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Promiscuity of the AlloHLA-A2 Restricted T Cell Repertoire Hampers the Generation of Minor Histocompatibility Antigen-specific Cytotoxic T Cells across HLA Barriers

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ABSTRACT

Hematopoietic system-specific miHAs are ideal targets for adoptive immunotherapy after allogeneic HLA (alloHLA)-matched SCT. Adoptive immunotherapy with cytotoxic T cells targeting hematopoietic system-specific miHAs restricted by alloHLA molecules is an attractive strategy to treat relapsed hematologic malignancies after HLA-mismatched SCT. As a proof of principle, we exploited 2 new strategies to generate alloHLA-A2-restricted miHA-specific T cells from HLA-A2^{neg} donors using a HLA/miHA multimer-guided approach. In one strategy, autologous DCs coated with HLA-A2/miHA complexes were used for in vitro generation of miHA-specific T cells from HLA-A2^{neg} male donors. In the other strategy, miHA-specific T cells were directly isolated from the peripheral blood of HLA-A2^{neg} parous females with HLA-A2^{pos} offspring. Both methods introduced recombinant HLA-A2/miHA complexes as the sole allogeneic target antigen. However, neither method yielded high avidity miHA-specific T cells or prevented the emergence of peptide-dependent promiscuous T cells. The latter T cells resembled miHA-specific T cells so closely with regard to tetramer binding and cytokine production that only extensive testing at a clonal level revealed their nonspecific nature. Therefore, promiscuity of the alloHLA-A2 T cell repertoire of HLA-A2^{neg} individuals hampers in vitro generation of genuine miHA-specific T cells and limits its use for adoptive immunotherapy after HLA-A2 mismatched SCT.

KEY WORDS

Stem cell transplantation • Immunotherapy • Cytotoxic T cells • Antigen recognition • Alloreac

INTRODUCTION

Allogeneic (allo) SCT is a well established treatment for a variety of hematologic malignancies [1]. The ideal stem cell (SC) donor in terms of reliable engraftment and minimal GVHD is a genotypically HLA-identical sibling [2]. Such donors are available for approximately 30% of patients [3]. The remaining patients are transplanted with SCs from haploidentical family donors or (partially) HLA-matched unrelated donors. SCT across HLA mismatches is feasible using T cell depletion [4,5]. However, the risk of leukemia relapse is high after T cell-depleted SCT [6,7] due to the absence of a graft-versus-leukemia (GVL) effect mediated by donor T cells [8,9].

The potency of the GVL effect is illustrated by the successful application of DLIs to treat relapsed leukemia after alloSCT [10]. Because the donor lymphocytes have not been selected for preferential reactivity against malignant cells, DLI therapy is often accompanied by GVHD. High rates of GVHD-associated morbidity and mortality limit the use of DLI after

HLA-mismatched SCT [11]. A potential approach to the treatment of relapsed leukemia after HLA-mismatched SCT comprises adoptive immunotherapy with leukemia-specific cytotoxic T cells. In this setting, a patient's mismatched HLA molecules are used as antigen-presenting molecules for hematopoietic system-specific antigens to generate non-self (allo) HLA-restricted T cells from the SC donor. Such alloHLA-restricted T cells should theoretically lyse a patient's hematopoietic cells only, including leukemic cells. Nonhematopoietic tissues that do not express the relevant antigen and graft-derived donor hematopoietic cells that lack the appropriate HLA restriction molecule should not be lysed. Previous studies have described in vitro generation of this type of T cells recognizing malignancy-associated or hematopoietic system-specific antigens presented by alloHLA molecules [12-18].

However, existing protocols for the generation of alloHLA-restricted cytotoxic T cells are complex and not clinically applicable, requiring the use of allogeneic cell lines as APCs or cloning by limiting dilution. Thus there is a real need for a simple, robust protocol for the generation of alloHLA-restricted T cells incorporating APCs that meet the requirements of good manufacturing practice.

To address this issue, we explored two new and different strategies to generate alloHLA-A2-restricted hematopoietic system-specific $CD8^+$ T cells. The first strategy was based on a recent study by Savage et al [14]. We explored the feasibility of using autologous DCs coated with recombinant trimeric HLA-A2/peptide complexes to stimulate alloHLA-A2-restricted $CD8^+$ T cells from HLA-$A2^{neg}$ donors. DCs are the most specialized APCs for induction and expansion of $CD8^+$ T cells [19,20]. Recombinant HLA-A2 complexes contained the previously described immunogenic T cell epitopes of the miHAs HA-1 or HA-2 [21]. Expression of HA-1 and HA-2 is restricted to the hematopoietic system, including leukemic cells and their progenitors [22-24]. Moreover, emergence of HA-1- and HA-2-specific T cells was shown to coincide with CR of relapsed leukemia or multiple myeloma after DLIs from HLA-matched HA-1- or HA-2-mismatched SC donors [25]. In addition, HA-1-specific cytotoxic T cells significantly inhibit human leukemia outgrowth in a translational NOD/SCID model [26]. Thus, HA-1 and HA-2 are suitable targets for adoptive immunotherapy after HLA-matched HA-1- and/or HA-2-mismatched SCT.

In the second strategy, we examined the feasibility of using HLA-A2/miHA tetramers to directly isolate alloHLA-A2-restricted HA-1- or HA-2-specific $CD8^+$ T cells from PBMCs of HLA-$A2^{neg}$ parous female donors who had delivered ≥1 HLA-$A2^{pos}$ child. Subsequent specificity and functional studies of in vitro induced and ex vivo isolated alloHLA-A2-restricted HA-1^{A2} or HA-2^{A2} tetramer binding $CD8^+$ T cells were performed at both the polyclonal and clonal levels.

METHODS

Blood Donors

Two healthy HLA-$A2^{neg}$ male blood donors with no record of prior blood transfusions were selected on the basis of successful in vitro generation of HLA-A2/HA-1-specific T cells in earlier experiments [13]. Five healthy HLA-$A2^{neg}$ multiparous female blood donors were selected on the basis of high levels of HLA-A2-specific antibody after delivery of an HLA-$A2^{pos}$ HA-2^{pos} child. HLA typing of the donors is listed in Table 1. Informed consent was obtained according to institutional guidelines. PBMCs were isolated by Ficoll-Isopaque density gradient centrifugation and stored in liquid nitrogen.

Synthetic Peptides and HLA-A2/miHA Peptide Complexes

HA-1, HA-2, HY, and CMV peptides were synthesized according to their reported sequences [27-30] and referred to as pHA-1, etc, in the figures. Biotinylated recombinant HLA-A2/peptide complexes were generated as described previously [31] and used as monomers for the coating of artificial antigen-presenting constructs (aAPC), as streptavidin (Molecular Probes, Leiden, the Netherlands) bound trimers for the coating of DCs, or as tetramers (peptide^{A2}) for analysis of miHA-specific T cells. The specificity and selectivity of HLA-A2/miHA peptide tetramers have been extensively tested and reported [32]. HPLC separation of a peptide pool eluted from HLA-A2 was performed using an 0.1% heptafluorobutyric acid gradient as described previously [33].

miHA-specific Polyclonal T Cell Lines and Clones, $CD4^+$ Th Cells, and DCs

In vitro generation of miHA-specific T cell lines and clones and $CD4^+$ Th cells is documented in detail elsewhere [20,34]. DCs were generated from peripheral blood-derived $CD14^+$ monocytes as described

Table 1. *HLA Class I Typing of Various Donors Used in this Study**

Donors	HLA-A	HLA-B	HLA-C
M#1	A*01, A*24	B*07, B*08	Cw*07
M#2	A*29, A*3001/ 3014L/3015	B*1302/1308, B*44	Cw*06
F#1	A*01, A*25	B*18, B*15	—
F#2	A*03, A*32	B*07, B*08	Cw*07
F#3	A*03	B*07, B*15	Cw*03, Cw*07
F#4	A1, A3	B7, B62	Cw3, Cw7
F#5	A*11, A*32	B*35, B*44	Cw*04, Cw*05

*Typing was performed serologically or by low-resolution PCR.

previously [35]. After 6 d of culture, DCs were maturated by overnight culture in the presence of 100 U/mL TNF-α (Peprotech, London, United Kingdom), 5 ng/mL IL-1β (Peprotech), 150 ng/mL IL-6 (Peprotech), 1 μg/mL prostaglandin E_2 (Sigma-Aldrich, Zwijndrecht, Netherland), and 800 U/mL GM-CSF (Novartis Pharma BV, Arnhem, the Netherlands).

HLA-A2/miHA Complex Coating on DCs

Mature DCs were incubated sequentially with biotinylated mouse anti-human CD1a (Serotec, Oxford, United Kingdom), anti-human HLA class I (BD Biosciences, Amsterdam, the Netherlands), anti-human HLA-DR (BD Biosciences), or anti-human CD45 antibodies (BD Biosciences; 3 μg/10^6 DCs), and with trimeric streptavidin-coupled HLA-A2/miHA complexes (5.64 μg/10^6 DCs) for 60 min at 4°C in 100 μL PBS per 10^6 DCs. DCs were washed with PBS after each incubation step. HLA-A2/miHA density of complex-coated DCs was validated by staining with a conformation-dependent HLA-A2 FITC-conjugated mouse antibody (BD Pharmingen) before use.

Generation of HLA-A2/HA-1-specific T Cells using Autologous HLA-A2/HA-1 Complex-coated DCs from HLA-$A2^{neg}$ Donors

PBMCs were depleted of various cell subsets using CD4, CD14, CD16, and CD19 magnetic beads (Miltenyi GmbH, Bergisch Gladbach, Germany) according to the supplier's protocol. The remaining $CD8^+$ cell fractions were stimulated with irradiated (30 Gy) autologous HLA-A2/HA-1 complex-coated DCs or with irradiated (30 Gy) alloHLA-$A2^{pos}$ HA-1 peptide-pulsed DCs, in the presence of autologous irradiated (30 Gy) $CD4^+$ Th cells at a CD8:DC:CD4 5:1:0.5 responder ratio in Iscove modified Dulbecco medium (IMDM) containing 10% pooled human serum (HS), 1 U/mL IL-12 (R&D Systems, Minneapolis, Minn), and 2 Cetus units (cU)/mL IL-2 (Cetus, Emeryville, Calif). The $CD8^+$ T cell lines were restimulated every 7 d with the same irradiated stimulators and $CD4^+$ Th cells. IL-2 (25 cU/mL) was added 24 h after each restimulation.

Direct Isolation and Culture of HLA-A2/HA-2-specific T Cells from PBMCs

The protocol used for detection of miHA-specific T cells in PBMCs was described previously [36]. Briefly, PBMCs from healthy multiparous female blood donors were depleted of various cell subsets using CD4, CD14, CD16, and CD19 magnetic beads (Miltenyi GmbH). Depleted fractions were stained with PE-conjugated HA-2^{A2} tetramers and subsequently with CD45RO-FITC (BD Biosciences) and CD8-allophycocyanin (-APC) conjugated antibodies (BD Biosciences). HA-2^{A2} tetramer binding $CD8^+$ cells were isolated on a FACS Vantage cell sorter (Becton Dickinson, San Jose, CA) using a double-sort protocol. First, cells were enriched for HA-2^{A2} tetramer- and CD8 antibody-binding cells using the "enrich mode." The enriched fraction was then reanalyzed and immediately resorted using the more stringent "normal-R" mode. Double FACS-sorted cells were expanded in the presence of 5×10^4 irradiated (30 Gy) autologous PBMCs, 1% phytohemagglutinin (Murex, Dartford, United Kingdom), and 30 cU/mL IL-2 (Cetus) in IMDM containing 10% HS.

For 2 donors, the enriched fraction was stained with relevant tetramers and resorted according to a "single cell per well" protocol. The resulting T cell clones were expanded in the presence of random blood donor-derived 5×10^4 irradiated (30 Gy) PBMCs and 5×10^3 irradiated (50 Gy) EBV-transformed lymphoblastoid cell lines (EBV-LCLs), 1% leukoagglutinin (Sigma-Aldrich), and 30 cU/mL IL-2 in IMDM containing 10% HS.

Generation of Artificial Antigen-presenting Constructs

The aAPCs were prepared as described previously [34]. In brief, polystyrene sulphate latex beads (Interfacial Dynamics, Portland, Ore) were incubated sequentially with streptavidin (10 μg/10^7 beads; Molecular Probes), recombinant human CD54/Fc-chimera (0.5 μg/10^7 beads; R&D Systems), and CD80/Fc-chimera (0.25 μg/10^7 beads; R&D Systems), 1% human albumin (Sanquin, Amsterdam, the Netherlands), and biotinylated HLA-A2/miHA complexes (2 μg/10^7 beads).

Functional Assays

Proliferation was determined by coculturing 5×10^4 irradiated (30 Gy) stimulator cells with 2.5×10^4 responder T cells for 48 h. The cultures were then labeled with 1.0 μCi ^{3}H-thymidine for 16 h, and ^{3}H-thymidine incorporation was measured using liquid scintillation counting. Cytotoxicity was evaluated in a chromium release assay by incubating 2500 chromium 51-labeled target cells with serial dilutions of effector T cells for 4 h. Supernatants were harvested for gamma counting: *percent specific lysis = (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100%*. Results are shown for an effector:target ratio of 10:1 unless stated otherwise. Proliferation and cytotoxicity assay results are expressed as the mean of duplicate samples. Error bars represent SEMs.

IFN-γ cytokine secretion was measured by stimulating 1×10^5 T cells with aAPCs or with natural stimulators (EBV-LCLs) for 4 h. Responding T cells were identified using the IFN-γ Secretion Assay Cell

Enrichment and Detection Kit (PE*, Miltenyi Biotec) according to the manufacturer's instructions. All flow cytometric analyses were performed on a FACSCalibur with Cellquest software (Becton Dickinson, San Jose, Calif). Gates were set on vital lymphocytes according to their typical forward- and side-scattering characteristics. If samples were stained with multiple tetramers, molar ratios were equalized. Results are displayed as mean fluorescent intensity.

RESULTS

Stimulation of miHA-specific T Cells by HLA-A2/miHA Complex-coated DCs

HLA-A2/miHA complex-coated DCs were generated by coupling HLA-A2/miHA complexes via a linker antibody to cell surface molecules expressed by DCs (see Methods). Four different antibodies were analyzed for their capacity to anchor HLA-A2/miHA trimers to the DC cell surface: anti-CD1a, anti-CD45, anti-HLA class I, and anti-HLA-DR. Linkage through HLA-DR was associated with the highest and most prolonged complex binding and displayed the strongest capacity to stimulate miHA-specific HLA-A2-restricted T cells (data not shown). FACS analysis with a conformation-dependent HLA-A2-specific antibody showed that HLA-A2/miHA complexes anchored via HLA-DR to HLA-A2^{neg} DCs were coated at densities equal to natural HLA-A2 levels expressed by HLA-A2^{pos} DCs (Figure 1A). We used HLA-A2/miHA complexes coupled via HLA-DR for further studies.

The capacity of HLA-A2/miHA complex-coated DCs to efficiently elicit cytolytic responses by HLA-A2-restricted miHA-specific T cells was analyzed in a cytotoxicity assay (Figure 1B). An HA-1- and an HA-2-specific T cell clone effectively lysed HLA-A2^{neg} DCs coated with HLA-A2/HA-1 or HLA-A2/HA-2 complexes in a dose-dependent manner. At higher coating densities, HLA-A2/miHA complex-coated DCs were lysed to the same extent as HLA-A2^{pos} miHA peptide-pulsed DCs. HLA-A2^{neg} DCs coated with irrelevant HLA-A2/miHA complexes, unbound

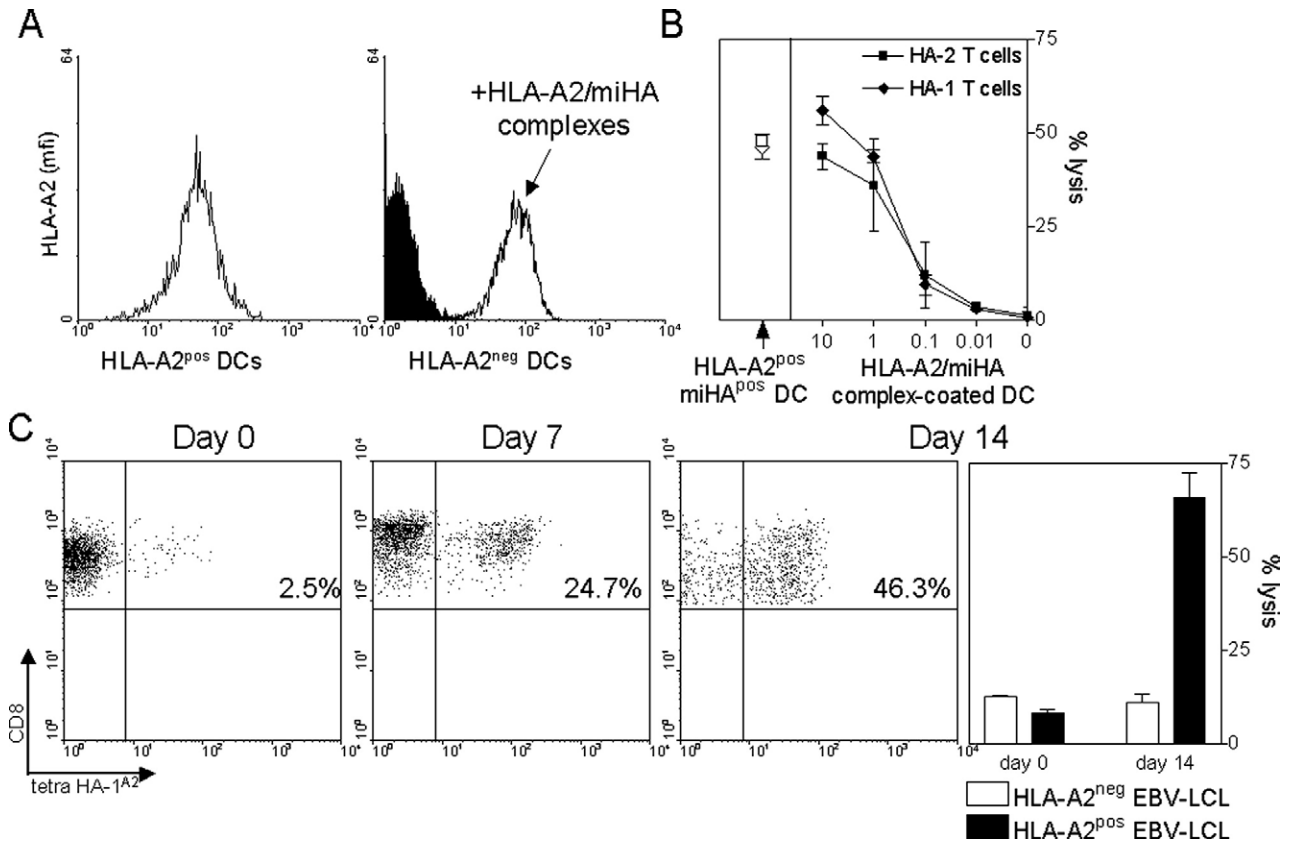

Figure 1. Stimulation of miHA-specific T cells with HLA-A2/miHA complex-coated DCs. A, HLA-A2 expression by HLA-A2^{pos} DCs (left) and HLA-A2/miHA complex-coated HLA-A2^{neg} DCs (right) before (filled histogram) and after (open histogram) complex coating. B, Cytolytic activity by an HA-1-specific T cell clone (filled diamonds) and an HA-2 specific T cell clone (filled squares) in response to DCs coated with various amounts of HLA-A2/HA-1 or HLA-A2/HA-2 complexes (micrograms per 10^6 DCs) or to HLA-A2^{pos} DCs pulsed with HA-1 or HA-2 peptides (open symbols). C, A polyclonal HA-1-specific T cell line generated from an HLA-A2^{pos} HA-1^{neg} donor was restimulated 2 times with autologous HLA-A2/HA-1 complex-coated DCs. HA-1^{A2} tetramer binding was determined on days 0, 7, and 14. The cytolytic activity directed against HLA-A2^{neg} EBV-LCLs (open bars) or HLA-A2^{pos} EBV-LCLs naturally expressing HA-1 (filled bars) was tested in parallel on days 0 and 14.

HLA-A2/miHA complexes, or HLA-A2/miHA complexes added together with HLA-A2^{neg} DCs in the absence of linking HLA-DR antibodies were not lysed (data not shown). Further, HLA-A2/HA-1 complex-coated DCs effectively enriched an HA-1-specific T cell line for functional HA-1-specific T cells upon 2 rounds of stimulation (Figure 1C). Collectively, these findings demonstrate that HLA-A2/miHA complex-coated DCs can be used as efficient stimulators for miHA-specific T cells.

Generation of AlloHLA-A2-restricted HA-1-specific T Cells from HLA-A2^{neg} Male Donors using Autologous HLA-A2/HA-1 Complex-coated DCs

HLA-A2/HA-1 complex-coated DCs were used as APCs for the generation of HA-1-specific CD8$^+$ T cells from HLA-A2^{neg} male donors M#1 and M#2 (Figure 2A,B). In parallel, we stimulated CD8$^+$ T cells from donors M#1 and M#2 with allogeneic HLA-A2^{pos} DCs pulsed with HA-1 peptide (Figure 2C,D). All 4 T cell lines were restimulated at weekly intervals (see Methods) and expanded well, resulting in 3- to 5-fold increases in absolute cell numbers on day 21 (data not shown). Emerging HLA-A2/HA-1-specific T cells were monitored with HA-1^{A2} tetramers and control tetramers comprising an irrelevant peptide. After 20 d of culture, HLA-A2/HA-1-specific T cells could be detected within the bulk T cell cultures of donors M#1 and M#2 stimulated with autologous HLA-A2/HA-1 complex-coated DCs. The frequency of tetramer binding T cells within the T cell line generated from donor M#1 increased with additional rounds of stimulation, whereas percent tetramer binding T cells remained constant in the T cell culture of donor M#2. Patterns of tetramer staining were different for each alloreactive T cell line, suggesting that distinct subsets of expanded T cells bound tetramers with different avidities. We could not detect HA-1^{A2} tetramer binding cells in T cell lines generated with allogeneic HA-1 peptide-pulsed HLA-A2^{pos} DCs.

The cytolytic activity of all 4 T cell lines was compared after 5 wk of continuous culture. The M#1 and M#2 T cell lines stimulated with autologous HLA-A2/HA-1 complex-coated DCs effectively lysed transporter associated with antigen-processing (TAP)-deficient T2 cells pulsed with HA-1 peptide, whereas unpulsed T2 cells were less efficiently lysed. These results suggested

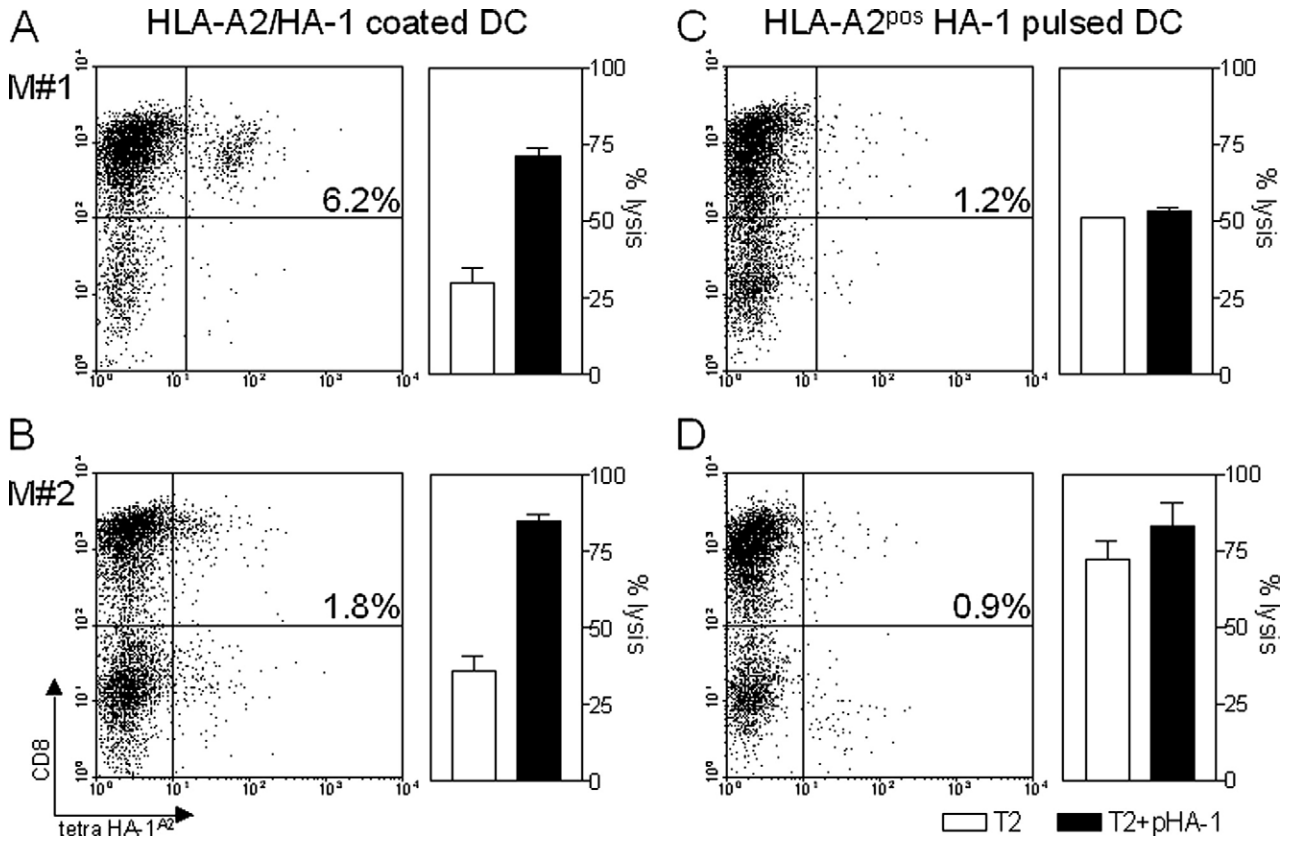

Figure 2. Generation of polyclonal alloHLA-A2/HA-1 specific T cells from HLA-A2^{neg} donors. CD8$^+$ T cells from HLA-A2^{neg} donors M#1 (A, C) and M#2 (B, D) were stimulated with autologous HLA-A2/HA-1 complex-coated DCs (A, B) or with alloHLA-A2^{pos} HA-1 peptide-pulsed DCs (C, D). HA-1^{A2} tetramer binding and cytolytic activity (effector:target ratio, 80:1) directed against TAP-deficient T2 cells (open bars) or HA-1 peptide-pulsed T2 cells (filled bars) were determined after 5 rounds of stimulation. A-D show preferential recognition of HA-1^{A2} by T cells stimulated with HLA-A2/HA-1 complex-coated DCs but not by T cells stimulated with HLA-A2^{pos} HA-1 peptide-pulsed DCs.

that T cell lines generated from donors M#1 and M#2 by autologous HLA-A2/HA-1 complex-coated DCs contained T cells with alloHLA-A2-restricted HA-1-specific reactivity. T cell lines generated with HA-1 peptide-pulsed allogeneic DCs displayed alloHLA-A2 reactivities of undetermined specificity only.

Specificity of In Vitro Generated AlloHLA-A2-restricted HA-1-specific T Cells

To further analyze the specificities of the alloHLA-A2-restricted HA-1-specific T cells generated with autologous HLA-A2/HA-1 complex-coated DCs, we isolated the HA-1^{A2} tetramer binding T cells from the polyclonal M#1 T cell line by flow cytometric cell sorting. The HA-1^{A2} tetramer binding T cells were enriched 26% to >90% (Figure 3A). The specificity of the HA-1^{A2} tetramer binding and nontetramer binding FACS-sorted fractions were assessed in a cytotoxicity assay (Figure 3B, left panel). Lysis profiles of the HA-1^{A2} tetramer binding T cells showed increased recognition of HLA-$A2^{pos}$ EBV-LCLs irrespective of the presence of HA-1, but decreased rec-

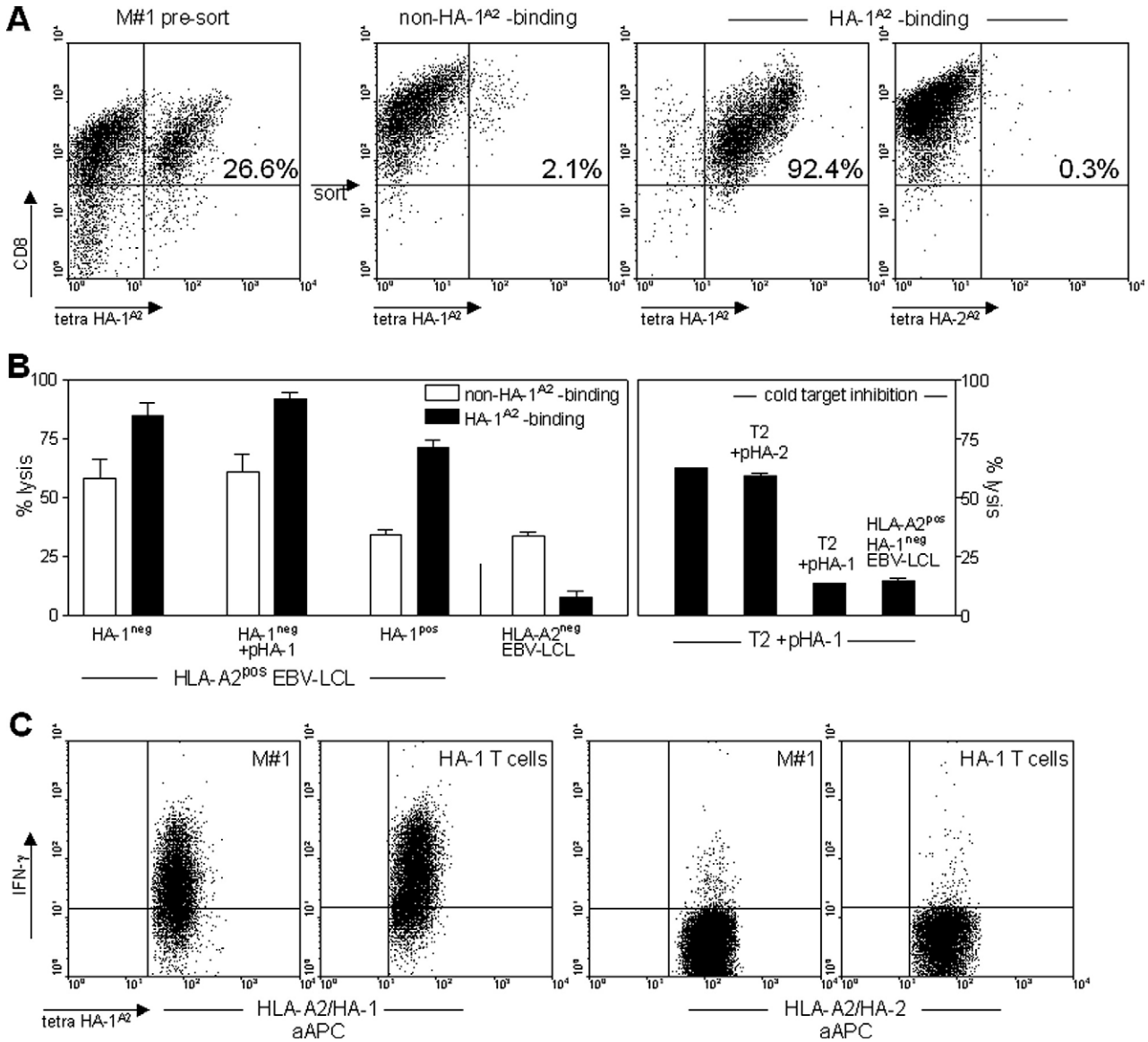

Figure 3. Specificity of polyclonal HA-1^{A2} tetramer binding T cells generated from donor M#1. A, tetramer binding profiles of HA-1^{A2} tetramer binding $CD8^+$ T cells isolated from donor M#1 before and after sorting. B, Left, Cytolytic activity of tetramer binding (filled bars) and tetramer-nonbinding (open bars) $CD8^+$ T cells isolated from donor M#1 directed against HLA-$A2^{pos}$ HA-1^{neg} EBV-LCLs, the same EBV-LCLs exogenously pulsed with HA-1 peptide, HLA-$A2^{pos}$ EBV-LCLs naturally expressing HA-1 or HLA-$A2^{neg}$ EBV-LCL. Right, Cold target inhibition of the cytolytic activity of the HA-1^{A2} tetramer binding $CD8^+$ T cells from donor M#1 in response to T2 cells pulsed with HA-1 peptide (filled bars). Unlabeled T2 pulsed with HA-2 or HA-1 peptide, or HLA-$A2^{pos}$ HA-1^{neg} EBV-LCLs were used as cold targets at a 10:1 cold:hot ratio. C, IFN-γ production by HA-1^{A2} tetramer binding $CD8^+$ T cells from donor M#1 or a HA-1-specific T cell clone in response to aAPCs presenting a single set of HLA-A2/HA-1 or HLA-A2/HA-1 complexes.

ognition of HLA-A2^{neg} EBV-LCLs. Apparently, the HA-1^{A2} tetramer-based flow cytometric cell sorting procedure had enriched for broad alloHLA-A2-reactive T cells but not for HLA-A2/HA-1-specific T cells. The lack of HA-1 peptide-specific lysis could not be attributed to increased NK reactivity because HLA class I^{neg} target cells (K562) were not lysed (data not shown).

Broad alloHLA-reactive T cells staining with multiple tetramers irrespective of peptide have been described before [15,17]. The HA-1^{A2} tetramer binding T cells did, however, not bind irrelevant peptide^{A2} tetramers or lysed T2 cells pulsed with these irrelevant peptides (data not shown). Cold target inhibition studies showed that lysis of HLA-A2^{pos} targets could be inhibited by addition of unlabeled T2 cells pulsed with HA-1 peptide or by addition of HLA-A2^{pos}/HA-1^{neg} EBV-LCLs, but not by addition of T2 cells pulsed with irrelevant HA-2 peptide (Figure 3B, right panel). These findings suggest that the HA-1^{A2} tetramer binding T cells generated from donor M#1 recognize HA-1, but also other undefined peptides presented in the context of HLA-A2, in a peptide-dependent manner.

To further validate this supposition, we analyzed the capacity of the HA-1^{A2} tetramer binding T cells to lyse T2 cells pulsed with fractions from an HPLC-separated HLA-A2-derived peptide pool. Although each of these fractions contains numerous peptides, only 4 of 50 fractions were recognized (data not shown). Another possible explanation for the presence of the HA-1^{A2} tetramer binding T cells of undefined antigen specificity could be peptide-independent T cell recognition of recombinant HLA-A2/peptide complexes due to slight variations in the folding of the recombinant HLA-A2 molecules. The presence of such T cells within the M#1-derived HA-1^{A2} tetramer binding T cell population could confound our findings. To exclude this possibility, we used aAPCs coated with HLA-A2/HA-1 or HLA-A2/HA-2 complexes to stimulate the HA-1^{A2} tetramer binding T cells from donor M#1. An established HLA-A2-restricted HA-1-specific T cell clone was tested in parallel (Figure 3C). The self HLA-A2- and the alloHLA-A2-restricted T cells from donor M#1 only produced IFN-γ when stimulated with HLA-A2/HA-1-expressing aAPCs, whereas nonspecific recognition of HLA-A2/HA-2 expressing aAPCs was not observed.

The T cell line generated from donor M#2 was enriched as described for donor M#1 and cytolytic function and specificity in response to various target cells were analyzed (Figure 4). Despite poor HA-1^{A2} tetramer binding, the FACS-sorted T cell population displayed increased lytic activity of HLA-A2^{pos} EBV-LCLs pulsed with HA-1 peptide. These results imply that the HLA-A2/HA-1-specific T cells obtained from donor M#2 are of low avidity but nonetheless display selective HA-1-specificity.

Direct Isolation of HLA-A2/HA-2-specific T Cells from Peripheral Blood of Multiparous Female Donors

The presence of circulating alloHLA-A2/HA-2-specific T cells in peripheral blood was analyzed in 5 HLA-A2^{neg} multiparous female donors (F#1 to F#5) who had delivered ≥1 HLA-A2^{pos} HA-2^{pos} child. We used the miHA HA-2 because of the high frequency of the HA-2V immunogenic allele in the Caucasian population [37]. HA-2^{A2} tetramer binding T cells were isolated from CD8^{+}-enriched PBMC fractions by FACS sorting. After the first enrichment sort, the

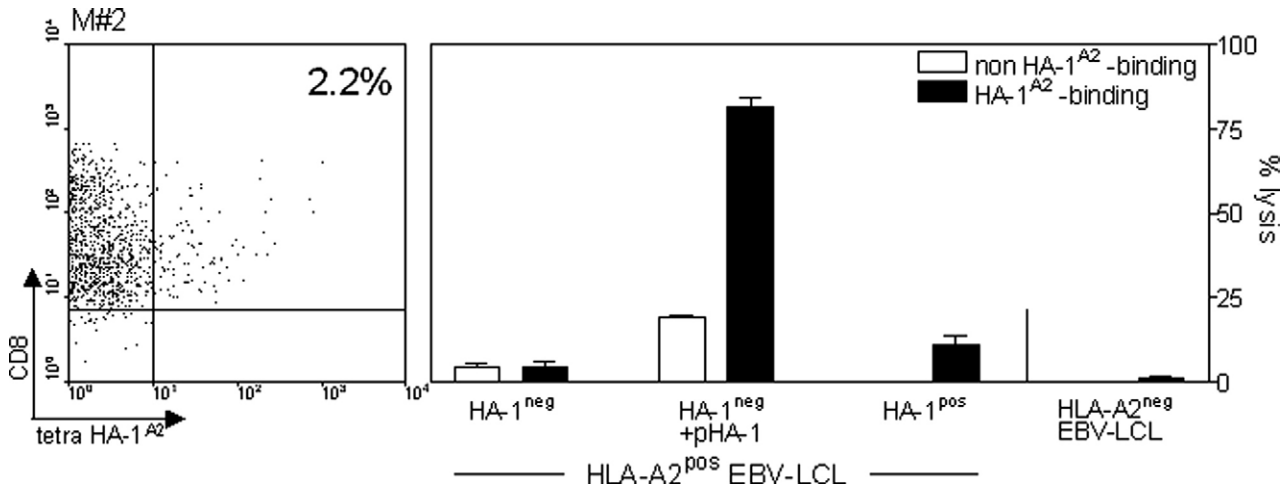

Figure 4. Left, Specificity of polyclonal HA-1^{A2} tetramer binding T cells generated from donor M#2. tetramer binding profile of HA-1^{A2} tetramer binding CD8^{+} T cells isolated from donor M#2 after sorting. Right, Cytolytic activity of tetramer binding (filled bars) and tetramer-nonbinding (open bars) CD8^{+} T cells isolated from donor M#2 directed against HLA-A2^{pos} HA-1^{neg} EBV-LCLs, the same EBV-LCLs exogenously pulsed with HA-1 peptide, HLA-A2^{pos} EBV-LCLs naturally expressing HA-1 or HLA-A2^{neg} EBV-LCL.

HA-2^{A2} tetramer binding profiles varied significantly from hardly any tetramer binding CD8^{+} T cells to binding of a clear population of CD8^{bright} T cells. FACS-sorted populations were nonspecifically expanded in vitro, omitting HA-2-specific stimulation (see Methods). After 28 d of culture, only the T cell populations sorted from donors F#1 and F#5 displayed HA-2^{A2} tetramer binding cells. The T cell culture obtained from donor F#1 contained >90% T cells that selectively bound HA-2^{A2} tetramers without appreciable crossreactivity with irrelevant peptide^{A2} tetramers (Figure 5A). However, HLA-A2^{pos} EBV-LCLs pulsed with HA-2 peptide or naturally expressing HA-2 were not lysed. Thus, the T cells obtained from donor F#1 were of low avidity or not HA-2 specific, despite their prominent tetramer binding profile.

Within the T cell culture established from donor F#5, 5%-8% of cells bound HA-2^{A2} tetramers in addition to HA-1^{A2} and CMV^{A2} tetramers (Figure 5B). These tetramers presumably bind the same T cell population because the percentage of tetramer binding T cells did not increase when the various tetramers were combined (Figure 5C, left panel). Interestingly, the T cell population did not bind HY^{A2} tetramers, indicating that the alloHLA-A2 recognition of these T cells was not peptide independent. When the T cell population was stained with a mixture of HA-1^{A2} and HA-2^{A2} tetramers, only the HA-1^{A2} tetramers were bound, indicating a higher TCR avidity for HLA-A2/HA-1 than for HLA-A2/HA-2 (Figure 5C, center panel). TCR avidity for HLA-A2/HA-1 and HLA-A2/CMV (Figure 5C, right panel) appeared to be similar, despite the absence of sequence homology between these peptides. The T cell line generated from donor F#5 was tested for alloHLA-A2 specificity in a cytotoxicity assay. The T cells lysed HLA-A2^{pos} target cells independently of the presence of HA-2, thus underlining the promiscuity of the T cells isolated from donor F#5 by HA-2^{A2} tetramers.

To further investigate the apparent different alloHLA-A2-reactive T cells populations present in donors F#1 and F#5, we cloned HA-2^{A2} tetramer binding T cells by single-cell-per-well FACS sorting. The

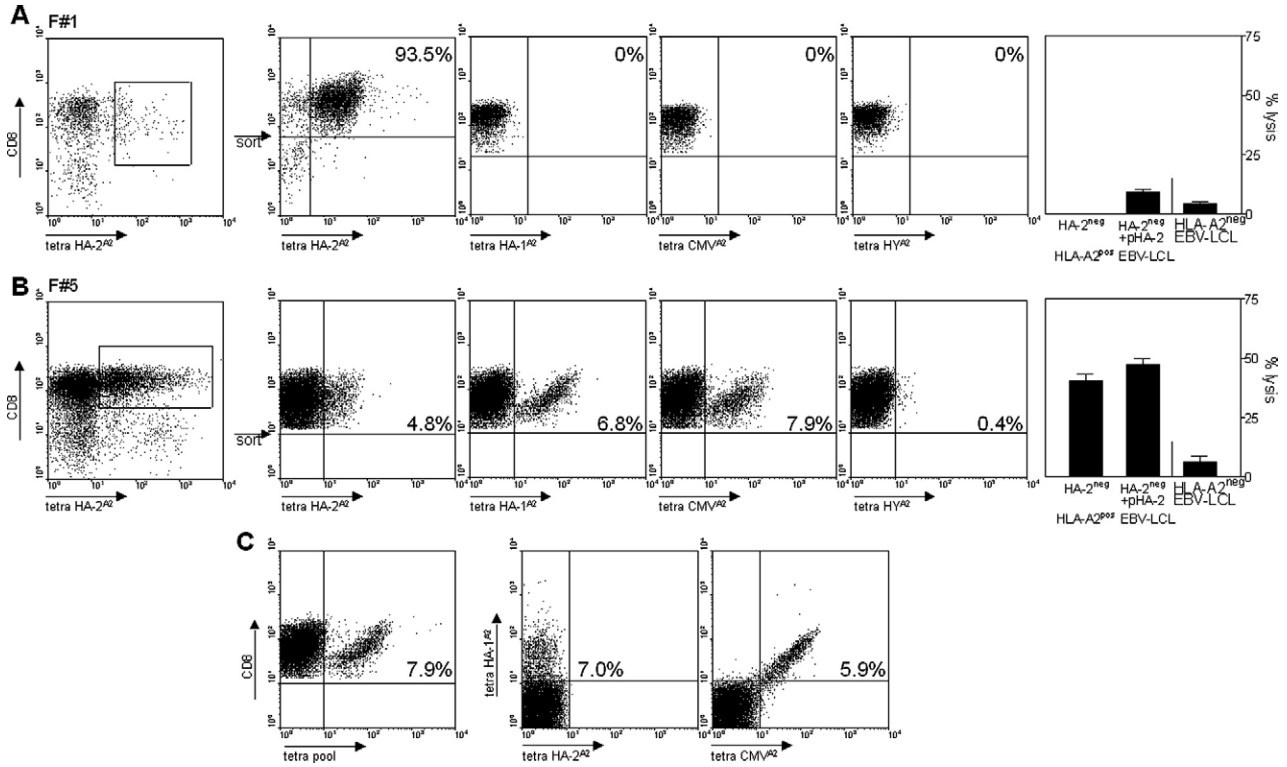

Figure 5. Direct isolation of alloHLA-A2/HA-2-specific T cells from HLA-A2^{neg} parous female donors F#1 and F#5. A, B, Left, Analysis of CD8^{+} cells obtained from the peripheral blood of HLA-A2^{neg} parous female donor F#1 or F#5 after a first enrichment sort for CD8^{+} and HA-2^{A2} tetramer binding cells. The rectangles represent the gate set for the second sort. Center, tetramer binding profiles (HA-2^{A2}, HA-1^{A2}, CMV^{A2}, HY^{A2}) of CD8^{+} T cells derived from donors F#1 or F#5 after 28 d of nonspecific expansion. Right, Cytolytic activity of these CD8^{+} T cells directed against HLA-A2^{pos} HA-1^{neg} EBV-LCLs, the same EBV-LCLs exogenously pulsed with HA-1 peptide or HLA-A2^{neg} EBV-LCL. C, Left, Staining of CD8+ T cells from donor F#5 with pooled tetramers. Center and right, Staining of the CD8+ T cell population from donor F#5 with HA-1^{A2} tetramer-APC and HA-2^{A2} tetramer-PE (center) or with HA-1^{A2} tetramer-APC and CMV^{A2} tetramer-PE (right). A single subset of the donor F#5-derived T cell population binds the HA-2^{A2}, HA-1^{A2}, and CMV^{A2} tetramers with varying affinities.

Table 2. *Functional Characteristics of T Cell Clones Generated from Parous Female Donors*

		Tetramer-staining Intensity*			IFN-γ Production†					
					aAPCs			EBV-LCLs		
		HA-2^{A2}	HA-1^{A2}	HY^{A2}	A2/HA-2	A2/HA-1	A2/HY	HLA-A2^{pos} −pHA-2	HA2^{neg} +pHA-2	HLA-A2^{neg}
F#1	I-3	+	+/−	+	0	6	4	0	0	0
	I-4	+	−	−	72	0	0	14	99	10
	I-28	−	−	−	0	0	0	0	0	0
	I-29	+	−	−	0	0	0	0	0	0
	I-45	+	−	+/−	74	1	8	91	93	0
F#5	I-51	−	−	−	0	0	0	1	1	1
	I-53	+/−	+	−	0	8	NT	25	24	0
	I-60	+	+/−	−	34	14	2	97	97	2

*Staining levels were measured in mean fluorescence units and compared with tetramer staining of established cytolytic HLA-A2-restricted miHA-specific T cell clones (+, equal staining; −, no staining; +/−, intermediate staining).
†IFN-γ production was measured as percent live CD8$^+$ cells producing IFN-γ.

sorted T cells were expanded and tested for their capacity to bind HA-2^{A2}, HA-1^{A2}, and HY^{A2} tetramers and to produce IFN-γ after ligand-specific stimulation. Stimulators were aAPCs presenting a single set of HLA-A2/HA-2, HLA-A2/HA-1, or HLA-A2/HY complexes or various EBV-LCLs. Table 2 summarizes the results. Three different types of T cell clones were generated from donors F#1 or F#5, ie, T cells that bound none of the tetramers tested (I-28, I-51), T cells that bound HA-2^{A2} tetramers and HA-1^{A2} and/or HY^{A2} tetramers (I-3, I-45, I-53, I-60), and T cells that bound HA-2^{A2} tetramers only (I-4). The strength of tetramer binding correlated with the capacity of the various T cell clones to produce IFN-γ in response to the various aAPCs. However, most T cell clones failed to discriminate between HA-2 peptide-pulsed or control unpulsed HLA-A2^{pos}/HA-1, HA-2, and HY^{neg} EBV-LCLs. Only T cell clone I-4 appeared to be HLA-A2/HA-2 specific in the latter assay and was subsequently tested in a cytotoxicity assay. Despite HA-2-specific tetramer binding (Figure 6A) and IFN-γ production (Figure 6B), T cell clone I-4 lysed HLA-A2^{pos}/HA-2^{pos} and HLA-A2^{pos}/HA-2^{neg} EBV-LCLs, but not HLA-A2^{neg} EBV-LCLs or HLA class I^{neg} target cells (Figure 6C).

Collectively, these results show that in vitro exposure through stimulation with artificial alloHLA-A2/miHA complexes and in vivo exposure to natural alloHLA-A2/miHA complexes through pregnancy yield T cells sharing functional characteristics. These T cell populations contain alloHLA-A2-restricted miHA-specific T cells with low TCR avidity or alloHLA-A2-restricted T cells that seem to be miHA specific, but crossreact with unknown peptides naturally presented by HLA-A2.

DISCUSSION

In this study we evaluated 2 different strategies to generate alloHLA-A2-restricted T cells specific for the miHA HA-1 or HA-2 for the purpose of cellular adoptive immunotherapy after putative HLA-A2-mismatched SCT. Aiming at clinical applicability, we chose isolation and expansion procedures meeting good manufacturing practice requirements. Both strategies used recombinant HLA-A2/miHA complexes as sole allogeneic antigens to isolate or expand alloHLA-A2-restricted miHA-specific T cells derived from PBMCs from HLA-A2^{neg} individuals. Neither stimulation with HLA-A2/miHA complex-coated DCs nor direct isolation from peripheral blood using HLA-A2/miHA tetramers yielded T cells displaying strict miHA-specific reactivity despite selective tetramer-staining profiles. Instead, we obtained miHA-specific T cells of low avidity or T cells displaying selective but not miHA-specific cytolytic capacities. The latter T cells were found at both the polyclonal and clonal levels. We conclude that promiscuity of the alloHLA-A2-reactive T cell repertoire hampers the use of HLA/miHA multimers as tools for the generation of alloHLA-A2-restricted HA-1- or HA-2-specific T cells in a reproducible, safe, and clinically applicable manner.

Previous studies have described in vitro generation of alloHLA-restricted antigen-specific cytotoxic T cells [14-18]. However, many of these alloHLA-restricted T cells were of low avidity or displayed additional reactivities against antigens other than the original target antigen [15-18]. These reactivities may have been due to the use of alloHLA-A2^{pos} cells expressing multiple potential allogeneic antigens such as APCs. In our study, we successfully applied and report for the first time the use of autologous HLA-A2^{neg} DCs as APCs. The DCs express HLA-A2/miHA complexes that are bound to the APC surface via anti-HLA-DR antibodies. Similarly, Savage et al [14] applied antibodies that specifically bind to the CD20 molecules expressed by B cells. We compared antibodies specific for CD1a, CD45, HLA class I, and HLA-DR for their capacity to anchor stimulatory

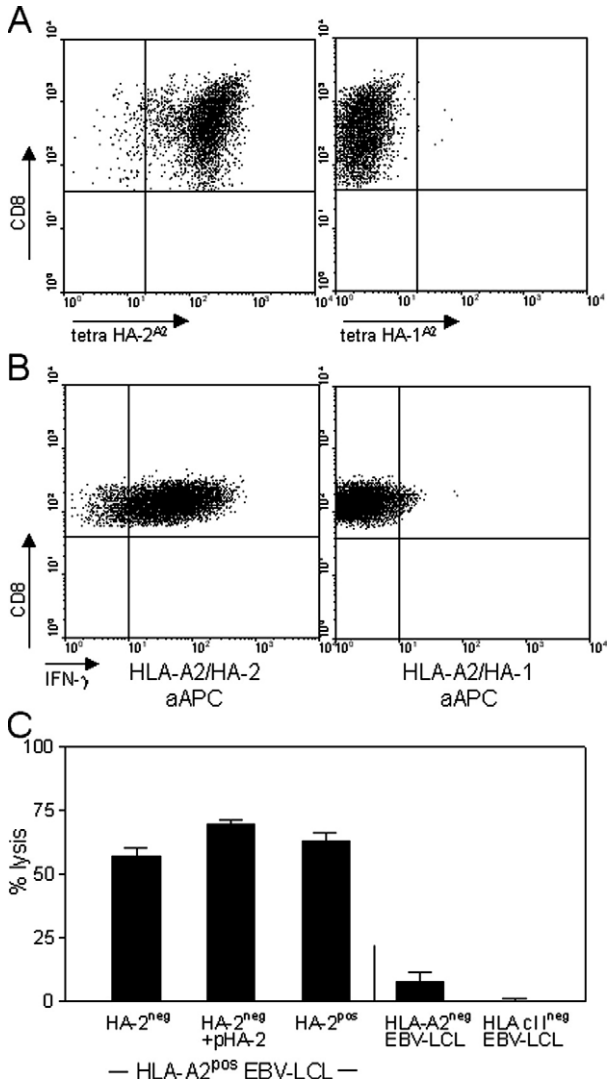

Figure 6. Specificity of HA-2^{A2} tetramer binding T cells derived from donor F#1. A, HA-2^{A2} and HA-1^{A2} tetramer binding by T cell clone I-4, derived from donor F#1 using a single-cell-per-well sorting procedure. B, IFN-γ production of T cell clone I-4 in response to aAPCs presenting a single set of HLA-A2/HA-2 (left) or HLA-A2/HA-1 (right) complexes. C, Cytolytic activity of T cell clone I-4 in response to varying EBV-LCLs displaying HA-2-independent recognition of HLA-$A2^{pos}$ target cells despite HA-1-specific tetramer binding and IFN-γ production shown in A and B.

ligand to DCs. The antibodies were evaluated in FACS analyses, cytotoxicity assays, and expansion procedures using established ligand-specific T cell lines and clones (data not shown). The comparative data showed that DCs anchoring ligand via HLA-DR were the most stimulatory for ligand-specific T cell lines and clones. Although this artificial form of antigen presentation could theoretically affect T cell function, we observed no loss of T cell cytotoxicity or specificity for any antibody tested.

A possible explanation for the high frequency of peptide-selective but not peptide-specific T cells observed in the present study is the application of linked HLA-A2/HA-1 complexes to mature DCs. High avidity alloHLA-A2-restricted HA-1-specific T cells have been generated from the same HLA-$A2^{neg}$ donors used in this study after limiting dilution and extensive testing [13]. The use of the latter optimal APCs as stimulator cells may have inadvertently expanded low affinity or cross-reactive T cells naturally present in the alloHLA-reactive T cell repertoire, thereby hampering the induction of miHA-specific T cells. Alternatively, environmental triggers may have affected the donors' alloHLA-reactive T cell repertoires in the time frame between the 2 studies. The association between "heterologous" antiviral T cell responses and alloHLA reactivity has previously been documented [38].

Our approach to isolate alloHLA-A2-restricted miHA-specific T cells was based on previous studies showing the feasibility of isolating alloHLA-A2-specific T cells by tetramer selection [15,17]. We chose female HLA-$A2^{neg}$ donors who had delivered ≥1 HLA-$A2^{pos}$ HA-2^{pos} child. miHA-mismatched pregnancy has been shown to immunize the mother, resulting in fetal miHA-specific T cells in the maternal circulation [36]. In that study, mother and child shared the HLA-A2 allele. In the HLA-A2-mismatched setting used in this study, only low avidity or cross-reactive T cells could be isolated, despite the use of the same isolation and culture protocols. Thus, our findings may point to a qualitative and/or quantitative difference between T cells generated in a setting wherein the HLA restriction molecule for miHA presentation is shared versus not shared.

Over the past decade, notions on the nature of the TCR-HLA/peptide interaction have changed considerably. Although this interaction was previously thought to be highly specific and restricted to a single HLA/peptide combination, evidence is emerging that TCR promiscuity is a common and important aspect of TCR recognition [39-42]. The balance between specificity and promiscuity likely represents a compromise between the necessity to ensure functional recognition of a large number of possible pathogenic epitopes and the need to avoid harmful autoreactivities. AlloHLA reactivity arises from the promiscuity of a T cell pool originally selected for reactivity to self-HLA molecules. During T cell maturation in the thymus, T cells are negatively selected (deleted) if they recognize ubiquitously expressed peptides in the context of self-HLA with high affinity. Selection for reactivity with peptides presented by alloHLA molecules does not occur. The alloHLA-reactive T cell repertoire is therefore large, up to 10% of the total peripheral T cell repertoire [43,44], and not very specific in terms of antigen recognition. Peptide-independent, peptide-dependent, and peptide-specific recognitions have all been documented [15,17,45-53].

The strategy of generating immunotherapeutic T cells recognizing peptides presented by alloHLA molecules is based on the assumption that the specificities of individual T cells are comparable for alloHLA/peptide complexes and self-HLA/peptide complexes [13]. Recombinant tetrameric HLA/peptide complexes bind to self-HLA-restricted peptide-specific T cells with high specificity [54] and tetramer binding usually correlates with peptide-specific cytolytic and cytokine-secreting functions [32,55]. Although HLA/peptide tetramers have also been used to visualize and isolate peptide-specific alloHLA-restricted T cells [15,17], both studies reported discrepancies between tetramer binding and cytolytic function. In line with these latter reports, we could not rely on HLA-A2/miHA tetramer binding as a selective tool to distinguish between alloHLA-restricted peptide-specific T cells and peptide-nonspecific T cells. Our results indicate that T cells recognize alloHLA/peptide and self-HLA/peptide complexes with different specificities and avidities. Promiscuity of miHA-specific T cells seems to occur predominantly in the alloHLA-restricted setting. The presence of such cross-reactivities seriously hampers the application of alloHLA-restricted T cells for adoptive immunotherapy because they may be detrimental to the patient. Naturally, our conclusions are exclusively based on in vitro analyses and do not allow direct interpretation for the in vivo situation. However, the underlying isolation and expansion protocols were executed as a feasibility study for the purpose of cellular adoptive immunotherapy in the HLA-mismatched SCT setting.

In summary, we show that the alloHLA-A2-restricted T cell repertoire is highly promiscuous. Exposing such T cells to single alloHLA/peptide complexes does not prevent the parallel induction of broad alloHLA-A2 reactivities. Moreover, polyclonal alloHLA-A2-restricted cross-reactive T cells cannot be readily distinguished from miHA-specific T cells by currently available technologies. Generation of alloHLA-A2-restricted hematopoietic system-specific T cells requires laborious and time-consuming limiting dilution protocols and extensive in vitro testing on a recipient's cells before such T cells can be safely applied in the clinical setting of HLA-mismatched SCT.

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