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ORIGINAL ARTICLE

Characterization of γδ T cell subsets in organ transplantation

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Summary

γδ T cells are innate-type lymphocytes that preferentially act as regulators of local effector immune responses. Recent reports found an altered distribution of the two main subpopulations of blood γδ T cells (Vδ1 and Vδ2) in operationally tolerant liver transplant recipients. Based on this, γδ T cells subset quantification was proposed as a biomarker of immunologic risk in liver transplantation. The specific characteristics of γδ T cell subsets in transplantation remain however unknown. We have investigated here the phenotype, repertoire and functional properties of γδ T cell subsets in a large population of allograft recipients. Our results indicate that alterations in the γδ T cell compartment are not restricted to tolerant liver recipients. In fact, most immunosuppressed liver and kidney recipients also display an enlarged peripheral blood γδ T cell pool mainly resulting from an expansion of Vδ1 T cells exhibiting an oligoclonal repertoire and different phenotypic and cytokine production traits than Vδ2 T cells. We propose that persistent viral infections are likely to contribute to these alterations. Our data provide novel insight in the biology of γδ T cells and a rationale for exploring these lymphocytes in more depth into the pathogenesis of viral infections in transplantation.

Introduction

αβ T lymphocytes, which are essential in pathogen clearance, are also known to play a critical role in various experimental models of allograft rejection and tolerance acting as both effector and suppressor T cells [1]. In contrast, unconventional lymphocytes such as γδ T cells appear to act more as regulators of local effector immune responses by delivering rapid stress-surveillance responses triggered by threats to tissue integrity. These responses, which can be elicited by pattern-recognition receptors independently of the T cell receptor (TCR), appear to be extremely pleiotropic and result in a variety of local and systemic effects, some of them immunoregulatory by limiting deleterious αβ T cell responses [2–4]. In contrast to αβ T cells, the contribution of γδ T cells to the effector and/or regulatory arms of the alloimmune response are unclear.

The main subset of γδ T cells in human peripheral blood expresses the Vδ2 TCR and recognizes nonpeptide antigens of bacterial origin. In contrast, the Vδ1 T cell subset predominates in tissues such as intestine, skin, spleen and liver, where they appear to limit inflammatory responses and prevent immunopathology [5–7]. The specific antigens recognized by Vδ1 T cells are still not clearly defined [8,9]. Two recent reports have described that in peripheral blood of operationally tolerant liver transplant recipients, but not in healthy individuals, Vδ1 T cells are significantly expanded and constitute the principal γδ T cell subpopulation [8,9]. These data have been interpreted

as indicating that in transplant recipients Vδ1 T cells could be exerting graft-protecting immunoregulatory functions. Furthermore, the ratio between the relative frequency of Vδ1 and Vδ2 T cells has been proposed as a candidate biomarker to identify liver recipients who could hypothetically discontinue immunosuppressive therapy (IS) [8,9]. The variables influencing γδ T cell expansion in transplant recipients and the mechanisms through which these lymphocytes could be exerting a graft-protective role however, have not been adequately assessed. In the current study we have expanded the original observations made in the context of selected operationally tolerant liver recipients and we have investigated in detail the number, phenotype, repertoire and functional properties of γδ T cell subsets present in peripheral blood of a large number of transplant recipients. In addition, we have addressed whether the type of organ being transplanted, the immunosuppressive therapy and the presence of persistent viral infections could influence the distribution of γδ T cell subsets. Finally, we have re-evaluated the clinical value of peripheral blood γδ T cell quantification as a biomarker to identify operationally tolerant transplant recipients.

Materials and methods

Patients and controls

Peripheral blood samples were collected from the following study subjects: 201 liver transplant recipients on maintenance IS (STA-Liver); 50 kidney transplant recipients on maintenance IS with normal creatinine serum levels and absence of proteinuria (STA-Kidney); 29 operationally tolerant liver transplant recipients (i.e. recipients off IS for at least 1 year and maintaining stable graft function; TOL); 50 patients with chronic end-stage liver disease listed for transplantation (ESLD); and 34 healthy volunteers (CONT) age-matched with transplanted patients. At the time of analysis STA-Liver recipients were receiving low-dose IS in monotherapy,while STA-Kidney were receiving double or triple IS therapy based on either cyclosporine A, tacrolimus or sirolimus. The local Ethics Committees of the participating centers approved all aspects of the study and all patients gave their informed consent. Table 1 summarizes the demographic characteristics of all patient groups included in the study. Data on the frequency of peripheral blood γδ T cell subsets from nine TOL described in the current study have been previously reported [9].

Table 1. Demographic and clinical characteristics of patient groups

Clinical diagnosis	Number	Age [A]	Gender	Time from transplantation (years) [A]	Treatment [B]	HCV infection	CMV infection	EBV infection	HSV infection	Center [C]
Operationally tolerant (TOL)	29	61 (29–75)	67% Male	13 (6–9)		32%	89%	91%	91%	B, R
Stable liver recipients (STA-Liver)	201	57 (24–78)	69% Male	8 (3–20)	47% FK 37% CsA 11.5% MMF 4% Rapa 0.5% AZA	37%	90%	95%	95%	B, R
Stable kidney recipients (STA-Kidney)	50	61 (32–81)	48% Male	10 (4–18)	48% Rapa based 48% CsA based 4% FK based	2%	85%	92%	88%	B
End stage liver disease (ESLD)	50	55 (26–79)	76% Male			10%	87%	91%	89%	B
Healthy controls (CONT)	34	56 (42–72)	40% Male							B

[A] Median (range)
[B] AZA, azathioprine; CsA, cyclosporine A; FK, tacrolimus; MMF, mycophenolate mophetil; Rapa, rapamycin
[C] B, Hospital Clinic Barcelona, Spain; R, University Tor Vergata Rome, Italy

Antibodies

For surface molecule staining fluorescent monoclonal antibodies directed against the following targets were used: CD3, CD4, CD8, CD25, CD28, CD56, CD16,

CD45RA, CD62L, CCR7, HLA-DR, γδ TCR, αβ TCR, NKG2D and PD1 (BD Biosciences, San Jose, CA, USA); NKG2A, NKG2C and GITR (R&D Systems, Minneapolis, MN, USA), Vδ1 TCR (ThermoScientific, Waltham, MA, USA); and Vδ2 TCR (Immunotech, Marseille, France). Intracellular staining experiments were performed employing antibodies against Foxp3 (eBioscience, San Diego, CA, USA), CTLA4, perforin IL-10, IL-17A and IFNγ (BD Biosciences, San Jose, CA, USA). Peripheral blood donor cell chimerism was analysed employing fluorescent monoclonal antibodies directed against HLA-A1 (One Lambda, Inc., Canoga Park, CA, USA) and HLA-A2 (BD Biosciences).

Surface and intracellular staining

For cell surface staining 100 μl of heparinized whole blood were incubated with the appropriate amount of labeled antibodies for 15 min at room temperature in the dark. Erythrocyte lysis was subsequently performed using BD FACS lysing solution (BD Biosciences) following the manufacturer's instructions. Cells were then washed and resuspended in 2.5% formaldehyde. Intracellular staining was conducted following permeabilization of surface-stained and fixed cells with Cytofix/Cytoperm (BD Biosciences). Background fluorescence was assessed with appropriate IgG isotypes for each of the tested antibodies. Flow cytometry data acquisition was performed using a BDFacs Canto II flow cytometer (BD Biosciences) and data analysis conducted using FlowJo Software (Tree Star Inc., Ashland, OR, USA).

Cytokine staining

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll density gradient (Histopaque 1077, Sigma-Aldrich, St. Louis, MO, USA). To perform IL-10 and IFNγ staining, 1×10^6 PBMCs were resuspended in 500 μl RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% male AB human serum (Biosera, East Sussex, UK), 1% pencillin-streptomycin and 10 mM L-glutamine, and cultured in 24-well flat bottom plates (Greiner Bio-one, Frickenhausen, Germany) for 4 h together with PMA (50 ng/ml, Sigma-Aldrich), ionomycin (1 μg/ml, Sigma) and brefeldin A (10 μg/ml, BD Biosciences). Cells were then harvested, washed and surface stained prior to intracellular staining using Caltag Fixation/Permeabilization kit (Invitrogen, Carlsbad, CA, USA). Nonstimulated cells were used as control. For IL-17A staining, PBMCs were first pre-stimulated for 1 week in the presence of IL-2 (100 IU/ml) following a protocol previously optimized in our laboratory and then processed as previously described.

Sequencing of the Vδ1 TCR chain complementary determining region 3

Total RNA was extracted from PBMCs collected from stable liver recipients employing TRIzol reagent (Invitrogen) and used for first-strand cDNA synthesis employing High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) following DNase digestion with Turbo Dnase (Ambion Inc., Austin, TX, USA). cDNA was then used to amplify the Vδ1 TCR chain complementary determining region 3 (CDR3) employing primers specific for either the variable Vδ1-region (designated VD1) or the constant Cδ-region (CD1) as described by Fujishima *et al.* [10]. Polymerase chain reaction (PCR) was performed for 40 cycles in a 25 μl reaction mixture containing 0.2 mM of each primer and 1 unit of Taq polymerase (Platinum Taq, Invitrogen) using the following PCR-conditions: denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min; extension at 72 °C for 1.5 min. The oligonucleotide sequences of the VD1 and CD1 primers were as follows: VD1, GTGGTCGCTAT TCTGTCAACT; CD1, AACAGCATTCGTAGCCCAAGC AC. PCR products were then cloned into the PCR2.1 TOPO cloning vector (Invitrogen), transformed into chemically competent *E. coli* (TOP10, Invitrogen) and plated on LB-Agar plates containing 50 μg/ml Ampicillin (Sigma). Plasmid-DNA was prepared using a Miniprep Kit (Qiagen, Hilden, Germany) and processed for sequencing using a Big-Dye Terminator Cycle Sequencing Kit (Version 3.1, Applied Biosystems). Sequence analysis was performed using an ABI Prism 3730 automated DNA sequencer (Applied Biosystems). To assess the complexity of the Vδ1 CDR3 TCR repertoire we computed the frequency of repetitive sequences exceeding 10% of the total pool of analysed amplicons (at least 14 per sample).

Statistical analysis

Flow cytometric data are shown as percentages of the gated lymphocyte population. Demographic and clinical variables are shown as mean percentage. Differences between two single patient groups were assessed employing *t*-test and Mann–Whitney. Comparisons among STA-Liver, TOL, STA-Kidney, ESLD and CONT were done by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) *post hoc* test. Non-parametric tests (Kruska–Wallis) were employed when appropriate. *P* values less than 0.05 were considered statistically significant. Reproducibility over time of the γδ TCR and subpopulations was estimated with the intra-class correlation coefficient for absolute agreement [11]. Values greater than 0.75 indicate an excellent agreement. Statistical analyses were performed using SPSS® 16.0 for

Windows (Chicago, IL, USA), GraphPad Prism 3.03 (San Diego, CA, USA) and MedCalc Software 10.4.0 (Mariakerke, Belgium).

Results

Most transplant recipients exhibit alterations in the peripheral blood γδ T cell compartment

To determine how organ transplantation influences the distribution of γδ T cell subsets we quantified the frequency of Vδ1, Vδ2 and γδ T cells in peripheral blood specimens obtained from both liver and kidney transplant recipients under maintenance IS (STA-liver and STA-Kidney, respectively), tolerant liver recipients not receiving IS for at least 1 year (TOL), nontransplanted liver patients (ESLD) and healthy individuals (CONT). All transplant recipients exhibited increased γδ T cell numbers as compared with age-matched healthy individuals (Fig. 1a) and this was mainly attributable to an expansion of the Vδ1 T cell subset (Fig. 1b). Vδ2 T cells were in fact reduced in both liver and kidney recipients in comparison with healthy individuals (Fig. 1c). As a result, Vδ1 T cells constituted the most abundant γδ T cell subset in peripheral blood of transplant recipients regardless of the type of organ being transplanted and of whether IS was administered or not (Fig. 1d). Similar differences between patient groups were observed upon computation of peripheral blood Vδ1, Vδ2 and γδ T cells in relation to absolute lymphocyte numbers (data not shown).

The altered distribution of peripheral blood γδ T cell subsets in transplant recipients is stable over time

To establish if the relative frequencies of peripheral blood Vδ1, Vδ2 and total γδ T cells are stable over time we performed two different measurements in blood samples collected 9–18 months apart from 30 STA-Liver recipients. Comparison of the two time points revealed that neither Vδ1, Vδ2, total γδ T cells nor the Vδ1/Vδ2 ratio significantly changed over time (Fig. 2). These data indicate that at least in liver recipients with stable graft function the size and subset distribution of the γδ T cell compartment remains constant over a substantial period of time.

Peripheral blood Vδ1 T cells from liver transplant recipients are phenotypically and functionally different from Vδ2 T cells

To understand the potential role of Vδ1 and Vδ2 T cells in transplantation we conducted an exhaustive phenotypic analysis of these T cell subpopulations in a subset of 19 liver recipients (including nine TOL and 10 STA-Liver). Vδ1 and Vδ2 T cells differed in their memory/naïve phenotype and in the expression of costimulatory molecules. Additionally, the expression of CD4/CD8 co-receptors, HLA class-II and NK receptors distinguished the two subsets (Table 2). Thus, while higher percentage of Vδ2 T cells exhibited a CD45RA-CCR7– effector memory (T_{EM}) phenotype, Vδ1 T cells predominantly displayed a

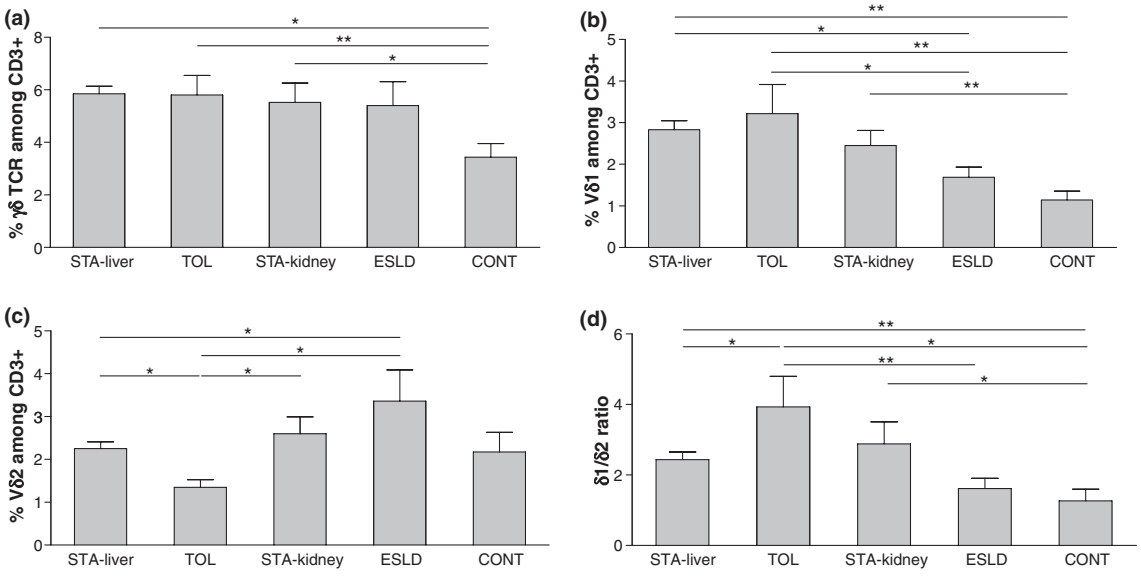

Figure 1 Quantitative differences of γδ T cell subsets between TOL, STA-Liver, STA-Kidney, ESLD and CONT patient groups. Proportion of (a) γδ, (b) Vδ1, (c) Vδ2 T cells among peripheral blood CD3+ mononuclear cells. (d) Calculated Vδ1/Vδ2 T cell ratio of peripheral blood mononuclear cells. Bar plots represent mean (±SEM) values from 201 STA-Liver, 29 TOL, 50 STA-Kidney, 50 ESLD and 34 CONT. Kruskall–Wallis test was employed in γδ TCR analysis. ANOVA and LSD were used for the residual parameters. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

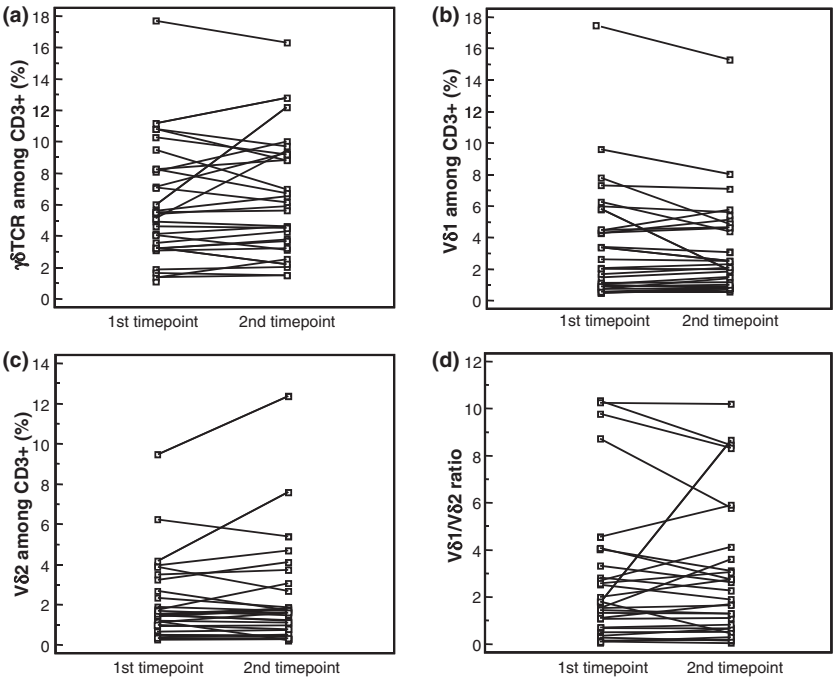

Figure 2 The frequencies of γδ T cell subsets are stable over time in a group of STA-Liver recipients. Comparison of the relative number of (a) γδ, (b) Vδ1, (c) Vδ2 T cells and (d) Vδ1/Vδ2 ratio in two sequential peripheral blood specimens obtained 14 months apart (range 9–18) from 30 STA-liver recipients. First and second timepoint measurements showed an excellent agreement, meaning intraclass correlation coefficient for absolute agreement over 0.75.

CD45RA+CCR7− phenotype characteristic of terminally differentiated effector memory T cells (T_{EMRA}). Moreover, as compared with the Vδ2 T cell subset, resting Vδ1 T cells expressed higher levels of HLA-DR, CD4, CD8, CTLA4 and perforin, as well as the NKG2C and KLRF1 receptors. In contrast, Vδ2 T cells exhibited high CD28 and NKG2A expression (Table 2). Regarding molecules involved in immunoregulatory pathways [12,13], neither Vδ1 nor Vδ2 T cells expressed Foxp3 (data not shown) and GITR and PD-1 was present at similar levels in both subsets (Table 2). The phenotypic differences between Vδ1 and Vδ2 from liver recipients were replicated in a group of eight age-matched nontransplanted healthy individuals (data not shown). *Ex-vivo* cytokine production of Vδ1 and Vδ2 T cells revealed that a higher frequency of Vδ2 T cells (mean 45.91%) produced IFNγ in comparison to Vδ1 T cells (mean 24.44%; Table 2). Conversely only a subset of Vδ1 T cells (mean 6.30%) secreted IL-17A and IL-10 was similarly produced by both γδ T cell subsets (Table 2). Additionally, similar statistical differences were reported when the analysis for the above described surface and intracellular markers was conducted on the basis of total lymphocyte count (Table 2).

T cell receptor diversity analysis reveals that Vδ1 T cells present in transplant recipients exhibit a skewed TCR-Vδ1 repertoire

In order to study the clonal diversity of the expanded Vδ1 T cell subpopulation, we cloned and sequenced the Vδ1-TCR complementary determining region 3 (CDR3) in peripheral blood specimens obtained from 12 hepatitis C virus (HCV) negative liver recipients. Specifically, this group included 6 TOL (83% male, mean age 59 years)

Table 2. Immunophenotypic profile and *ex-vivo* cytokine production of Vδ1 and Vδ2 T cell subsets 19 from liver transplanted recipients.

	Vδ1 T cells [%(SEM)]	Vδ2 T cells [%(SEM)]	*P* value
CD4	2.11 (0.47)	0.86 (0.26)	0.0276
CD8	21.85 (5.04)	3.44 (1.16)	0.0014
CD45RA-CCR7-	11.68 (1.73)	92.18 (0.95)	<0.0001
CD45RA+CCR7-	83.10 (2.46)	5.80 (0.77)	<0.0001
NKG2A	23.69 (3.79)	57.94 (3.98)	<0.0001
NKG2C	21.71 (3.69)	4.73 (0.86)	0.0001
NKG2D	86.04 (2.61)	92.54 (2.87)	ns
HLA-DR	8.17 (1.61)	3.88 (1.16)	0.0375
CD28	12.74 (2.71)	75.71 (4.51)	<0.0001
KLFR1	63.59 (5.63)	23.46 (6.34)	<0.0001
GITR	4.76 (1.17)	4.13 (1.00)	ns
PD-1	11.43 (2.24)	5.61 (2.51)	ns
CTLA-4	8.98 (1.16)	4.16 (0.72)	0.001
Perforin	11.26 (2.85)	4.09 (1.10)	0.0221
INFg	24.44 (2.85)	45.91 (4.89)	0.0006
IL10	8.62 (1.36)	8.64 (2.55)	ns
IL17-A	6.30 (0.90)	2.11 (0.32)	0.0002
Mean absolute cell count (10^9/L)	57.52 (16.90)	36.64 (7.23)	

ns: non significant

and six STA-Liver (66% male, mean age 51 years). Furthermore, six healthy volunteers were also analysed (66% male, mean age 55 years). CDR3 repertoire analysis revealed that in all six TOL liver recipients Vδ1 T cells exhibited a skewed TCR repertoire with 58.3% of repetitive sequences consisting of clonotypes harboring identical nucleotide sequences (Table 3). This contrasted with a rate of 30% and 31.6% repetitive sequences displayed by STA-Liver and CONT, respectively. The repetitive clonotypes were unique to each individual and were not found by conducting a BLAST search at the NCBI GenBank database (private sequences). To determine whether expanded Vδ1 T cells shared recurrent CDR3 aminoacid motifs indicative of antigen-driven selection by structurally related antigens, we aligned and compared all Vδ1-TCR sequences derived from TOL, STA-Liver and CONT. Therefore, they were translated into the corresponding aminoacid sequences, but this did not reveal a common CDR3 motif (Table 3). The absence of public clonotypic sequences or aminoacid motifs shared by different recipients argue against a single epitope or family of structurally-related epitopes as being responsible for oligoclonal Vδ1 T cell expansion. When TOL, STA-Liver and CONT were compared, Vδ1 T cells from TOL recipients displayed a significantly more biased repertoire than those from STA-Liver recipients or CONT (Fig. 3).

Peripheral blood donor cell chimerism does not account for the alterations observed in the γδ T cell compartment in liver recipients

Small quantities of donor-derived 'passenger' lymphocytes can be detected in the peripheral blood of graft recipients many years after liver transplantation [16]. To address whether persistence of donor-derived lymphocytes could account for the altered distribution of γδ T cells observed in liver recipients, we conducted additional flow cytometry experiments employing fluorescent monoclonal anti-

Table 3. Aminoacid sequences of repeated polyclonal Vδ1 CDR3 TCR clonotypes in 6 TOL, 6 STA-Liver recipients and 6 healthy individuals.

Status	Vδ1	N-D-N	Jδ1	Colony frequency	CDR3 length
TOL	CALG	DGSGVL	DKLIFGKG	10/33	13
	CALGE	KEWELLGDN	TDKLIFGKG	7/33	18
TOL	CALG	DPPNLGGYP	YTDKLIFGKG	27/40	18
	CALGE	VVGPTVGDLHH	TDKLIFGKG	4/40	22
TOL	CALGE	PYINAFLLTGGFDLKVP	YTDKLIFGKG	6/35	27
	CALGE	LTPTFLLLALGAS	DKLIFGKG	4/35	21
TOL	CALG	DSTDGEWGGL	YADKLIFGKG	10/33	19
	CALGE	PPPSYESQCWGIGPLCG	TDKLIFGKG	10/33	26
	CALG	ASTFLLWGIRT	YTDKLIFGKG	4/33	20
TOL	CALG	GPTSYRIFSYWGIGW	TDKLIFGKG	13/27	23
	CALGE	PGFLRFYWGIR	TDKLIFGKG	5/27	20
TOL	CALG	DPLSRSTGGYRRGQA	DKLIFGKG	8/33	22
	CALGE	PFLGPT	KLIFGKG	6/33	13
STA-Liver	CALG	VYKEGLNWGIRKYLS	DKLIFGKG	6/34	22
	CALGE	PYRPAEGENP	YTDKLIFGKG	5/34	20
STA-Liver	CALG	DRLWGPGPLALTAQ	LFFGKG	13/34	19
STA-Liver	CALG	DPGGKTATGGL	YTDKLIFGKG	6/31	20
STA-Liver	CALG	NSHPTGYWGILRW	TDKLIFGKG	4/25	21
	CALG	TQIPRRVSGDHVRSWVGDML	TDKLIFGKG	4/25	28
STA-Liver	CALG	DTSLPTLTGGYPTRP	LIFGKG	5/19	19
STA-Liver	CALGE	HDPPWGIS	TDKLIFGKG	3/19	17
	CALGE	RRGYLK	YTDKLIFGKG	3/19	16
CONT				0/26	
CONT	CALG	SHHGSSSKYWGV	YTDKLIFGKG	6/23	19
CONT	CALGE	LPPGD	YTDKLIFGKG	5/20	15
	CALG	GPLPPLGWGIRG	YTDKLIFGKG	3/20	21
CONT	CALG	NTYRRWGIGETF	TDKLIFGKG	6/20	20
	CALG	LSTVGIRTYWGIFVG	TDKLIFGKG	3/20	23
CONT	CALGE	SLPTNGIRGSRP	LIFGKG	2/14	18
	CALGE	PVRTSFSWDTRQMF	FGTG	2/14	18
CONT	CALGE	PRRRRYSGGSV	TDKLIFGKG	2/14	20
	CALGE	LRPGSYALLGTPLSSWDTRQMF	FGTG	2/14	26

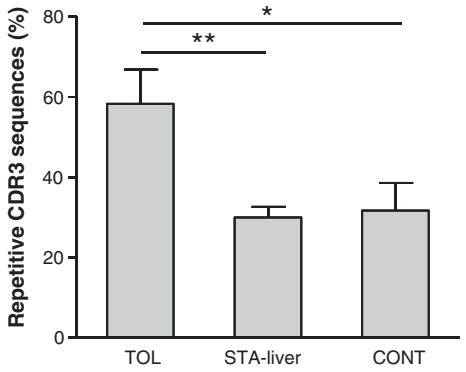

Figure 3 In TOL recipients Vδ1 T cells exhibit a more restricted CDR3 TCR repertoire than STA-Liver recipients and CONT. To assess the degree of repetitive sequences comprised in the Vδ1 TCR repertoire, CDR3 segments from six TOL, six STA-Liver recipients and six healthy controls were cloned and sequenced. The bar plot represents the mean (±SEM) frequency of repetitive CDR3 sequences exceeding 10% of the totally analyzed amplicons (at least 14 per sample). $*P < 0.05$; $**P < 0.01$ (*t*-test).

bodies directed against donor/recipient mismatched HLA class I molecules in a subgroup of seven TOL recipients. In all seven liver recipients analysed, less than 1% of peripheral blood γδ T cells were stained positive for the donor-type HLA (this was not found to be significant as compared with background) and similar findings were obtained for CD4, CD8, and NK cells (Table 4). These results do not exclude the presence of peripheral blood donor cell microchimerism, which flow cytometry might not be sensitive enough to detect. They indicate however that the changes observed in the γδ T cell compartment in liver recipients cannot be attributed to a significant expansion of donor-derived lymphocytes.

Persistent viral infections influence the distribution of peripheral blood γδ T subsets in liver transplant recipients

Considering our findings indicating that most Vδ1 T cells present in transplant recipients exhibited a terminally differentiated phenotype resembling the effector memory RA αβ CD8+ T cells (T_{EMRA}) found in the context of persistent viral infections, we then investigated whether persistent and/or past exposure to viral infections could account for the altered distribution of γδ T cell seen in liver transplant recipients. To conduct such analysis on a large homogeneous group of patients, we focused on STA-Liver recipients and correlated the frequency of peripheral γδ, Vδ1, Vδ2 T cells and Vδ1/Vδ2 T cell ratio with seropositivity for CMV, HSV, EBV and HCV. As compared with HCV-negative liver recipients, HCV-positive recipients exhibited a trend towards a decreased number of peripheral blood Vδ2 T cells and increased number of Vδ1 T cells, altogether resulting in a significantly increased Vδ1/Vδ2 T cell ratio ($P = 0.046$; Fig. 4a). CMV seropositivity was also associated with an expansion of Vδ1 T cells (3.22% vs. 1.02%; $P < 0.001$; Fig. 4c) and an increased Vδ1/Vδ2 T cell ratio ($P = 0.006$; Fig. 4b). In addition, recipients seropositive for CMV also showed an increased in the total number of γδ T cells ($P = 0.007$; Fig. 4d). Both, HCV-positive and HCV-negative recipients displayed a very high prevalence of CMV infection (91% and 90%, respectively). This indicates that the differences observed in Vδ1/Vδ2 T cell ratio between the two groups of recipients could not be attributed to

Table 4. Percentage of donor origin cells among PBMC subsets from 7 TOL recipients.

	Donor HLA [%(SEM)]
	TOL
γδ TCR	0,61 (0,23)
CD4	0,31 (0,17)
CD8	0,22 (0,10)
NK	1,32 (0,60)
NKT	1,26 (0,43)

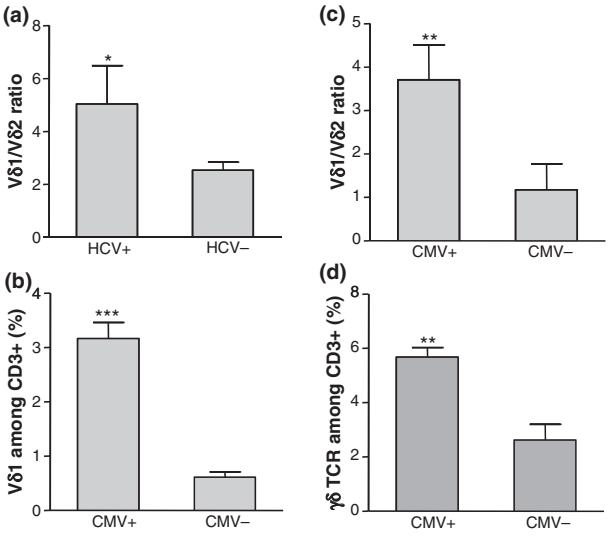

Figure 4 Peripheral blood γδ T cells, Vδ1 and Vδ2 subsets and the subsequent ratio are quantitatively altered in HCV-positive and CMV-positive in a set of liver recipients receiving maintenance IS. (a) Bar plot shows mean (±SEM) calculated peripheral blood Vδ1/Vδ2 T cell ratio from 70 HCV-positive [69% male, mean age 62 years (31–78)] and 110 HCV-negative [66% male, mean age 55 years (24–75)] STA-Liver recipients. (b) Computed Vδ1/Vδ2 T cell ratio, (c) relative amount of Vδ1 subset and (d) γδ T cell population from 112 CMV-positive [74% male, mean age 57 years (24–78)] and 13 CMV-negative [66% male, mean age 53 years (30–72)] STA-Liver recipients. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (*t*-test and Mann–Whitney test).

dissimilarities in the status of CMV infection. In contrast, EBV and HSV status did not influence the number of peripheral blood γδ T cell subsets (data not shown).

Quantification of peripheral blood γδ T cell subsets does not allow for the accurate discrimination between tolerant liver recipients and those recipients requiring maintenance immunosuppression

Quantification of peripheral blood Vδ1 and Vδ2 T cells has been proposed as a biomarker to discriminate operationally tolerant liver recipients from recipients requiring IS. In agreement with previously published data [8,9], in our current study the Vδ1/Vδ2 ratio significantly differed between TOL and STA-Liver recipients (Fig. 1d). However, when we plotted receiver operating characteristic curves (ROC) for Vδ1, Vδ2 and Vδ1/Vδ2 ratio to determine the diagnostic utility of these parameters, the resulting areas under the curve (AUC) were less than 0.7 in all cases, indicating that none of the measurements was capable of discriminating TOL from STA-liver recipients with acceptable sensitivity and specificity. To establish if specific phenotypic traits of Vδ1 and Vδ2 T cells could further help in the search for biomarkers of operational tolerance we compared the detailed immunophenotype and cytokine secretion profile of γδ T cells from nine TOL and 10 STA-Liver recipients. Vδ1 and Vδ2 T cell subsets from both TOL and STA-Liver recipients expressed similar surface markers and cytokine production (Table 5). The only significant differences observed were an increased CD28 expression in Vδ2 T cells from TOL recipients (86.65% in TOL vs. 66.96% in STA-Liver; $P = 0.0248$) and more abundant perforin-producing Vδ1 T cells in STA-Liver recipients (5.30% in TOL vs. 17.21% in STA-Liver; $P = 0.0315$). The overlap in the distribution of these markers between TOL and STA-Liver recipients, however, prevented their use as potentially diagnostic biomarkers.

Discussion

Clinically-applicable biomarkers of operational tolerance constitute a pre-requisite for the widespread implementation of tolerogenic therapies in human organ transplantation. Liver transplantation is a unique clinical setting for the search and validation of such biomarkers, given that operational tolerance spontaneously occurs in about 20% of stable liver recipients. Recent reports from two different laboratories have documented that operationally tolerant liver recipients exhibit a significant alteration in the distribution of the two main peripheral blood γδ T cell subsets, consisting in an expansion of Vδ1 T cells and a shift in the Vδ1/ Vδ2 T cell ratio [8,9]. These concordant observations, derived from studies conducted on highly dissimilar transplant recipient populations, suggested that γδ T cells and particularly the Vδ1 subset could be involved in the development of spontaneous operational tolerance.

Table 5. Differences in γδ T cell subset phenotype and cytokine secretion between TOL and STA-Liver patients.

	Vδ1 T cells [%(SEM)]			Vδ2 T cells [%(SEM)]		
	TOL ($n = 9$)	STA-Liver ($n = 10$)	*P* value	TOL ($n = 9$)	STA-Liver ($n = 10$)	*P* value
CD4	2,11 (0,83)	2,11 (0,58)	ns	0,78 (0,34)	0,93 (0,38)	ns
CD8	29,29 (11,02)	16,44 (3,19)	ns	2,67 (1,63)	4,06 (1,67)	ns
CD45RA-CCR7-	11,73 (3,16)	11,64 (2,04)	ns	93,83 (1,09)	90,99 (1,37)	ns
CD45RA+CCR7-	82,26 (4,44)	83,71 (2,91)	ns	4,05 (0,96)	7,04 (1,04)	ns
NKG2A	19,22 (5,22)	26,53 (5,24)	ns	58,59 (7,28)	57,49 (4,81)	ns
NKG2C	22,80 (7,03)	21,02 (4,34)	ns	4,72 (1,62)	4,73 (1,02)	ns
NKG2D	84,99 (4,35)	86,80 (3,38)	ns	88,40 (6,27)	95,85 (0,91)	ns
HLA-DR	9,95 (2,31)	6,74 (2,23)	ns	1,82 (0,43)	5,53 (1,94)	ns
CD28	9,87 (4,14)	14,83 (3,62)	ns	86,65 (2,80)	66,96 (6,71)	0.0248
KLFR1	70,79 (9,53)	58,55 (6,20)	ns	25,15 (12,93)	22,13 (5,99)	ns
GITR	5,98 (1,86)	3,39 (1,30)	ns	4,98 (1,69)	3,27 (1,10)	ns
PD-1	12,68 (3,33)	10,18 (3,13)	ns	9,27 (4,79)	1,96 (0,64)	ns
CTLA-4	9,72 (1,05)	8,31 (2,02)	ns	4,76 (1,19)	3,66 (0,88)	ns
PERFORIN	5,30 (1,89)	17,21 (4,69)	0.0315	2,30 (1,07)	5,69 (1,77)	ns
INFg	22,71 (3,34)	25,98 (4,63)	ns	47,46 (7,25)	44,53 (7,00)	ns
IL-10	10,27 (2,41)	7,34 (1,53)	ns	11,31 (4,36)	6,27 (2,86)	ns
IL17-A	7,05 (1,30)	5,44 (1,26)	ns	2,13 (0,29)	2,10 (0,64)	ns
Mean absolute cell count (10^9/L)	69.11 (36.64)	48.82 (12.67)		34.94 (7.49)	37.91 (11.64)	

ns: non significant

In recent years there has been a growing interest in the study of γδ T cells, particularly in autoimmune diseases, viral infections and malignancy [14–16]. Thus considerably insight has been gained on their tissue location, recognition pattern and effector functions. In experimental animal transplantation γδ T cells appear to possess an immunoregulatory function [17,18]. Similarly, in a clinical context, γδ T cells could also contribute to allogenic engraftment in both bone marrow transplantation [19] and kidney transplantation [20]. Furthermore, γδ t cells have been extensively studied in the context of CMV infection in renal transplant recipients [21,22].

However, the repertoire, functional and phenotypic characteristics of these lymphocytes had not been previously explored in detail in clinical liver transplantation. This precluded the understanding of the role of γδ T cell subsets in clinical allograft tolerance.

Our results, obtained on peripheral blood samples collected from both liver and kidney transplant recipients, show that an expansion of Vδ1 T cells (leading to an increased total number of γδ T cells and to a shift in the ratio between Vδ1 and Vδ2 T cells) is a common finding in transplant recipients regardless of the type of allograft being transplanted and of whether IS drugs are chronically administered or not. Furthermore, in liver transplantation the phenotypic traits of γδ T cells are similar in operationally tolerant recipients and in those receiving maintenance IS. These observations imply that rather than a specific consequence of liver allograft tolerance, the expansion of Vδ1 T cells is more likely to be related to the transplantation procedure *per se* and/or to the chronic exposure to pharmacological IS.

As compared with Vδ2 T cells that resemble effector memory αβ T cells, the expanded Vδ1 T cells appear to be terminally differentiated lymphocytes expressing higher levels of perforine and of activator killer-like receptors such as NKG2C, KLRF1 and lower levels of inhibitory NKG2A. The terminally differentiated phenotype exhibited by Vδ1 T cells in transplant recipients parallels the phenotypic characteristics of effector memory RA αβ CD8+ T cells (T_{EMRA}) [23] found in the context of persistent viral infections [24]. While we lack data on the specificity of the expanded γδ T cells for our patients, their phenotypic traits are very similar to expanded γδ T cell subset, CMV specific, recently identified in kidney recipients [25]. Furthermore, the expanded pool of peripheral blood Vδ1 T cells display a markedly skewed Vδ1-CDR3 TCR repertoire characterized by multiple repeated sequences within each individual that is reminiscent of previous clonal expansions driven by peripheral antigens. Vδ1 T cells recognize heterogeneous and not well-defined antigens, which in most instances appear to be self-antigens whose expression is induced by cell stress, bacterial and particularly viral infections [26]. Expansion of peripheral blood γδ T cells and/or skewed TCR repertoires of Vδ1 T cells have indeed been observed in patients with various viral infections [10,27–30]. For instance, Vδ1 cells substantially expand in the course of HIV [31,32] and CMV [27] infections. In these cases it is still unclear whether Vδ1 T cells expand in response to pathogen antigens or triggered by endogenous gene products upregulated by infection. In the case of hepatotropic viruses previous analyses of the peripheral blood γδ T cell repertoire conducted on non-transplanted HBV- or HCV-infected patients failed to show an association between viral infection with selective expansion of Vδ1 T cells [32,33]. Our results in a large cohort of liver recipients confirm the association between CMV infection and peripheral blood Vδ1 T cell expansion. In addition, and in contrast to the studies conducted on nontransplanted patients, our data reveal for the first time that in liver transplantation HCV infection is also associated with an altered distribution of γδ T cells due to an increase in Vδ1 T cells and particularly to a significant decrease in Vδ2 T cells. Taken these data together, our findings support the hypothesis that in transplant recipients viral infections constitute the main force shaping the repertoire of peripheral blood γδ T cells and provide a rationale for exploring in more depth the influence of these lymphocytes in the pathogenesis of persistent viral infections such as CMV and HCV in transplantation.

Our study does not allow us to elucidate why operationally tolerant liver recipients exhibit a more substantial alteration in the distribution of γδ T cell subsets than other transplant recipients. The immune reconstitution taking place following the successful discontinuation of IS drugs in tolerant recipients could be preferentially influencing Vδ1 T cells. Alternatively, the expression of Vδ1 TCR ligands could be different between tolerant and non-tolerant liver recipients. The latter hypothesis would be consistent with the finding that the Vδ1 TCR repertoire is more restricted in TOL than in STA-Liver recipients. An alternative hypothesis could be a direct immunosuppressive effect of CMV infection, with Vδ1 expression being just an epiphenomenon of CMV status. This explanation is however unlikely, given that both TOL and STA-Liver recipients displayed a similar prevalence of CMV seropositivety and that no cases of CMV reactivation were clinically detected in any of our recipients in the course of the study.

Despite the differences observed between the studied cohorts it is clear from our findings that quantification of peripheral blood γδ T cell subsets by flow cytometry is unlikely to constitute a useful biomarker of operational liver allograft tolerance, given that neither total γδ, Vδ1

and Vδ2 T cell numbers nor the Vδ1/Vδ2 ratio accurately discriminated between TOL and STA-Liver recipients regardless of the threshold employed. Indeed, γδ T cell subset quantification only appears to capture a portion of the liver allograft tolerance clinical spectrum by correctly identifying a small fraction of TOL recipients in cases of a very high Vδ1/Vδ2 threshold (Fig. 1d). However, a definitive answer to this question will require the performance of a prospective weaning study in which γδ T cells subsets are quantified before and after immunosuppression is withdrawn.

Authorship

I. Puig-Pey performed research, analysed data and co-wrote the manuscript; F. Bohne performed research and co-wrote the manuscript; C. Benítez supervised sample collection; M. López contributed to data interpretation; M. Martínez-Llordella performed research and contributed to data interpretation; F. Oppenheimer provided study material and supervised sample collection; J. J. Lozano contributed to data interpretation; J. González-Abraldes contributed to statistical analysis; G. Tisone provided study material and clinical data; A. Rimola contributed to the conception of the study; A. Sánchez-Fueyo contributed to the conception of the study, designed experiments and wrote the manuscript.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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