In Vivo T-Cell Clonal Amplification at Time of Acute Graft-Versus-Host Disease

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In a series of patients transplanted with HLA-matched allogeneic bone marrow grafts (alloBMT), we previously showed that a few T-cell receptor (TCR) Vα and Vβ gene segment transcripts were overexpressed in skin compared with blood at the time of acute graft-versus-host disease (aGVHD). Here, in one selected patient with overexpressed Vβ16 and Vα11 transcripts in skin, we analyzed the junctional variability of these transcripts in donor blood, patient blood, and skin collected at aGVHD onset. A unique junctional region sequence accounted for 81% of in frame Vβ16 transcripts (13 of 16) in skin and 59% (13 of 22) in patient blood. Similarly, two recurrent junctional region sequences were found in skin Vα11 transcripts, one accounting for 66% (21 of 32) and the other for 16% (5 of 32). These recurrences were also found in patient blood (36% and 15% of Vα11 transcripts, respectively). To extend our analysis, a polymerase chain reaction (PCR)-based method was used to precisely determine TCR β transcript length in run-off reactions using uncloned bulk cDNA samples. All Vβ-Cβ PCR products analyzed in donor blood, as well as the majority of those analyzed in patient blood, included transcripts with highly diverse junctional region sizes. As expected from the sequence data, most Vβ16-Cβ PCR products in skin and patient blood were of the same size (ie, same junctional region). In addition, Vβ3, Vβ5, and Vβ17 transcripts in skin were shown to display highly restricted size variability. The clonality of the Vβ16-Cβ and Vβ17-Cβ transcripts was further supported by the results of run-off reactions using 13 Jβ specific primers. We have identified several recurrent TCR transcripts in skin, some of them also present in patient blood. These data support the view that several T-cell subpopulations are clonally expanded in vivo at the time of aGVHD onset in this case of related HLA-matched alloBMT.

T LYMPHOCYTES play a key role in acute graft-versus-host disease (aGVHD), as shown by the efficacy of T depletion in preventing this disease and the predominant presence of α/β T cells at the site of aGVHD target organs.[1-4] Their precise contribution in initiating this alloreaction directed against host major or minor histocompatibily antigens (miHA) is a field of intense research, warranted by the intricate relationships between GVHD and graft versus leukemia (GVL) reaction and by the predominant involvement of few target organs (skin, liver, and digestive tract).[5-7]

Mature α/β T lymphocytes recognize antigenic peptides presented by major histocompatibility complex (MHC) molecules through their heterodimeric α/β surface receptor T-cell receptor (TCR). This specific recognition is dependent on interaction between a MHC/peptide complex and the variable region of TCR molecules. During T-cell differentiation, unique variable region genes are created by recombination of variable (V), diversity (D) and joining (J) segments for the β locus, and V and J segments for the α locus. In addition to this combinatorial variability, TCR diversity is greatly increased by nibbling of V, D or J segments, as well as addition of N-region nucleotides between V and D or D and J segments during recombination (junctional diversity).[8] The three-dimensional structure of the TCR backbone is presently unknown, but it is likely that the TCR antigen binding site has an antibody-like structure. Complementary determining regions (CDR) 1, 2, and 3 have accordingly been defined for TCR molecules. CDR3 regions are encoded by the hypervariable V-J or V-D-J junctions and are essential for binding to the antigenic peptide presented by a MHC molecule. Expression of unique rearranged TCR gene products thus determines the specificity of a given T cell,[9,10] and identification of recurrent TCR transcripts (same CDR3) in large T-cell populations indicates antigen-driven expansion of the corresponding T-cell clones.

In a series of HLA-matched allogeneic bone marrow transplants (alloBMT), we previously showed an unrestricted TCR Vα and Vβ gene segment usage in both skin and blood lymphocyte RNA samples collected at the time of GVHD onset with overexpression of a few Vα and Vβ gene segments in skin compared with blood.[11] These overexpressions varied from one patient to another. In these patients, a nonspecific inflammatory response could mask clonal T-cell expansion, and we selected one patient with Vβ16 and Vα11 TCR transcripts overexpressed in skin compared with blood in an attempt to identify putative in vivo T-cell clonal expansions. The junctional variability of polymerase chain reaction (PCR) amplified Vα and Vβ gene segment transcripts was analyzed by two methods, sequencing of cloned cDNA and determination of CDR3 region size distribution on bulk cDNA. We found evidence for at least five TCR gene segment transcript recurrences. These data support the view that several T-cell subpopulations are clonally expanded in vivo at the time of aGVHD onset in this case of related HLA-matched alloBMT.

MATERIALS AND METHODS

Patient. As previously reported,[11] this 23-year-old woman underwent a related HLA-matched alloBMT for an accelerated phase

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of chronic myelogenous leukemia in 1990. Total body irradiation, cytarabine, and melphalan (TAM) regimen was the conditioning treatment. GVHD prophylaxis consisted of cyclosporine and a short course of methotrexate. Four samples were available for Vα and Vβ gene segment usage analysis: donor blood collected before alloBMT, patient blood, and two skin samples collected at the onset of cutaneous grade II acute GVHD (day 25). Note that the two skin biopsies have been performed in clinically involved and noninvolved areas. Skin taken in clinically noninvolved fields had a normal histologic pattern, with little, if any, T-lymphocyte infiltration, and no TCR transcript could be amplified, even after 40 PCR cycles.[11]

Cloning and sequencing analysis. Using reverse transcriptase-polymerase chain reaction (RT-PCR) with a panel of experimentally validated oligonucleotides,[12] we previously showed apparently unrestricted TCR Vα and Vβ gene segment usage by peripheral blood mononuclear cells (PBMC) and skin lymphocytes at the time of aGVHD onset in this patient.[11] Some PCR-amplified Vα and Vβ cDNA were chosen to further characterize their junctional diversity. For the cloning of Vβ sequences, the primers were respectively Vβ16 5′-GCCTGCAGAACTGGAGGATTCTGG-3′ (position 192 from ATG), Vβ19 5′-TCCTCTCACTGTGACATCGGCCCA-3′ (position 279 from ATG) in the V region, and oli 75 5′-ACCAGCTCAGCTCCGCGGGGTCGG-3′ in the Cβ region. For the cloning of Vα sequences, the primer were respectively Vα4 5′-TTGGTATCGACAGCTTCACTCCCA-3′ (position 153 from ATG), Vα11 5′-CGCTGCTCATCCTCCAGGTGCGGG-3′ (position 254 from ATG) in the V region, and oli 76 5′-GTTGCTCCAGGCCGCGGCACTGTT-3′ in the Cα region. Both Cα and Cβ primers contained an artificial *Sac*II cloning site. The first cDNA/RNA heteroduplex strand was submitted to two rounds of 30 cycles amplification on a thermocycler. Between the two rounds of amplification, the material was size purified on 1% low-melting agarose gel. Following ethanol precipitation, one-half of the second amplification product was digested by *Sac*II and purified on 2% agarose gel. Bands with the expected size were isolated, following adsorption on glass powder beads (Geneclean, BIO 101, Inc, La Jolla, CA). The material was ligated to Bluescript SK+ (Stratagene, La Jolla, CA) digested with *Sac*II and *Eco*RV and used to transform XL1-blue *Escherichia coli* strains (Stratagene). The white colonies were screened using a dot blot technique and a third nested Cα or Cβ ^{32}P-labeled oligonucleotide as a probe. Plasmid DNA was extracted from positive colonies and sequenced with Sequenase 2.0 (United States Biochemicals, Cleveland, OH) using the dideoxy-chain termination procedure. Particular care was taken to avoid sample contamination. All solutions were aliquoted, and aliquots were used only once. A mock sample was processed along with each set of experimental samples during the entire procedure. This control was checked by hybridization with the Cα or Cβ probe to rule out any contamination.

CDR3 size analysis. Experiments were performed as previously described.[13,14] Briefly cDNA copies of 0.1 μg total RNA were amplified in 40 cycles Vβ-Cβ PCR in 50 μL, and 2-μL aliquots were copied in one cycle run-off reactions (volume, 10 μL) primed with fluorescent (Applied Biosystems fluorophor Fam, Foster City, CA) labeled oligonucleotides specific for Cβ (Fam-CACAGCGACCTCGGGTGGG), or for each Jβ, eg, Jβ1.2 (Fam-ACAACGGTTAACTTGGTCCCCGAA), or Jβ2.3 (Fam-TGCCTGGGCCAAAATACTGCG). The run-off products were then subjected to electrophoresis on an ABI sequencer in the presence of fluorescent size markers and analyzed by Immunoscope software (Pannetier, Institut Pasteur, Paris, France, unpublished data). A normal transcript size distribution reflecting polyclonal cDNAs is bell-shaped and contains 6 to 8 peaks with a mean CDR3 size of 27 to 33 nucleotides (nt) or nine to 11 amino acids. On the other hand, emergence of one or more dominant peaks reveals the presence of one or more cDNAs with a similar (ie, same size) or identical in frame junctional region.[14]

RESULTS

Identification of recurrent TCR transcripts in skin and blood. The presence of recurrent TCR transcripts in skin and blood of one patient with aGVHD was determined by cloning and sequencing PCR-amplified cDNA. Considering the Vβ16 specificity (overexpressed in skin compared with blood), a predominant recurrent transcript with a unique Vβ16-ERGGI-Jβ1.2 rearrangement accounted for 81% (13 of 16) of Vβ16 in frame transcripts in skin and 59% (13 of 22) in patient blood (Table 1). Less striking recurrences were also found in skin (a Vβ16-QEGRI-Jβ2.3 rearrangement, 19%) and in blood (Vβ16-LRD-Jβ2.1, 14%). Controls included donor blood Vβ16 and a non-overexpressed specificity in skin (Vβ19).[11] Both of these PCR products were devoid of significant junctional region sequence recurrence. Two transcript recurrences were identified by analyzing Vα11 (overexpressed in skin) (Table 2): Vα11-LN-JHAP10 (66% in skin, 37% in blood) and Vα11-PL-IGRJA14 (16% both in skin and blood) rearrangements. Minor recurrences (15%) were also observed in a non-overexpressed PCR product in skin (Vα4),[11] used as a control. Overall, the high rate of identical junctional regions found for some Vβ and Vα specificities overexpressed in skin demonstrates that recurrent TCR species are transcribed in this aGVHD patient.

CDR3 size analysis of bulk cDNA. To extend the analysis, we examined CDR3 size distribution in a series of Vβ transcripts (Vβ1, 2, 3, 5, 7, 9, 13, 14, 16, 17, 19, 23) present in the three RNA samples (donor blood, patient blood, and skin). The corresponding Vβ-Cβ PCR products were copied in run-off reactions primed with a nested fluorescent Cβ (Fig 1) or Jβ (Fig 2) oligonucleotide. The sizes of the fluorescent run-off products were determined by electrophoresis on an automated DNA sequencer as previously described.[13,14] The signals (or peaks) corresponded to a defined run-off species separated from one another by three nucleotides (ie, one CDR3 amino acid coding triplet), because 95% of V-(D)-Jβ rearrangements are in frame in blood or tissue samples.[15]

For elongation with the Cβ fluorophor (Fig 1), CDR3 size distribution in all Vβ transcripts analyzed from donor blood showed bell-shaped patterns of about eight peaks separated by three nt. (one codon) with the major peaks corresponding to CDR3 sizes of 9 to 11 amino acids, as found in healthy human donors (unpublished data) and in mice.[14] In contrast, the Vβ16-Cβ profiles, both in patient blood and skin, showed an identical prominant peak (143 nt), matching exactly the size of the recurrent Vβ16-ERGGI-Jβ1.2 sequence (see Table 1). A clearly predominant peak is likely to represent a distinct recurrent TCR transcript characterized by its CDR3 size. Some of the other Vβ specificities analyzed in skin also included predominant transcript species with a defined junctional region size (see peaks for Vβ3, Vβ5 and Vβ17), while others displayed a polyclonal pattern (Vβ14 and Vβ19). Polyclonal patterns were usually observed in patient blood, except for Vβ16 and Vβ17 (Fig 1).

Analysis of Vβ16 and Vβ17 PCR products was further refined in run-off reactions with the 13 Jβ fluorescent primers. Here again, predominant peaks, whose nt sizes corresponded exactly to the Vβ16-ERGGI-Jβ1.2 recurrent tran-

Table 1. Analysis of TCR-β Chain Transcripts Expressed in Patient Skin and Blood and in Donor Blood at Time of GVHD Onset

	Sequences Occurring More Than Twice					Sequences Occurring 1 or 2 Times		
			Junctional Sequence				Jβ1	Jβ2
Sample Sequence	No. of Occurrences (%)	No. of Vβ	NDβN		Jβ	No. of Occurrences	123456	1234567
Patient skin								
Vβ16	16	13 (81%)	CASS	ERGGI	YGYTFG (Jβ 1.2)	0		
		3 (19%)	CASS	QEGRI	STDTQYFG (Jβ 2.3)			
Patient skin								
Vβ19	18	0				18	300000	6120006*
Patient blood								
Vβ16	22	13 (59%)	CASS	ERGGI	YGYTFG (Jβ 1.2)	6	010000	1010201
		3 (14%)	CASS	LRD	SYNEQFFG (Jβ 2.1)			
Donor blood								
Vβ16	10	0				10†	020200	2020002

* Two sequences occurring two times.

† No sequence recurrence.

script (in both blood and skin) and the less recurrent Vβ16-QEGRI-Jβ2.3 rearrangement (in skin) sequences (102 nt and 92 nt, respectively), were detected in bulk cDNA samples (Fig 2). When Vβ17 PCR products were copied with the 13 Jβ primers, no signal was observed except with the Jβ2.3 fluorescent primer. Using Jβ2.3 primer (Fig 2), a single peak was found in skin, whose size corresponded to the predominant Cβ run-off product shown in Fig 1, taking into account the position of the Jβ2.3 primer. Note that the predominant peak observed for Vβ17-Jβ2.3 in skin was not the same one as in blood. On the other hand, the CDR3 size distributions from donor blood were polyclonal. Together, these results illustrate the expression of at least five recurrent TCR transcripts in this patient, which is highly suggestive of multiple antigen-driven T-cell clonal expansions taking place in vivo.

DISCUSSION

The pivotal role of T cells in initiating GVHD has been clearly established by clinical, epidemiologic, as well as biologic studies.[1-5] Recent experimental data support the view that a subset of T cells mediating GVHD may be different from those responsible for GVL effect.[7,16,17] Thus, in the near future, selective T-cell depletion using Vβ specific monoclonal antibodies appears a possible strategy to prevent GVHD while keeping GVL effect. This implies that GVHD should be mediated by a limited set of T-cell subpopulations recognizable by their TCR V region. To evaluate the extent of in vivo TCR diversity during GVHD, we have analyzed the TCR Vα and Vβ chain structure of T cells present in blood and skin at time of GVHD onset in patients grafted with HLA-identical transplant. We have previously reported that some Vα and Vβ gene segment subfamilies are overexpressed in skin compared with blood, but it was not determined if such slightly skewed TCR repertoire corresponded to clonal T-lymphocytes expansions.[11] The present study was designed to analyze the junctional variability of TCR Vα and Vβ gene segment transcripts in both skin and blood of a selected patient. Several T-cell clonal expansions were identified, some of them being preferentially or exclusively expressed in skin.

In this study, the human TCR repertoire was directly analyzed in small skin biopsies and lymphopenic blood samples,

Table 2. Analysis of TCR-α Chain Transcripts Expressed in Skin and Blood at Time of GVHD Onset

	Sequences Occurring More Than Twice					Sequences Occurring 1 or 2 Times	
			Junctional Sequence				
Sample	No. of Sequence	No. of Occurrences (%)	Vα	N	Jα	No. of Occurrences	Jα
Patient skin							
Vα11	32	21 (66%)	YCAV	LN	DYKLSFG (JHAP10)	6	IGRJA05* (2), IGRJA09,
		5 (16%)	YCAV	PL	NYQLIWG (IGRJA14)		JAD17, JHA29, JAG212
Patient skin							
Vα4	20	3 (15%)	VYYC	N	NTDKLIFG (JHAP02)	14	JHAP08* (4), JHAP44 (2),
		3 (15%)	VYYC	ILGA	FGNEKLTFG (JAA13)		JHAVT27 (2), JAA13, IGRJA04
							IGRJA05, IGRJA12* (2), JGERMD
Patient blood							
Vα11	19	7 (37%)	YCAV	LN	DYKLSFG (JHAP10)	9	JHAP29* (2), JHAP58
		3 (16%)	YCAV	PL	NYQLIWG (IGRJA14)		JAB11, JAB19, JAE212
							IGRJA04, IGRJA14, JHJGC

* Four sequences occurring two times.

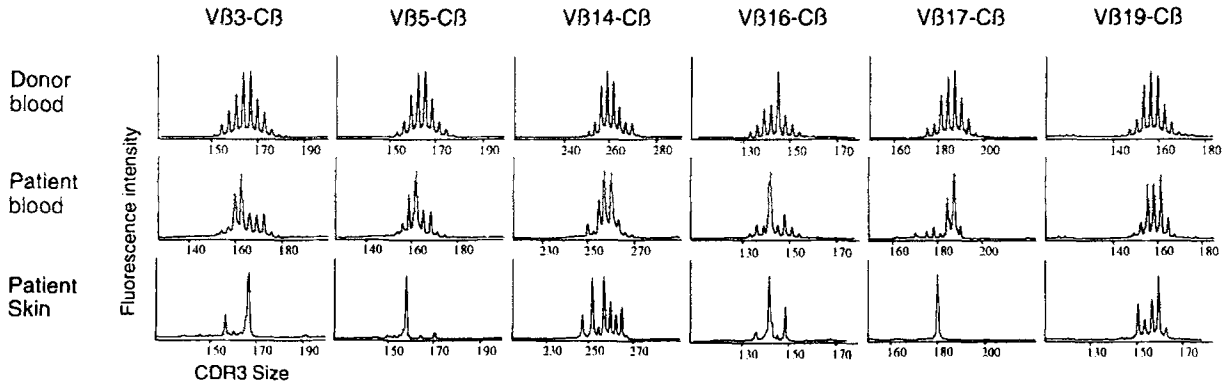

Fig 1. CDR3 size distribution patterns of fluorescent Vβ-Cβ run-off products. Total RNA from tumor and peripheral blood lymphocytes was reverse transcribed and amplified by PCR with Vβ and Cβ primers. Amplified DNA was copied by a fluorescent Cβ primer in a run-off reaction and subjected to electrophoresis on an automated sequencer. The pattern obtained show the size and intensity distribution of in frame Vβ-Cβ amplification products according to their migration time from left to right.[14] The Vβ3, Vβ5, Vβ14, Vβ16, Vβ17, and Vβ19-Cβ amplification products from donor blood, patient blood, and patient skin were copied with a fluorescent Cβ primer.

without any cell separation or culture. Several aspects of this approach deserve discussion. Firstly, the initial Vβ-Cβ PCR reactions are not quantitative because amplification efficacy is known to vary from one primer pair to another. Nonetheless, use of this panel of primers has enabled us to focus on Vα and Vβ gene segment subfamilies overexpressed in skin compared with blood (same primer pair used).[11] Some of these overexpressions are shown here to correspond to a high incidence of overrepresented TCR rearrangements, possibly as a result of in vivo antigen-driven clonal T-cell expansion. Secondly, both the sequence analysis and run-off reactions are strictly quantitative within a given V subfamily. PCR products were cloned and randomly picked for sequence analysis from large numbers of recombinant colonies. In the run-off reactions, peak sizes are rigorously proportional to the initial number of amplified cDNAs.[14] The high resolution of this novel approach has enabled us to quantitatively study bulk cDNA, circumventing the cumbersome process of sequencing very large numbers of TCR transcripts. The high consistency of results obtained independently with the same PCR products by these two methods has eventually secured their quantification properties.

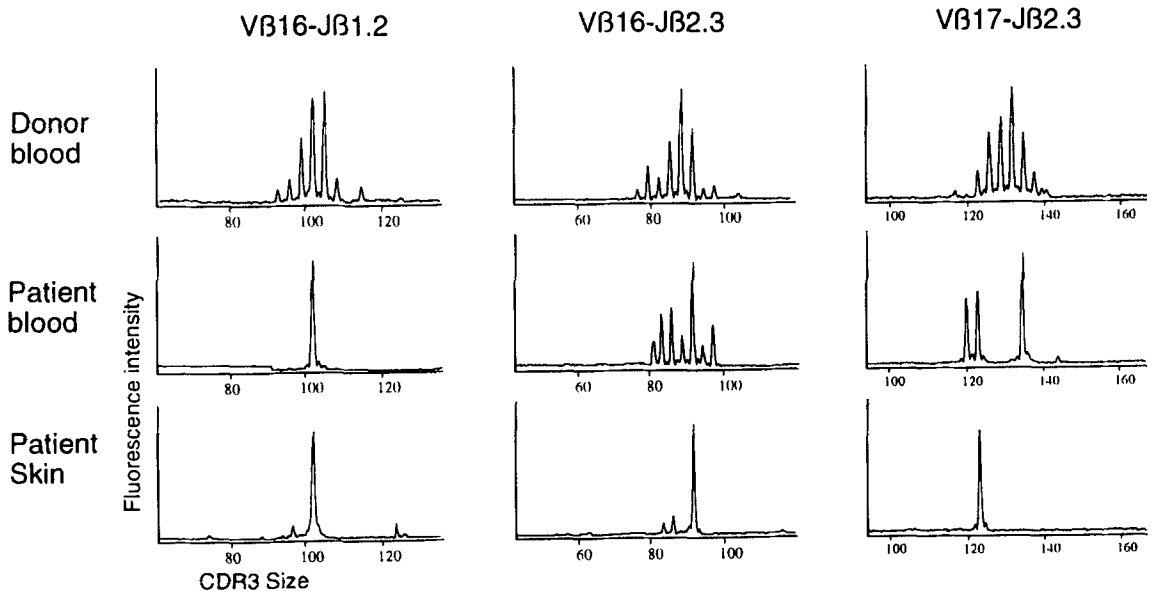

Fig 2. CDR3 size distribution patterns of fluorescent Vβ-Jβ run-off products. Total RNA from tumor and peripheral blood lymphocytes were reverse transcribed and amplified by PCR with Vβ and Cβ primers. Amplified DNA was copied by a fluorescent Jβ primer in a run-off reaction and subjected to electrophoresis on an automated sequencer. The pattern obtained show the size and intensity distribution of in frame Vβ-Jβ amplification products according to their migration time from left to right.[14] The Vβ16-Cβ amplification products from donor blood, patient blood, and patient skin were copied with fluorescent Jβ1.2 and Jβ2.3 primers. The Vβ17-Cβ amplification products from donor blood, patient blood, and patient skin were copied with a fluorescent Jβ2.3 primer.

Thirdly, one may ask if the minimal size of the lymphocyte sample can yield relevant and reproducible results, especially in terms of recurrence frequencies. In the aGVHD skin biopsy studied here, we have previously shown a significant $CD3^+$ T-lymphocyte infiltrate by immunohistologic analysis,[11] and it may be assumed that these T cells contained a sufficient number of TCR β transcripts, leading to the amplification of enough cDNA copies to avoid stochastic variations. Another argument against stochastic PCR amplification is the finding that some recurrent skin transcripts (eg, Vβ16-ERGGI-Jβ1.2) were also identified in blood, where material from more than 3×10^6 T cells was initially included in the PCR sample. In addition, in the same skin sample where recurrent transcripts have been identified, other Vβ-Cβ PCR products have been shown to be highly diverse in terms of junctional regions.

Overall, our data suggest that such direct approaches, where the resident T-cell repertoire is studied without any in vitro manipulation (ie, cell subpopulation enrichment and/or cell culture), are appropriate for the identification of in vivo antigen-driven clonal T-cell amplification. Indeed, in other clinical situations (a case of regressive melanoma and a case of renal cell carcinoma), this approach has demonstrated that recurrent TCR transcripts observed in vivo at the tumor site or in draining lymph node corresponded to specific cytotoxic T-lymphocyte clones generated in vitro,[18,19] (and unpublished results). Therefore, we would like to emphasize the interest to combine in further studies such in situ TCR molecular analyses to more conventional functional studies,[16,17,20-23] in an attempt to identify the nature of human miHA.

In this study, the presence of several recurrent TCR transcripts support the view that a process of antigen-driven clonal T-cell expansion was taking place at the time of sample collection. The clonal expansions observed here may relate to complex phenomenoma occurring during bone marrow transplant (BMT) course. Infections are a frequent complication after BMT, and bacterial, viral, or fungal microorganisms are good candidates to induce a strong cellular immune response. Whether such a response induces clonal T-cell expansions characterized by dominant peaks, as found here, is not known at the present time. Perhaps more importantly, clonal expansion of some T-lymphocyte subpopulations early posttransplant may be the result of a regenerating T-cell system.[24] We also observed few T-cell clonal expansions in blood of four individuals undergoing either autologous (two cases) or allogeneic (two cases) transplant without GVHD development (data not shown), using the same methods described here.

In the context of aGVHD occurring after HLA-matched transplant, such clonal expansions may also reflect the donor's immune response against patient miHA.[25,26] Albeit not yet characterized, human miHA are recognized in the context of MHC molecules (mostly class I),[22,26] and cytotoxic T lymphocytes (CTL) against miHA have been shown to be implicated in GVHD.[20] Skin is frequently involved in aGVHD, and T cells infiltrating an aGVHD target organ are likely to be implicated in GVH reaction. Interestingly, in our study, salient clonal T-cell expansions were preferentially or exclusively observed in skin (see Vβ3, Vβ5 and Vβ17 in Fig 1, and Vβ17-Jβ2.3 in Fig 2). Both the clinical tropism of aGVHD to skin and the presence of these T-cell clonal expansions in skin may be due to a preferential expression of some miHA in this tissue.[21] Alternatively, T cells initially expanded in blood may selectively home to skin because of the high level of adhesion molecules or MHC molecules expression (necessary for miHA presentation to T cells).[27,28] In any case, our data showing that some recurrent transcripts were predominantly observed in skin compared with blood are consistent with GVHD-related antigen driven T-cell clonal expansion. Further studies are needed to determine if such clonal expansions detected in vivo actually correspond to T-cell clones with specific cytotoxicity against miHA. On the other hand, TCR molecular analyses as described here, can help to assess the biologic relevance of anti-miHA CTL generated in vitro, and thus guide the research for characterizing human miHA. Finally, the results of the present study indicate that the prevention of GVHD by selective T-cell depletion using Vβ specific monoclonal antibodies, remains a complex issue because the expansion of at least four T-cell clones, each of them expressing a different Vβ specificity, has been observed in this patient.

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