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Alloreactivity Across HLA Barriers Is Mediated by Both Naïve and Antigen-Experienced T Cells

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T cell responses to allogeneic targets arise predominantly from the naïve pool. However, in humans, the risk of graft-versus-host disease is increased if the donor has circulating T cells recognizing multiple persistent DNA viruses, suggesting that memory T cells also contribute to the alloresponse. To examine HLA alloreactivity, we used flow cytometry–based proliferation and cytokine production assays. We identified the clonal identity of virus-specific T cells cross-reacting with HLA-disparate targets by sequencing the T cell receptor β chains in virus-specific T cell lines restimulated with cognate and HLA-disparate targets and sorting these chains according to cytokine response. We confirmed that naïve T cells from cord blood and adult individuals responded to HLA-mismatched target cells. In addition, in adults, we identified memory T cells responding by cytokine release to HLA-mismatched targets both in direct assays and after 8 days of culture with allogeneic stimulator cells. Epstein-Barr virus–specific and cytomegalovirus-specific T cells, tested against a panel of 30 T cell antigen-presenting cells with a broad coverage of the most prominent HLA types, displayed specificity for certain mismatched HLA alleles. Sequencing of the T cell receptor β chain demonstrated a clonotypic identity of cells that responded to both viral and allogeneic stimulation. These findings show conclusively that alloresponses in humans are not confined to the naïve T cell subset, and that memory viral antigen–specific T cells can cross-react with specific mismatched HLA–peptide complexes not presenting with cytomegalovirus or Epstein-Barr virus peptides.

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INTRODUCTION

Transplantation of donor hematopoietic cells or solid organs into a partially HLA-matched recipient activates $CD4^+$ and $CD8^+$ T cells recognizing allogeneic tissues. The high frequency of such alloresponses, on the order of 0.1%-10% of all T cells [1], has puzzled investigators. This T cell alloresponse has been proposed to represent either major histocompatibility complex (MHC)- [2] or peptide-focused [3] recognition by the T cell receptor (TCR). The consensus is that such alloreactivity is both MHC-restricted and peptide-specific, with T cells recognizing either a peptide in the nonself MHC [4-9] or, alternatively, a nonself MHC–derived peptide presented and recognized in the context of self MHC [10-13].

Allloreactivity can be identified in murine and human T cells directly ex vivo. In murine models, naïve, but not memory, T cells display alloreactivity in vivo and in vitro [14-17], although recent data in animal models of graft-versus-host disease (GVHD) suggest that the memory pool can exert nonself MHC reactivity as well [18,19]. Based on the findings from murine T cell allostimulation, where naïve T cells produce tumor necrosis factor (TNF)-α but not interferon (IFN)-γ, it was assumed that any TNF-α produced by human T cells stimulated ex vivo with HLA-mismatched targets originated from naïve T cells [20]. However, evidence using cloned T cells suggests that virus-specific T cells can recognize nonself peptide MHC (pMHC) [21-28]. Because the human T cell memory pool is largely dominated by reactivities against common DNA viruses, such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex

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virus, and varicella-zoster virus [29-32], the possibility of frequent cross-reactivity of antigen-experienced T cells with foreign pMHC is high despite the relative rarity of individual cross-reactivities.

The distinction between naïve and memory T cell alloreactivity is important in allogeneic hematopoietic stem cell transplantation (HSCT). Although umbilical cord blood (UCB) for HSCT contains >99% naïve T cells, which should be capable of strong alloreactivity, it confers less GVHD than transplants from similarly mismatched adult sources of bone marrow or peripheral blood, conversely suggesting a role for memory T cells in alloresponses causing GVHD. Indeed, clinical observations in HSCT indicate an association between DNA virus reactivity and GVHD [33,34].

In the present study, we evaluated the ability of both naïve and antigen-experienced CD4 and CD8 T cell subsets to recognize and respond to MHC-mismatched antigen-presenting cells (APCs). Our findings indicate that both memory and naïve T cells recognize allogeneic targets.

MATERIALS AND METHODS

Samples

UCB cells for this research were provided by the New York Blood Center. Peripheral blood cells were collected from hematopoietic stem cell transplant donors and from healthy paid volunteers under National Heart, Lung, and Blood Institute Institutional Review Board–approved protocols. Informed consent was obtained in accordance with the Declaration of Helsinki. UCB and adult peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density-gradient centrifugation and cryopreserved in liquid nitrogen using standard procedures. PBMCs were thawed and rested overnight at 37°C and 5% CO_2 in complete medium (IMDM; Cambrex, Walkersville, MD) supplemented with 10% heat-inactivated human AB serum (Gemini Bio-Products, Woodland, CA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA) with 50 U/mL of DNase I (Roche, Indianapolis, IN) before functional assays.

Reagents for Flow Cytometry

The following fluorochrome-conjugated monoclonal antibodies (mAbs) were purchased from commercial vendors: (1) αCD3 cyanin-7-allophycocyanin (Cy7APC), αCCR7 biotin, αCD45RA allophycocyanin (APC), αCD45RO APC, αCCR5 phycoerytherin (PE), αCD3 Cy7PE, αCD4 peridinin chlorophyll protein, αCD4 APC, αCD137 PE, αCD137 biotin, αCD38 Cy5PE, αCD69 fluorescein isothiocyanate (FITC), interleukin (IL)-2 FITC, and APC- or Alexa 647–conjugated IFN-γ (BD Biosciences, San Diego, CA); (2) αCD4 Cy5.5PE, αCD8 Alexa 750APC, αCD14 Pacific blue, αCD19 Pacific blue, αCD57 FITC, αTNFα PE, and αIL-2 APC (Invitrogen, Burlingame, CA); (3) αCD27 Cy5PE and αCD45RO PE (Beckman Coulter, Miami, FL); and (4) αCD4 Cy5.5- peridinin chlorophyll protein (Biolegend, San Diego, CA). For some experiments, mAbs were conjugated in-house; αCD8 and αCD45RA (BD Biosciences) were conjugated to Quantum Dot 585 and 705 (Invitrogen), respectively, and αCD107a was conjugated to Alexa 594. Streptavidin PE (BD Biosciences) was used to identify biotinylated mAb-labeled cells. The fixable violet amine reactive dye (ViViD; Invitrogen/Molecular Probes, Eugene, OR) or Via-Probe (7AAD; BD Biosciences) was used to eliminate dead cells from the analysis [35]. For intracellular cytokine detection (ICD)–mixed lymphocyte reaction experiments, cells were labeled with the green fluorescent dye carboxyfluorescein diacetate, succinimidyl ester (CFSE; Invitrogen) [36]. Magnetic beads coated with mAb toward CD27, CD45RO, CD45RA, CD57, and CD62L were obtained from Miltenyi (Bergisch Gladbach, Germany).

Isolation and Expansion of Antigen-Specific T Cells

PBMCs were stimulated overnight in complete medium with irradiated (75 Gy) autologous EBV-transformed lymphoblastoid cell lines (EBV-LCL) or a custom-synthesized CMV pp65 peptide library as described previously [37]. Controls were unstimulated PBMCs. On the next day, the cells were stained with αCD14 and αCD19 Pacific blue, αCD69 FITC, and either αCD137 PE or αCD137 biotin, followed by streptavidin PE, washed, and resuspended in sterile FACS buffer (phosphate-buffered saline supplemented with 2% fetal calf serum and 0.05% sodium azide; Sigma-Aldrich, St Louis, MO), to which 7AAD was added. Live ($7AAD^-$) $CD14^-CD19^-$ lymphocytes brightly expressing CD69 and CD137 were sorted and further expanded by restimulation with the same antigen in complete medium supplemented with 100 IU of recombinant human IL-2 (Tecin; Roche). After 4 weeks, the T cells were tested for reactivity by ICD with a panel of HLA-disparate PBMCs or activated T cells (T-APCs) as APCs [37] (Table 1).

Isolation of T Cell Subsets

PBMCs were labeled with magnetic bead-coupled mAbs (Miltenyi) specific for CD27, CD45RO, CD45RA, CD57, and CD62L in combinations of CD57 plus CD45RO, CD57 plus CD45RA, CD57 plus CD62L, and CD27 plus CD45RO to obtain naïve, central memory, effector memory, and effector T cells, respectively. These fractions were applied to

Table 1. High-Resolution HLA Typing of Responder and Stimulator Cells Used in This Study

	HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DRBn	
Subject	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
1	02*	03*	07*	44*	0501	0702	1501		06*		5*00	
2	0201		0702	62	1	7	1501	1402	0602	0301	52	
3	2601	6801	3503	3901	0401	120301	14*	16*	05*		3*02	5*02
4	240201	240301	380101	5001	0602	1203	1101	1502	0301	0601	3*0202	5*0102
5	2	3	44	51			0401	1101	0301		52	53
6	1	0201	35	62	3V		1001	14*	0501	05*	52	53
7	0206	24	35	3906			0404	1406	0301	0302	52	53
8	020101	110101	3501	4001	0304	0401	0101	1302	0501	06*	3*0301	
9	01	25	18	57	06		07	15	0201	06	53	
10	24*	29*	1517	44*	07*	16*	0701	1201			3*00	4*01
11	020101	260101	130201	570101	0602	0701	07*		0201	0303	4*01	
12	2	28	7	8	7		0301	0701	0201	0303	52	53
13	02*	3001	13*	44*	05*	06*	0101	0301	0201	0501	3*0101	
14	01	24	15	44			0401	0801	0302	04	53	
15	0101	68*	08*	27*	0202	0701	0301	1101	02*	03*	3*01	3*02
16	0201	3101	51*				0101	0402	03*	0501	4*01	
17	2501	6601	07*	41*			0801	1303	0301	04*	52	
18	0201	0203	13*	38*	03*	07*	1202	1602	03*	05*	3*00	5*00
19	0201	0203	13*	38*	03*	07*	1202	1602	03*	05*	3*00	5*00
20	03*	30*	42*	57*	0701	1701	0302	1302	0402	0502	3*0101	3*0301
21	030101	110101	3503	510101	040101		0407	1104	0301	0302	4*01	3*0202
22	2401	3303	3801	5001	0602	1203	1401		05*		3*02	
23	0201	11	35	58			3	13	1	2		
24	240201	290201	3901	4403	120301	160101	0701	1501	0201	0602	4*01	
25	29*	32*	35*	44*	04*	16*	0103	0701	0202	0301		4*0101
26	0211	3201					0101	07*	0501	0303	53	
27	3002	68*	18*	3905	05*	07*	0301	0802			3*00	
28	2301	2403	4901	1501			1501		06*			
29	26*	6901	35*	38*	12*		03*	13*	02*	06*		
30	11	33	51	52			01	16	05*			
31	0205	6601	44*	50*			14*	1501	05*	06*	52	
32	010101	6802	080101	140201	0701	0802	03*	13*	02*	0301	3*01	3*01
33	03*	30*	42*	57*	0701	1701	0302	1302	0402	0502	3*0101	3*0301
34	020101	6802	150101	570101	030301	0602	0701		0303			4*01

*Stimulators 18 and 19 and stimulators 20 and 33 are HLA-identical siblings.

an LS column (Miltenyi Biotec), and the unbound (ie, unlabeled) cells were collected, washed, and cultured for 8 days with irradiated (50 Gy) HLA-mismatched targets. The purity of the fractions exceeded 90%.

ICD

Donor PBMC and T cell subsets were first primed by stimulation for 8 days with irradiated HLA-mismatched PBMCs in complete medium and 5% CO_2 at 37°C. Cells were then labeled with CFSE and restimulated with the same target cells or the autologous control for 6 hours in the presence of brefeldin A, monensin, and αCD28 and αCD49d (BD Biosciences). The cells were washed, stained with ViViD and cell surface mAb, and fixed and permeabilized using the BD Cytofix/Cytoperm Fixation and Permeabilization Kit following the manufacturer's instructions. The cells were stained with anticytokine mAb, washed twice with Cytoperm buffer, fixed for 10 minutes at room temperature with 4% formaldehyde (Tousimis, Rockville, MD), washed once with FACS buffer, and then resuspended in FACS buffer for acquisition. Virus antigen-specific T cells were similarly examined for alloreactivity by ICD using a panel of 30 T-APCs (Table 1). For the direct ex vivo assessment of allo-HLA reactivity of $CD4^+$ and $CD8^+$ T cells, the responder cells were labeled with CFSE and stimulated for 6 hours at a 1:1 ratio with (unlabeled) stimulator cells as described earlier.

Flow Cytometry

Stained cells were acquired on either a (modified) LSR II or a Canto II flow cytometer (BD Biosciences). A minimum of 2×10^5 events was collected for each condition. Compensation and data analysis were performed as described previously [35]. Forward scatter-area versus forward scatter-height properties were used to exclude cell aggregates; live T cells were separated from dead cells, monocytes, and B cells using a ViViD/CD14/CD19 (dump channel)-versus-CD3 bivariate plot. Lymphocytes were identified in a forward scatter-area versus side scatter-area plot, and responder cells were identified in the $CFSE^{hi}$ fraction after restimulation of CFSE-labeled primed allospecific or virus antigen–specific T cells in an ICD

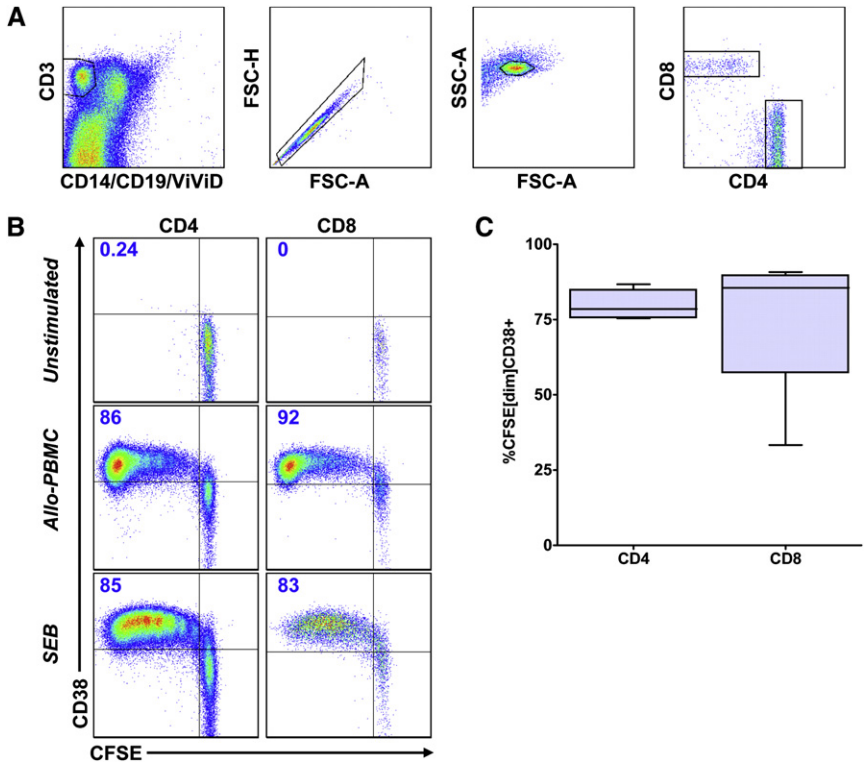


Figure 1. UCB $CD4^+$ and $CD8^+$ T cells contain a large alloreactive T cell population. A, Gating strategy. Live T cells were identified in a bivariate plot of CD14/CD19/ViViD versus CD3, and single cells were identified in a forward scatter-area versus forward scatter-height plot. Single intact lymphocytes were gated in a forward scatter-area versus side scatter-area plot, and $CD4^+$ and $CD8^+$ T cells were identified in the next plot. B, Reactivity of $CD4^+$ and $CD8^+$ T cells in 1 of 5 umbilical cord blood mononuclear cells. Responding T cells were identified as $CD3^+$ T cells that had serially diluted CFSE and acquired CD38 expression. Note the lack of response in unstimulated cells and the reactivity of T cells with the positive control staphylococcus enterotoxin B (SEB). C, Summary of the reactivity of UCB T cells with allogeneic PBMCs. In this box-and-whiskers plot, the horizontal bar represents the median, the borders are the 25th (lower) and 75th percentiles, and the whiskers are the minimum and maximum values.

experiment. Gating was standardized within individual samples to generate a fully comparative dataset.

TCR Sequencing

Virus antigen–specific T cells were restimulated with the cognate antigen and with T-APCs to which they displayed reactivity, and then electronically sorted on a modified BD Biosciences Aria sorter based on ICD. Sorted cells were collected in a dry collection tube, and genomic DNA was extracted as described previously [38]. TCR-Vβ sequences were amplified, cloned, and sequenced as described previously [36,38], and the composition of the third complementarity-determining region was analyzed using the international Immunogenetics Information System (http://imgt.cines.fr/).

Statistics

GraphPad Prism v4 (GraphPad, La Jolla, CA) was used to determine the difference in the magnitude of the response of alloantigen-primed donor T cells toward PBMCs or T-APCs in the second stimulation using Wilcoxon's two-tailed signed-rank test.

RESULTS

UCB T Cells Respond to HLA-Disparate Target Cells

We first tested the alloreactive potential of naïve T cells by stimulating UCB T cells, which are predominantly phenotypically naïve [39], with allogeneic stimulator cells and determining the fraction that had proliferated and acquired expression of the activation marker CD38 by day 8. In 5 UCB samples, a median of 79% of $CD4^+$ T cells and 86% of $CD8^+$ T cells were $CFSE^{dim}CD38^+$ by day 8 (Figure 1), confirming that naïve T cells respond to HLA-disparate target cells.

Both Naïve and Memory T Cell Subsets Contain Alloreactive Precursor Cells

Next, we separated adult PBMCs into various functional subsets by immunomagnetic depletion and stimulation with a pool of irradiated allogeneic PBMCs. In our approach, T cell subsets were purified free of bound antibody by negative immunomagnetic selection, and T cell subsets were identified using well-established marker combinations [40]. Thus, naïve T cells were

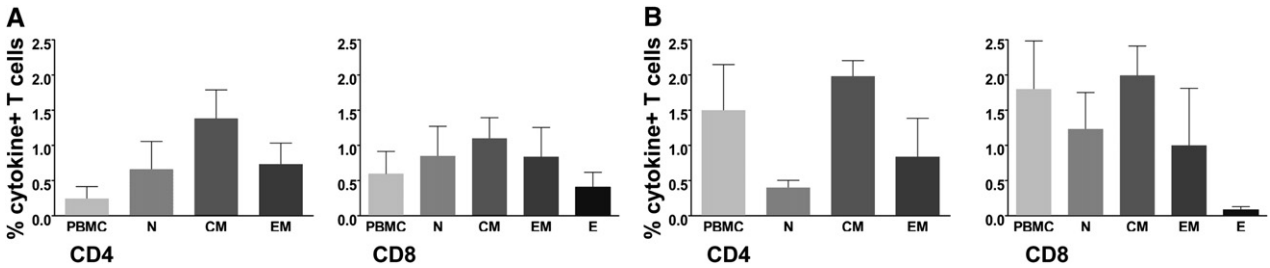


Figure 2. Adult donor memory T cell subsets respond to allogeneic target cells (PBMCs and T-APCs). Unfractionated PBMCs and purified naïve (N), central memory (CM), effector memory (EM), and effector (E) T cell subsets were primed for 8 days with irradiated allogeneic PBMCs and then restimulated with the original PBMCs for 6 hours to assess cytokine (IL-2/IFN-γ and/or TNF-α) production by the alloantigen-primed T cells. A, Reactivity of the primed donor $CD4^+$ and $CD8^+$ T cell fractions with allogeneic PBMCs showing allo-pMHC reactivity by all subfractions. Shown are the background-subtracted average and standard error of the mean (SEM) in each $CD4^+$ (left) and $CD8^+$ (right) T cell subset. B, Reactivity of the same primed donor T cells with allogeneic T-APCs demonstrating essentially the same reactivity pattern. Again, the average and SEM in each $CD4^+$ (left) and $CD8^+$ (right) T cell subset are shown. None of the donors had phenotypically effector $CD4^+$ T cells.

defined as $CD45RA^+CD27^+$ and lacking CD57 and CD45RO; central memory T cells were $CD45RO^+CD27^+$, lacking CD45RA and CD57; effector memory T cells were $CD45RO^+CD27^{+/-}$, lacking CD62L and CD57; and effector T cells were $CD27^-CD45RO^-CD45RA^+CD57^+$. Consequently, naïve T cells were obtained by depleting cells expressing CD57 and CD45RO, central memory T cells were obtained by depleting cells expressing CD45RA and CD57, effector memory T cells were obtained by depleting cells expressing CD62L and CD57, and effector T cells were obtained by depleting cells expressing CD27 and CD45RO.

T cells can proliferate in response to common γ chain signaling cytokine stimulation alone [41]. Such cytokines, including IL-2, are abundantly produced in a mixed pool of allogeneic PBMCs and could contribute to a TCR-independent T cell proliferation in the mixed lymphocyte reactions. Thus, in the next set of experiments, we first primed PBMC and T cell subsets from 3 donors with HLA-mismatched targets for 8 days, followed by restimulation with the original donor PBMCs, and measured antigen response based on the flow cytometric detection of cytokine (IL-2 and/or TNF-α) production (via ICD) in response to secondary stimulation. Unstimulated allo-HLA–primed T cells served as a negative control. In all 3 individuals (Figure 2A), naïve, central, effector memory, and in some cases effector T cells were primed and responsive to restimulation with allogeneic target cells in the secondary 6-hour stimulation.

To exclude the possibility that the memory T cells were merely responsive to EBV or CMV viral antigens present in PBMCs, we repeated the same experiment using activated T-APCs, which do not present these viral antigens. The pattern was largely the same, except that the magnitude of the response to restimulation with T-APCs was significantly greater than that with PBMCs ($P > .05$, Wilcoxon's two-tailed signed-rank test) (Figure 2B), probably because T-APCs express high levels of costimulatory molecules plus HLA class II [37,42]. Collectively, these results show that both naïve and memory T cells contribute to the alloresponse, suggesting that although it removes undesired self-reactivity from the T cell repertoire, negative thymic selection does not prevent responses to antigens presented by nonself MHC.

Both Naïve and Memory T Cells Recognize HLA-Disparate Targets Directly Ex Vivo

Because memory T cells were found to recognize unrelated pMHC complexes, they are likely to exert their effector function with fast kinetics and should be detectable in a short-term stimulation-based assay using a standard overnight ICD procedure. We first stimulated PBMCs from 6 responders individually overnight with 5 HLA-mismatched and one autologous control PBMC samples in the presence of cytokine secretion inhibitors, and then stained them for IL-2, IFN-γ, and TNF-α. Five of 6 $CD4^+$ T cell fractions and 2 of 6 $CD8^+$ T cell fractions demonstrated reactivity with HLA-mismatched targets (Figure 3A). We next assessed the alloreactivity of naïve, memory, and effector $CD4^+$ and $CD8^+$ T cells. T cells readily responded to the HLA-mismatched target cell stimulation by producing cytokines (Figure 3B), accounting for up to 2.35% of $CD8^+$ T cells and 0.23% of $CD4^+$ T cells. Direct alloreactivity, predominantly by naïve ($CD27^+CD45RO^-CD57^-$) and end-stage effector ($CD27^-CD45RO^-CD57^+$) T cells, was demonstrated in the $CD4^+$ and $CD8^+$ T cell populations, respectively (Figure 3B). These findings indicate that both naïve and effector T cells can respond with fast kinetics to HLA-disparate target cells.

Virus Antigen–Specific $CD4^+$ and $CD8^+$ T Cells Recognize Structurally Disparate Peptide–HLA Complexes

To extend these observations to T cells with known specificity, we stimulated donor T cells for up to 24 hours with either autologous EBV-LCL or

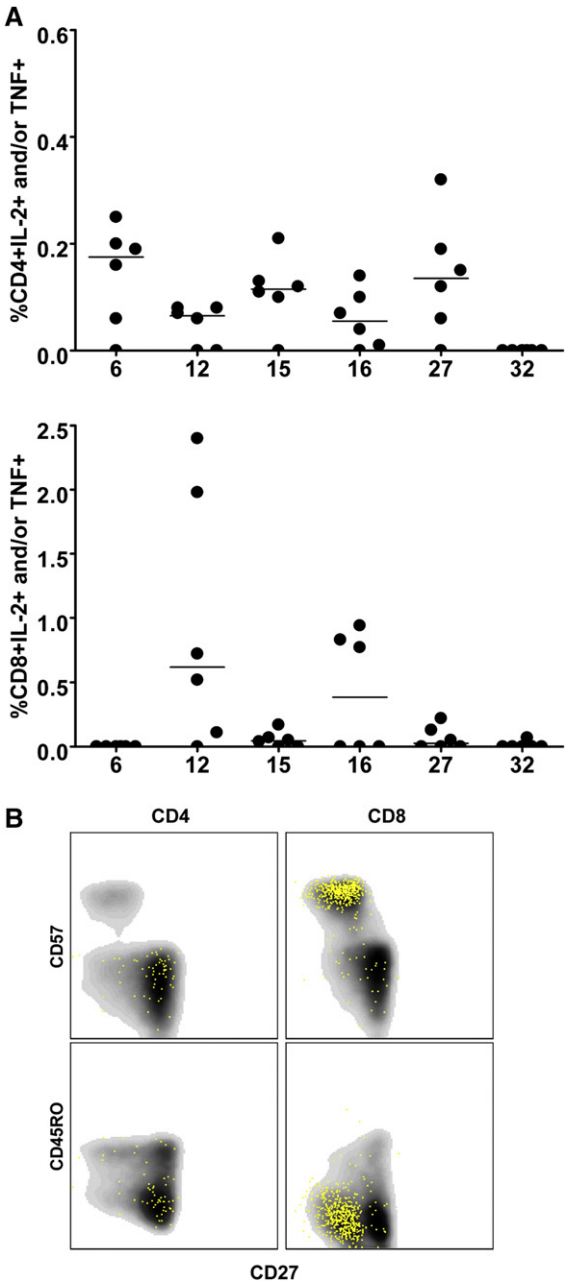


Figure 3. Alloreactive T cell precursors are readily identifiable in both naïve and memory T cells directly ex vivo. A, Frequencies of $CD4^+$ (top) and $CD8^+$ T cells from 6 donors (denoted by numbers on the *x*-axis corresponding to subject numbers listed in Table 1) responding with HLA-mismatched target cells. The frequencies displayed are background-subtracted, that is, after correction for cytokine production in the absence of allogeneic stimulator cells. Responder $CD8^+$ T cells were identified as cells producing TNF-α and/or IFN-γ, and responder $CD4^+$ T cells as cells producing TNF-α and/or IL-2. B, A representative example of one such analysis demonstrating direct ex vivo cytokine production. Shown is the phenotype of $CD4^+$ and $CD8^+$ T cells from donor 11 as a density plot, with the phenotype of alloreacting T cells overlad in yellow. The figure shows that alloresponding CD4 T cells lack CD57 expression, and that the bulk of the responder cells express CD27 but lack CD45RO expression, indicative of their naïve nature. However, a smaller, but sizeable, fraction also expresses CD45RO, indicating that some $CD4^+$ T cells responding to nonself MHC are contained in the memory ($CD27^+$ $CD45RO^+$) pool. On the other hand, the majority of the alloreactive $CD8^+$ T cells express CD57 but not CD27 or CD45RO, indicating that most alloreactive T cells originate from the end-stage effector pool.

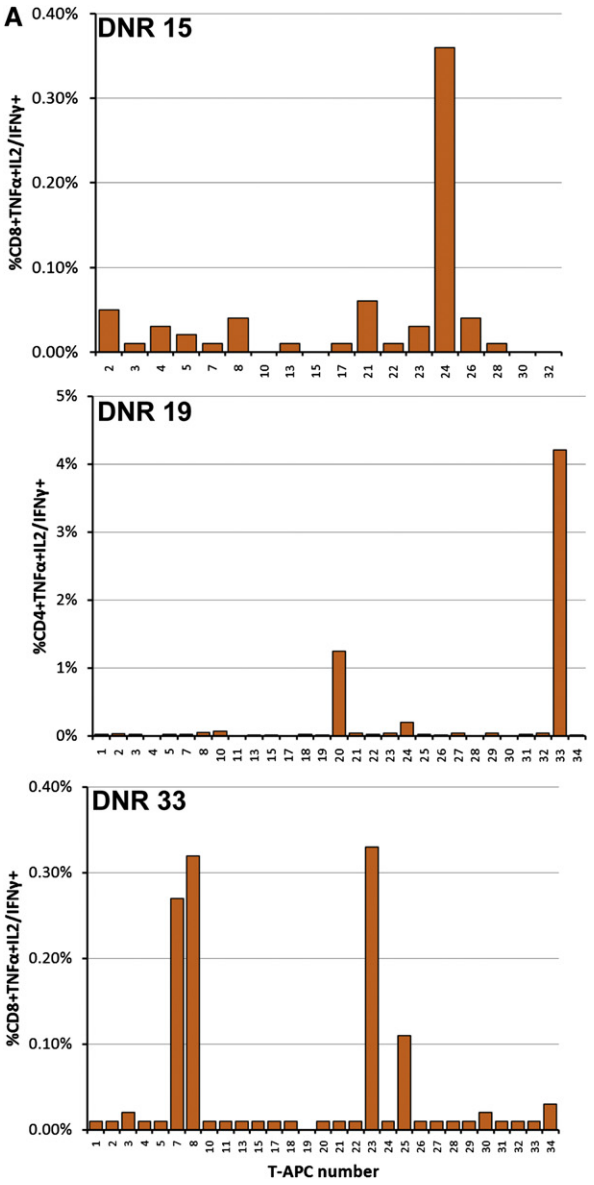
a pp65 peptide library known to elicit both $CD4^+$ and $CD8^+$ T cell responses [37], and then sorted and expanded activated ($CD69^+CD137^+$) cells for 4 weeks. We then tested these antigen-specific T cell lines against a panel of T-APCs expressing the most common HLA class I and II molecules (Table 1) in an ICD experiment. The virus antigen–specific $CD4^+$ and $CD8^+$ T cells displayed specificity for certain restricted target cells, recognizing between 1 and 4 APCs (Figures 4A, S1, and S2). The CMV pp65-specific $CD4^+$ T cells of donor 19 cross-reacted with targets 20 and 33 (HLA-identical siblings; Figure 4A, middle panel)). Although stimulators 20 and 33 were genotypically HLA identical, it is notable that the alloresponse to APC 33 was 3-fold greater than the alloresponse to the HLA-identical sibling, suggesting differences in the self-peptides presented by the 2 individuals.

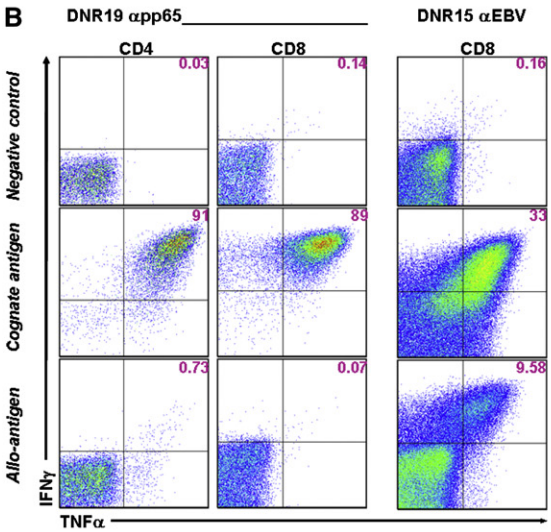
Identical T Cell Clones Respond to DNA Virus and Allogeneic pMHC Complexes

To demonstrate that the same T cell clones can respond to both viral and allogeneic pMHC stimulation, we electronically sorted $CD4^+$ and $CD8^+$ T cells from the EBV- and CMV-responsive T cell lines described earlier, based on the intracellular detection of both TNF-α and IFN-γ after stimulation with cognate (viral) antigen and with an allogeneic T-APC to which they also responded. Figure 4B shows the response of two such T cell lines to cognate antigen (pp65 or EBV) and to allogeneic targets in the CD4 and/or CD8 T cell subsets. To restrict the study to pure viral-specific T cells, we selected only T cells producing both TNF-α and IFN-γ, because no unstimulated cultured T cells were double positive for these cytokines. (Figure 4B). Alloresponding $CD4^+$ T cells from a third, anti–EBV-LCL T cell line were selected based on the expression of TNF-α and/or IFN-γ, because this gave the greatest difference with unstimulated cells (Figure S3). Sequencing the TCR-β chains in these samples using our well-established methodology [38,43] identified identical sequences in viral- and alloantigen-stimulated cells (Figures 4C and S3), providing strong evidence of the cross-reactivity of virus-specific $CD4^+$ and $CD8^+$ T cells with unrelated antigens.

DISCUSSION

It is well accepted that naïve T cells can mount a response to any new antigen, whether pathogen-derived or an allogeneic pMHC complex; however, the contribution of memory cells to allo-HLA reactivity is less well defined. Numerous previous studies have demonstrated the alloreactivity of virus antigen-specific $CD4^+$ [23,26-28,44] and $CD8^+$ [21-25,44] T cell





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Donor	Subset	Antigen	Tube	TCRBV	CDR3	TCRBJ	%
15	CD8	EBV	A	12.4	CASSFSWTEEAF	1.1	75
				5.8	CASSLIEGPWDNSPLHF	1.6	25
			B	12.5	CASGPGTGPGTQYF	2.5	75
				5.6	CASQPGRRFSTDTQYF	2.3	8
				27	CASSSASGVYQETQYF	2.5	8
				29.1	SIYLCCVAQYF	2.5	8
			C	29.1	SIYLCSVGAGDYEQYF	2.7	91
				27	CASSSGYLYYEQYF	2.7	4
				29.1	SIYLCSVGSGEDYEQYF	2.7	4
		allo	A	5.8	CASSLIEGPWDNSPLHF	1.6	100
			B	19	CASSAASDLGYEQYF	2.3	100

Donor	Subset	Antigen	Tube	TCRBV	CDR3	TCRBJ	%
19	CD4	pp65	A	6.1	CASSEGSANYGYTF	1.2	75
				19	CASSTTTNTEAF	1.1	19
				6.4	CASTTRTGRGEKLF	1.4	6
			B	6.1	CASDSGGVQRTQYF	2.5	73
				11.2	CASRYGAITDTQYF	2.3	18
				27	CASSDTGLGIF	2.1	9
			C	28	CATVDRGPFGELF	2.2	100
		allo	A	6.1	CASSEGSANYGYTF	1.2	100
			B	11.2	CASRYGAITDTQYF	2.3	100

Figure 4. Virus-specific CD4$^+$ and CD8$^+$ T cells recognize allogeneic pMHC. A, CD4$^+$ and CD8$^+$ T cells from donors 15, 19, and 33 responded to selected T-APCs with production of IL-2/IFN-γ and TNF-α. B, EBV and CMV pp65-specific T cells from donors 15 and 19 were sorted based on the expression of both TNF-alpha and IFN-gamma upon stimulation with cognate antigen or allogeneic target. The same T cell clone recognized both cognate antigens presented in self-MHC and allogeneic pMHC. Selected and expanded T cells were stimulated with cognate antigen or allogeneic T-APCs, stained for surface markers and cytokines, and sorted based on cytokine production. The TCR sequences were amplified in 3 separate polymerase chain reaction (PCR) tubes, each containing the full set of Vβ PCR primers but a different set of Jβ (BJ) primers. Tube A contained primers specific for TCRBJ 1.1–1.6; tube B contained primers specific for TCRBJ 2.1, 2.3, 2.4, and 2.5; and tube C contained primers specific for TCRBJ 2.2, 2.6, and 2.7. Given that we examined the TCR repertoire in 3 separate PCR reactions, we indicate clonal dominance per tube (A, B, and C). We obtained a total of 224 in-frame TCR sequences in this experiment, including 40 and 66 sequences for donor 15's CD8$^+$ T cell response against the cognate and alloantigen, respectively, and 56 and 62 sequences for donor 19's CD4$^+$ T cell response against the cognate and alloantigen, respectively. The clonotype composition in the sorted fraction shows identical clonotypes in the respective cell lines, indicated in matching colors for identical clonotypes.

clones (reviewed in [45]), but none of those studies addressed this issue at the population level as we have done in the present work. Furthermore, depletion of naïve T cells has been proposed as a method of avoiding GVHD after allogeneic HSCT [15,46]. We measured cytokine production at the single-cell level by flow cytometry in allo-MHC–primed T cells after short-term (6 hours) restimulation with the same allogeneic PBMCs and confirmed their alloreactive potential. The functional readout might not accurately reflect the true number of alloresponding T cells, however. In these experiments, we chose to analyze TNF-α and IFN-gamma/IL-2 production after allogeneic or syngeneic secondary stimulation; it is possible that responder frequencies might be different when analyzing other effector functions. Thus, although our results do not reflect overall allo-HLA responder T cell frequencies, they do show that responder frequencies to

allo-HLA stimuli are comparable in naïve and memory T cells.

Previous assays aimed at demonstrating the alloreactive potential of T cells ex vivo have assumed that such reactivity resides exclusively in the naïve pool [14,20]. Using ICD, we show that such allo-pMHC reactivity can be identified in healthy donor T cells ex vivo. Importantly, however, by combining such assays with the phenotypic identification of naïve, memory, and effector T cell subsets in a polychromatic flow cytometry approach, we have demonstrated conclusively that naïve $CD4^+$ T cells and effector $CD8^+$ T cells are the predominant subsets reacting with pMHC targets.

Because the DNA viruses are latently present in circulating B cells (eg, EBV [47]) and myeloid cells (eg, CMV [48]), the analysis of allo-pMHC reactivity of memory T cells could be confounded by responses to viral antigens presented by the HLA-disparate stimulators. However, activated T cells, which do not carry EBV or CMV, also elicited alloresponses, excluding the possibility that the responses were directed against these common DNA viruses.

The identification of alloreactivity in the memory pool raises the question of whether cross-reactivity of viral antigen–specific $CD4^+$ and $CD8^+$ T cells occurred with mismatched virus antigen-free pMHC complexes. Most previous studies have examined only a few clones against a limited panel of allogeneic EBV-LCL [27,28,49] or PBMCs [20] as APCs. We earlier established that the likelihood of finding alloreactivity depended on the complexity of the responder cell population (Melenhorst et al., unpublished observations) and on the extent of HLA diversity of the T-APC panel. Activation marker-selected and expanded virus-specific T cell lines were analyzed for clonal composition on restimulation with the cognate antigen (the immunodominant antigen from CMV, pp65, or a more complex source of antigen, EBV-LCL, which are known to express approximately 8 EBV-encoded proteins [50]) and with an allogeneic target.

To refine our selection of pure virus-specific T cells, we sorted only those T cells that produced both TNF-α and IFN-γ in response to cognate or alloantigen stimulation, because the proportion of T cells producing both of these inflammatory cytokines in the absence of restimulation was negligible, whereas single-cytokine producers were present in the unstimulated populations. TCR-β sequence analysis of $CD4^+$ and $CD8^+$ T cells responding to cognate antigen stimulation showed that the responder populations were highly oligoclonal, confirming previous reports [51,52]. The examination of the TCR-β sequences in the same T cell lines responding to an allogeneic target identified shared clonotypes with the cognate antigen-responsive population.

To expand our findings with the clonotype analysis of virus-specific T cell lines reacting with allogeneic APCs, we subjected a third, $CD4^+$ EBV-LCL–reactive T cell line to the same procedure. Here, we also identified a shared clonotype between virus-responding and alloresponding cells (Figure S3). But because the background production of cytokines by this cell line (ie, in the absence of antigen stimulation) was substantial, we cannot formally prove the dual reactivity of the clone identified in both reactivities. Collectively, our data do allow us to draw the conclusion that DNA virus antigen–specific T cells, which can compose 10% or more of the circulating post-HSCT T cell population in HSCT recipients [51] and in elderly donors [32], are commonly reactive with unrelated pMHC complexes.

Our findings have clinical relevance. First, the adoptive transfer of virus-specific T cell lines and clones to treat reactivating viruses in (partially) matched recipients [53] may risk sporadic but powerful alloreactions against the recipients. In the present study, we used T-APCs and PBMCs as representative GVHD targets because they were readily available; however, it is possible that these targets are not as representative of GVHD targets as fibroblasts or cells from GVHD target tissues such as the skin, gastrointestinal tract, and the liver. Amir et al. [44] recently used an EBV-LCL, HLA-transduced erythroleukemic cell line and phytohemagglutinin blasts to demonstrate that a large proportion of virus antigen–specific T cell clones reacted with nonself pMHC complexes. Those findings should be interpreted with caution, however; in vitro allo-HLA reactivity of virus-specific T cells might not directly translate to the alloreactivity in the form of GVHD, because other factors (eg, target antigen expression, homing, in vivo expansion) can affect clinical outcome. Indeed, the adoptive transfer of virus-specific T cell lines in the partial HLA-disparate setting [53,54] was not found to result in GVHD [55]. Furthermore, a recent study by the Riddell group [56] suggests that even minor histocompatibility antigen–specific T cells selected for reactivity with hematopoietic targets (EBV-transformed B cells) but nonreactivity with patient fibroblasts still exerted antilung reactivity in the patient, indicating that even fibroblasts might not express the relevant repertoire of target antigens of GVHD. Second, as others have demonstrated, cross-reactivity by viral antigen–specific TCRs with various nonself pMHC complexes [24] implies that when targeting an epitope presented in one particular HLA (eg, $WT1_{RMF}$ presented in the context of HLA-A*0201), TCRs from multiple clones should be available for such therapeutic applications, because, depending on the HLA makeup of the TCR donor, these complexes might recognize unrelated pMHC complexes. The same principles may apply to any TCR challenged with pMHC complexes not encountered during thymic selection in the original host [57]. Finally, because alloreacting memory

T cells, with their rapid proliferation kinetics, may kill their target within hours of engagement, they may contribute to the hyperacute GVHD observed mainly in partially HLA-matched HSCT recipients [58]. Although end-stage effector T cells are short-lived in vitro [59], they arise from the memory pool and may continue to replicate [60].

In conclusion, T cell reactivity with unrelated pMHC complexes can originate from any postthymic T cell population. Alloreactivity is common and is mediated by the same TCR that recognizes viral and possibly self antigens.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbmt.2010.12.711.

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