

Variances in Antiviral Memory T-Cell Repertoire of CD45RA- and CD62L-Depleted Lymphocyte Products Reflect the Need of Individual T-Cell Selection Strategies to Reduce the Risk of GvHD while Preserving Antiviral Immunity in Adoptive T-Cell Therapy

Caroline Mangare^a Sabine Tischer-Zimmermann^a Agnes Bonifacius^a
Sebastian B. Riese^a Anna Christina Dragon^a Rainer Blasczyk^a
Britta Maecker-Kolhoff^b Britta Eiz-Vesper^a

^aInstitute of Transfusion Medicine and Transplant Engineering, Hannover Medical School, Hannover, Germany;

^bDepartment of Pediatric Hematology and Oncology, Hannover Medical School, Hannover, Germany

Keywords

Naïve T-cell depletion · Low precursor frequency · T-cell response · Alloreactivity

Abstract

Introduction: Viral infections and reactivations still remain a cause of morbidity and mortality after hematopoietic stem cell transplantation due to immunodeficiency and immunosuppression. Transfer of unmanipulated donor-derived lymphocytes (DLI) represents a promising strategy for improving cellular immunity but carries the risk of graft versus host disease (GvHD). Depleting alloreactive naïve T cells (T_N) from DLIs was implemented to reduce the risk of GvHD induction while preserving antiviral memory T-cell activity. Here, we compared two T_N depletion strategies via CD45RA and CD62L expression and investigated the presence of antiviral memory T cells against human adenovirus (AdV) and Epstein-Barr virus (EBV) in the depleted fractions in relation to their functional and immunophenotypic characteristics. **Methods:** T-cell responses against ppEBV_EBNA1, ppEBV_Consensus and ppAdV_Hexon within T_N -depleted (CD45RA⁻/CD62L⁻) and T_N -enriched (CD45RA⁺/CD62L⁺) fractions were quantified by interferon-gamma (IFN- γ) ELISpot assay after short- and long-term *in vitro* stimulation. T-cell frequencies and immunophenotypic composition were assessed in all

fractions by flow cytometry. Moreover, alloimmune T-cell responses were evaluated by mixed lymphocyte reaction. **Results:** According to differences in the phenotype composition, antigen-specific T-cell responses in CD45RA⁻ fraction were up to 2 times higher than those in the CD62L⁻ fraction, with the highest increase (up to 4-fold) observed after 7 days for ppEBV_EBNA1-specific T cells. The CD4⁺ effector memory T cells (T_{EM}) were mainly responsible for EBV_EBNA1- and AdV_Hexon-specific T-cell responses, whereas the main functionally active T cells against ppEBV_Consensus were CD8⁺ central memory T cells (T_{CM}) and T_{EM} . Moreover, comparison of both depletion strategies indicated that alloreactivity in CD45RA⁻ was lower than that in CD62L⁻ fraction. **Conclusion:** Taken together, our results indicate that CD45RA depletion is a more suitable strategy for generating T_N -depleted products consisting of memory T cells against ppEBV_EBNA1 and ppAdV_Hexon than CD62L in terms of depletion effectiveness, T-cell functionality and alloreactivity. To maximally exploit the beneficial effects mediated by antiviral memory T cells in T_N -depleted products, depletion methods should be selected individually according to phenotype composition and CD4/CD8 antigen restriction. T_N -depleted DLIs may improve the clinical outcome in terms of infections, GvHD, and disease relapse if selection of pathogen-specific donor T cells is not available.

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Published by S. Karger AG, Basel

Introduction

Hematopoietic stem cell transplantation and solid organ transplantation are sometimes associated with infectious complications due to conditioning and immunosuppression. Post-transplant mortality is caused by graft versus host disease (GvHD) in 12% of cases and by infections in 11% of cases [1, 2]. Viral infections and reactivations frequently encountered among immunocompromised patients mainly involve endogenous herpesviruses, such as cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV6), and lytic pathogens, such as human adenovirus (AdV) and BK polyomavirus. EBV persistently infects more than 90% of the human adult population and can cause infectious mononucleosis, which is highly symptomatic [3, 4]. EBV is categorised into 3 latency types and has a potent B-cell growth-transforming ability linked to various EBV-associated tumours, including endemic Burkitt lymphoma, Hodgkin lymphoma and diffuse large B-cell lymphoma, and is associated with post-transplant lymphoproliferative disease in immunocompromised patients [5–8]. Among viral infections, AdV is the most common infectious complication in children with reported incidences varying from 6 to 28% after hematopoietic stem cell transplantation [9] followed by up to 6% in adults [10, 11]. Reduction of immunosuppression is a standard approach to restoring antiviral immunity in these patients, while antiviral drug therapy is administered to prevent or control viral infection or reactivation [12]. Downstream complications, adverse drug reactions and the appearance of disease-resistant mutants are major limitations of these drugs.

During the past years, the therapeutic potential of virus-specific memory T cells (VSTs) was demonstrated using unmanipulated donor lymphocyte infusions (DLIs) from seropositive donors. However, application of DLIs resulted in the same cases in the development of severe GvHD due to the presence of alloreactive T cells in the naïve T-cell (T_N) compartment, which accounted for up to 10% of circulating T cells [13]. These limitations led to the development of methods for VST-enriched products, which were shown to have beneficial therapeutic effects against severe viral infections, particularly CMV, EBV and AdV, and, consequently, this treatment approach has now been extended to other viral infections [14, 15]. VSTs can be isolated via an interferon-gamma (IFN- γ)-based cytokine capture system, directly selected via reversible peptide-major histocompatibility complex (pMHC) multimers [16, 17], or generated by *in vitro* expansion, starting with a small number of antiviral precursor T cells in the presence of specific target antigens [18]. Even so, the potential to generate VSTs by these promising strategies is limited by conditions required for effective stimulation

and enrichment and a good knowledge of immunodominant viral proteins and epitopes and the availability of good manufacturing practice quality grade antigens. Furthermore, low immunogenicity of some T-cell epitopes (e.g., Epstein-Barr nuclear antigen 1, EBNA1) also causes problems [19]. Consequently, strategies to refine DLIs through selective T_N depletion were developed to obtain antiviral memory T-cell products with the potential to protect patients from viral infection/reactivation while reducing the risk of GvHD occurrence [20–22].

Antiviral memory T cells are a heterogeneous population differing in phenotype, function and protective capacity against different viruses [23, 24], but their contribution to alloreactivity is unknown. Strong correlations between the frequency, the phenotypic profile and the protective efficacy of antiviral T cells have been described [25]. These characteristics may vary among individuals, resulting in different strengths of antiviral responses. We and other researchers demonstrated that CMV-specific T-cell responses are dependent on CD8⁺ late effector memory RA T cells (T_{EMRA}) [26, 27], while other studies reported that central memory T cells (T_{CM}) are responsible for immune responses against hepatitis C virus (HCV) and effector memory T cells (T_{EM}) for human immunodeficiency virus (HIV) [27, 28]. CD4⁺ and CD8⁺ antiviral memory T-cell functions have also been described in different viral antigen settings. CD4⁺-specific T cells are reportedly responsible for the alleviation of Dengue virus [29], West Nile virus [30], Zika virus [31] and Friend virus [32], and CD8⁺-specific T cells for lymphocytic choriomeningitis virus [33], influenza A virus [34], CMV [27] and some EBV epitopes from latent membrane proteins (LMP), LMP2 and LMP1 [35, 36]. Therefore, as new T-cell therapy modalities enter into clinical trials, it is important to understand the precise contribution of CD4⁺ and CD8⁺ naïve and memory T cells and the circumstances that predict a greater likelihood of success in improving antiviral T-cell responses and reducing the risk of GvHD.

T-cell phenotypes are characterised according to their cell surface markers. T_N are CD45RA⁺/CD62L⁺, T_{CM} CD45RA[−]/CD62L⁺, T_{EM} CD45RA[−]/CD62L[−], and T_{EMRA} CD45RA⁺/CD62L[−] [37]. These differences allow for selective T_N depletion using CD45RA and CD62L microbeads as major T_N depletion strategies. CD45RA depletion has been the subject of most clinical trials performed to date, in spite of the subsequent loss of CD8⁺ T cells and T_{EMRA} [21, 26, 35, 36]. On the other hand, T_{CM} are lost in CD62L-based depletion. Nevertheless, precise information about the contribution of CD45RA^{+/−}, CD62L^{+/−} and CD4/CD8 T-cell proportion against various viral antigens has not been described. In a previous study, we demonstrated that the magnitude of CMV-specific T-cell responses depends on the immunophenotypic composition

of the lymphocyte product [26]. T-cell fractions that had a higher proportion of CD8⁺ T_{EMRA} elicited significantly higher T-cell responses against ppCMV_pp65 and ppCMV_IE1. We extended this approach to the current study, which aimed to elucidate the most efficient T_N depletion strategy in terms of preserving functional antiviral memory T cells with low precursor frequencies and their protective capacity while using human AdV and EBV as viral targets of interest. Moreover, we examined the potency of alloreactive responses among the T-cell fractions to detect T-cell subsets with the least alloreactivity.

Materials and Methods

Ethics Statement and Donors

All experiments were performed with residual blood samples from platelet apheresis disposable kits used for routine platelet collection from regular healthy blood donors of the Institute of Transfusion Medicine and Transplant Engineering at the Hannover Medical School (MHH). Informed consent as approved by the Ethics Committee of the MHH was obtained from all donors. All donors belong to alloCELL registry (www.allcell.com) and were pre-tested for EBV and AdV serology as described previously using commercially available IgG Western blot and IgG ELISA kits (recomLine EBV IgG and alphaWell Adenovirus IgG ELISA, both from Mikrogen) according to the manufacturer's instructions [38, 39].

Isolation of Peripheral Blood Mononuclear Cells and T Cells

Isolation of the different T-cell fractions was performed as described previously [26, 40]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from EBV- and AdV-seropositive donors by discontinuous-gradient centrifugation. Untouched CD3⁺ T cells were enriched by magnetic cell sorting (MACS) using the Pan T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. CD3⁺ T cells were collected as flow-through and bead-loaded non-T cells were collected from columns as eluate. Further, the isolated CD3⁺ T cells were used for T_N depletion using CD45RA or CD62L immunomagnetic microbeads according to the manufacturer's instructions (Miltenyi Biotec). Negative fractions (flow-through) and positive fractions (eluate) were collected by LS columns (Miltenyi Biotec) and termed as follows: memory/negative fractions (CD45RA_NF and CD62L_NF) and naïve/positive fractions (CD45RA_PF and CD62L_PF).

Short- and Long-Term *in vitro* Stimulation Assays to Assess EBV- and AdV-Specific T-Cell Response in T_N-Depleted and T_N-Enriched Fractions

EBV- and AdV-specific memory T cells in T_N-depleted and -enriched fractions were evaluated by phenotypic and functional assays. Briefly, freshly isolated CD45RA and CD62L T-cell fractions (CD45RA_NF/PF and CD62L_NF/PF) were rested overnight (37°C, 5% CO₂) at a density of 1×10^7 cells/well (24-well plate, Sarstedt) prior to short- (1 day) or long-term (7 days) *in vitro* stimulation. 1×10^7 CD3-depleted PBMCs/well (24-well plate) were loaded overnight with the peptide pool (pp) ppEBV_EBNA1, ppEBV_Consensus or ppAdV_Hexon, respectively (1 µg/per peptide/mL; Miltenyi Biotec). On day 1, T cells at a density of 5×10^5 were co-cultured with 5×10^4 antigen-loaded CD3-depleted PBMCs in T-cell culture medium (TCM) RPMI1640 (Lonza) with

10% heat-inactivated human AB serum (C.C.pro) supplemented with 50 U/mL interleukin (IL)-2, 10 ng/mL IL-7 and 10 ng/mL IL-15 (all PeproTech) over 7 days.

Detection of EBV- and AdV-Specific T-Cell Response by IFN-γ Enzyme-Linked ImmunoSpot (ELISpot) Assay

Functionality of the different T-cell fractions was analysed by a target cell-dependent IFN-γ ELISpot assay after short- and long-term *in vitro* stimulation, as previously described [38, 41]. On day 0 (short-term stimulation) as well as on day 6 (long-term stimulation), 1×10^7 CD3-depleted PBMCs/well (24-well plate) were loaded overnight either with ppEBV_EBNA1, ppEBV_Consensus or ppAdV_Hexon (1 µg per peptide/mL) in TexMACS medium (Miltenyi Biotec) followed by co-cultivation with the different T-cell fractions (effector cells) in an effector to target (E:T) ratio of 1:1 and 5:1 in pre-coated anti-IFN-γ ELISpot plates (Lophius Biosciences). Negative and positive controls were carried out by using either solitary medium, effector T cells and target cells alone or 1 µg/mL staphylococcal enterotoxins B (SEB, Sigma Aldrich). IFN-γ secretion was detected using streptavidin-alkaline phosphatase (Mabtech) and revealed by 5-13 bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT Liquid Substrate, Sigma-Aldrich). Spots were counted using AID ELISpot 8.0 on an AID iSpot spectrum reader system (both from AID). Resultant findings are indicated as number of spots per well (spw). The cut-off for positive response was at least 2 times higher than the negative control.

Alloreactivity in Mixed Lymphocyte Reaction Cultures

T-cell-mediated alloreactivity was measured in all T-cell fractions (CD45RA_NF/PF and CD62L_NF/PF) by allogeneic mixed lymphocyte reaction (MLR) assay. T-cell fractions (effector cells) were labelled with carboxyfluorescein succinimidyl ester (CFSE, ThermoFisher Scientific) at a final concentration of 4 µM. At a cell density of 1×10^5 , CFSE-labelled T-cell fractions (responder) were co-cultured with irradiated (1×30 Gy) HLA-matched autologous or HLA-mismatched pooled PBMCs (stimulator, PBMCs from 3 to 5 donors) at an E:T ratio of 1:1 in TCM supplemented with 50 U/mL IL-2, 10 ng/mL IL-7 and 10 ng/mL IL-15. As negative controls, target or effector cells were cultured alone, while T-cell fractions co-cultured with anti-CD3/CD28 beads (ThermoFisher Scientific) in a 1:1 ratio served as positive control. After 7 days, T-cell frequencies and phenotype were analysed by flow cytometry. Proliferation of CFSE-labelled T-cell fractions co-cultured with HLA-mismatched allogeneic PBMCs was compared with proliferation in relation to HLA-matched autologous controls determined by CFSE dilution. The difference in T-cell proliferation between the autologous and allogeneic setting was used as readout for alloreactivity.

Flow Cytometry Analysis

T-cell frequencies, CD4/CD8 ratio and the phenotypic T-cell composition were assessed by flow cytometry (FACSCanto 10c, FACSDiva V8.0.1 software, BD Biosciences) directly after isolation and T_N depletion (PBMCs, CD3⁺ T cells and CD45RA_NF/PF, CD62L_NF/PF) as well as after long-term *in vitro* stimulation (day 6). Assessment of T-cell frequency, CD4/CD8 ratio and phenotypic T-cell composition was carried out using the following monoclonal antibodies (mAbs): anti-CD3-Peridinin-Chlorophyll (PerCP), anti-CD8Alexa Fluor (AF) 700, anti-CD45RA-brilliant violet (BV) 510 and anti-CD62L-allophycocyanin/Cyanin 7 (APC/Cy7) (all BioLegend). Gating was carried out in accordance with the light scatter properties of lymphocytes and CD3⁺ T-cell populations. With at least 30,000 events in the CD3⁺ gate, the focus of the gating strategy was set on the CD3⁺, CD3⁺CD8⁺ and CD3⁺CD4⁺

Table 1. Assessment of cellular composition including T-cell frequencies and phenotypes among healthy blood donors following naive T-cell depletion

T-cell subset	PBMCs	CD3 ⁺	CD45RA_NF	CD62L_NF	CD45RA_PF	CD62L_PF
CD3 ⁺ , %	64.4 (47.4–74.9)	96.0 (95.2–97.3)	97.5 (94.8–99.7)	96.2 (94.2–98.3)	94.9 (91.8–97.9)	96.0 (94.9–99.5)
T _N , %	34.0 (16.4–49.3)	47.0 (33.1–58.7)	0.2 (0.04–0.48)	13.5 (0.54–45.1)	72.7 (65.2–78.4)	57.6 (39.6–64.0)
T _{CM} , %	20.7 (9.63–30.0)	24.3 (18.3–31.1)	57.5 (52.1–62.7)	2.5 (0.11–7.68)	0.2 (0.01–0.43)	35.6 (26.0–56.0)
T _{EM} , %	21.5 (15.3–30.7)	18.6 (10.1–29.6)	42.1 (37.2–47.6)	63.8 (23.5–85.0)	0.1 (0.01–0.28)	3.11 (1.02–6.49)
T _{EMRA} , %	23.9 (11.2–48.4)	10.2 (5.79–14.9)	0.14 (0.042–0.31)	20.2 (11.6–27.4)	27.0 (21.0–34.8)	4.0 (0.98–10.6)
CD4 ⁺ , %	84.33 (78.6–91.1)	71.62 (65–79.4)	83.93 (74.4–89.5)	64.35 (57.0–83.3)	65.85 (60.6–75.7)	23.28 (10.4–29.7)
CD8 ⁺ , %	15.67 (8.92–21.4)	28.38 (20.6–35.0)	16.07 (10.5–25.6)	5.65 (16.7–43.0)	34.15 (24.3–39.4)	24.66 (3.6–46.6)
CD4/8 ratio	5.38	2.52	5.22	1.81	1.93	0.9

T-cell frequencies and phenotypes in different T-cell fractions, as determined by flow cytometry (mean, range; $n = 6$) in naive T-cell-depleted and naïve-enriched T-cell fractions. T-cell frequencies are expressed as % mean and range of CD3⁺, CD4⁺, and CD8⁺ T cells.

T cells in order to assess their phenotype using CD45RA and CD62L as specific markers. Data were analysed by using the FlowJo V10.6.1 software (Becton Dickinson).

Statistics

Statistical analysis was performed using the Prism v8.0 software (GraphPad). The results are displayed as mean, range $\pm$ standard deviation (SD). Generated data were analysed using non-parametric Mann-Whitney test and Wilcoxon matched-pairs signed-rank test (* $p < 0.05$, ** $p < 0.01$, not significant [ns]).

Results

Efficacy of T_N Depletion Using Immunomagnetic CD45RA and CD62L Microbeads

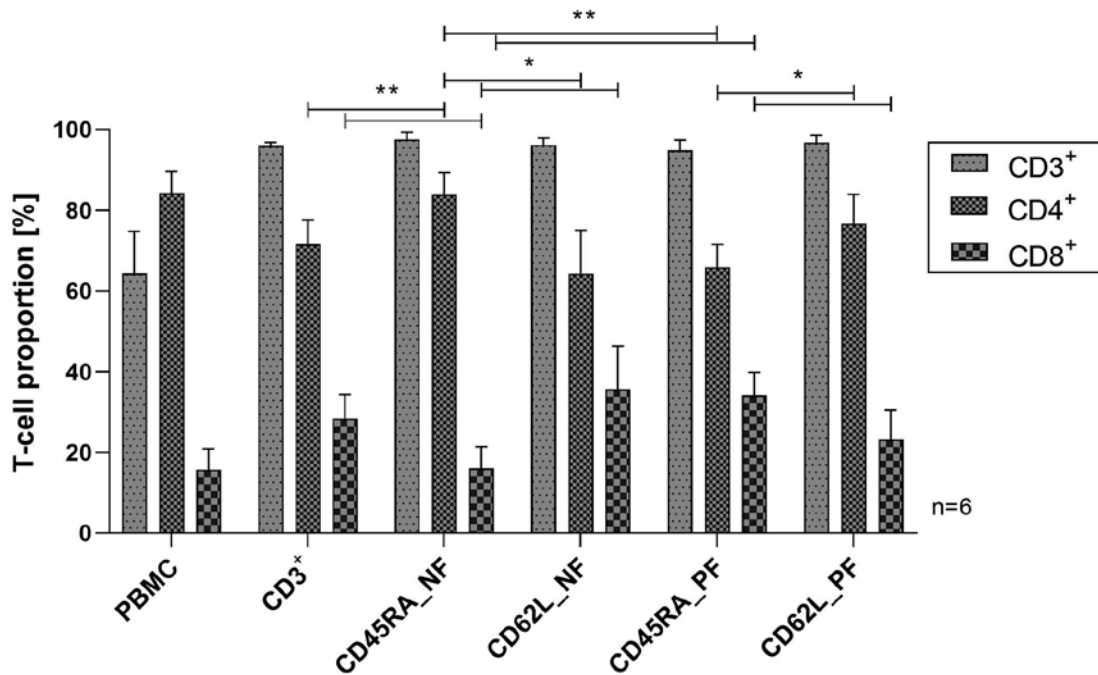
Small-scale experiments with PBMCs from healthy blood donors were performed to assess the feasibility of T_N-depleted DLI using CD45RA and CD62L microbeads. The CD45RA_NF/PF as well as CD62L_NF/PF fractions were analysed for purity and phenotype (shown in Fig. 1). The mean CD3⁺ T-cell frequency in CD45RA_NF and CD62L_NF was 97.5 and 96.2%, 83.9 and 64.3% for CD4⁺ T-cell subsets, and 16.1 and 35.7% for CD8⁺ T-cell subsets, respectively (shown in Table 1, Fig. 1A). Hence, the depletion of T_N led to a significant reduction of CD8⁺ T cells in CD45RA_NF (CD4/CD8 ratio 5.2, $p < 0.01$), but not in CD62L_NF (CD4/CD8 ratio 1.8, shown in Fig. 1A). Furthermore, the proportion of CD4⁺ and CD8⁺ T cells was significantly different between CD45RA and CD62L fractions (NF/PF, $p < 0.05$). Regarding the memory fractions gated on CD3⁺ T cells, CD45RA_NF consisted mainly of T_{CM} (mean 57.5%) and T_{EM} (mean 42.1%), whereas CD62L_NF consisted mainly of T_{EM} (mean 63.8%) and T_{EMRA} (mean 20.2%), with 13.5% T_N contamination (shown in Fig. 1B). Conversely, in the naïve fractions, CD45RA_PF contained predominantly T_N and T_{EMRA}, while CD62L_PF contained mainly T_N and T_{CM}

with only a small proportion of T_{EM} and T_{EMRA}. Overall, CD45RA microbeads proved to be more suitable for T_N depletion, because of a higher purity of the memory T-cell fraction (NF).

Functional Signatures of Antiviral Memory T Cells within CD45RA_NF/PF and CD62L_NF/PF Fractions

Based on observations of viral infections *in vivo*, there is a strong correlation between the phenotypic profiles of antiviral T cells, which dictate the magnitude of T-cell responses, and their protective efficacy against different viruses [25, 26, 42]. With respect to low-frequency antiviral T cells, we evaluated EBV- and AdV-specific T-cell responses in T_N-depleted and T_N-enriched T-cell fractions following short-term (1 day; shown in Fig. 2A, B and Fig. 3A–C) and long-term *in vitro* stimulation (7 days; shown in Fig. 3A–C) using a target cell-dependent IFN- γ ELISpot assay as the readout system. T-cell responses were evaluated in the T_N-depleted fractions since these fractions were comprised of memory T-cells depending on the depletion strategy used, and it was anticipated that they would elicit antiviral T-cell responses. Overall, after short-term stimulation, T-cell responses, measured as number of spw, were highest against the ppEBV_Consensus among all T-cell fractions with CD45RA_NF showing the highest T-cell response (mean 319 spw, range 118–790 spw). On the other hand, T-cell responses against ppEBV_EBNA1 and ppAdV_Hexon following short-term stimulation were absent or low, with the lowest number of spots detected in CD62L_PF (ppEBV_EBNA1, mean 10 spw, range 0–52 spw; ppAdV_Hexon mean 7 spw, range 0–16 spw). However, the highest T-cell responses for both peptide pools were detected among CD45RA_NF, with a mean of 42 spw (ppEBV_EBNA1) and 39 spw (ppAdV_Hexon), respectively, as shown in Figure 2A, B. Low-frequency T cells against EBV_EBNA1 and AdV_Hexon were considerably better detectable by

A



B

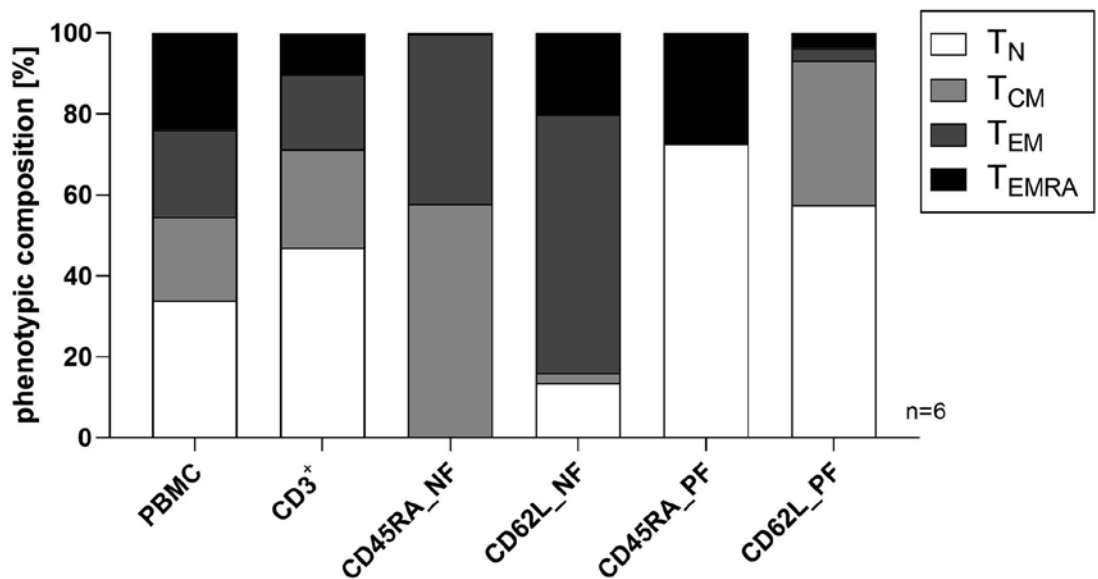


Fig. 1. Evaluation of T-cell frequencies and phenotypes following T_N depletion. CD3⁺ T cells were isolated from PBMCs of 6 healthy blood donors followed by T_N depletion using CD45RA and CD62L microbeads. Immunophenotypic analysis was performed by flow cytometry before and after depletion. **A** Frequencies of CD3⁺, CD4⁺ and CD8⁺ T cells within the different T-cell fractions. **B** T-cell phenotypes were described as: naïve (T_N: CD45RA⁺CD62L⁺), central memory (T_{CM}: CD45RA⁻CD62L⁺), effector memory (T_{EM}: CD45RA⁻CD62L⁻) and late effector memory T cells re-expressing

CD45RA (T_{EMRA}: CD45RA⁺CD62L⁻). The isolated fractions consisted mainly of CD45RA_PF (T_N and T_{EMRA}) and CD45RA_NF (T_{CM} and T_{EM}) as well as CD62L_PF (T_N and T_{CM}) and CD62L_NF (T_{EMRA} and T_{EM}). Data represent the means of 6 donors. Memory/negative fractions: CD45RA_NF and CD62L_NF, naïve/positive fractions: CD45RA_PF and CD62L_PF. Results are displayed as means ± SD. Asterisks indicate statistically significant differences between T-cell fraction in the T-cell proportion (Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$).

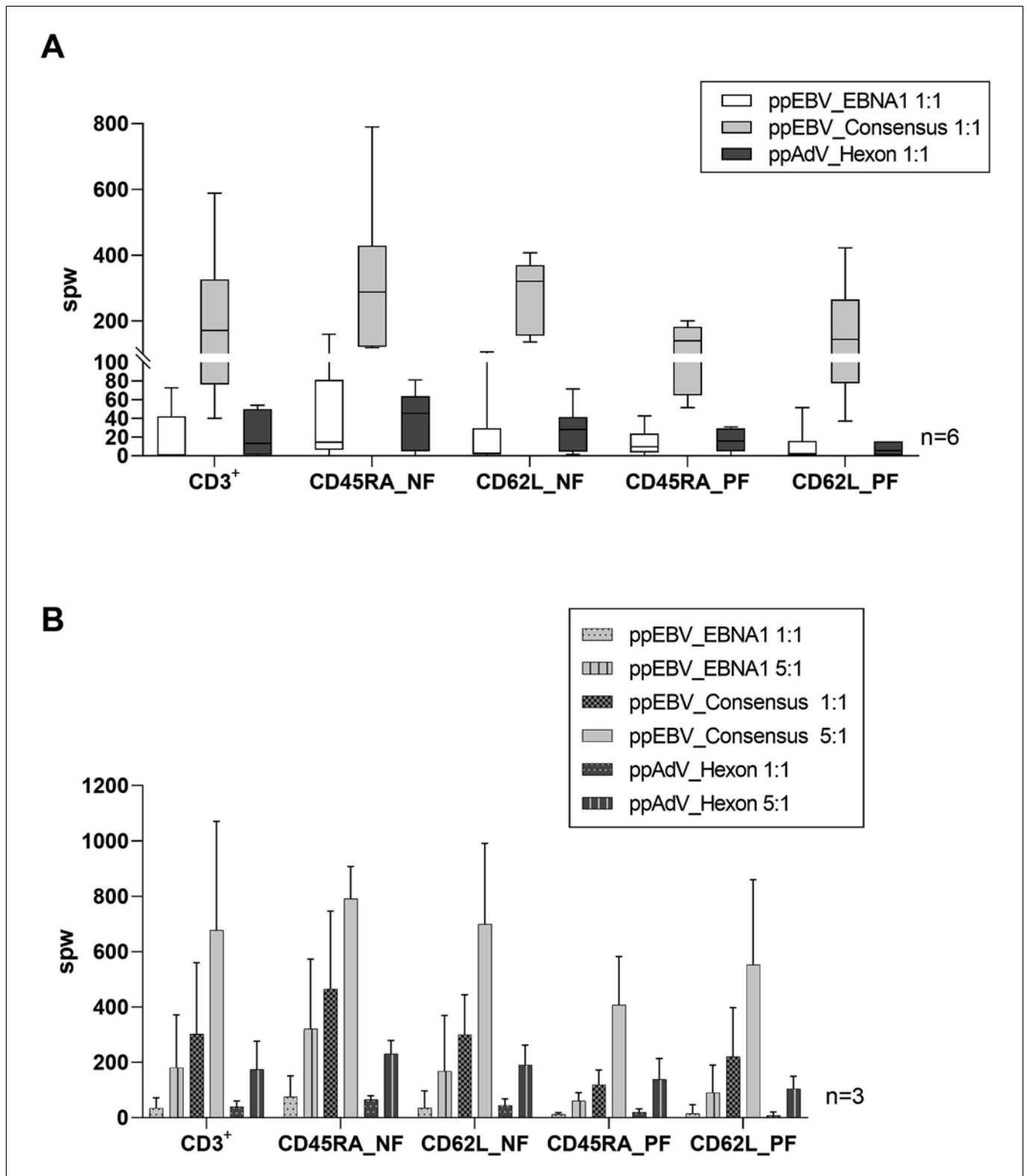


Fig. 2. T-cell responses to ppEBV_EBNA1, ppEBV_Consensus and ppAdV_Hexon after short-term *in vitro* stimulation using IFN- γ ELISpot assay. Antiviral T-cell responses were determined by target cell-dependent IFN- γ ELISpot assay. CD3⁺ T cells were isolated from PBMCs of 6 healthy blood donors followed by T_N depletion using CD45RA and CD62L microbeads. Antigen-specific T-cell responses detected by IFN- γ ELISpot assay after short-term *in vitro* stimulation (1 day) with antigen-loaded CD3-de-

pleted PBMCs (target cells) loaded with either ppEBV_EBNA1, ppEBV_Consensus or ppAdV_Hexon (1 μ g per peptide/mL) at an E:T ratio of 1:1 ($n = 6$) (**A**) and at an E:T ratio of 1:1 and 5:1 ($n = 3$) (**B**). T-cell fractions cultured alone served as control. Results are displayed as means $\pm$ SD and were calculated by subtracting the observed values from the control. Memory/negative fractions: CD45RA_NF and CD62L_NF, naïve/positive fractions: CD45RA_PF and CD62L_PF; spw, spot per well; pp, peptide pool.

using an E:T ratio of 5:1 for IFN- γ ELISpot assay (shown in Fig. 2B). Since T-cell responses against ppEBV_EBNA1 and ppAdV_Hexon were low or not detectable after short-term stimulation, long-term stimulation of the different T-cell fractions was also performed. Longer stimulation induced a high increase in antiviral T-cell responses against ppEBV_EBNA1 and ppAdV_Hexon in CD45RA_NF, with maximum mean responses of 306 ($p < 0.05$) and 104 spw, respectively. Additionally, long-term stimulation produced a significant increase in T-cell responses in CD62L_PF, which had shown no or low T-cell responses after short stimulation (shown in Fig. 3A, $p < 0.01$, and Fig. 3C). On the other hand, only marginal differences in T-cell responses against ppEBV_Consensus between short- and long-term stimulation were observed (shown in Fig. 3B); the highest T-cell response occurred in CD62L_PF (mean of 355 spw). Furthermore, the amplitude of antiviral T-cell response, as detected by IFN- γ ELISpot assay, correlated nicely with the total cell counts (data not shown). However, the lowest fold changes occurred in CD45RA_PF followed by CD62L_NF. This might be due to presence of T_{EMRA}, which have low proliferative capacities [25], as shown in online suppl. Figure S1A (see www.karger.com/doi/10.1159/000516284 for all online suppl. material).

Phenotypic Plasticity of Antiviral T Cells within CD45RA_NF/PF and CD62L_NF/PF following Antigenic Stimulation

Here, we evaluated the immunophenotypic composition of the different T-cell fractions after short- and long-term antigenic stimulation. Stimulation drove T cells towards a more differentiated phenotype, and a substantial increase in T_{EM} was observed within all T-cell fractions. Memory T cells (T_{EM} and T_{CM}) are known to be more efficient producers of IFN- γ than T_N following stimulation [43]. This is also reflected by the antiviral T-cell response detected by target cell-dependent IFN- γ ELISpot assay after short- and long-term stimulation [43]. Following stimulation with ppEBV_EBNA1 and ppAdV_Hexon, T_{EM} frequencies increased to a mean of 74.9 and 67.4% in CD45RA_NF, and to a mean of 79.8 and 76.9%, respectively, in

CD62L_NF (shown in online suppl. Fig. S1A). On the other hand, T cells stimulated with ppEBV_Consensus resulted in a slightly higher proportion of T_{CM} within CD62L_PF (mean of 44.7%) and higher proportions of T_{EM} in the other T-cell fractions (shown in online suppl. Fig. S1A). Following stimulation with ppEBV_Consensus, CD8⁺ T-cell frequencies increased in all T-cell fractions except CD45RA_NF, while CD4⁺ T-cell frequencies increased following stimulation with ppEBV_EBNA1 and ppAdV_Hexon in all fractions except CD62L_NF as compared to the control (effector cells co-cultured with unloaded target cells, shown in online suppl. Fig. S1B). Overall, ppEBV_EBNA1 and ppAdV_Hexon induced a shift towards CD4⁺ T cells and T_{EM}, while after expansion with ppEBV_Consensus, CD8⁺ T cells, T_{CM} and T_{EM} phenotype predominated (shown in online suppl. Fig. S1A, B).

Contributions of CD45RA_NF/PF and CD62L_NF/PF Fractions in Alloreactive CD4⁺ and CD8⁺ T-Cell Responses

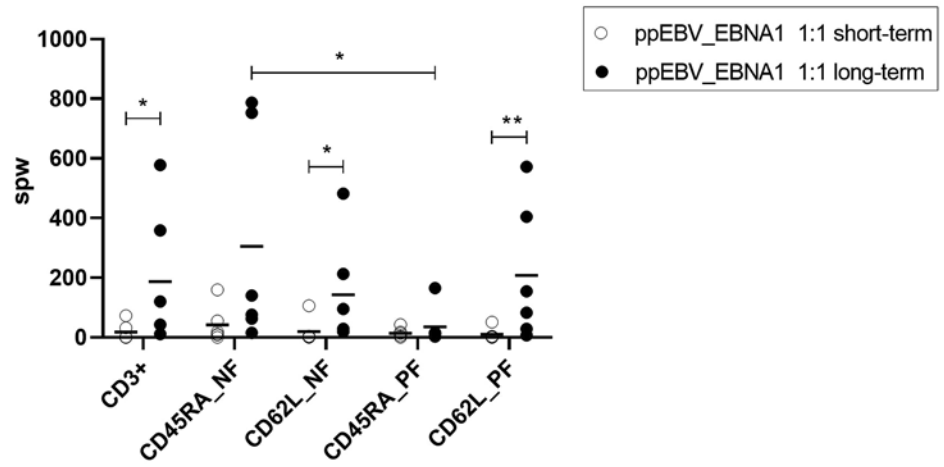
Previous studies provided evidence that alloreactive lymphocytes are not only restricted to the naïve T-cell pool, but that they could also be present in memory T-cells compartment due to subliminal pre-sensitization following blood transfusion or pregnancy [44, 45]. Therefore, we analysed alloreactivity induced by the different T-cell subsets in the previously described T_N-depleted and enriched T-cell fractions (responders) co-cultured with HLA-mismatched allogeneic PBMCs (stimulators) and compared with proliferation in relation to HLA-matched autologous controls determined by CFSE dilution (shown in Fig. 4). Overall, differences in T-cell proliferation in the T_N-depleted memory fractions (NF) co-cultured with autologous versus allogeneic stimulators were lower than those in unmanipulated CD3⁺ controls and the T_N-enriched fractions (PF), as shown in Figure 4. In the memory fractions, lower alloreactivity was detected in CD45RA_NF compared to CD62L_NF (2.5 vs. 3.8% difference in proliferation). The lowest alloreactivity was shown for CD45RA_NF (2.5% difference in proliferation), while CD62L_PF resulted in the highest alloreactivity (28.3% difference in proliferation, $p < 0.05$). Additionally,

Fig. 3. T-cell responses to ppEBV_EBNA1, ppEBV_Consensus and ppAdV_Hexon detected by IFN- γ ELISpot assay after short-term and long-term *in vitro* stimulation. Antiviral T-cell responses were determined by target cell-dependent IFN- γ ELISpot assay. CD3⁺ T cells were isolated from PBMCs of 6 healthy blood donors followed by T_N depletion using CD45RA and CD62L microbeads. EBV-specific (A, B) and AdV-specific (C) T-cell responses detected by IFN- γ ELISpot assay after short-term (1 day) and long-term *in vitro* stimulation (7 days) with antigen-loaded CD3-depleted PBMCs (target cells) loaded with either ppEBV_EBNA1, ppEBV_

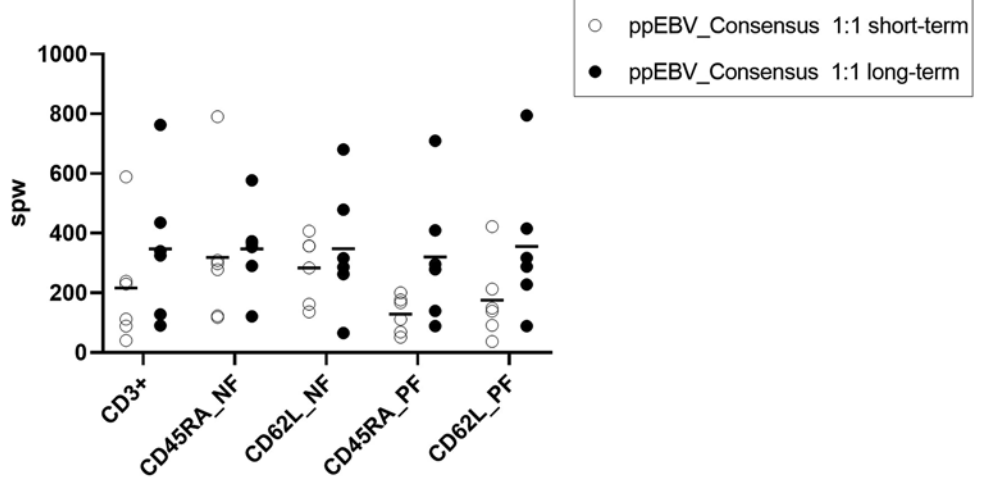
Consensus or ppAdV_Hexon (1 μ g per peptide/mL) at an E:T ratio of 1:1. As a control, T-cell fractions were cultured alone in the presence of IL-2, IL-7 and IL-15. Data are expressed as means of 6 donors and were calculated by subtracting the observed values from the control. Memory/negative fractions: CD45RA_NF and CD62L_NF, naïve/positive fractions: CD45RA_PF and CD62L_PF; spw, spot per well; pp, peptide pool. Asterisks indicate statistically significant differences between the T-cell fractions and short- and long-term stimulation (Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$).

(For figure see next page.)

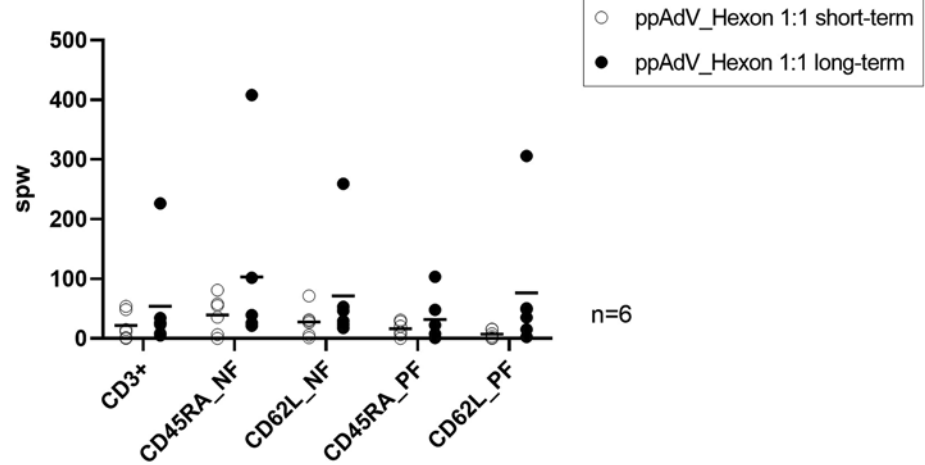
A



B



C



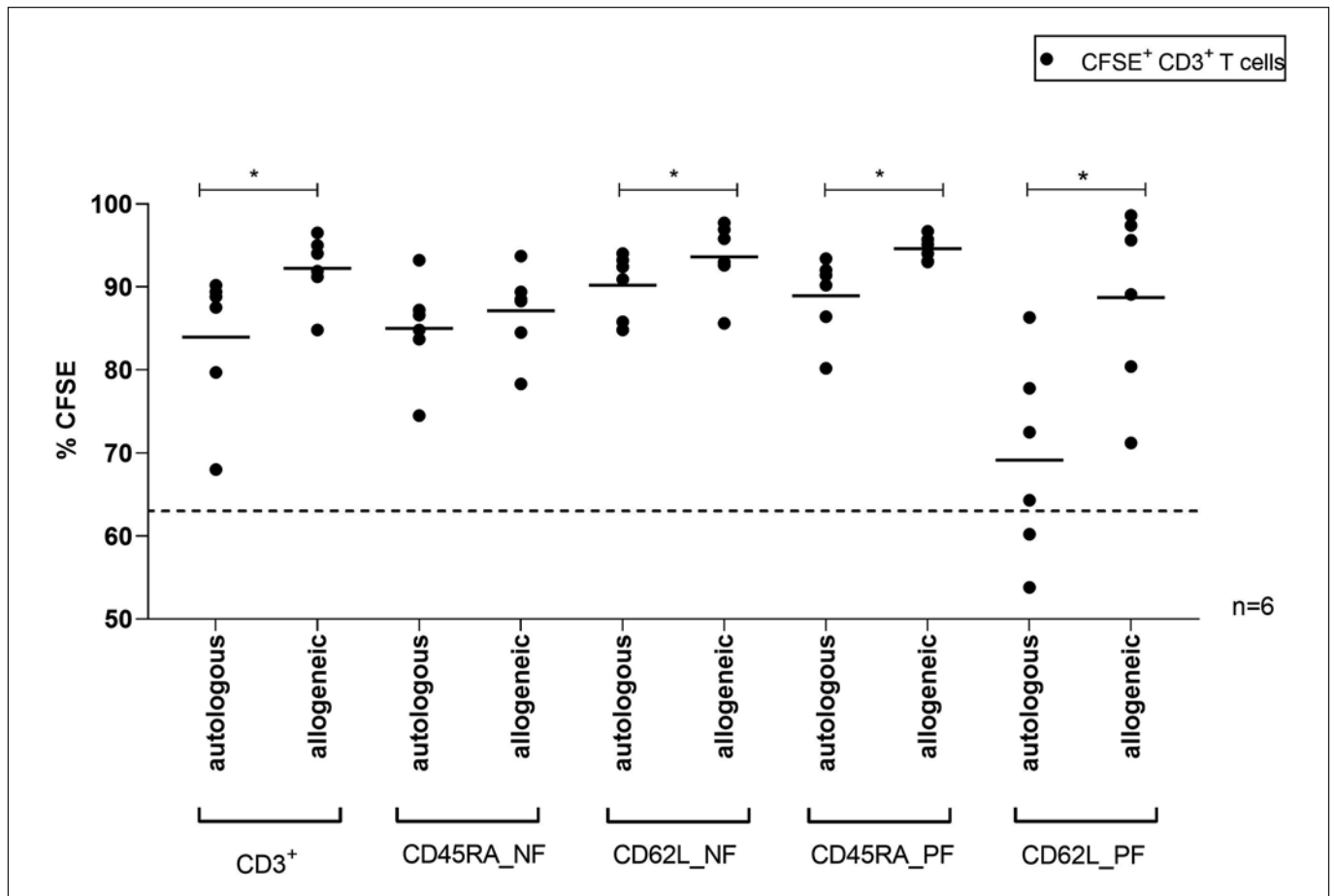


Fig. 4. Attrition of alloreactivity following naïve T-cell depletion. T-cell-mediated alloreactivity measured in the T_N -depleted and enriched fractions (CD45RA_NF/PF and CD62L_NF/PF) by allogeneic mixed lymphocyte reaction (MLR). Effector cells were labelled with carboxyfluorescein succinimidyl ester (CFSE, 4 μ M). 1×10^5 CFSE-labelled T cells (responder) were co-cultured with irradiated (1×30 Gy) autologous or pooled PBMCs (stimulator, from 3 to 5 donors) at an E:T ratio of 1:1 supplemented with IL-2, IL-7 and IL-15. As negative controls, target or effector cells were cultured in the presence of IL-2, IL-7 and IL-15 alone, while T-cell

fractions co-cultured with anti-CD3/CD28 beads served as positive control. Background reaction indicated by the mean frequency of proliferating CFSE+CD3+ T cells in the negative control (effector cells) is shown as dashed line. Memory/negative fractions: CD45RA_NF and CD62L_NF, naïve/positive fractions: CD45RA_PF and CD62L_PF. Data represent the mean of $n = 6$ donors. Asterisks indicate statistically significant differences between autologous and allogeneic calculated using Wilcoxon matched-pairs signed-rank test (* $p < 0.05$).

CD45RA fractions had consistently lower differences in T-cell proliferation between the autologous and allogeneic setting than CD62L fractions (NF: 2.5 vs. 3.8% and PF: 6.4 vs. 28.3% difference in proliferation, shown in Fig. 4). The naïve fractions exhibited a phenotype shift towards T_{CM} and T_{EM} following co-culture with HLA-mismatched allogeneic pooled stimulators, highlighting why the alloreactive responses were not significantly higher in the T_N fractions (shown in online suppl. Fig. S2A–C). The greatest differences between autologous and allogeneic settings were detected in CD45RA_PF compared to CD62L_PF. Furthermore, CD4+ and CD8+ T-cell subsets displayed similar proportions of proliferation for CD62L fractions (NF/PF) in response to allostimulation but higher proliferation for CD45RA fractions, as reflected by the CD4/

CD8 ratios following proliferation in the MLR cultures (CD62L_NF: 1.46 vs. 1.44 and CD62L_PF: 2.05 vs. 1.82; CD45RA_NF: 4.36 vs. 7.26 and CD45RA_PF: 1.84 vs. 2.88 CD4/CD8 ratio in the autologous vs. allogeneic setting, shown in online suppl. Fig. S2D).

Discussion

Adoptive transfer of DLI and VSTs is an effective strategy shown to rapidly restore virus-specific T-cell immunity to prevent or control viral infections following transplantation. DLIs contain memory T cells against a wide spectrum of pathogens, but therapeutic success is limited by the presence of alloreactive T_N capable of causing

GvHD. Adoptive transfer of VSTs represents a more specific approach which has been successfully applied over the past years [13, 15, 16, 46]. Nonetheless, the potential to generate VSTs can be limited if immunodominant epitopes of the target virus are unknown, if antigens required for stimulation and enrichment are not available in good manufacturing practice quality, or if no at least partially HLA-matched seropositive donor with sufficient VST frequencies is available. Alternative strategies for clinical-grade T-cell production are urgently required to overcome these limitations. Selective depletion of alloreactive T_N using immunomagnetic CD45RA and CD62L microbeads represents a promising strategy for preserving memory T cells against a broad variety of pathogens while reducing the risk of GvHD. In a previous study, we demonstrated that T_N depletion with CD62L microbeads was preferable for CMV due to the presence of $CD8^+ T_{EMRA}$ and T_{EM} [26]. Variable differences in immunophenotypic composition of T-cell fractions between donors led to differences in CMV-specific T-cell responses, indicating that the T_N depletion strategy should be selected according to the specific T-cell phenotypes mirrored in the donor [25, 26]. Based on our previous experience, the present study was designed to identify the most suitable strategy for generating T_N -depleted lymphocyte products containing low-frequency functionally active antiviral memory T cells using EBV and AdV as viral models. The results of this study provide comprehensive data on human antiviral T-cell responses against AdV and EBV following T_N depletion, which indicate that T_N depletion strategies via CD45RA and CD62L represent feasible alternatives for generating memory T-cell products that will provide a broad, pathogen-specific T-cell repertoire and minimise risk of GvHD.

Complexity of Antiviral T-Cell Responses in Relation to T-Cell Frequencies and Phenotype

Following long-term *in vitro* stimulation, CD4/CD8 ratios and immunophenotypic compositions of the various T-cell fractions differed depending on the type of viral antigen used. T-cell fractions stimulated with ppEBV_EBNA1 and ppAdV_Hexon were characterized by a marked increase in $CD4^+$ T-cell subset and T_{EM} , which were the most dominant phenotype, particularly in CD45RA_NF. T-cell fractions stimulated with ppEBV_Consensus displayed slightly higher amounts of T_{CM} in CD62L_NF and of the T_{EM} phenotype in the other T-cell fractions, with higher-level activation of $CD8^+$ T cells; this was essentially caused by HLA class I-restricted epitopes within ppEBV_Consensus. Previous studies consistently report that EBV_EBNA1-specific T cells are mainly $CD4^+$ restricted and contribute to controlling EBV infection and EBV-associated malignancies [47]. EBV latent-cycle proteins were shown to display a dis-

tinct, marked hierarchy of immunodominance for $CD4^+$ T-cell responses (EBNA1, EBNA3C $\gg$ LMP1, LMP2) and categorical differences with respect to $CD8^+$ T-cell responses (EBNA3C $>$ EBNA1 $>$ LMP2 $\gg$ LMP1) from the same EBV proteins [47, 48]. Moreover, the slight increase in $CD4^+$ T-cell frequency observed following stimulation with ppAdV_Hexon in the present study represented the major responding T-cell population, and the respective healthy adult or convalescent subjects developed long-lived $CD4^+$ T-cell responses [49, 50]. Apparently, these AdV-specific $CD4^+$ T cells are able to recognize conserved antigens and can exhibit cross-reactivity with diverse serotypes [50, 51]. Our results suggest that products with a higher content of $CD4^+$ T cells, such as CD45RA_NF, will be more efficient in providing ppEBV_EBNA1- and ppAdV_Hexon-specific T cells since these are primarily mediated by $CD4^+$ T cells. In a clinical study by Triplett et al. [52], CD45RA_NF conferred specific protection against adenoviral colitis, despite reduced use of cidofovir, and additionally reduced EBV reactivation: only 1 of 67 patients developed post-transplant lymphoproliferative disease due to the efficacy of B-cell depletion in this T-cell fraction. In general, the magnitude of the EBV-specific T-cell responses was consistently greater than that of the AdV-specific responses in the same individuals. These differences are likely due to the fact that EBV is a persistent virus that provides chronic stimulation to circulating memory T cells, while AdV causes non-persistent infections that are cleared by the immune response [52].

A Simple Model to Demonstrate the Magnitude of T-Cell Responses of Low-Frequency Antiviral T Cells after Long-Term Stimulation

Reduction in viral loads often correlates with expansion of T cells that recognize targeted viral antigens [46, 53]. Therefore, the magnitude of T-cell responses of specific low-frequency antiviral T cells against ppEBV_EBNA1 and ppAdV_Hexon was measured after short- and long-term *in vitro* stimulation in this study. Short-term stimulation mainly resulted in no or low T-cell responses in the naïve fractions, and slightly higher responses in the memory fractions. On the other hand, T-cell responses against ppEBV_Consensus were consistently higher responses in all T-cell fractions. These findings are consistent with those of previous reports that highlight the fact that EBV_EBNA1-derived epitopes are of low frequency [54, 55] and that only about 1–2% of circulating T cells are specific for EBV in healthy EBV seropositive individuals [56–58]. Interestingly, following long-term *in vitro* stimulation, the highest increase in T-cell response, as detected by IFN- γ ELISpot assay, was obtained with ppEBV_EBNA1 (up to 4-fold), and the increase correlated with the cell count; ppEBV_Consensus, on the other hand, resulted in a pro-

foundly low, marginal difference. These results consistently confirm the high proliferative and differentiation potentials of low-frequency EBV- and AdV-specific memory T cells, which shifted to a more T_{EM} phenotype, producing effector molecules that were sufficient to elevate T-cell responses, particularly in CD45RA_NF. These findings are in line with those of other studies reporting that infusions with low frequencies of donor-derived cytotoxic T lymphocytes were able to sufficiently and dynamically expand *in vivo*, with clinical benefits persisting for more than 3 years [59–61].

Dynamics and Functional Signatures of T-Cell Phenotypes in Antiviral T-Cell Immunity among the Different CD45RA_NF/PF and CD62L_NF/PF Fractions

The findings of the present study demonstrate that the investigated CD45RA and CD62L depletion strategies deplete different parts of the memory T-cell phenotypes. CD45RA additionally depletes CD8⁺ T_{EMRA}, while CD62L depletes T_{CM}. These differences resulted in variable antiviral T-cell responses against different viral antigens in the CD45RA_NF/PF and CD62L_NF/PF. Memory T cells display diversity in terms of antigenic phenotype, which correlates to their functional capacity. We have shown that EBV_EBNA1 and AdV_Hexon-specific T-cell responses are mainly mediated by CD4⁺ T_{EM}, while CD8⁺ T_{CM} and T_{EM} are responsible for T-cell responses against ppEBV_Consensus. In a previous study, we observed variable differences in phenotypic composition between donors, which led to differences in CMV-specific T-cell responses and subsequent stratification of donors into donors with expected response and donors with unexpected response [26]. This classification was mainly due to the observed differences in CD45RA fractions. However, our tested donors were not stratified in this manner since the EBV_EBNA1 and AdV_Hexon-specific T-cell responses were mainly observed in CD45RA_NF. For this reason, we conclude that T_N depletion using CD45RA is suitable for ppEBV_EBNA1 and ppAdV_Hexon due to the presence of CD4⁺ T cells and T_{EM} in CD45RA_NF, while depletion with CD62L is beneficial in the case of CMV due to the fact that CD8⁺ T cells and T_{EM} are retained in CD62L_NF [26]. Therefore, clinicians should use a balanced approach to selecting the T-cell phenotype that is most suitable for control of the viral infection in question according to the specific phenotypic composition of the donor.

Attrition of Alloreactivity following Naïve T-Cell Depletion with CD45RA and CD62L Microbeads

HLA mismatch antigens are major targets of alloreactive T cells in HLA-incompatible cellular T-cell products; this can trigger severe GvHD and, thus, reduce survival

in transplant recipients. The frequency of alloreactive T cells capable of reacting with any unique allogeneic MHC haplotype represents a substantial proportion of the naïve T-cell repertoire and is estimated to range from 0.1 to 10% [62–65]. Mouse models revealed that CD4⁺ and CD8⁺ T cells are capable of mediating lethal GvHD [66]. T_N caused severe GvHD, T_{CM} induced milder GvHD, and T_{EM} did not cause significant GvHD [67–70]. However, the relative contributions of CD4⁺ and CD8⁺ naïve and memory T cells to alloimmune response in HLA-incompatible transplants in humans are still unclear. Here, we used MLR cultures in order to assess and identify T-cell fractions with reduced *in vitro* reactivity to mismatched HLA. We found significantly higher proliferation in the allogeneic setting compared to the autologous setting for all T-cell fractions with the exception of CD45RA_NF. Essentially, T_N depletion with CD45RA was more effective in reducing alloimmune T-cell responses than CD62L, which showed variability among the donors. These findings suggest that depletion with CD45RA is preferable for preventing the development of GvHD. These data are consistent with those reported by Distler et al. [71], who compared several markers for T_N depletion and found out that CD45RA was more efficient than others, e.g., CD45RO, CCR7 and CD62L. Our results indicate that CD8⁺ populations tend to proliferate at slightly higher frequencies in response to allostimulation among the CD62L_PF/NF fractions in both the autologous and the allogeneic setting. It is noteworthy that in our previous analysis, CD45RA_NF had a higher amount of T_{reg} cells, which are known to be potent suppressors regulating alloreactive donor T-cell responses [72–75]. Thus, it could be postulated that T_{regs} in CD45RA_NF might contribute to the decreased alloreactive T-cell responses. In agreement with a study reported by Teschner et al. [36], T_N depletion via CD45RA decreased the number of alloreactive CD8⁺ T cells in CD45RA_NF and, hence, induced lower alloreactivity levels than the starting DLI. Therefore, although T_N depletion with CD45RA is less effective in CD8⁺ T cells, it may decrease the overall GvHD induction potential *in vivo*. Contrary to our expectations, we observed curtailed alloreactivity levels in the T_N fractions, particularly in CD62L_PF. Thus, we performed phenotype analysis following MLR culture and found that the T_N population had substantially decreased and differentiated into effector T lymphocytes, which may explain the aforementioned observation. Indeed, a recent clinical investigation by Bleakley et al. [76] revealed that although T_N-depleted PBMCs did not reduce the incidence of acute GvHD following infusion, the patients were responsive to corticosteroids and, remarkably, the occurrence of chronic GvHD was 9% compared to 50% with T-cell-depleted grafts.

Conclusion

This study demonstrates the feasibility of T_N depletion using both CD45RA and CD62L immunomagnetic beads and highlights differences between the two approaches. T_N depletion with CD45RA results in high CD4/CD8 ratios due to enrichment of CD4⁺ and depletion of CD8⁺ T cells, while T_N depletion with CD62L retains CD8⁺ T cells. EBV_EBNA1- and AdV_Hexon-specific T cells are mainly of the CD4⁺ T_{EM} phenotype; hence, T_N depletion with CD45RA will be preferable for CD4⁺ antiviral T cells. T_N depletion with CD62L will be suitable for CD8⁺ antiviral T cells, such as those stimulated with CMV-derived antigens, which are mainly CD8⁺ T_{EM}; dismally, this occurs with a loss of T_{CM}. Taken together, our results indicate that CD45RA is more suitable for generating T_N-depleted products consisting of memory T cells against ppEBV_EBNA1 and ppAdV_Hexon than CD62L in terms of product purity, T-cell functionality and allo-reactivity. This study underlines the need to select precise T_N depletion strategies according to the immunophenotypic T-cell profile and the CD4/CD8 antigen restriction. This concept will ensure the production of effective lymphocyte products that will provide a broad, pathogen-specific T-cell repertoire and minimise the risk of GvHD. Efficacy and safety enhancement is essential to improving the therapeutic benefit of adoptive immunotherapy and the clinical outcome.

Acknowledgements

The authors would like to thank Nicole Neumann, Dörthe Rokitta, Sarina Lukis, Elvira Schulde, Sophie Meyer and Bach Uy Vu for their excellent technical assistance.

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Statement of Ethics

The study was approved by the Institutional Review Board of Hannover Medical School (MHH, approval number 3331_2016, 3639_2017).

Conflict of Interest Statement

The authors declare no conflict of interest.

Funding Sources

C.M. was supported by the Kenyan-German Postgraduate program in conjunction with DAAD, the Hannover Biomedical Research School (HBRS). This study was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), project ID 158989968-SFB 900/B11.

Author Contributions

C.M. participated in the design of the study, carried out the experiments for T-cell isolation, functionality assays and immunofluorescence staining, performed the data and statistical analysis and drafted the manuscript; S.T.-Z. participated in the design of the study, provided helpful input to overall discussions, data and statistical analysis, and co-wrote the manuscript; A.B. contributed with helpful discussion and helped to draft the manuscript; S.B.R. contributed with helpful discussion and helped to draft the manuscript; A.C.D. contributed with helpful discussion and helped to draft the manuscript; B.M.-K. contributed with helpful discussion and helped to draft the manuscript; R.B., the head of the institute, contributed with helpful discussion and helped to draft the manuscript; B.E.-V. conceived the study and participated in its design and coordination, designed the experiments for T-cell isolation, functionality assays and immunofluorescence staining, participated in the statistical analysis and co-wrote the manuscript.

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