A Good Manufacturing Practice procedure to engineer donor virus-specific T cells into potent anti-leukemic effector cells

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ABSTRACT

A sequential, two-step procedure in which T-cell-depleted allogeneic stem cell transplantation is followed by treatment with donor lymphocyte infusion at 6 months can significantly reduce the risk and severity of graft-*versus*-host disease, with postponed induction of the beneficial graft-*versus*-leukemia effect. However, patients with high-risk leukemia have a substantial risk of relapse early after transplantation, at a time when administration of donor lymphocytes has a high likelihood of resulting in graft-*versus*-host disease, disturbing a favorable balance between the graft-*versus*-leukemia effect and graft-*versus*-host disease. New therapeutic modalities are, therefore, required to allow early administration of T cells capable of exerting a graft-*versus*-leukemia effect without causing graft-*versus*-host disease. Here we describe the isolation of virus-specific T cells using Streptamer-based isolation technology and subsequent transfer of the minor histocompatibility antigen HA-1-specific T-cell receptor using retroviral vectors. Isolation of virus-specific T cells and subsequent transduction with HA-1-T-cell receptor resulted in rapid *in vitro* generation of highly pure, dual-specific T cells with potent anti-leukemic reactivity. Due to the short production procedure of only 10-14 days and the defined specificity of the T cells, administration of virus-specific T cells transduced with the HA-1-T-cell receptor as early as 8 weeks after allogeneic stem cell transplantation is feasible. *(This clinical trial is registered at www.clinicaltrialsregister.eu as EudraCT number 2010-024625-20).*

Introduction

Patients with hematologic malignancies can be successfully treated with human leukocyte antigen (HLA)-matched allogeneic stem cell transplantation (SCT).[1] To reduce the development of graft-*versus*-host disease (GvHD), donor T cells can be depleted from the stem cell graft, and re-administered preemptively after the allogeneic SCT.[2] Although this two-step procedure of T-cell-depleted allogeneic SCT and donor lymphocyte infusion (DLI) reduces the incidence and severity of GvHD compared to non-T-cell-depleted allogeneic SCT, GvHD remains an important cause of morbidity and mortality, particularly in the setting of HLA-mismatched transplantation. The risk of inducing GvHD is even higher when DLI is administered early after allogeneic SCT. Patients with high-risk leukemia are likely to relapse early after transplantation, at a time when administration of DLI is likely to result in GvHD. Treatment options are scarce for this patient population and new therapeutic modalities are required to allow early administration of T cells capable of exerting a graft-*versus*-leukemia (GvL) effect without causing GvHD.

Adoptive transfer of T cells with defined anti-leukemia specificity is a strategy to dissect GvHD responses from GvL responses. It has been demonstrated that donor T cells recognizing minor histocompatibility antigens (MiHA) selectively expressed on hematopoietic cells mediate anti-leukemic reactivity after allogeneic SCT without causing severe GvHD.[3,4] The HA-1-T-cell receptor (TCR) is specific for the MiHA HA-1, which is presented in the context of HLA-A*0201[5] and was among the first MiHA described to be expressed solely on cells of the hematopoietic system and to be present on clonogenic leukemic precursor cells.[6-8] HA-1 MiHA expression can induce high-affinity T-cell responses *in vivo* in HLA-A*0201+ and HA-1$^+$ patients who received an allogeneic SCT from a HLA-A*0201$^+$ but HA-1$^-$ donor.[9-12] Previously, a direct association was shown between the emergence of MiHA HA-1 tetramer$^+$ cytotoxic T cells and the complete disappearance of malignant recipient cells in MiHA HA-1 incompatible donor–recipient pairs.[4] We have recently presented the results of our phase I clinical study in which the toxicity and the potential anti-leukemic effect of treatment with HA-1-specific cytotoxic T lymphocyte lines was examined in three patients with a leukemic relapse following allogeneic SCT.[14] The administration of HA-1-specific T-cell lines was demonstrated to be safe without induction of GvHD. However, HA-1-specific T-cell lines lacked *in vivo* persistence and *in vivo* anti-leukemic reactivity. This lack of persistence and anti-leukemic reactivity may be explained by the long culture period of at least 4 weeks.

TCR gene transfer is an attractive strategy to modify T cells with well-defined specificities in a short time period. Recently, the effectiveness of TCR transfer was demonstrated in patients with melanoma or synovial cell sarcoma who were treated with TCR-modified autologous T cells.[15-17] To engineer T cells that exert selective GvL without GvHD, we prefer to transfer the HA-1-TCR into virus-specific T cells instead of polyclonal T cells. It has been described that both cytomegalovirus (CMV)-specific[18-23] and Epstein-Barr virus (EBV)-specific[24-29] donor T cells can be safely reinfused into immunodeficient patients at risk of developing CMV disease,

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EBV reactivation or EBV-positive B-cell lymphomas, respectively. This adoptive transfer was demonstrated not only to be effective in preventing or curing the viral diseases but also to be safe without inducing GvHD. In addition, long-term persistence of the virus-specific donor T cells was demonstrated.[26] We hypothesize that *in vivo* activation of the endogenous TCR by viral antigens can result in both increased numbers of TCR-modified T cells, as well as in increased introduced TCR expression, as T-cell stimulation is followed by increased activation of the retroviral promotor.[30-32] Previously, we demonstrated that we could reprogram virus-specific T cells into anti-leukemic effector T cells using TCR gene transfer without loss of their original anti-virus specificity.[33,34] Another possible advantage of the use of virus-specific T cells is the exclusion of regulatory T cells from the pool of TCR-modified lymphocytes that can possibly disturb the immune reaction. Since virus-specific T-cell populations consist of a restricted TCR repertoire,[35,36] the number of different mixed TCR dimers formed will be limited and from *in vivo* data this appears a viable strategy to prevent neoreactivity[37] caused by mixed TCR dimers.[37,38] Furthermore, we have modified the HA-1-TCR both to improve cell surface expression of the HA-1-TCR, and to diminish mixed TCR dimer expression with unknown and potentially unwanted reactivity.[38,39] For the clinical study, we will selectively isolate permissive virus-specific T cells that highly express HA-1-TCR after gene transfer (Table 1).[39,40]

Recently, Streptamers were used to selectively isolate CMV-specific T cells.[41] CMV-specific T cells were transferred directly after Streptamer-based isolation into patients with CMV reactivation without toxicity, and patients were able to manage CMV virus thereafter.[41] Here, we describe a Good Manufacturing Practice (GMP) procedure to rapidly generate dual-specific, donor virus-specific T cells with high avidity anti-leukemic reactivity. The process of Streptamer-based isolation of pure populations of virus-specific T cells and transduction with GMP-grade retroviral supernatant encoding the HA-1-TCR has been validated with four large-scale test procedures in the cleanroom. All HA-1-TCR-transduced, virus-specific T-cell products met the criteria for in process testing and quality control testing, and were highly reactive against HA-1-positive leukemic cells.

Methods

Selection and isolation of virus-specific T cells

This study was approved by the Leiden University Medical Center institutional review board and written informed consent was obtained according to the Declaration of Helsinki. From donor leukocytes from a leukapheresis product or total peripheral blood mononuclear cells either one or two virus-specific T-cell populations were isolated using Streptamers (Table 1) (Stage Therapeutics, Götingen, Germany) according to the manufacturer's instructions. Streptamer-incubated donor leukocytes were purified using autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' protocol, or in the case of the test-runs using a CliniMACS instrument (Miltenyi Biotec) with the "CD34 selection 1" program. Streptamers were dissociated from the eluted cells with 1 mM D-biotin. Eluted cells purified by either auto-MACS or CliniMACS were cultured with irradiated, non-selected, autologous peripheral blood mononuclear cells (ratio 1:5) in T-cell medium consisting of IMDM supplemented with 10% ABOS, 100 IU/mL interleukin-2 (Chiron, Amsterdam, the Netherlands), and 10 ng/mL interleukin-15 (Peprotech, Rocky Hill, NJ, USA). Anti-CD3/CD28 beads (ratio 5:1, Dynabeads, Invitrogen) were added in some of the experiments.

Transduction of the virus-specific T cells

Some of the virus-specific T cells were transduced 2-3 days after MACS-isolation with vectors containing only a NGF-R marker gene, or with GMP-grade retroviral supernatant encoding the HA-1-TCR (EUFETS GmbH, Idar Oberstein, Germany), as previously described using GMP-grade retronectin (end concentration 30 μg/mL; Takara Biotec, Otsu, Japan).[39] For this purpose, 24-well clear flat-bottomed ULA microplates (Corning Inc, Corning, NY, USA) were pre-coated with retronectin (0.5 mL/well). Viral supernatant (0.5 mL/well) was spun down at 3,000 rpm for 20 min at 4°C. T cells ($0.5x10^6$ per well) were added in 0.5 mL T-cell medium and incubated for at least 4 h.

Tetrameric HLA class I-peptide complexes, flowcytometric analyses

Phycoerythrin- or allophycocyanin-conjugated tetrameric complexes were constructed with minor modifications, as described previously.[42] Table 1 provides an overview of the peptides and multimers used in this study, and the monoclonal antibodies used are described in more detail in the *Online Supplementary Data*. For flowcytometric analyses, cells were labeled with tetramers for 1 h, and monoclonal antibodies for 30 min at 4°C. Cells were analyzed on a FACS Calibur, and data were analyzed using FlowJo software. For the combinatorial coding analysis,[43,44] cells were analyzed on a FACS LSR-II, and data were analyzed using FACS-Diva software.

Functional analysis

At the end of the culture period (day 10-14), purified virus-specific T cells transduced with the HA-1-TCR were tested for antigen-specific interferon (IFN)-γ production in a standard enzyme-linked immunosorbent assay (CLB, Amsterdam, the Netherlands). Five thousand T cells were tested against 20,000 target cells. In addition, T cells were co-cultured with target cells at different effector-to-target ratios and cytotoxic reactivity was determined after 5 h.[40] The tests were done in triplicate. Target cells used were HA-1 or virus peptide (Table 1) pulsed T2 cells, HLA-A*0201$^+$ HA-1$^+$ or HLA-A*0201$^+$ HA-1– primary acute lymphoblastic leukemia (ALL) cells and primary acute myeloid leukemic (AML) cells.

Statistics

Experimental data were evaluated in a paired fashion using the Student T-test. Reported *P* values are two-sided and were considered statistically different if <0.01.

Table 1. List of different peptide-HLA complexes used for FACS analysis and MACS-isolation.

Protein	Peptide	HLA-restriction
CMV pp50	VTEHDTLLY	A*0101
CMV pp65	NLVPMVATV	A*0201
CMV pp65	RPHERNGFTVL	B*0702
CMV IE-1	QIKVRVDMV	B*0801
EBV BMLF-1	GLCTLVAML	A*0201
EBV EBNA3A	RPPIFIRRL	B*0702
EBV BZLF-1	RAKFKQLL	B*0801
HA-1	VLHDDLLEA	A*0201

Results

Enrichment of virus-specific T-cell populations after Streptamer incubation followed by MACS-separation

The HA-1-TCR-transduced T-cell product will be adoptively transferred in an allogeneic setting. We, therefore, attempt to minimize the amount of polyclonal T cells with potential GvHD-inducing specificity that we infuse. In this regard, it is important that we transduce purified virus-specific T-cell populations that harbor a known and harmless specificity.

To test whether we could isolate highly enriched virus-specific T cells using Streptamers, we performed 12 test isolations (Table 2). The frequency of virus-specific T cells in the starting material ranged from 0.023% - 2.6% (median 0.69%) of total lymphocytes. Peripheral blood mononuclear cells were incubated with either one or two different Streptamers simultaneously, and virus-specific T cells were purified over a MACS column. Directly after isolation, Streptamers were dissociated from the isolated T cells using D-biotin, and T cells were stained using relevant tetramers and analyzed by flow cytometry. The purity of isolated virus-specific T cells directly after isolation ranged from 34.9% - 92.1% (median 64.0%) for single virus-specific T-cell enrichments and 60.9% - 80.8% (median 71.9%) for double virus-specific T-cell enrichments (Table 2). We observed a correlation between purity of virus-specific T cells after isolation and the frequency of virus-specific T cells in peripheral blood. For example, the lowest purity of 34.9% after single virus-specific T-cell enrichment was observed after isolation from peripheral blood mononuclear cells containing a relatively low frequency of 0.03% pp50^{A1}-specific T-cells. These results indicate that we can efficiently enrich virus-specific T-cell populations from peripheral blood mononuclear cells of healthy individuals using Streptamers followed by MACS-isolation, provided that a profound population (>0.05%) is present in the starting material.

MACS-separated virus-specific T cells can be efficiently transduced

Next, we studied whether we could transduce the virus-specific T cells after Streptamer-based MACS-isolation. For efficient transduction, T cells need to proliferate. The MACS-isolated virus-specific T cells were, therefore, stimulated with either anti-CD3/anti-CD28 beads, irradiated autologous feeder cells, 10 ng/mL interleukin-15 and 100 IU/mL interleukin-2, or only with autologous feeders and cytokines (Figure 1). Not only could anti-CD3/anti-CD28-stimulated Streptamer-isolated T cells be transduced efficiently (Figure 1; 54.6%, 52.6% and 13.0%, respectively), but also T cells cultured with autologous feeder cells and cytokines were efficiently transduced (Figure 1; 53.0%, 43.3% and 15.4%, respectively). When the purity of the Streptamer-based MACS-isolated T cells was low directly after isolation (Figure 1C; 34.9%), the purity of virus-specific T cells within the anti-CD3/anti-CD28-stimulated T cells was lower (Figure 1C; 64%) than Streptamer-isolated T-cells that received no additional stimulation (Figure 1C; 99.1%). In addition, a substantial part (20%) of contaminating T cells was transduced (7.4% of 36.4%). Not only can these contaminating non-virus-specific T cells potentially induce GvHD, but an increased number of mixed TCR dimers can also be formed. These results indicate that anti-CD3/anti-CD28 stimulation can be dismissed for effective transduction of Streptamer-isolated T cells, and can even prevent preferential outgrowth of virus-specific T cells by non-discriminative stimulation of both virus-specific and non-specific T cells.

Using MACS, we isolated ten different virus-specific T-cell subsets from peripheral blood of seven healthy individuals, dissociated the Streptamers and transduced these T cells without an additional anti-CD3/anti-CD28 stimulation 2-3 days after isolation using retroviral supernatant encoding NGF-R (Table 3). Transduced T cells were analyzed for antigen-specificity and transduction efficiency. The purity of virus-specific T cells directly after MACS-

Table 2. Virus-specific T cells present at different frequencies in peripheral blood can be efficiently purified using Streptamer-based MACS-isolation.

#	Donor	Streptamers used[#] Specificity 1	Specificity 2	Percentage (%) virus-specific T cells in PBMC[§] Specificity 1	Specificity 2	Percentage (%) virus-specific T cells directly after MACS-isolation Specificity 1	Specificity 2	SUM[‡]
1	BST	BMLF-1^{A2}		1.16		82.5		82.5
2	CGT	pp50^{A1}		0.74		51.5		51.5
3a	CBS	IE-1^{B8}		0.51		94.9		94.9
3b	CBS	pp50^{A1}		0.77		92.7		92.7
4	EHB	pp50^{A1}		0.03		34.9		34.9
5	JBC	pp50^{A1}		1.01		77.7		77.7
6	UGW	pp50^{A1}		0.44		54.0		54.0
7	AKO	BMLF-1^{A2}		0.73		44.9		44.9
8	UQN	BMLF-1^{A2}	pp65^{A2}	0.71	0.95	33.0	45.6	78.6
9	HVU	BMLF-1^{A2}	pp65^{A2}	0.10	0.22	29.6	31.3	60.9
10	ALN	pp65^{A2}	pp65^{B7}	0.023	0.68	0.2	66.9	67.1
11	CVO	pp50^{A1}	IE-1^{B8}	0.32	2.6	4.8	76.0	80.8

[#]Streptamers used for isolation were selected based on HLA, seropositivity for EBV or CMV and precursor frequency of virus-specific T cells in donor leukocytes. [§]The percentage of virus-specific T cells, as measured with virus tetramers using flowcytometry and gated on total lymphocytes. [‡]SUM, percentage of virus-specific T cells calculated as the sum of Specificity 1 and 2. PBMC: peripheral blood mononuclear cells.

isolation ranged from 44.9%-92.1% (median 74.2%). All virus-specific T-cell lines displayed ≥10% transduction efficiency, as measured with anti-NGF-R monoclonal antibodies after an additional 8-10 days of culture (range 11.1% - 53%, median 30.7%). Moreover, at this time-point all ten T-cell lines contained more than 60% virus-specific T cells (range 63.9% - 98.5%, median 79.2%). These results confirm that for efficient transduction of Streptamer-isolated virus-specific T cells no additional anti-CD3/anti-CD28 stimulation is required.

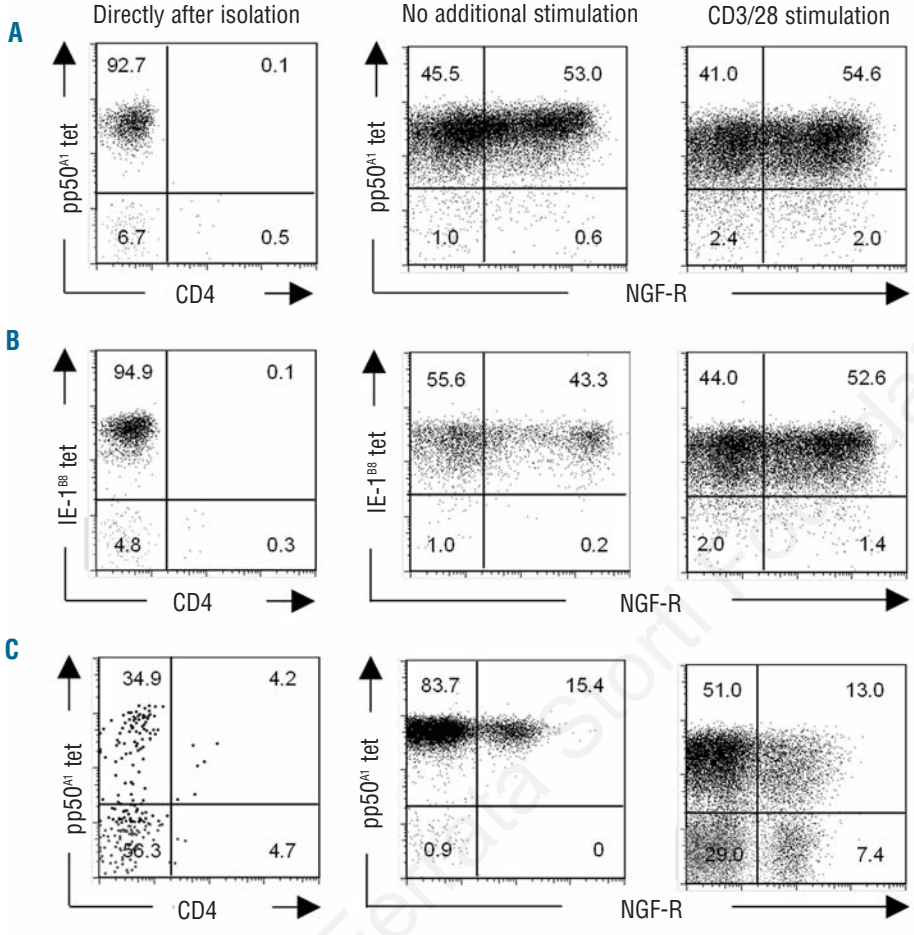

Figure 1. Anti-CD3/anti-CD28 stimulation is not required for efficient transduction of Streptamer-based isolated T cells. To study whether Streptamer-based isolated T cells could be transduced, (A) pp50^{A1} or (B) IE-1^{B8} were isolated from healthy individual, CBS, or (C) pp50^{A1} specific T cells were isolated from healthy individual, EHB. After Streptamer-based MACS-isolation, T cells were either cultured with irradiated autologous feeder cells and cytokines (no stimulation), or stimulated using CD3/28 beads (CD3/28 stimulation), irradiated autologous feeder cells and cytokines, and 2 days thereafter transduced with NGF-R. T cells were analyzed for purity directly after isolation (first panel), or analyzed for purity and transduction efficiency 10 days after transduction (second and third panels) by flow cytometry, and numbers indicate percentage of cells per quadrant. Representative examples of four different transduced virus-specific T cells derived from three different healthy individuals are depicted.

Table 3. Virus-specific T cells purified using Streptamer-based MACS-isolation can be efficiently transduced.

#	Donor	Streptamers used[#] Specificity 1	Specificity 2	Percentage (%) virus-specific T cells in PBMC[§] Specificity 1	Specificity 2	Percentage (%) virus-specific T cells directly after MACS-isolation Specificity 1	Specificity 2	SUM[‡]	Percentage (%) virus-specific T cells and transduced virus-specific T cells at day 8-12 after isolation Specificity 1	Specificity 2	SUM[‡]	Transduced[&]
1	UGW	pp50^{A1}	-	0.41	-	54.0	-	54.0	66.2	-	66.2	11.1
2	JBC	pp50^{A1}	-	1.01	-	82.2	-	82.2	72.7	-	72.7	33.2
3	JBC	pp50^{A1}	IE-1^{B8}	1.37	0.2	47.3	39.1	86.4	34.0	29.9	63.9	33.8
4	CGT	pp50^{A1}	-	0.74	-	66.0	-	66.0	68.4	-	68.4	44.4
5	BST	BMLF-1^{A2}	-	1.16	-	74.4	-	74.4	81.8	-	81.8	12.3
6	AKO	BMLF-1^{A2}	-	0.73	-	44.9	-	44.9	74.3	-	74.3	26.4
7	CVO	pp50^{A1}	IE-1^{B8}	0.39	2.6	4.8	75.7	80.5	25.1	49.5	74.6	15.8
8	CBS	pp50^{A1}	-	0.77	-	92.1		92.1	98.5		98.5	53.0
9	CBS	IE-1^{B8}	-	0.51	-	87.2		87.2	94.3		94.3	43.3
10	CBS	pp50^{A1}	IE-1^{B8}	0.77	0.51	40.0	34.2	74.2	53.9	21.9	75.8	34.0

[#]Streptamers used for isolation were selected based on HLA, seropositivity for EBV or CMV and precursor frequency of virus-specific T cells in donor leukocytes. [§]The percentage of virus-specific T cells, as measured with virus tetramers using flow cytometry and gated on total lymphocytes. [‡]SUM, percentage of virus-specific T cells calculated as the sum of Specificity 1 and 2. [&]The percentage of transduced virus-specific T cells, as measured with anti-NGF-R monoclonal antibodies using flow cytometry and gated on virus-specific T cells. PBMC: peripheral blood mononuclear cells.

Streptamer-based MACS-separation induces proliferation of virus-specific T cells

To confirm that without additional TCR-stimulation Streptamer-based MACS-isolated, virus-specific T cells were able to proliferate, thereby enabling transduction, T cells were isolated from peripheral blood mononuclear cells of five healthy individuals using different Streptamers. Directly after isolation, Streptamers were dissociated and T cells were cultured with irradiated autologous feeder cells and cytokines. T cells were counted both directly after isolation as well as 1 week after isolation to determine their proliferation rate (Figure 2A) and their purity was analyzed (Figure 2B). Of the five purified virus-specific T cells, four (JBC, CGT, BST and CBS) were able to proliferate without anti-CD3/anti-CD28 stimulation with, on average, a 3-fold increase in cell numbers within a week, whereas one (AKO) demonstrated little or no proliferation (Figure 2A). In addition, all five virus-specific T-cell lines demonstrated increased purity in time (Figure 2B). From these results we concluded that T cells purified using Streptamer-based MACS-isolation proliferate without additional TCR stimulation.

To exclude that dissociation of the Streptamers was incomplete and virus-specific T cells were stimulated by residual Streptamers attached to their TCR, we performed tetramer staining of virus-specific T cells before Streptamer-based selection, and after selection either with or without dissociation (Figure 2C). The tetramer-staining of unselected virus-specific T cells that had not been incubated with Streptamers was considered 100%. Streptamer-isolated T cells that were not incubated with D-biotin demonstrated significantly less tetramer staining than virus-specific T cells that were not stained with Streptamers (Figure 2C), indicating that bound Streptamers hampered tetramer staining. In contrast, D-biotin-treated Streptamer-isolated T cells demonstrated comparably high tetramer staining to T cells that had not been incubated with Streptamers (Figure 2C). These results indicate that D-biotin removed Streptamers very efficiently from the cell surface. Furthermore, Streptamer-isolated T cells incubated with D-biotin did not produce IFN-γ within 24 h after isolation, and no TCR down-regulation was observed 24 h and 48 h after isolation (*data not shown*), indicating that Streptamer-based MACS-isolation did not result in vigorous TCR stimulation. These results demonstrate that D-biotin removed Streptamers very efficiently from the cell surface, and it is therefore unlikely that proliferation observed after Streptamer-based MACS-isolation is due to residual Streptamer binding to TCR of virus-specific T cells.

Streptamer selection and transduction method can be successfully scaled up and translated into a Good Manufacturing Practice-grade procedure

Based on the previous results, we studied whether we could scale up this procedure for clinical purposes resulting in a rapid procedure to engineer therapeutically relevant numbers of pure, virus-specific T cells transduced with the HA-1-TCR. To obtain therapeutic cell numbers after the total procedure, donor leukocytes will be incubated with one or two Streptamers consisting of the relevant CMV and EBV peptide-HLA complexes for which profound T-cell populations are present in the donor. For this purpose, we performed four test procedures using $1x10^9$ peripheral blood mononuclear cells derived from leukapheresis products of four healthy individuals, donors

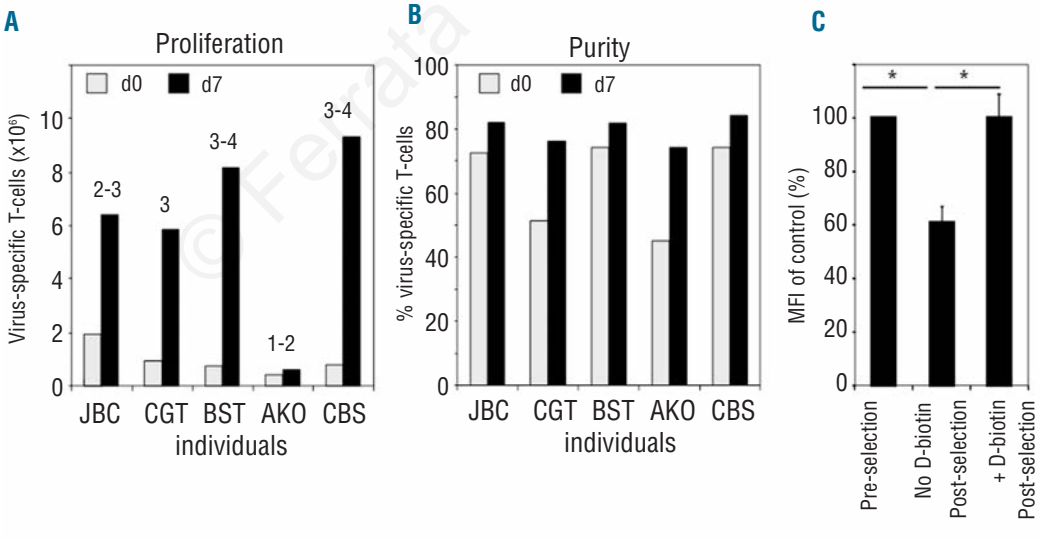

Figure 2. Streptamer-based isolated T cells cultured only with irradiated autologous feeders are able to proliferate. To study whether Streptamer-based isolated virus-specific T cells could be expanded in culture without additional stimulation, isolated virus-specific T cells were cultured by adding irradiated autologous feeder cells in a 5:1 ratio and cytokines. Virus-specific T cells were isolated from healthy individuals JBC and CGT, using pp50^{A1} streptamers, from BST and AKO using BMLF-1^{A2} streptamers, and from CBS using pp50^{A1} and IE-1^{B8} streptamers. (A) Cells were counted directly after isolation (day 0; gray bars) and 1 week after isolation (day 7; black bars). Numbers of divisions of the isolated virus-specific T cells are indicated above the bars. (B) In addition, T cells were stained with virus tetramers and checked for purity at day 0 (gray bars) and day 7 (black bars) after isolation using flow cytometry. (C) To study whether residual Streptamer was bound to Streptamer-based isolated T cells, we determined tetramer staining of virus-specific T cells before and after Streptamer-based MACS-isolation, and of virus-specific T cells after Streptamer-based MACS-isolation and a D-biotin dissociation step. The mean fluorescence intensity (MFI) of virus-specific T cells before Streptamer-based MACS-isolation that were not incubated with Streptamers was set at 100%. *P* values <0.01, which are statistically different, are indicated with an asterisk.

JBC, UPB, UHO and UBQ (Figure 3 and Table 4). The frequencies of virus-specific T cells in the leukapheresis material of healthy individuals JBC, UPB and UHO varied between 0.26% - 0.77% of lymphocytes (Figure 3A-C). Sensitive combinatorial coding analysis demonstrated that frequencies of virus-specific T cells of interest in the leukapheresis material of healthy individual UBQ were very low (Figure 3D). Leukocytes were incubated with the relevant Streptamers, and purified using CliniMACS (Figure 3A-D and Table 4). Directly after isolation, T cells were incubated with D-biotin, and analyzed for purity using flow cytometry. As depicted in Figure 3A-D and summarized in Table 4, all positive fractions contained ≥60% virus-specific T cells even when starting material had low frequencies of virus-specific T cells (Figure 3D). Based on the frequencies and the amount of cells in the starting material, the expected number of antigen-specific T cells that could be isolated was calculated (Table 4). For all four test procedures the positive fraction had a recovery rate of virus-specific T cells present in the starting material of nearly 60% (Table 4). After isolation by CliniMACS, the positive fractions were cultured in T-cell medium containing irradiated autologous feeders (1:5 ratio) and cytokines. Part of the Streptamer-enriched cell lines was not transduced, whereas the largest fraction of the cell lines was transduced with GMP-grade retroviral supernatant encoding the HA-1-TCR 2-3 days after isolation. After an additional culture period of 8-12 days, transduced T cells were analyzed for transduction efficiency and purity using HA-1 and virus-tetramers. All four Streptamer-enriched cell lines that were not transduced were ≥97% pure as measured with virus-tetramers (Figure 3E-H). Transduction efficiencies of the four HA-1-TCR transduced Streptamer-enriched cell lines ranged between 22.5% and 54.2% (Figure 3E-H and Table 4). T cells within the HA-1-TCR transduced virus-specific T cells that stained positive with the HA-1 tetramer dominantly expressed the HA-1-TCR and expressed reduced levels of the virus TCR due to competition for cell surface expression.[39,45] At the end of the culture period (day 14 after isolation) all T-cell products were harvested and viable cells were counted (Table 4). Test procedures on cells from JBC, UPB and UHO resulted in ≥15x10^6 highly pure antigen-specific T cells. The test procedure on cells from UBQ with low frequencies of virus-specific T cells in the starting material resulted in 2x10^6 antigen-specific T cells at the end of the culture period (Table 4). In conclusion, these results demonstrate that using GMP-grade isolation methods, virus-specific T cells can be enriched with a high recovery rate from thawed peripheral blood mononuclear cells, and efficiently transduced.

HA-1-TCR transduced virus-specific T-cell populations exert dose-dependent HA-1-specific effector functions

HA-1-specific functionality was tested in a peptide titration assay for three of the HA-1-TCR transduced virus-specific T cells (JBC, UHO, UBQ), and IFN-γ production was measured. All three transduced virus-specific T-cell lines demonstrated equal HA-1-specific dose-dependent IFN-γ production, comparable to the HA-1-specific control T-cell clone. In addition, no HA-1-specific IFN-γ production of non-transduced T-cells was observed. To study whether HA-1-TCR transduced virus-specific T cells were able to recognize malignant primary leukemic cells presenting endogenously processed HA-1 antigen, HA-1-TCR transduced virus-specific T-cells were tested against HLA-A2$^+$ primary ALL cells which were either HA-1$^+$ or HA-1$^-$. As can be observed in Figure 4B, all HA-1-TCR-transduced virus-specific T-cell lines but not non- transduced virus-specific T cells were able to produce IFN-γ after stimulation with HA-1$^+$ primary ALL cells, whereas no IFN-γ was produced after stimulation with HA-1$^-$ primary ALL cells. Both the HA-1-TCR-transduced virus-specific T-cell lines and non-transduced virus-specific T cells produced IFN-γ after stimulation with T2 cells pulsed with viral peptides. In addition, all four HA-1-TCR-transduced virus-specific T cells were tested for HA-1-specific cytotoxic reactivity against virus or HA-1 peptide pulsed T2 cells, or against HLA-A2$^+$ primary ALL and AML cells either HA-1$^+$ or HA-1$^-$ (Figure 4C). Results demonstrate that HA-1-TCR transduced virus-specific T cells efficiently lysed HA-1 peptide-pulsed T2 cells, as well as HLA-A2$^+$ and HA-1$^+$ ALL and AML cells. In addition, they showed comparable cytotoxic reactivity against virus peptide-pulsed T2 cells as non-transduced virus-specific T cells.

These results demonstrate that reproducible production of dual-specific T cells with potent anti-leukemic reactivity is feasible using a GMP-grade production process.

Table 4. The four test runs performed demonstrate the feasibility of generating relatively pure virus-specific T cells that are efficiently transduced with the HA-1-TCR using GMP-grade procedures and materials.

Test run		Streptamers used[#]		Percentage (%) virus-specific T cells in PBMC[$]		Percentage (%) virus-specific T cells directly after MACS-isolation			Calculated and actually isolated virus-specific T cells			Percentage (%) virus-specific T cells and transduced HA-1-tetramer positive T cells at day 8-14 after isolation		
#	Donor	Specificity		Specificity		Specificity			Expected # of cells	Actually isolated # of cells	Recovery (%)	HA-1 tetramer (%)	Virus tetramer (%)	Cell # end of culture[&]
		1	2	1	2	1	2	SUM[‡]						
1	JBC	pp65^{A2}	pp50A1	0.26	0.77	22.6	76.1	98.7	1x10^7	7x10^6	63.0	22.5	72.7	51.4x10^6
2	UPB	BMLF-1^{A2}	-	0.49	-	92.3	-	92.3	6.4x10^6	4.7x10^6	73.4	54.2	44.9	16.8x10^6
3	UBQ	pp65^{A2}	BMLF-1^{A2}	0.008	0.03	6.2	63.0	69.2	5.2x10^5	3.5x10^5	67.3	27.2	70.5	2.1x10^6
4	UHO	pp65^{A}2	BMLF-1^{A2}	0.75	0.31	62.5	20.2	82.7	1.3x10^7	8x10^6	59.3	53.3	39.6	45.5x10^6

[#]Streptamers used for isolation were selected based on HLA, seropositivity for EBV or CMV and precursor frequency of virus-specific T cells in donor leukocytes. [$]The percentage of virus-specific T cells, as measured with virus tetramers using flow cytometry and gated on total lymphocytes. [&]Cell number at end of culture is corrected for the % antigen-specific T cells, calculated as the sum of HA-1 tetramer-positive T cells and HA-1 tetramer-negative but virus tetramer-positive T cells. PBMC: peripheral blood mononuclear cells.

Discussion

Here, we describe a GMP-grade procedure to generate dual-specific T cells with potent anti-leukemic reactivity. Using Streptamer-based MACS-isolation, we were able to reproducibly isolate highly pure virus-specific T-cell populations. In addition, we demonstrate that Streptamer-based MACS-isolation induced weak T-cell proliferation, resulting in two to three T-cell divisions that enabled transduction of these purified T cells without requiring additional stimulation using anti-CD3/anti-CD28 beads. We performed four test procedures. All HA-1-TCR-transduced virus-specific T cell products contained ≥60% antigen-specific T cells as measured with virus- and HA-1-tetramers, and were efficiently transduced as measured with HA-1-tetramers. In addition, HA-1-TCR expression correlated with potent effector functions directed against HA-1^{+} target cells. We think that this rapid procedure for generating high numbers of anti-leukemic T cells with well-defined specificities is an attractive option for treating patients with high-risk acute leukemia who are likely to relapse within 6 months after allogeneic SCT when it is not yet safe to administer DLI.

To be able to transfer a donor T-cell product safely early after allogeneic SCT, we want to retrovirally transfer the well-characterized HA-1-TCR into virus-specific T cells with a known and harmless specificity. For this purpose, it is crucial that relatively pure virus-specific T-cell populations are transduced. In some of the enrichments, the frequencies of virus-specific T cells directly after isolation were too low for clinical purposes. A low purity directly after Streptamer-based MACS-isolation was correlated with a low frequency of virus-specific T cells in peripheral blood before isolation. Nevertheless, some of these T-cell populations with low purities directly after isolation can convert to >95% pure, virus-specific T-cell populations after additional culturing. In addition, our large scale test procedures that were performed on CliniMACS demonstrate higher purity directly after isolation (Table 4) than after the small-scale procedures that were performed on AutoMACS (Table 2). Possibly, the absolute number of contaminating non-specific T cells that are isolated

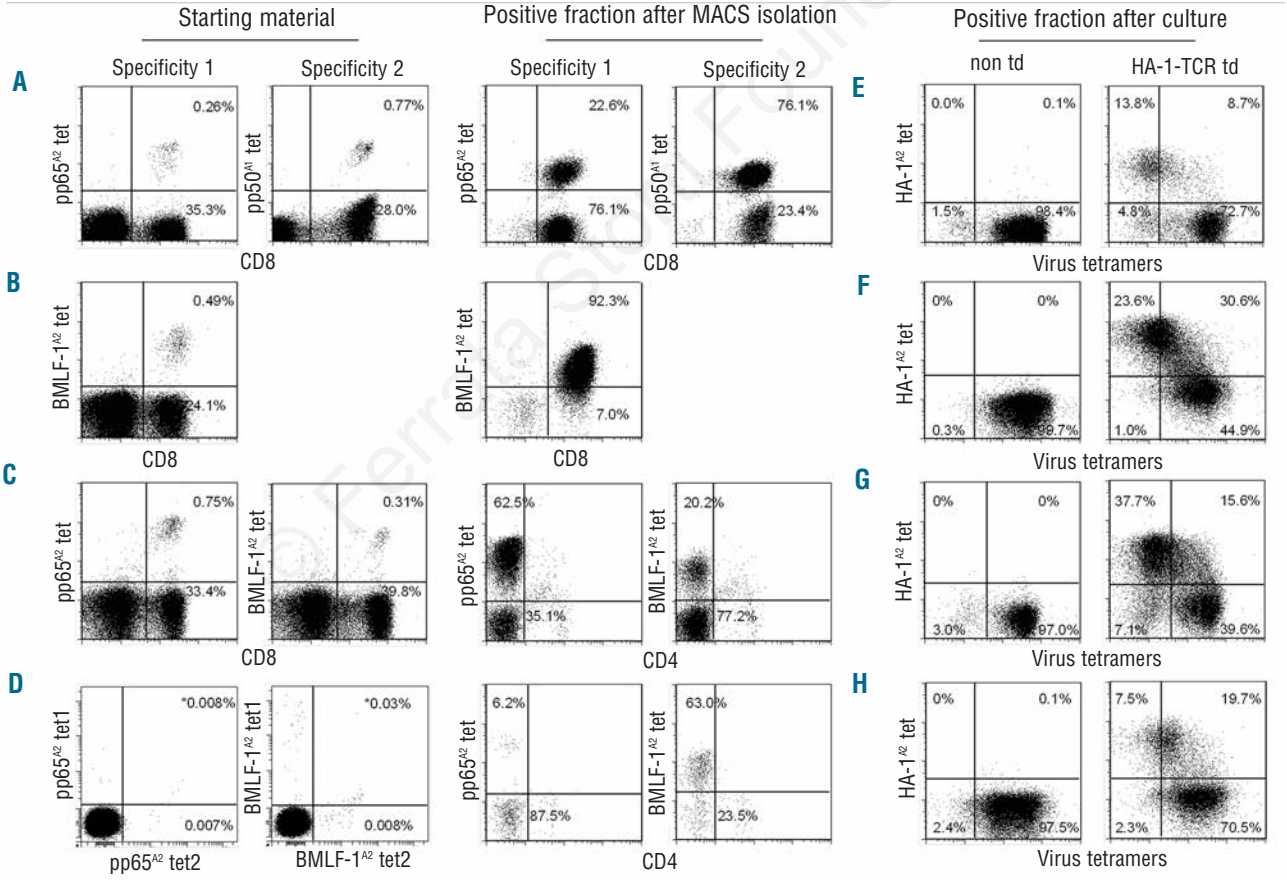

Figure 3. Virus-specific T cells can be purified and transduced using a Streptamer-based selection procedure on a Clini-MACS device. To test whether we could scale up the procedure, virus-specific T cells were purified using Streptamers and a Clini-MACS device and 2-3 days after isolation either transduced with the HA-1-TCR (HA-1-TCR td) or non-transduced (non td). For this purpose, 1x10^{9} peripheral blood mononuclear cells (PBMC) were thawed from healthy donors JBC (A+E), UPB (B+F), UHO (C+G), and UBQ (D+H). (A-D) Before and directly after CliniMACS-separation donor PBMC were stained with tetramers and the frequencies of virus-specific T cells were measured in the starting material as well as in the positively isolated fraction using flow cytometry after a D-biotin dissociation step. (E-H) One week after transduction and 12-13 days after MACS-isolation, antigen-specificity of both the non td and HA-1-TCR td cell lines was measured using HA-1- and virus-tetramers. Percentages indicate frequencies of virus-specific or HA-1-specific T cells in that particular quadrant. *Sensitive combinatorial coding analysis demonstrated that leukapheresis material of UBQ contained 0.008% pp65^{A2} specific T cells and 0.03% BMLF-1^{A2} specific T cells.

remains identical when starting with high ($1x10^9$) or low ($5x10^7$) cell numbers.

We demonstrated that we can transduce Streptamer-isolated virus-specific T cells 2-3 days after MACS-isolation by adding only irradiated autologous feeder cells and cytokines. This is a striking finding, as T cells need to proliferate for efficient transduction. This indicates that apparently Streptamer-isolated virus-specific T cells were proliferating after the isolation procedure. Correspondingly, we show moderate proliferation of Streptamer-isolated T cells (Figure 2A; 3-4 fold), consistent with a rather mild instead of a vigorous stimulation of the T cells. A key feature of the Streptamer-isolation procedure is that all isolation reagents can be completely removed from the cells with an excess of D-biotin and isolated T cells should be left phenotypically and functionally indistinguishable from untreated cells. Potentially, in our experiments the dissociation step was incomplete, and residual Streptamer bound to the virus-specific TCR triggered the TCR. However, tetramer analysis (Figure 2C) confirmed previously published data that the D-biotin dissociation step very efficiently removes Streptamers from the cell surface of the T cells,[46] as we observed no difference in fluorescence between virus-specific T cells that were not pre-incubated with Streptamers and T cells that were pre-incubated with Streptamers but were treated with D-biotin to dissociate the Streptamers after MACS-isolation. In addition, we could not demonstrate TCR stimulation by residual Streptamer-binding, as we observed no TCR down-regulation or IFN-γ production 4 h, 24 h and 48 h after Streptamer-based MACS-isolation (*data not shown*).

Alternatively, dissociated Streptamers might disintegrate, resulting in free peptide binding in HLA expressed on the T cells. In this way, T cells presenting the peptide in their HLA would be able to stimulate neighbor T cells. However, we could not demonstrate that Streptamer-isolated and D-biotin-treated EBNA3A^{B8} and IE-1^{B8} specific T cells were able to stimulate EBNA3A^{B8} and IE-1^{B8} specific T-cell clones in a stimulation assay (*data not shown*). Nevertheless, our data show that Streptamer-isolated T cells proliferate moderately (Figure 2A; 3-4 fold), and can be efficiently transduced without additional stimulation (Figure 1). We, therefore, hypothesize that the selection procedure as a whole results in a mild T-cell stimulation, possibly due to very low levels of free peptide or small temperature fluctuations within 4-10°C that enable mild TCR-triggering. Alternatively, Streptamer binding could have supported the formation of TCR microclusters that

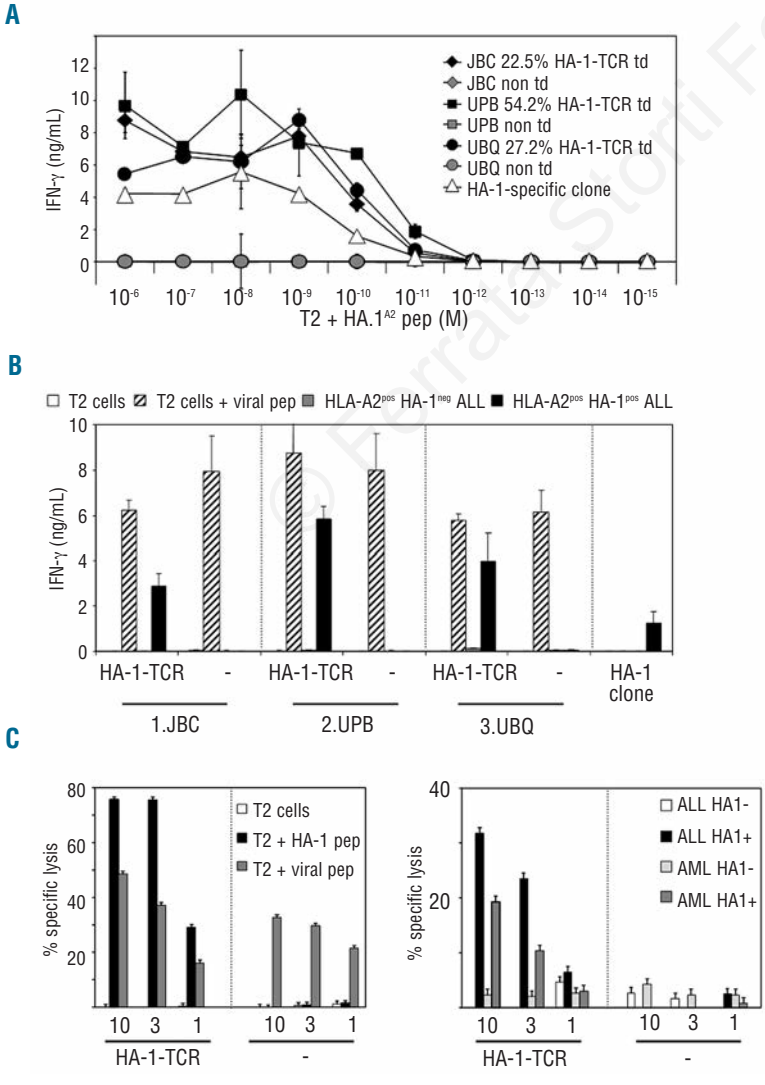

Figure 4. HA-1-TCR tranduced, virus-specific T cells demonstrate dose-dependent HA-1 specific reactivity and recognize HA-1-positive primary leukemic cells. (A) Both non-transduced (gray symbols; non td) and HA-1-TCR transduced (black symbols; HA-1-TCR td) virus-specific T cells of three different test procedures (JBC, UPB and UBQ) were tested for their HA-1-specific reactivity in standard IFN-γ ELISA using T2 cells pulsed with different concentrations of HA-1 peptide. Five thousand virus-tetramer$^+$ or 5,000 HA-1-tetramer$^+$ transduced T cells were tested against 20,000 T2 cells. (B) In addition, the same T cells were tested for their capacity to recognize HA-1$^+$ target cells presenting endogenously processed HA-1. Target cells were T2 cells unpulsed (white bars) or pulsed with relevant viral peptides (viral pep; black striped bars), or HLA-A2$^+$ primary ALL cells either HA-1$^-$ (gray bars) or HA-1$^+$ (black bars). As a control, a HA-1-specific T-cell clone was included. (C) Both non-transduced and HA-1-TCR transduced virus-specific T cells of all four different test procedures (JBC, UPB, UHO and UBQ) were tested for their HA-1-specific reactivity in a chromium release assay using different effector-to-target ratios. As a representative example, cytotoxic reactivity of HA-1-TCR transduced virus-specific T cells of healthy individual UHO is depicted. Target cells were T2 cells unpulsed (white bars) or pulsed with either HA-1 peptide (black bars) or relevant viral peptides (viral pep; gray bars) (left panel), or HLA-A2$^+$ primary ALL cells that were either HA-1$^-$ (white bars) or that were HA-1$^+$ (black bars), or HLA-A2$^+$ primary AML cells either HA-1$^-$ (light gray) or HA-1$^+$ (dark gray) (right panel).

have been described to be able to mediate some stimulatory activity even in the absence of bound ligand.[47] In our hands, anti-CD3/anti-CD28 beads that non-discriminatively stimulate both the virus-specific as well as contaminating T cells did not result in higher purity or greater transduction efficiency. Therefore, for our clinical GMP procedure we will not use additional stimulation with anti-CD3/anti-CD28 beads after Streptamer-based MACS-isolation.

The GMP-grade retroviral supernatant encodes for the HA-1-TCR without a marker gene. We have previously shown that introduced TCR compete with endogenous TCR.[45] T cells that are profoundly HA-1-TCR$^+$ will, therefore, hardly express the virus-TCR.[34,45] Thus, to determine the purity of the T-cell product we can add up the percentages of HA-1 tetramer$^+$ and virus tetramer$^+$ T cells. To be able to discriminate between HA-1-TCR-transduced virus-specific T cells that have down-regulated the endogenous virus-TCR and HA-1-TCR-transduced contaminating non-virus-specific T cells, a small sample will not be transduced after MACS-isolation and will be used as the internal control. If, directly after MACS-isolation, the purity of virus-specific T cells is < 50% as measured by tetramer staining, we will restart with the CliniMACS isolation procedure.

Recently, our group has shown that some virus-specific CD8$^+$ T cells can also exhibit allogeneic HLA reactivity, directed against non-self HLA class I molecules.[48] If an HLA-matched HLA-A2$^+$ HA-1$^-$ donor is lacking for an HLA-A2$^+$ HA-1$^+$ patient, the patient can potentially be transplanted with cells from a single HLA-mismatched HLA-A2$^-$ donor. In these particular cases, the virus-specific T cells of the donor will be tested for allogeneic HLA reactivity directed against the patient's cells. Only virus-specific T cells that are unreactive towards patient-derived dendritic cells will be selected for the HA-1-TCR modified virus-specific T-cell product.

In conclusion, we describe here a rapid GMP-grade procedure to generate a pure cell product containing HA-1-TCR-transduced virus-specific T cells. These engineered T cells were comparably effective, or even more effective than the parental HA-1-specific T-cell clone in antigen-specifically recognizing HA-1$^+$ malignant cells. This pure antigen-specific T-cell product may be safely administered early after allogeneic SCT exerting GvL effects without GvHD activity.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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