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CMV-, EBV- and ADV-Specific T Cell Immunity: Screening and Monitoring of Potential Third-Party Donors to Improve Post-Transplantation Outcome

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Adoptive immunotherapy with virus-specific T lymphocytes can efficiently reconstitute antiviral immunity against cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus (ADV) without causing acute toxicity or increasing the risk of graft-versus-host disease. To gain insight into antiviral T cell repertoires and to identify the most efficient antigens for immunotherapy, the frequencies of CMV-, EBV- and ADV-specific T cells in 204 HLA-typed healthy donors were assessed using viral peptides and peptide pools. Confirmatory testing for CMV serology by Western blot technique revealed 19 of 143 (13%) false-positive results. We observed highly significant individual and overall differences in T cell frequencies against CMV, EBV, and ADV antigens, whereas antigen-specific T cells were detected in 100% of CMV- seropositive donors, 73% of EBV- seropositive donors, and 73% of ADV-seropositive donors. At least 124 (61%) potential T cell donors were identified for each virus. Among the tested antigens, frequencies for CMV*pp65* and EBV*BZLF1* peptide pools were highest. Short-term in vitro peptide stimulation revealed that a donor response to a certain ADV- and EBV-derived peptide may not be determined without prior stimulation. A modified granzyme B ELISpot was used to detect T cell specificity and alloreactivity. Treatment with allogeneic virus-specific cytotoxic T lymphocytes from seropositive third-party donors may be a feasible therapeutic option for infections following cord-blood stem cell transplantation or hematopoietic stem cell transplantation from virus-seronegative donors.

INTRODUCTION

Major complications after hematopoietic stem cell transplantation (HSCT), such as graft rejection and graft-versus-host disease (GVHD), are encountered by HLA-high resolution donor-recipient matching and T cell depletion from stem cell grafts and treated by increased immunosuppression [1]. The conditioning regimens, even reduced-intensity conditioning, account for a prolonged immune recovery period of 3 to 6 months, during which the patient is highly susceptible to infections normally controlled by T cell immunity. Thus, infectious complications mainly caused by endogenous herpesviruses, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV), or lytic viruses, such as adenovirus (ADV), are common and associated with momentous morbidity and mortality rates. CMV and EBV infections occur in about 60% to 70% and 10% to 26% of SCT recipients, respectively, and ADV infections in about 3% to 20%, with significantly higher rates in pediatric patients [2-4]. Pre-emptive therapy of CMV with ganciclovir or other medication and treatment of EBV with rituximab are possible but do not yield restoration of the T cell repertoire.

Donor lymphocyte infusions can be used to treat both viral infections and leukemia relapses, but are associated with potentially life-threatening GVHD and are unavailable for patients receiving cord blood transplants. Furthermore, treatment with donor lymphocyte infusions is not a therapeutic option for high-risk patients with seronegative donors. It has recently been shown that the adoptive transfer of antiviral cytotoxic T lymphocytes (CTLs) directed against CMV [5-9], EBV [10-13], and ADV [14-16] isolated from seropositive donors can rapidly reconstitute antiviral immunity after HSCT and organ transplantation without significant toxicity or GVHD. Infusions of T lymphocyte lines enriched in multivirus (CMV, EBV, and ADV)-specific CTLs reproducibly controlled infections by all 3 viruses and may form the basis of future adoptive immunotherapy trials in patients at risk for multiple infections [17-20].

Methods for direct selection of virus-specific T cells without the need for ex vivo manipulation are attractive to generate clinical-grade antiviral CTLs. The 2 main approaches include the use of peptide-MHC (pMHC) multimers [21-24] and cytokine secretion assays (eg, interferon-gamma [IFN-γ] secretion assay) [7-9,13,14,25]. The pMHC multimer technology requires knowledge of immunodominant HLA-restricted peptides. Reversible pMHC multimer technologies (streptamers, histamers) were developed to isolate antigen-specific T cells without altering their functional status [26,27]. Cytokine secretion assays are not subject to any HLA

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restrictions when using proteins or peptide pools consisting of overlapping peptides spanning whole immunodominant proteins. They enable the generation of $CD4^+$ and/or $CD8^+$ T cells responsive to multiple epitopes [25].

The isolation of antiviral T cells by the above-described assays is restricted to antigens for which moderate or high T cell frequencies are present in the peripheral blood of potential T cell donors. Furthermore, it is still difficult, laborious, and time-consuming to generate antigen-specific T cells from naïve T cell donors [28-30]. Therefore, allogeneic third-party T cell donors may serve as an alternative for patients receiving allogeneic cord blood transplants, transplants from virus-seronegative donors, or solid organ transplants with limited HLA-match, for which donor blood generally is not available. To gain more insight into virus-specific memory T cell repertoires and to identify the most efficient target antigens for adoptive immunotherapy, we determined the frequencies of antiviral T cells in 204 healthy donors via IFN-γ enzyme-linked immunospot (ELISpot) assay and by flow cytometric analysis using pMHC multimers. Using these well-established methods of T cell monitoring [31,32], we assessed the T cell frequencies against peptides and peptide pools available in good manufacturing practice (GMP) quality, derived from viral proteins previously demonstrated to be immunodominant or subdominant, eg, phosphoprotein 65 (pp65) and immediate early (IE)-1 for CMV [33-35], BZLF1, nuclear antigen [EBNA]-1) and latent membrane protein 2A (LMP2 A) for EBV [36], and the ADV major capsid protein, hexon [37]. Furthermore, a modified granzyme B (GrB) ELISpot was established to determine the T cell specificity and alloreactivity.

MATERIALS AND METHODS

Study Subjects and Preparation of Peripheral Blood Mononuclear Cells

Informed consent as approved by ethics committee of the Medical School Hannover was obtained from 204 healthy donors (mean age, 42 years; range, 20 to 69 years) with a wide HLA diversity, no prior history of blood transfusion, and no signs of acute infection. Age and gender distribution were as follows: male, n = 156; mean age, 43 years; range 20 to 69 years; female, n = 48; mean age, 40 years; range, 22 to 65 years. In all donors, HLA class I and class II (4-digit) typing was performed by sequence-based typing. Peripheral blood mononuclear cells (PBMCs) were resuspended in culture medium (CM) consisting of RPMI1640 (Lonza, Vervies, Belgium) with 10% human AB serum (C.C.pro, Oberdorla, Germany). Samples were cryopreserved in liquid nitrogen for further analysis.

Serological Testing by ELISA and Western Blot

The ELISA-based Abbott Architect CMV-IgG assay (Abbott Diagnostic, London, UK) was routinely used to detect CMV-specific antibodies in donor plasma. Additionally, plasma samples were tested using a commercial Western blot assay designed as a confirmatory assay to determine anti-CMV (recomBlot CMV IgG) or anti-EBV (recomLine EBV IgG, both from Mikrogen, Neuried, Germany) IgG antibodies. The alphaWell Adenovirus IgG ELISA (Mikrogen) was used for qualitative determination of IgG antibodies against ADV. Serological testing was performed at the time of the baseline T cell assay and at 4 weeks and 3 months of follow-up. All donors were retrospectively tested for anti-EBV IgG antibodies by ELISA (Enzygnost anti-EBV-IgG, Dade Behring, Newark, Denmark).

Flow Cytometric Analysis of Antiviral T Cells

Briefly, 1×10^6 freshly isolated PBMCs were stained with the phycoerythrin-conjugated pentamers (Proimmune, Oxford, UK) representing 6 common HLA molecules from different immunodominant proteins of CMV (A*02:01/NLVPMVATV [$pp65_{495-504}$, shortened mA02-pp65] and A*01:01/YSEHPTFTSQY [$pp65_{363-373}$, mA01-pp65]); EBV (A*02:01/CLGGLLTMV [$LMP2_{426-434}$, mA02-LMP2], B*08:01/RAKFKQLL [$BZLF1_{190-197}$, mB08-BZLF1], B*08:01/FLRGRAYGL [$EBNA\text{-}3A_{193-201}$, mB08-EBNA-3A], B*35:01/HPVGEADYFEY [$EBNA\text{-}1_{407-417}$, mB35-EBNA-1]); and ADV (A*01:01/TDLGQNLLY [$hexon_{886-894}$, mA01-Hex], A*24:02/TYFSLNNKF [$hexon_{37-45}$, mA24-Hex] and B*07:02/KPYSGTAYNAL [$hexon_{114-124}$, mB07-Hex]), followed by staining with allophycocyanin-conjugated monoclonal anti-CD8 antibody (mAb, Beckman Coulter, Miami, Florida) and phycoerythrin-Cy7-conjugated anti-CD19 (BD Biosciences, Heidelberg, Germany). Flow cytometric analyses were performed using a FACS-CantoII flow cytometer (BD Biosciences) and FACSDiva Software (V6.1.2). At least 50,000 events in the lymphocyte gate were analyzed and the proportion of virus-specific pentamer$^+$/$CD8^+$ cells was expressed as percentage of all $CD19^-CD8^+$ lymphocytes analyzed. The gating strategy used for the detection of virus-specific pentamer$^+$/$CD8^+$ cells (A) and representative dot plots for each pentamer (B) are shown in Supplementary Figure S-F1. For a population to be considered positive, 1 of 2 conditions had to be met: (1) well-defined cell population, and/or (2) ≥ .3% pentamer$^+$/$CD8^+$ cells. Scattered groups of stained cells were not sufficient to be considered a positive population.

IFN-γ ELISpot Assay for High-throughput Screening

MultiScreen filter plates (MSIPS4W10, Millipore, Bedford, MA) were coated overnight with 1 μL/mL anti-IFN-γ mAb 1-D1 K (Mabtech, Stockholm, Sweden) and blocked with CM for 1 hour at 37°C. Before performing ELISpot assays, the optimal cell number was validated by cell titration using 1×10^5, 2×10^5, 2.5×10^5, 3×10^5, 4×10^5, and 5×10^5 PBMCs/well isolated from 4 different donors (Supplementary Figure S-F2). Because 2.5×10^5 PBMCs/well were identified in the range of linearity for all used peptide pools and the classification of the donors into the different responder groups by using 2.5×10^5 PBMCs/well remained stable at higher cell concentrations (Supplementary Table S-T1), this cell number was used in all further ELISpot assays.

The 2.5×10^5 PBMCs were plated in 125 μL CM/well and incubated overnight with 10 μg/mL CEF pool (positive control covering HLA class I–restricted T cell epitopes of CMV, EBV, and flu virus (CEF peptides), PANATecs, Tuebingen, Germany), 10 μg/mL peptide and .6 nmoL of each peptide/mL peptide pool (pp), respectively. Pools of the following overlapping peptides were used to screen donors independent of HLA type: ppCMV*pp65*, ppCMV*IE-1*, ppEBV*EBNA-1*, ppEBV*LMP2 A*, ppEBV*BZLF1*, and ppADV5*hexon* (Miltenyi Biotec, Bergisch Gladbach, Germany).

IFN-γ secretion was detected using biotin-conjugated antihuman IFN-γ antibodies (mAb 7-B6-1-biotin, Mabtech) and streptavidin-alkaline phosphatase (Mabtech) and revealed by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT Liquid Substrate, Sigma-Aldrich, Hamburg, Germany). Spots were counted using ImmunoScan Core Analyzer and ImmunoSpot 5.0 Academic software (both from Cellular Technology Ltd., Bonn, Germany). Means of duplicate wells were calculated and expressed as spots per well (spw)/2.5×10^5 PBMCs. Donors were divided into 3 groups as follows: high responders (≥50 spw), low responders (10 spw to 50 spw), and nonresponders (≤10 spw).

Peptide-specific Expansion of Antiviral T Cells

Frequencies of virus-specific T cells of 30 donors were determined before stimulation (day 0) by HLA-matched pentamer staining and ELISpot using the respective HLA-matched peptides. The following peptides were used with regard to the donor's HLA type: (1) CMV, A*02:01/NLVPMVATV ($pp65_{495-504}$, shortened pA02-pp65) and A*01:01/YSEHPTFTSQY ($pp65_{363-373}$, pA01-pp65); (2) EBV, A*02:01/CLGGLLTMV ($LMP2_{426-434}$, pA02-LMP2), B*08:01/RAKFKQLL ($BZLF1_{190-197}$, pB08-BZLF1), B*08:01/FLRGRAYGL ($EBNA\text{-}3A_{193-201}$, pB08-EBNA-3A), and B*35:01/HPVGEADYFEY ($EBNA\text{-}1_{407-417}$, pB35-EBNA-1); and (3) ADV, A*01:01/TDLGQNLLY ($hexon_{886-894}$, pA01-Hex), A*24:02/TYFSLNNKF ($hexon_{37-45}$, pA24-Hex), and B*07:02/KPYSGTAYNAL ($hexon_{114-124}$, pB07-Hex) (all ProImmune).

PBMCs were resuspended in CM supplemented with 50 U/mL IL-2 (PeproTech, Hamburg, Germany) at a concentration of 1×10^7 cells/mL and stimulated with 10 μg/mL peptide. On day 7, cells were analyzed by pentamer staining and IFN-γ ELISpot using the specified peptides for overnight stimulation.

Detection of IFN-γ Secreting Cells by Cytokine Secretion Assay

Cytokine secretion assays were performed using PBMCs from 4 donors, diluted at 1×10^7 cells/mL in CM and stimulated for 4 hours with ppCMV*pp65*, ppEBV*BZLF1*, and ppADV5*hexon* at a final concentration of 1 μg/mL of each peptide/mL pp. Additionally, PBMCs from 7 donors were stimulated with either ppCMV*IE-1*, ppEBV*EBNA-1*, and/or ppEBV*LMP2*A. The assays were performed according to the manufacturer's instructions (IFN-γ Secretion Assay—Detection Kit, Miltenyi Biotec). Anti-CD8- allophycocyanin and anti-CD4-FITC mab (BD Biosciences) were used for staining. At least 20,000 events were acquired in the live gate for each analysis. Gates were set based upon the scatter properties of lymphocytes and on IFN-γ^+/$CD3^+$, IFN-γ^+/$CD8^+$, and IFN-γ^+/$CD4^+$ T cell populations.

Granzyme B and IFN-γ ELISpot Assays to Determine Specificity and Alloreactivity

T cells stimulated for 7 days with the respective peptides were tested for killing of autologous and allogeneic PBMCs in IFN-γ and GrB ELISpot assays. IFN-γ ELISpot plates were coated and developed as already described. The