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Lymphoid reconstitution

Identification of the T cell clones expanding within both $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subsets in recipients of allogeneic hematopoietic cell grafts and its implication in post-transplant skewing of T cell receptor repertoire

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Summary:

We have previously reported that skewed repertoires of T cell receptor-β chain variable region (TCRBV) and TCR-α chain variable region (TCRAV) are observed at an early period after allogeneic hematopoietic cell transplantation. Furthermore, we found that T lymphocytes using TCRBV24S1 were increased in 28% of the recipients of allogeneic grafts and an increase of TCRBV24S1 usage was shown to result from clonal expansions. Interestingly, the arginine residue was frequently present at the 3′ terminal of BV24S1 segment and was followed by an acidic amino acid residue within the CDR3 region. These results suggest that these clonally expanded T cells are not randomly selected, but are expanded by stimulation with specific antigens. This study was undertaken to elucidate the mechanisms of the post-transplant skewing of TCR repertoires. Since the $CD8^+CD28^-CD57^+$ T cell subset has been reported to expand in the peripheral blood of patients receiving allogeneic hematopoietic cell grafts, we examined the TCRAV and TCRBV repertoires of the $CD8^+CD28^-$ T cell and $CD8^+CD28^+$ T cell subsets, and also determined the clonality of both T cell populations. In all three recipients examined, the $CD8^+CD28^-$ T cell subset appeared to define the post-transplant TCR repertoire of circulating blood T cells. Moreover, the CDR3 length of TCRBV imposed constraints in both $CD8^+CD28^-$ T cell and $CD8^+CD28^+$ T cell subsets. The DNA sequences of the CDR3 region were determined, and the same clones were identified within both $CD8^+CD28^-$ and $CD8^+CD28^+$ T cell subsets in the same individuals. These results suggest that the clonally expanded $CD8^+CD28^-$ T cells after allogeneic hematopoietic cell transplantation derive from the $CD8^+CD28^+$ T cell subset, possibly by an antigen-driven mechanism, resulting in the skewed TCR repertoire. *Bone Marrow Transplantation* (2001) **27,** 731–739.
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The CD28 molecule is a disulfide-linked homodimer expressed on the surface of T cells and binds to the natural ligand B7 family members, CD80/CD86, expressed on antigen-presenting cells, resulting in costimulation of T cell activation.[1] In humans, all thymocytes and the vast majority of peripheral blood T cells at birth express CD28. The proportion of $CD8^+$ T cells lacking CD28 expression increases during human aging, so that approximately 30% of $CD8^+$ T cells are negative for CD28 in adults.[2] Most of the $CD8^+CD28^-$ T cells express CD57.[2] A number of reports have shown that the proportion of $CD8^+CD28^-$ T cells is increased in HIV-infected patients.[3,4]

The $CD8^+CD28^-CD57^+$ T cell subset is also expanded in the peripheral blood of patients receiving allogeneic bone marrow transplants.[5,6] However, the mechanisms and biological relevance of this expansion remain to be elucidated. Moreover, it is still uncertain whether the $CD8^+CD28^-$ T cell subset arises from the $CD8^+CD28^+$ T lymphocytes or whether both subsets belong to distinct T cell lineages.

We have previously reported that the skewed TCRAV and TCRBV repertoires are observed at an early period after allogeneic hematopoietic cell transplantation.[7] We also found that the TCR usage appeared to be limited and T cells using TCRBV24S1 were increased in 28% of the patients receiving allogeneic hematopoietic cell grafts. An increase of the TCRBV24S1 usage was shown to be the result of clonal T cell expansions. Interestingly, in four out of six patients examined, the arginine residue was present at the 3′ terminal of BV24S1 segment and was followed by an acidic amino acid residue, such as glutamic acid and aspartic acid, within the CDR3 regions. These results suggest that these clonally expanded T cells are not randomly selected, but are expanded by stimulation with specific antigens. Further evidence in support of this includes oligoclonal expansion of $CD8^+CD57^+$ T lymphocytes pre-

viously reported in marrow transplant recipients.[8,9] If the clonally expanded $CD8^+CD28^-$ T cells following transplantation originated from $CD8^+CD28^+$ T cells that have been exposed to certain antigens, it is possible that identical T cell clones could be demonstrated in both $CD28^-$ and $CD28^+$ subsets. In this study, we present evidence for this hypothesis and discuss the biological relevance of our findings.

Materials and methods

Patients

Informed consent was obtained from the patients and donors before blood samples were collected. All the patients were conditioned with myeloablative chemoradiotherapy, mostly consisting of fractionated total body irradiation (12 Gy in six fractions) and cyclophosphamide (60 mg/kg/day for 2 days), followed by infusion of allogeneic marrow or blood stem cell grafts from HLA-matched donors. All the patients received cyclosporin A and short-term methotrexate for prophylaxis of acute graft-versus-host disease (GVHD).[10] Engraftment was achieved in patients, and confirmed by recovery of hematopoiesis and the presence of donor-derived sex chromosome or mismatched antigens on red cells. Clinical grading of acute GVHD was determined according to the criteria reported by Glucksberg *et al*.[11] Patients were monitored for cytomegalovirus (CMV) infection by weekly CMV antigenemia assays[12] from when the granulocyte count reached 500/μl until day 100 after transplantation. Patients who were positive for CMV antigenemia received prophylactic ganciclovir (5 mg/kg/day, 3 days a week) from when the granulocyte count was greater than 1000/μl.[13]

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by the Ficoll/Conray gradient centrifugation method from heparinized blood. PBMCs were stained with FITC- or PE-conjugated monoclonal antibodies and analyzed using a flow cytometer (Cytron Absolute, Ortho Diagnostics, Tokyo, Japan). Monoclonal antibodies used in this study were as follows: anti-CD3 (SK7, IgG1; Becton Dickinson, San Jose, CA, USA); anti-CD4 (MT310, IgG1; DAKO, Glostrup, Denmark); anti-CD8 (DK25, IgG1; DAKO); anti-CD28 (CD28.2, IgG1; Beckman Coulter, Fullerton, CA, USA); anti-CD57 (HNK-1, IgM; Beckman Coulter); anti-HLA-DR (L243, IgG2a; Becton Dickinson); anti-CD25 (ACT-1, IgG1; DAKO); anti-CD122 (TU27, IgG1; Becton Dickinson); and control mouse IgG (X40, IgG1; DAKO). In some experiments, $CD4^+CD28^+$, $CD4^+CD28^-$, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subsets were collected by FACS sorting.[14] Each population was >98% pure.

TCRAV and TCRBV repertoire analysis

Analysis of TCRAV and TCRBV repertoires was performed by an adaptor ligation PCR-based microplate hybridization assay, as reported previously.[15] Briefly, total RNA was extracted from PBMCs and converted to double-strand cDNA using the SuperScript cDNA synthesis kit (BRL, Bethesda, MD, USA). The P10EA/P20EA adaptors were

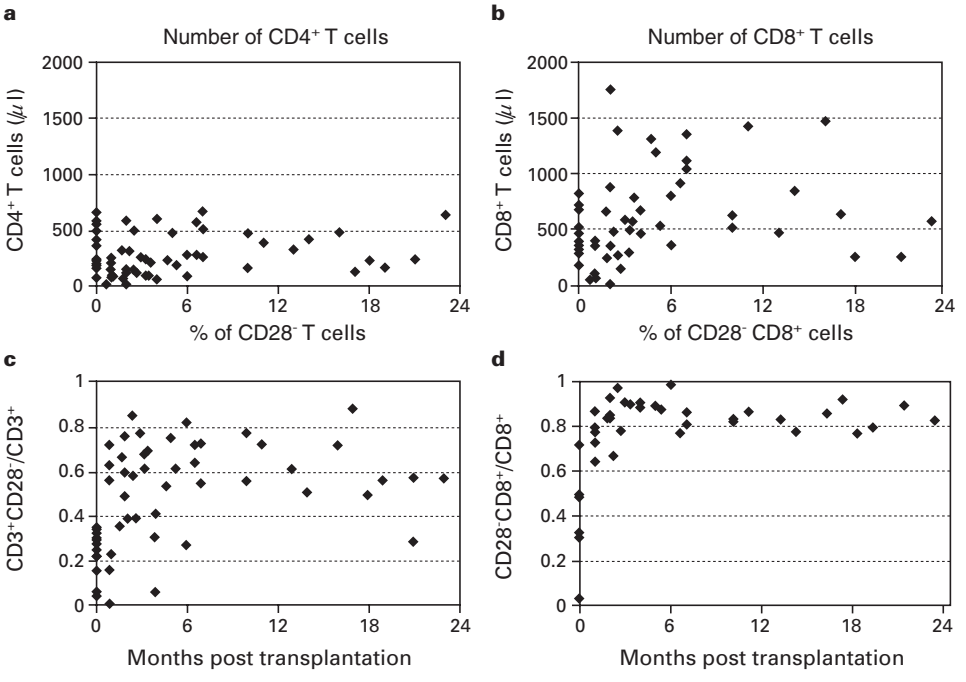

Figure 1 Recovery of the $CD28^-$ T lymphocyte subset after allogeneic bone marrow transplantation. Collected data from 23 patients receiving allogeneic marrow grafts are graphically presented. Panels (a) and (b) show the absolute counts of $CD4^+$ and $CD8^+$ T cells, respectively. Panels (c) and (d) show the fraction of $CD28^-$ cells in $CD3^+$ and $CD8^+$ subsets, respectively.

ligated to the 5′ end of cDNA prepared from PBMCs, and PCR was performed using either a biotinylated TCRCA-specific or TCRCB-specific primer, and a P20EA primer. Biotinylated PCR products were hybridized with immobilized TCRAV- or TCRBV-specific primers in 96-well microtiter plates. Subsequently, alkaline phosphatase-conjugated streptavidin was added to each well and a colorimetric assay was performed. Skewing of the TCR repertoire was defined as a significant increase of TCRAV and TCRBV subfamilies with greater percentage than the mean plus 3 standard deviation (s.d.) of 20 healthy individuals, and exceeded 5% of total circulating blood T cells.[7,15]

PCR amplification and CDR3 size distribution analysis of the TCR-β chain

The procedure for CDR3 size analysis (spectratyping) for the TCR-β chain has been described elsewhere.[16–18] Total RNA was extracted from PBMCs using a RNeasy Total RNA Kit (Qiagen, Hilden, Germany) and was used for first-strand cDNA synthesis with an oligo-dT primer (First-Strand cDNA Synthesis Kit, Amersham Pharmacia Biotech, Uppsala, Sweden).[19,20] Aliquots of the cDNA were amplified with a Vβ-specific primer and a Cβ-specific primer. Primer sequences were previously described.[18,21] PCR amplification was performed for 40 cycles in a 20 μl reaction mixture containing 0.2 μM of each primer and 0.5 U of Taq polymerase (TaKaRa, Osaka, Japan). Conditions for the PCR were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. Following the 40 cycles of PCR, an additional extension at 72°C for 15 min was performed. The PCR buffer was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.2 mM of each dNTP.

Aliquots (4 μl) of the unlabeled V β–Cβ PCR products were subjected to one cycle of elongation (runoff reaction) with a FAM-labeled nested Cβ primer (FAM-CB3) under the following conditions: denaturation at 94°C for 2 min, annealing at 55°C for 1 min, and extension at 72°C for 15 min. The reaction buffer was the same as that described above. The labeled PCR products were mixed with the size marker (GeneScan-500 TAMRA; Applied Biosystems, Warrington, UK), and loaded on to 5% polyacrylamide sequencing gels for determination of size and fluorescence intensity using an automated DNA sequencer (ABI 377, Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

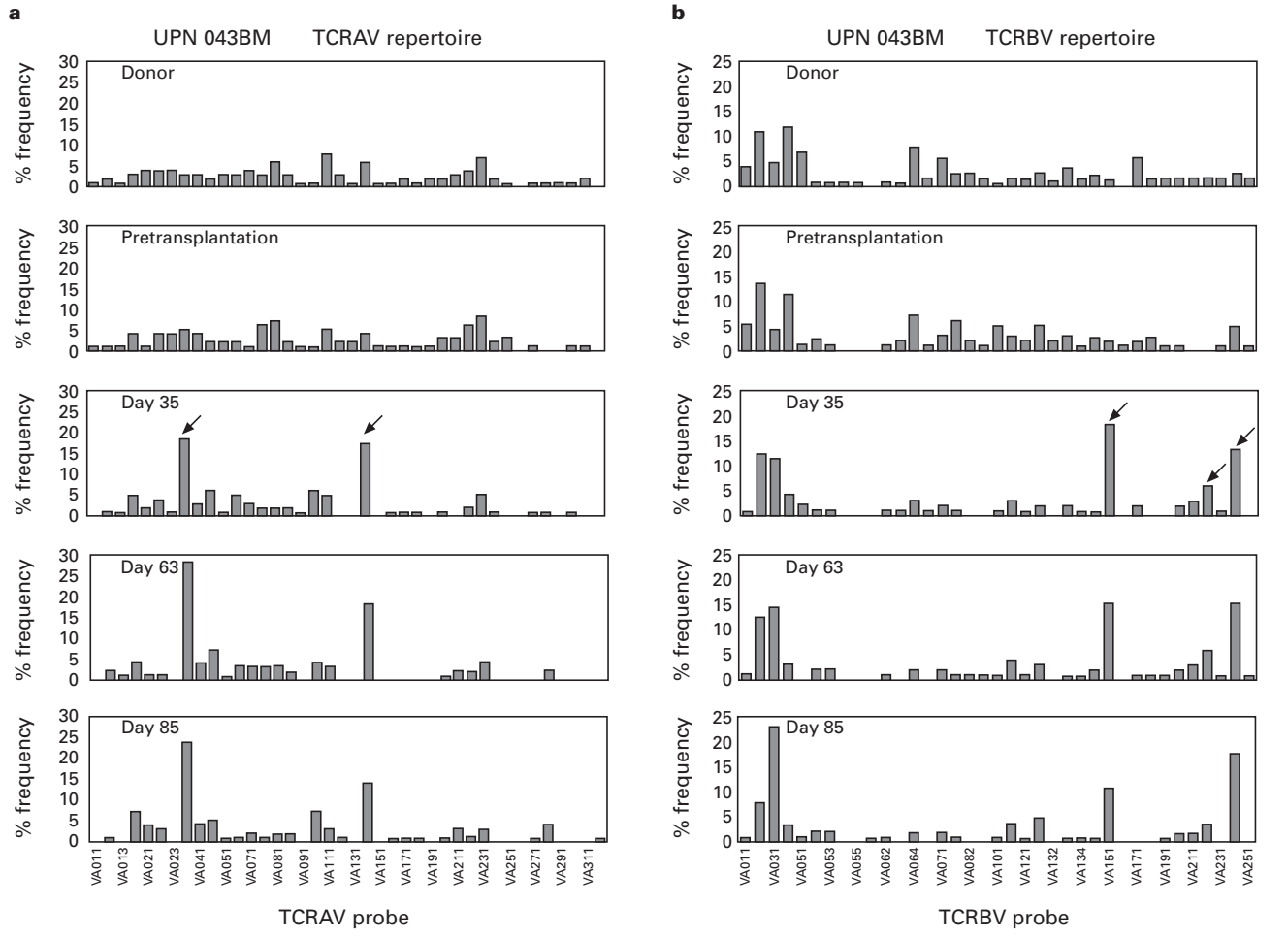

Figure 2 Skewing of TCRAV (a) and TCRBV (b) repertoires after allogeneic marrow transplantation. Blood samples were drawn from the recipient (UPN 043BM) before and after transplantation at indicated time points. TCRAV and TCRBV subfamilies with an increased frequency are indicated by arrows. Skewing of TCR repertoires was determined according to the criteria described in Materials and methods.

Table 1 Immunophenotype of circulating T lymphocytes from recipients of allogeneic hematopoietic cell transplants

	UPN 042BM	*UPN 043BM*	*UPN 02PB*
Date of analysis (after transplant)	Day 150	Day 60	Day 90
Lymphocyte subset (%)[a]			
$CD3^+$	67.5	73.6	60.3
$CD3^+CD28^-$	52.3	56.1	33.6
$CD3^+CD57^+$	37.9	ND	17.7
$CD3^+DR^+$	46.4	46.2	20.3
$CD3^+CD25^+(IL2R\alpha)$	0.5	1.2	3.7
$CD3^+CD122^+(IL2R\beta)$	0.3	0.2	0.2
$CD4^+CD28^+$	10.1	8.0	17.7
$CD4^+CD28^-$	8.7	8.1	9.5
$CD8^+CD28^+$	5.7	3.7	7.0
$CD8^+CD28^-$	45.1	45.1	29.1

[a]Each value represents the percentage of the indicated subset in total blood lymphocytes. All patients were seropositive for CMV.
ND = not determined.

Data were analyzed using GeneScan software (Perkin-Elmer Applied Biosystems).

Sequencing of CDR3 region in the TCR-β chain

PCR products of the TCR-β chain were cloned into the PCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced using a Big-Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems).[22] Sequence analysis was performed using an Applied Biosystems 377A automatic DNA sequencer.

Results

$CD8^+CD28^-$ T cells as an initially repopulated T cell subset after allogeneic bone marrow transplantation

During the first few months after transplant the recipients show lymphocytopenia and reduced CD4 counts. In agreement with previous reports, there was a marked increase of $CD28^-$ T cell fraction early after bone marrow transplantation (Figure 1c). Since the CD4/CD8 ratio is invariably

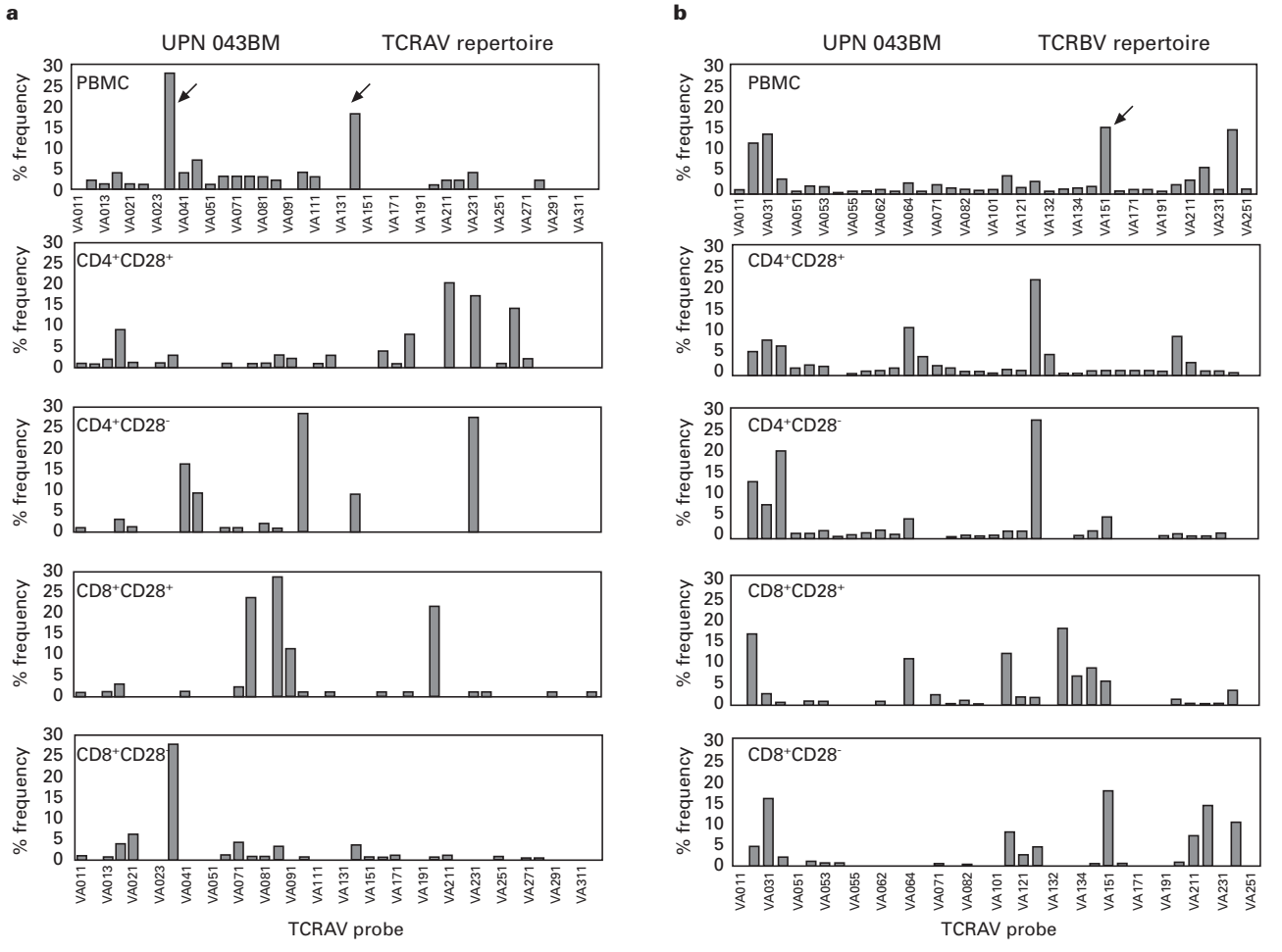

Figure 3 TCRAV (a) and TCRBV (b) repertoires of $CD28^+$ and $CD28^-$ T cell subsets. The blood donor was the same as in Figure 2 and the blood sample was collected on day 60.

low at this time, the majority of the T cells during the early immune reconstitution have the CD8$^+$CD28$^-$ phenotype. We have previously reported that the skewed TCRAV and TCRBV repertoires are observed at an early period after allogeneic hematopoietic cell transplantation.[7] We therefore asked whether the TCR repertoire of CD8$^+$CD28$^-$ T cell subset would impose a skew upon the post-transplant TCR repertoire of circulating blood T cells.

TCRAV and TCRBV repertoires of the CD8$^+$CD28$^-$ T cell subset defines the skewed TCR repertoire of circulating T lymphocytes

PBMCs were collected from three recipients of allogeneic hematopoietic cell grafts, and the T cells were separated into four subsets by FACS sorting depending upon their expression of CD4, CD8 and CD28. TCRAV and TCRBV repertoires were analyzed on PBMC and these four T cell subsets. Consistent with our previous report,[7] all three patients showed skewed TCRAV and TCRBV repertoires at an early phase of engraftment. Results of one representative patient are shown in Figure 2. In this patient, the TCR VA3–1, VA14–1, VB15–1, VB22–1 and VB24–1 subfamilies were increased following transplantation, although there was no difference in TCR repertoires between the donor and the recipient pre-transplant. The CD8$^+$CD28$^-$ T cell subset was the major population of the T cells in this patient (Table 1). The TCRAV and TCRBV repertoires of the CD8$^+$CD28$^-$ T cell subset were most similar to those of the PBMCs among other subsets, and the increased TCRAV and BV subfamilies were enriched in this population (Figure 3). In the same manner, two other patients were examined and similar results were found (Table 1, Figure 4). Moreover, a skew of the TCR repertoire in the CD8$^+$CD28$^-$ T cell subset was found within both TCRAV and TCRBV. Again, these results suggest that the expansion of CD8$^+$CD28$^-$ T cells was the result of recognizing antigens in the context of MHC/peptide but not the way of recognition of superantigens.[23]

Clonal diversity of CD28$^+$ and CD28$^-$ T cell subsets with skewed TCRBV repertoire

If the expansion of CD8$^+$CD28$^-$ T lymphocytes is imposed by the stimulation with classical peptide antigens, one would expect to find CDR3 length constraints.[24] We meas-

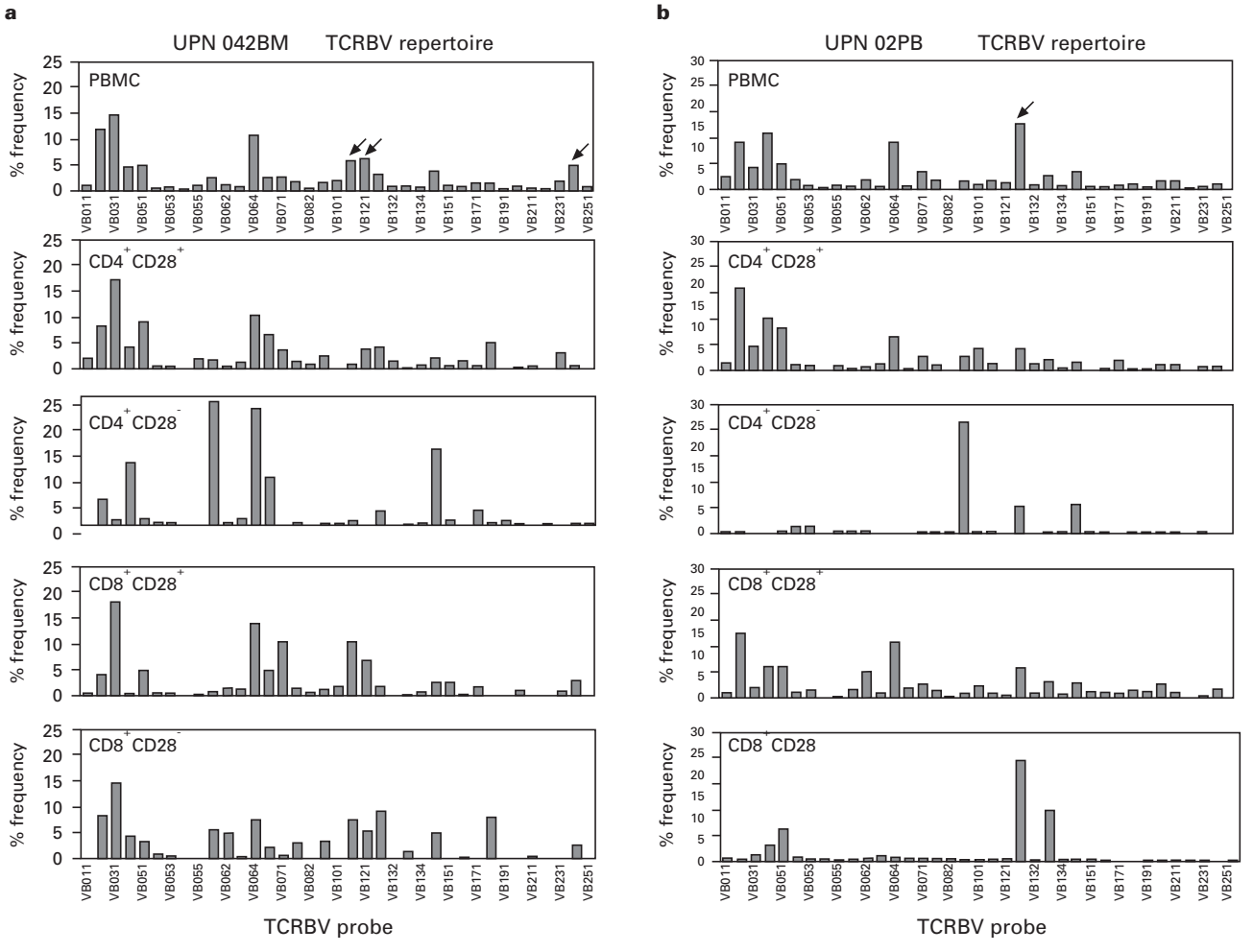

Figure 4 TCRBV repertoires of CD28$^+$ and CD28$^-$ T cell subsets in the UPN 042BM (a) and UPN 02PB (b) patients. Note that the TCRBV repertoire of the PBMC is similar to that of the CD28$^-$CD8$^+$ T cell subset in both patients.

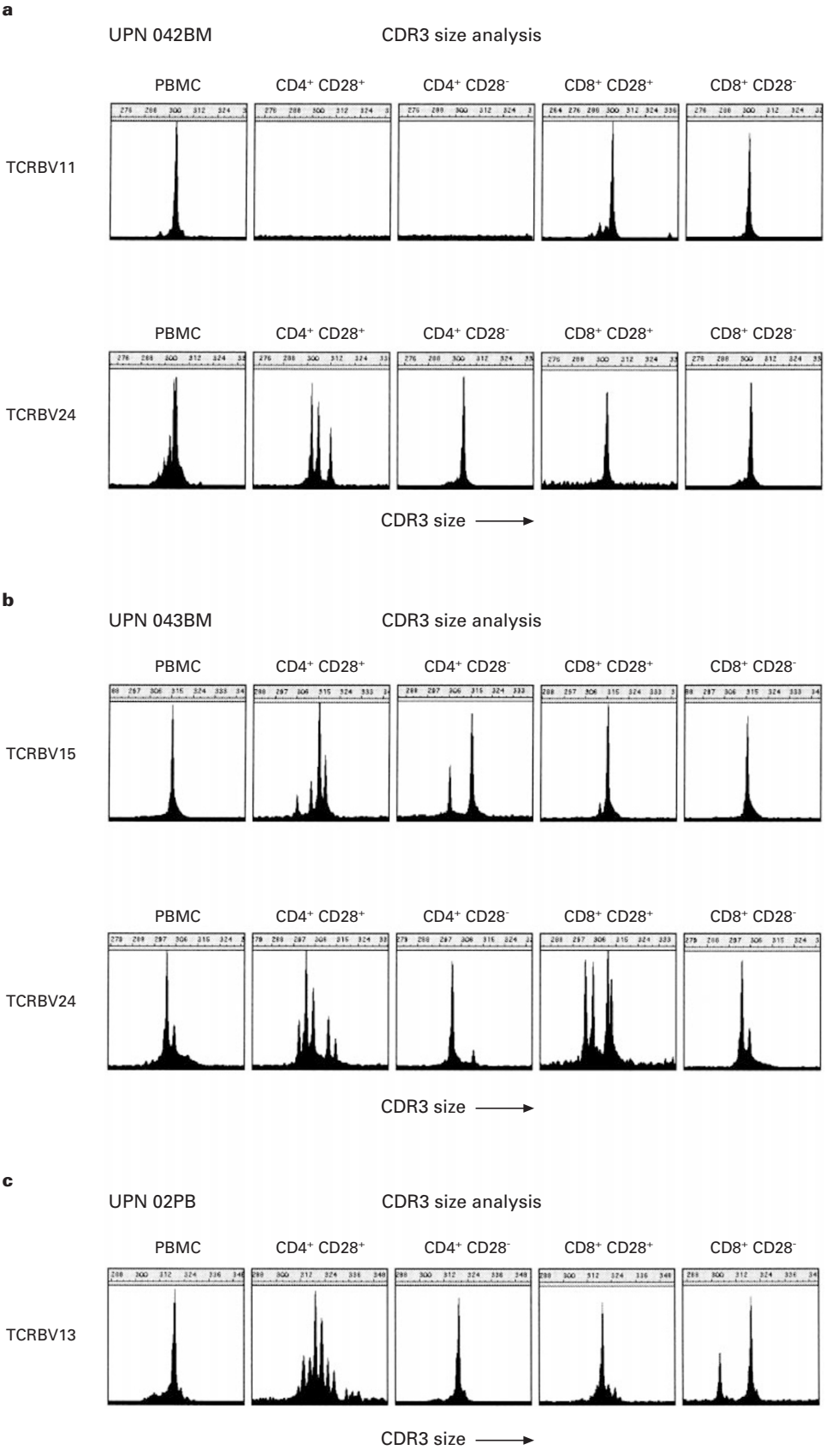

Figure 5 CDR3 size distribution pattern of skewed TCRBV subfamilies in the CD28$^+$ and CD28$^-$ T cell subsets. (a) UPN 042BM; (b) UPN 043BM; (c) UPN 02PB.

ured the CDR3 size of skewed TCRBV in each T cell subset. Clearly, the expansion of $CD8^+CD28^-$ T cells was clonal or oligoclonal (Figure 5), which supports the hypothesis mentioned above that the post-transplant expansion of $CD8^+CD28^-$ T cells was antigen-driven.[24,25] In addition, the diversity of the $CD8^+CD28^+$ T cell subset was also restricted as well as the $CD8^+CD28^-$ T cell population (Figure 5). The CDR3 length of the dominant peak was similar in both $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells in a given patient, suggesting that there may exist identical clones within the two subsets.

Demonstration of identical clones within both $CD8^+CD28^-$ and $CD8^+CD28+$ T cell subsets

If the $CD8^+CD28^-$ T cells originated from the $CD8^+CD28^+$ T cell subset, one would expect the identification of the same clones within both subsets. We subcloned PCR products of skewed TCR-β chains to see if the identical CDR3 sequences could be found in both $CD8^+CD28^-$ and $CD8^+CD28^+$ subsets. We found identical clones in both subsets in all three patients examined (Table 2).

Discussion

The present study demonstrates the presence of identical expanded clones within both $CD8^+D28^+$ and $CD8^+CD28^-$ T cell subsets in recipients of allogeneic hematopoietic stem cell grafts. Our observation suggests that the clonally expanded $CD8^+CD28^-$ T cells following allogeneic blood and marrow transplantation originate from the $CD8^+CD28^+$ T cells that have been exposed to antigens. The transition between the two subsets could be unidirectional, since $CD8^+CD28^-$ T lymphocytes are infrequent in cord blood, thymus and lymph nodes but this subset increases during human aging.[27] $CD8^+CD28^-$ T cells have been demonstrated to have shorter telomeres than $CD8^+CD28^+$ T cells.[28] Moreover, it has been recently reported that $CD8^+CD28^-$ T cells can be generated from chronically stimulated $CD8^+CD28^+$ T cells.[29] Mugnaini *et al*[30] have also reported the presence of identical expanded clones within both $CD8^+CD28^-$ and $CD8^+CD28^+$ T cell fractions in HIV patients. Thus, it has become more evident that $CD8^+CD28^-$ and $CD8^+CD28^+$ T cell subsets belong to the same T cell lineage with just a distinct phenotype.

We have previously observed the skewed TCRAV and TCRBV repertoires at an early period after human allogeneic blood and marrow transplantation.[7] We have also found that there was a strong correlation between delta scores of TCRAV and those of TCRBV. Delta scores signify the extent of alteration of the TCR repertoire.[31] In 28% recipients of allogeneic grafts, there was a clonal expansion of the T cells carrying TCRBV24S1. DNA sequence analysis revealed that an arginine residue followed by acidic amino acid residues is frequently observed in the CDR3 of TCR-β chain containing BV24S1, suggesting that skewing of the TCR repertoire is antigen-driven. To support this, measurement of the CDR3 length of TCRBV clearly demonstrated the clonal expansions in the $CD8^+CD28^-$ T cell subset in our study. Since the $CD8^+CD28^-$ T cell fraction is the major population among T cells during the first several months after transplantation, the TCR repertoire of this subset imposes a great impact on the repertoire of circulating blood T lymphocytes.

Antigen specificity of the clonally expanded $CD8^+CD28^-$ T cells following allogeneic transplantation remains to be determined. The $CD8^+CD28^-$ T cell population has been shown to contain virus-specific memory cytotoxic T lymphocytes that respond to human cytomegalovirus (CMV) and human immunodeficiency virus (HIV).[32–37] Thus, clonally expanded $CD8^+CD28^-$ T cells following transplantation may be derived from the T cells recognizing antigens that persistently exist in the host, and may contain CTL clones specific for viral antigens such as herpesviruses.

Post-transplant immune reconstitution should be also addressed at an antigen-specific level. Quantitative analysis of antigen-specific T cells has now become feasible by

Table 2 Deduced amino acid sequences of CDR3 regions of the TCR-β chain in $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells

Patient No.	*TCRBV segment*[a]	*Cells*	*TCRBV*	*N-D-N*	*TCRBJ*	*TCRBJ segment*	*Colony frequency*
1 UPN 042BM	BV11S1	$CD28^+$ $CD8^+$	SQYLCAS	RRGQ	TQYFGPGTRL	J2S5	8/8
		$CD28^-$ $CD8^+$	SQYLCAS	RRGQ	TQYFGPGTRL	J2S5	5/8
			SQYLCAS	TAG	DTQYFGPGTR	J2S3	3/8
	BV24S1	$CD28^+$ $CD8^+$	MYLCATS	YGTGA	YEQYFGPGTR	J2S7	9/9
		$CD28^-$ $CD8^+$	MYLCATS	RGQLSD	EQFFGPGTRL	J2S1	8/8
2 UPN 043BM	BV15S1	$CD28^+$ $CD8^+$	ALYFCAT	RGAGR	RAKNIQYFGA	J2S4	6/6
		$CD28^-$ $CD8^+$	ALYFCAT	RGAGR	RAKNIQYFGA	J2S4	7/7
3 UPN 02PB	BV13S1	$CD28^+$ $CD8^+$	VYFCASS	EWVR	YQPQHFGDGT	J1S5	2/7
			VYFCASS	YF	GSNQPQHFGD	J1S5	2/7
			VYFCA	SSDGTG	YEQYFGPGTR	J2S7	2/7
			VYFCA	SSYGGNG	NQPQHFGDGT	J1S5	1/7
		$CD28^-$ $CD8^+$	VYFCASS	YF	GSNQPQHFGD	J1S5	6/6

[a]TCRBV gene segments are described according to the nomenclature reported by Concannon *et al*.[26]

using fluorescence-conjugated major histocompatibility complex (MHC)-peptide tetramers, although this approach is limited to patients with certain HLA types.[38,39] Antigen specificity of the clonally expanded CD8$^+$CD28$^-$ T cells following allogeneic transplantation may be determined by taking advantage of this technique.

As yet, we are unable to draw any conclusions regarding the relationship between the magnitude of clonal expansion of CD8$^+$CD28$^-$ T cells and the immune status of blood and marrow transplant recipients. Quantitative analysis of antigen-specific CD8$^+$ T cells may provide insights into our understanding of clonal expansions of T cells following allogeneic hematopoietic cell transplantation.

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