Party. Bone Marrow Transplant. 2005;36:947-950.
26. Tichelli A, Gratwohl A, Wursch A, Nissen C, Speck B. Late haematological complications in severe aplastic anaemia. Br J Haematol. 1988; 69:413-418.
27. Socié G, Amar MH, Bacigalupo A, et al. Malignant tumors occurring after treatment of aplastic anemia. N Engl J Med. 1993;329:1152-1157.
28. Ohara A, Kojima S, Hamajima N, et al. Myelodysplastic syndrome and acute myelogenous leukemia as a late clonal complication in children with acquired aplastic anemia. Blood. 1997;90:1009-1013.

Ataxia telangiectasia の臨床的特徴

—全国調査から明らかになったこと—

東京医科歯科大学大学院発生発達病態学分野

森尾　友宏

小児科臨床別刷

61：2008—9

特集

9．Ataxia telangiectasia の臨床的特徴

—全国調査から明らかになったこと—

東京医科歯科大学大学院発生発達病態学分野　森尾友宏（もりお ともひろ）

KEY WORDS
ataxia telangiectasia
ATM
diagnosis
cerebellar ataxia
immunodeficiency

Tomohiro Morio

はじめに

Ataxia telangiectasia（以後 AT と省略）は「毛細血管拡張性小脳失調症」と訳されている，頻度は高くないが，幼児期からの小脳失調症で鑑別すべき重要な疾患である。毛細血管拡張症は遅れて出現するが，進行性の小脳失調症に加えて，高頻度の悪性腫瘍発生，免疫不全症が臨床上大きな問題になる[1)2)]。

発症頻度は欧米では40,000～100,000人に1人とされている。したがって保因者は100人から160人に1人程度であると推測される。責任遺伝子は11番染色体上の ATM（Ataxia telangiectasia mutated）であり，常染色体劣性の遺伝形式をとる。

Ⅰ．病態生理[1)3)]

ATM は DNA 損傷修復反応（DNA damage response：DDR），特に二重鎖 DNA（double strand DNA：dsDNA）切断修復に重要な役割を果たす分子であり，生体にとって危害の大きい dsDNA 切断に際して活性化し，下流の様々な鍵となる分子をリン酸化することにより，細胞周期を制御し，DNA 切断修復あるいはアポトーシスに関与する。dsDNA 切断は，Mre11/Rad50/NBS 1（MRN 複合体）によって感知され，ATM を損傷の場に誘導する。ATM は MRN 複合体をリン酸化するとともに，Chk 2，SMC 1，p53などの分子をリン酸化して，ダ

〒113-8519　東京都文京区湯島1-5-45

メージの加わった細胞周期や，刺激の種類に応じて，さらに細胞周期停止，非相同 DNA 末端結合，相同組換え，細胞死などを誘導する。

DNA 損傷は電離放射線，紫外線，薬剤などの外的要因のみならず，日々の細胞代謝（活性酸素産生，複製停止など）において観察される事象である。1つの細胞において1日に10,000のプリン残基が欠失しているとされている。dsDNA 切断はその回数はさらに少ないが，細胞分裂や，遺伝子再構成・体細胞突然変異など免疫学的多様性を生み出す機構において必須で，そのたびに DDR が起きていることになる。

このように ATM は，DNA 損傷を受ける場において発動・活性化し，腫瘍化のバリアとして働いているので，その欠損は高頻度悪性腫瘍発生という表現型として観察される。また V（D）J 再構成，免疫グロブリンクラススイッチ（Immunoglobulin class switch recombination：IgCSR）においても，ATM がシナプス形成や切断修復に関与するために，リンパ球発生や CSR に関与する。一方，ATM は免疫グロブリン体細胞突然変異（Immunoglobulin somatic hypermutation：SHM）には不可欠でないことが示されている[4]。

一番大きな問題である小脳失調に関しては，Purkinje 細胞の異常が認められるものの，なぜ小脳に比較的特異的に症状が出現するのかは未だに大きな謎である。ATM 欠損マウスで明らかな小脳失調が現れないことも研究の進展を阻んでいるといえる。

II．日本での AT 患者全国調査

日本での AT 患者の実態が明らかでなかったために，2005年9月に厚生労働省難治性疾患克服研究事業「原発性免疫不全症候群に関する調査研究」班の活動のなかで，初めての全国調査を行った。全国665の病院の神経内科，小児科，血液腫瘍科，リハビリ科などを中心とする1,223部局にアンケートを送付したところ，92名の AT の可能性のある患者について一次情報が集まった。さらに二次アンケートを行い，より詳細な情報を収集したところ（**表1**），重複患者，AT ではない方を除外して合計89名の AT 患者が登録された。そのうち10名では，説明と同意の元に，ATM タンパク質発現解析および遺伝子変異解析を行った。

神経内科医，小児科医フォローアップの比率はほぼ半々であるが，小児科では神経専門医による診療が多く，初発症状からも理解できる。悪性腫瘍の発生，感染症の反復をもって血液・腫瘍・免疫専門医と併診になることが多いようである。

表1　二次アンケート内容

基本情報
- 生年月，性別
- 家族歴（悪性腫瘍）
- 診断年月日，経過および死亡原因

臨床症状
- 小脳失調（体幹失調，構語障害，流涎，眼球運動の失行あるいは眼振，車いすの使用，舞踏病，振戦）と発症時期
- 毛細血管拡張，部位と発症時期
- 感染症の種類と発症時期
- 悪性腫瘍の種類・表現型・遺伝子異常・治療の有害事象，治療結果
- 自己免疫疾患・内分泌疾患（糖尿病，甲状腺機能，そのほか）
- そのほかの特記すべき症状

検査所見
- 遺伝子変異
- 染色体異常とその種類
- 免疫学的所見：リンパ球サブセット・TCR レパートア，免疫グロブリン値・EBV 抗体価
- 検査データ（α-fetoprotein，CEA）
- 脂質・代謝関連所見およびデータ
- 知能テスト・画像所見

治療内容