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Activated CD4+ T Cells and Highly Differentiated Alloreactive CD4+ T Cells Distinguish Operationally Tolerant Liver Transplantation Recipients

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Spontaneous operational tolerance to the allograft develops in a proportion of liver transplantation (LT) recipients weaned off immunosuppressive (IS) drugs. Several studies have investigated whether peripheral blood circulating T cells could play a role in the development or identify operational tolerance, but never characterized alloreactive T cells in detail due to the lack of a marker for these T cells. In this study, we comprehensively investigated phenotypic and functional characteristics of alloreactive circulating T cell subsets in tolerant LT recipients (n = 15) using multiparameter flow cytometry and compared these with LT recipients on IS drugs (n = 23) and healthy individuals (n = 16). Activation-induced CD137 was used as a marker for alloreactive T cells upon allogenic stimulation. We found that central and effector memory CD4+ T cells were hyporesponsive against donor and third-party splenocyte stimulation in tolerant LT recipients, whereas an overall hyperresponsiveness was observed in alloreactive terminally differentiated effector memory CD4+ T cells. In addition, elevated percentages of circulating activated T helper cells were observed in these recipients. Lastly, tolerant and control LT recipients did not differ in donor-specific antibody formation. In conclusion, a combination of circulating hyperresponsive highly differentiated alloreactive CD4+ T cells and circulating activated T helper cells could discriminate tolerant recipients from a larger group of LT recipients.

Liver transplantation (LT) is the sole treatment option for end-stage liver disease. Over the last few decades immunosuppressive (IS) drugs substantially improved short-term graft and patient survival.[1] However, long-term use of IS drugs leads to various serious side effects and adversely affects quality of life.[2-4] Therefore, most transplantation centers attempt to gradually reduce or even completely cease IS regimen over time.[5] Several clinical trials have shown that some LT recipients that are completely weaned off IS regimen develop operational tolerance toward their graft, a long-term state defined by absence of (acute) rejection episodes while free of IS drugs.[6-8]

Compared with other solid organ grafts, the transplanted liver facilitates operational tolerance.[9] Preformed or de novo donor-specific antibodies (DSAs) against donor human leukocyte antigen (HLA) types

Abbreviations: aTh, activated T helper cells; allTh, all T helper cells; aTreg, activated regulatory T cells; allTreg, all regulatory T cells; CM, central memory T cells; CMV, cytomegalovirus; CTLA4, cytotoxic T lymphocyte antigen 4; CTRL, control LT recipient group; DSAs, donor-specific antibodies; EM, effector memory T cells; EMRA, terminally differentiated effector memory T cells; HC, healthy control; HLA, human

have been associated with an increased risk of acute and chronic rejection.[10] Nevertheless, many DSA-positive LT recipients do not experience rejection, and DSAs have even been detected in tolerant LT recipients.[11,12]

Several studies have investigated whether immune system–related peripheral blood markers could identify the LT recipients that have developed immunological tolerance toward their graft. Higher relative numbers of circulating CD4+CD25high T cells,[13-15] CD4+FoxP3+ T cells,[14] and CD4+CD25++CD127dim cells,[16] and a higher Vδ1:Vδ2 γδT cell ratio[13,14,17] in blood of adult or pediatric recipients were implied to discriminate between tolerant LT recipients without IS regimen and (nontolerant) LT recipients with IS regimen. These data suggest that regulatory T cells and γδT cells might play a role in the development and/or maintenance of operational tolerance. However, many of these studies lack matching of parameters that are known to influence the composition of circulating immune cells, such as cytomegalovirus (CMV) infection,[18,19] when comparing tolerant with control groups of LT recipients.

While donor-specific T cells critically contribute to liver graft rejection, their association with operational tolerance after LT is underinvestigated. Lack of proliferation of total CD4+ T cells upon stimulation with donor antigens when compared with third-party antigens (donor-specific hyporesponsiveness) was reported,[16,20] but donor-specific responses of CD4+ or CD8+ T cells have never been studied in more detail in tolerant LT recipients. CD137 is expressed by activated CD4+ and CD8+ T cells upon interaction with antigen-presenting cells, and it has been proven that this marker can identify all alloreactive T cells in kidney and LT recipients.[21-23]

The purpose of this study is to comprehensively investigate phenotypic and functional characteristics of circulating (anti-donor antigen-specific CD137+) T cell subsets and DSAs in operationally tolerant LT recipients and compare these immunological markers with well-matched control groups.

Patients and Methods

STUDY DESIGN AND PARTICIPANTS

In this study cohort, all adult operationally tolerant LT recipients (TOL; n = 15) followed at the outpatient clinic at the Erasmus University Medical Centre between 2014 and 2020 were included. TOL LT recipients were completely weaned from IS regimen for medical reasons or noncompliance between 2008 and 2019 (Table 1). Four LT recipients were prospectively completely weaned from IS (Supporting Table 1). Recipients were defined as operationally tolerant when the IS regimen was completely ceased for at least 1 year without the occurrence of a rejection episode. Protocol biopsies after complete weaning of IS drug were not taken in this study because of possible complications related to the procedure and patient reluctance. Therefore, acute rejection was defined as at least a 2-fold increase in serum bilirubin, aspartate aminotransferase or alanine transaminase, alkaline phosphatase, or gamma-glutamyltransferase. A liver biopsy was performed in 5 tolerant LT recipients because of possible rejection, as indicated by increasing liver enzymes, at a mean of 3.1 (standard deviation 2.2) years after complete weaning. In all cases rejection was excluded using BANFF criteria. A control group of stable LT recipients (CTRL) on regular IS regimen (n = 23) and a healthy control (HC) group (n = 16) were included in the study and both were matched with the TOL group based on their sex, age, and CMV seropositivity. For the CTRL

TABLE 1. Characteristics of the HC Group, Control Group (CTRL), and Study Group (TOL)

	HC	CTRL	TOL	*P* Value
Demographics	n = 16	n = 23	n = 15	
Male	75.0	65.2	73.0	0.58*
Age, in years, at end follow-up	53.3 ± 15.0	49.7 ± 17.7	52.9 ± 16.4	1.00†
Years LT - end follow-up	NA	14.6 ± 5.4	16.1 ± 5.4	0.62‡
Years LT - complete IS drug weaning	NA	NA	12.3 ± 6.7	
Years complete IS drug weaning - end follow-up	NA	NA	3.6 ± 2.9	
Primary disease				0.84*
Cholestatic disease	NA	21.7	33.3	
Virus related	NA	34.8	26.7	
Hepatocellular carcinoma	NA	21.7	26.7	
Cryptogenic cirrhosis	NA	13.0	13.3	
Drug induced	NA	4.3	0.0	
Metabolic related	NA	4.3	0.0	
CMV+ serostatus				
Recipient before LT	NA	47.8	46.7	1.00§
Recipient or HC at end follow-up	62.5	73.9	66.7	0.74*
Donor	NA	43.5	46.7	1.00§
Acute rejection				
<2 years after LT	NA	39.1	6.7	0.06§
>2 years after LT	NA	8.7	0.0	0.51§
HLA mismatches recipient/donor				
A + B	NA	3.0 ± 0.9	3.5 ± 0.7	0.09‡
DR + DQ	NA	2.8 ± 1.0	2.7 ± 1.0	0.68‡
IS drug last used				0.35*
Tacrolimus	NA	65.2	46.7	
Cyclosporine A	NA	4.4	6.7	
Mycophenolate mofetil	NA	8.7	6.7	
Azathioprine	NA	0.0	13.3	
Tacrolimus and mycophenolate mofetil	NA	8.7	13.3	
Prednisolone and mycophenolate mofetil	NA	4.4	0.0	
Prednisolone and tacrolimus	NA	8.7	0.0	
Azathioprine and cyclosporine A	NA	0.0	6.7	
Unknown	NA	0.0	6.7	

NOTE: Data are presented as % or mean ± standard deviation.
*Statistical analyses were performed with the chi-square test.
†Statistical analyses were performed with the Bonferroni test.
‡Statistical analyses were performed with the Mann-Whitney U test or ANOVA.
§Statistical analyses were performed with Fisher's exact test.

group, additionally, time after LT and primary disease were matched with the TOL group. No other inclusion or exclusion criteria were applied. Heparinized blood samples were collected from all participants. From the TOL group, blood samples were collected at a time point at least 1 year after complete IS drug weaning. From the CTRL group, blood samples were collected at matched time points with TOL for time after LT. The CTRL LT recipients did not experience rejection episodes for at least 5 years before and 4 years after blood collection. From the LT recipients, prospectively weaned blood was collected before the start, during, and 6 months after IS drug weaning. Clinical and laboratory data were retrieved from electronic patient records. From all participants written informed consent was obtained. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of Erasmus MC (MEC 2014-232; MEC-2012-022).

DONOR AND THIRD-PARTY T CELL STIMULATION

For each donor-specific stimulation, donor splenocytes that had been collected and stored in liquid nitrogen at the time of LT were used. To account for nonspecific HLA stimulation, third-party splenocytes with the same number of but different HLA mismatches with the recipient, as between recipient and donor, were used. Recipient peripheral blood mononuclear cells (PBMCs) and splenocytes were thawed according to our standard protocol. Splenocytes were depleted of CD3+ cells using MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Efficiency of T cell depletion (>98.0% was accepted for further use) was determined by staining with CD3 fluorescein isothiocyanate–conjugated antibody (clone sk7; BD Biosciences, San Jose, CA) and measured using a BD FACS Canto II flow cytometer (BD Biosciences). After resting, PBMCs and CD3-depleted donor or third-party splenocytes were cocultured in a 1:1 ratio with 2 million cells each overnight (±14 hours) in Roswell Park Memorial Institute 1640 medium (RPMI-1640) GlutaMAX (Thermo Fisher Scientific, Waltham, MA) with human serum (Sanquin, Amsterdam, the Netherlands). As a negative control, PBMCs only were included. As a positive control, PBMCs were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, St. Louis, MO) and 1 µg/mL ionomycin (IONO; Sigma Aldrich). All cell cultures were costimulated with 1 µg/mL α-CD49d (purified NA/LE mouse; BD Biosciences) and 1 µg/mL α-CD28 (low endotoxin; Bioconnect). In addition, 1:400 Golgi stop (BD Biosciences) was added to each culture. Alloreactive recipient T cells were detected by measuring activation-induced CD137.

For information on antibody staining and flow cytometry and donor-specific HLA Class I and II antibodies, see the Supporting Information.

ANTIBODY STAINING AND FLOWCYTOMETRY

See Supporting Information.

DONOR-SPECIFIC HLA CLASS I AND CLASS II ANTIBODIES

See Supporting Information.

STATISTICAL ANALYSES

Statistical analyses were carried out with GraphPad Prism 8, version 8.4.3 (GraphPad Software Inc., San Diego, CA) or IBM SPSS software, version 25 (SPSS Inc., Chicago, IL). The normality of the distribution of the data was determined by the Shapiro-Wilk normality test. Differences between 2 groups were analyzed by either the t test or the Mann-Whitney U test. Statistical analyses of ≥3 independent groups were performed with 1-way analysis of variance (ANOVA) or the Kruskal-Wallis test, with a Bonferroni or Dunn's posttest. Statistical analyses within groups were performed with 1-way ANOVA or the Friedman test, with a Bonferroni or Dunn's posttest. Differences in discrete nominal data between groups were analyzed by 2-sided Fisher's exact test or the Pearson chi-square test. Figures were created with GraphPad Prism 8, version 8.4.3. Principal component analysis was performed using IBM SPSS software, version 25.

Results

PATIENT CHARACTERISTICS

In this study, operationally tolerant (TOL) LT recipients were compared with a control (CTRL) group of stable LT recipients on regular IS regimen and a HC group. All 3 groups were matched for age, sex, and CMV serostatus; additionally, CTRL and TOL were also matched for time after LT and primary disease. Therefore, no significant differences between these parameters were observed (Table 1). The TOL group completely ceased use of IS drugs 12.3 ± 6.7 years after LT and has been IS drug and rejection free for 3.6 ± 2.9 years. The number of recipients with biopsy-proven acute rejection episodes early after LT (<2 years after LT) in the TOL and CTRL groups did not significantly differ. The numbers of HLA mismatches between recipient and donor were similar between both groups for HLA Class I and II.

TOLERANT LT RECIPIENTS HAVE HIGHER RELATIVE NUMBERS OF CIRCULATING ACTIVATED T HELPER CELLS

Percentages of circulating CD3+ cells (data not shown) and CD4+ and CD8+ T cells did not differ between HC,

CTRL, and TOL (Fig. 1A). Several studies reported a higher proportion of CD4+FoxP3+CD25+ T cells in TOL compared with control groups.[13-15] Indeed, in the TOL group the proportion of FoxP3+CD25+ T cells within CD4+ T cells was significantly higher than that of the CTRL group (Fig. 1B), but only a higher trend was observed versus HC. Because CD4+FoxP3+CD25+ T cells could contain regulatory T cells (Tregs) and activated T helper cells (aTh), we elucidated this further by discriminating aTh, resting Tregs (rTregs), and activated Tregs (aTregs) within CD4+ T cells using

FoxP3 and CD45RA expression[24] (Fig. 1C). In TOL a significantly higher percentage of circulating aTh was present compared with CTRL and HC, whereas percentages of aTreg and rTreg were similar among these groups. Although a higher percentage of aTh is observed in TOL, their capacity to produce interferon γ (IFNγ) and interleukin 17 (IL17) was similar among all groups (Fig. 1D). No significant differences in percentages of circulating CD4+LAG3+CD49b+ type 1 regulatory T cells, late differentiated CD4+ or CD8+ CD28− T cells, and CD4+ or CD8+ T cells expressing
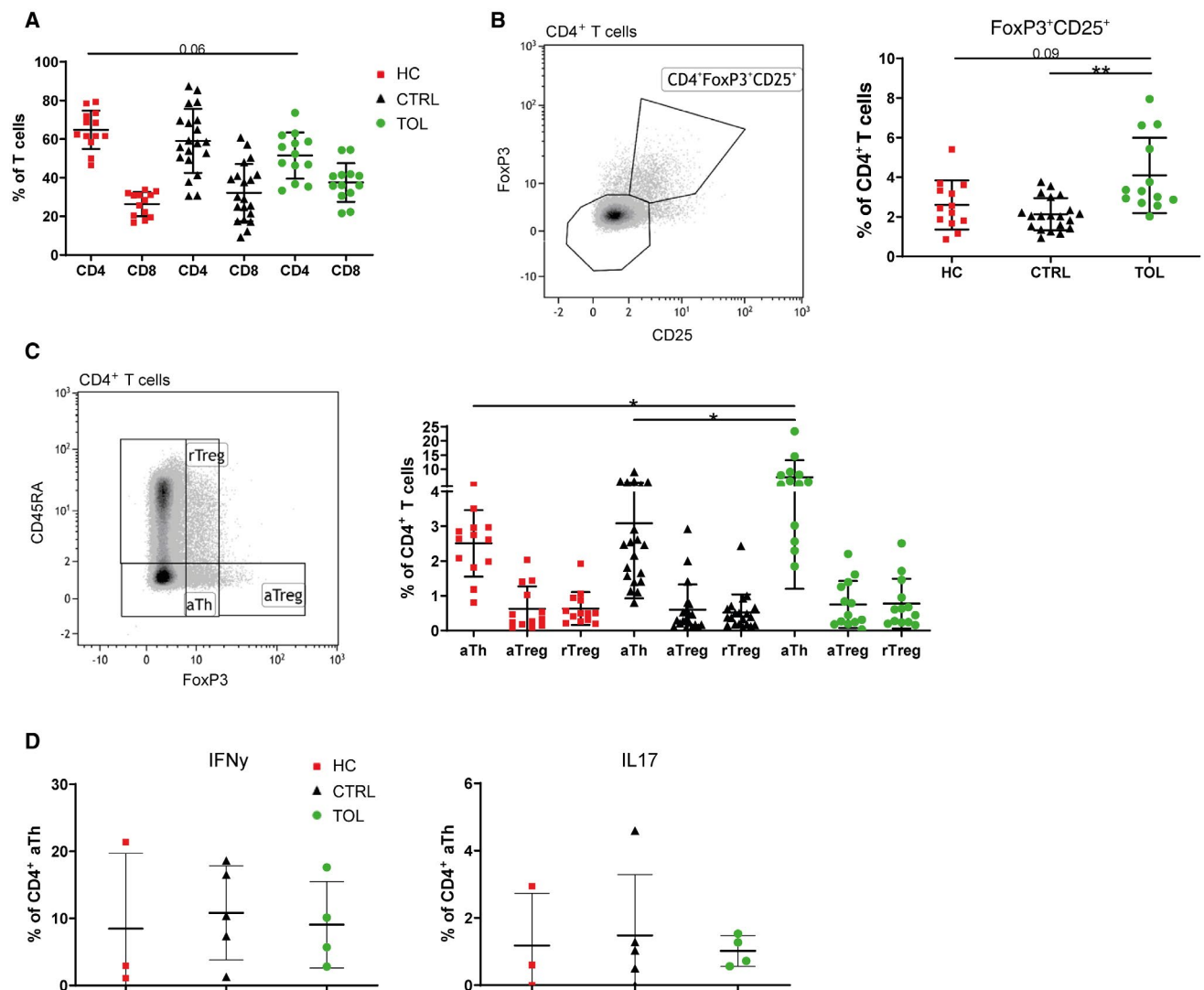
FIG. 1. Higher relative numbers of circulating activated T helper cells in tolerant LT recipients. Percentages of (A) CD4+ and CD8+ T cells, (B) CD4+FoxP3+CD25+ T cells and gating strategy, (C) aTh, aTreg, and rTreg defined by gating strategy with FoxP3 and CD45RA, and (D) IFNγ- or IL17-positive cells in PMA/IONO-stimulated aTh are presented. HC, n = 13; CTRL, n = 20; and TOL, n = 13. Statistical analyses were performed with 1-way ANOVA or Kruskal-Wallis and posttests. *P < 0.05 and **P < 0.01.

costimulatory immune checkpoint inducible T cell costimulator (ICOS) or coinhibitory immune checkpoints programmed death 1 (PD1) or cytotoxic T lymphocyte antigen 4 (CTLA4; Supporting Fig. 1A-F) were observed between groups, except for higher numbers of CD4+CXCR5+ICOS+ follicular T helper cells in TOL compared with HC. Furthermore, no significant differences in differentiation status of circulating CD4+ or CD8+ T cells, and perforin and/or granzyme B-expressing T cells were observed between groups (Supporting Fig. 2A,B).

CMV-SEROPOSITIVITY IS ASSOCIATED WITH AN INCREASED Vδ1:Vδ2 γδT CELL RATIO IN LT RECIPIENTS

Several studies reported that a higher Vδ1:Vδ2 γδT cell ratio could discriminate tolerant from control or nontolerant LT recipients.[13,14,17] We did not find a significant difference in the Vδ1:Vδ2 γδT cell ratio between CTRL and TOL (Fig. 2A). However, this ratio was significantly lower in HC compared with both
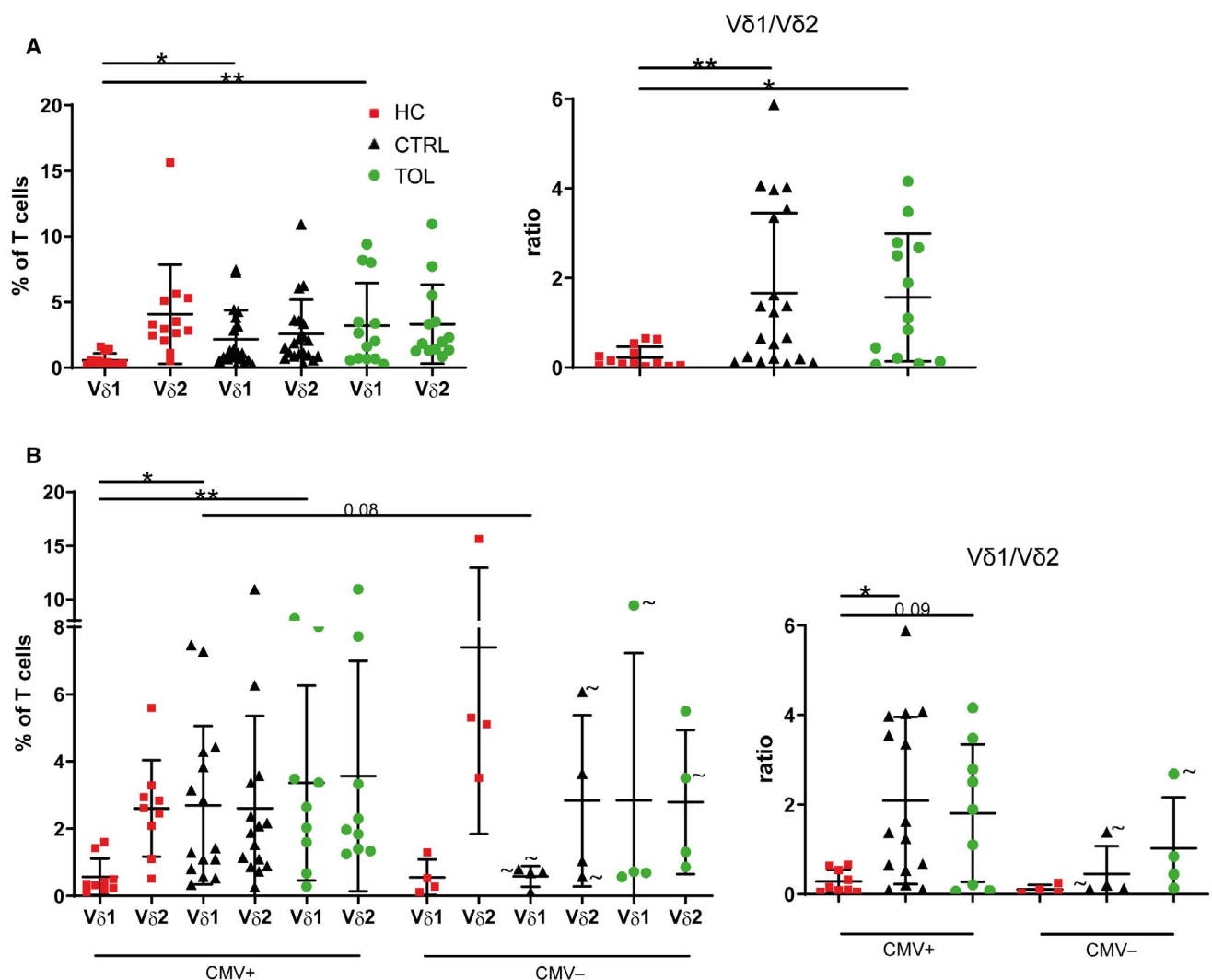
FIG. 2. CMV-positive serostatus is associated with a higher Vδ1:Vδ2 γδT cell ratio in all LT recipients. Percentages and ratios of Vδ1 and Vδ2 γδT cells of (A) entire groups and (B) in CMV+ and CMV− individuals sorted by serostatus at the end of follow-up are presented. ˜These individuals are CMV seronegative but were transplanted with a CMV+ donor. HC, n = 13; CTRL, n = 20; and TOL, n = 13. Statistical analyses were performed with 1-way ANOVA, Kruskal-Wallis or Friedman, and posttests. *P < 0.05 and **P < 0.01.

TOL and CTRL, and this was due to a significantly lower percentage of Vδ1 T cells (Fig. 2A) within CD3$^+$ T cells. In our study we chose to match the groups for, among others, the CMV serostatus, as a CMV infection profoundly influences the composition of circulating immune cell subsets.[19] When we sorted the individuals according to the CMV serostatus at the end of follow-up (Fig. 2B), high Vδ1:Vδ2 γδT cell ratios were indeed predominantly found in CMV-seropositive LT recipients of both CTRL and TOL groups. Within the CMV seropositive cases, both TOL and CTRL have (significantly) higher percentages of Vδ1 γδT cells and Vδ1:Vδ2 γδT cell ratios compared with HC. This indicates that a CMV-seropositive serostatus in LT recipients is associated with an increased proportion of Vδ1 γδT cells and thereby an enhanced Vδ1:Vδ2 γδT cell ratio.

ALLOREACTIVE MEMORY CD4$^+$ T CELLS ARE MORE TERMINALLY DIFFERENTIATED IN TOLERANT LT RECIPIENTS

Using activation-induced CD137 expression as a surrogate marker for antigen-specific T cells, we investigated T cell responses against donor or HLA-mismatched third-party splenocytes (Fig. 3A; Supporting Fig. 3 for full gating strategy). Responses of CD4$^+$ and CD8$^+$ CD137$^+$ T cells against donor and third-party splenocytes were detected in our assay, as the ratios of percentages of CD137-expressing T cells upon allogenic stimulation over nonstimulated T cells were higher than 1 for most individuals in all 3 groups. However, no significant differences were observed in ratios of donor (D) or third-party (T) CD4$^+$ or CD8$^+$ CD137$^+$ T cell responses against nonstimulated (−) T cells between groups (Fig. 3B; Supporting Fig. 4). No significant differences were observed in donor against third-party ratios (D/T) of CD4$^+$ or CD8$^+$ CD137$^+$ T cell responses. Within CD4$^+$CD137$^+$ T cells the proportions of Tregs and Th that responded to donor or third-party splenocytes were similar (Fig. 3C; Supporting Fig. 5). Functional alloreactive responses by measuring IFNγ-producing CD137-expressing T cells were assessed. The ratios of CD137$^+$ IFNγ-producing CD4$^+$ and CD8$^+$ T cells after stimulation with donor or third-party splenocytes were similar between CTRL and TOL (Fig. 3D). Furthermore, the maximum production capacity of IFNγ within CD4$^+$ or CD8$^+$ CD137$^+$ T cells upon stimulation with PMA/IONO was similar among all groups (Fig. 3E). Differentiation statuses of CD4$^+$ or CD8$^+$ CD137$^+$-expressing T cells were also assessed. Ratios of alloreactive CD4$^+$ naive T cells did not differ in TOL and CTRL (Fig. 3F). In the TOL group, CD4$^+$ central memory T cells (CMs) and effector memory T cells (EMs) responded significantly less to both donor and third-party splenocytes compared with CTRL. By contrast, CD4+ terminally differentiated effector memory T cells (EMRA) responded significantly more to both donor and third-party splenocytes in TOL compared with CTRL (Fig. 3F). Within CD8$^+$ T cells no differences were observed in ratios of percentages of activation-induced CD137 expression in different differentiation statuses (Supporting Fig. 6). Altogether, these results indicate that alloreactive memory CD4$^+$ T cells of TOL are more differentiated compared with those of CTRL.

A TOLERANCE PROFILE DISCRIMINATING TOLERANT LT RECIPIENTS COULD BE ESTABLISHED

To investigate whether the phenotypic and functional differences in circulating (alloreactive CD137$^+$) T cells between TOL and CTRL could identify operational tolerance, a heat map with hierarchical clustering analysis of these parameters was created (Fig. 4A). Interestingly, TOL and CTRL completely separated based on these differences in circulating T cells. Increased relative numbers of circulating aTh and elevated alloreactive responses of CD4 EMRA T cells were the most discriminative characteristics of TOL compared with CTRL. TOL were clustered into 2 different groups, of which group I clearly displayed characteristics of overall hyporesponsiveness in CM and EM CD4$^+$ T cells, whereas group II showed more variability. This could indicate that LT recipients may develop spontaneous operational tolerance in more than 1 way. Group III and IV consisted of CTRL LT recipients and differed in relative numbers of aTh, whereas the other markers were quite variable in both groups. Heat map with hierarchical clustering analysis including alloreactive responses of HCs, but without the donor-reactive response, did not result in complete separate clustering of TOL and CTRL LT recipients, suggesting a contribution of donor-specific CD4$^+$ T cell subset responses (Supporting Fig. 7).

Principal component analysis of all significantly different parameters between TOL and CTRL revealed 2 components that completely separated the 2 groups and in combination accounted for 70.6% of the variance (Fig. 4B). In conclusion, the combination of alloreactive hyporesponsive and hyperresponsive
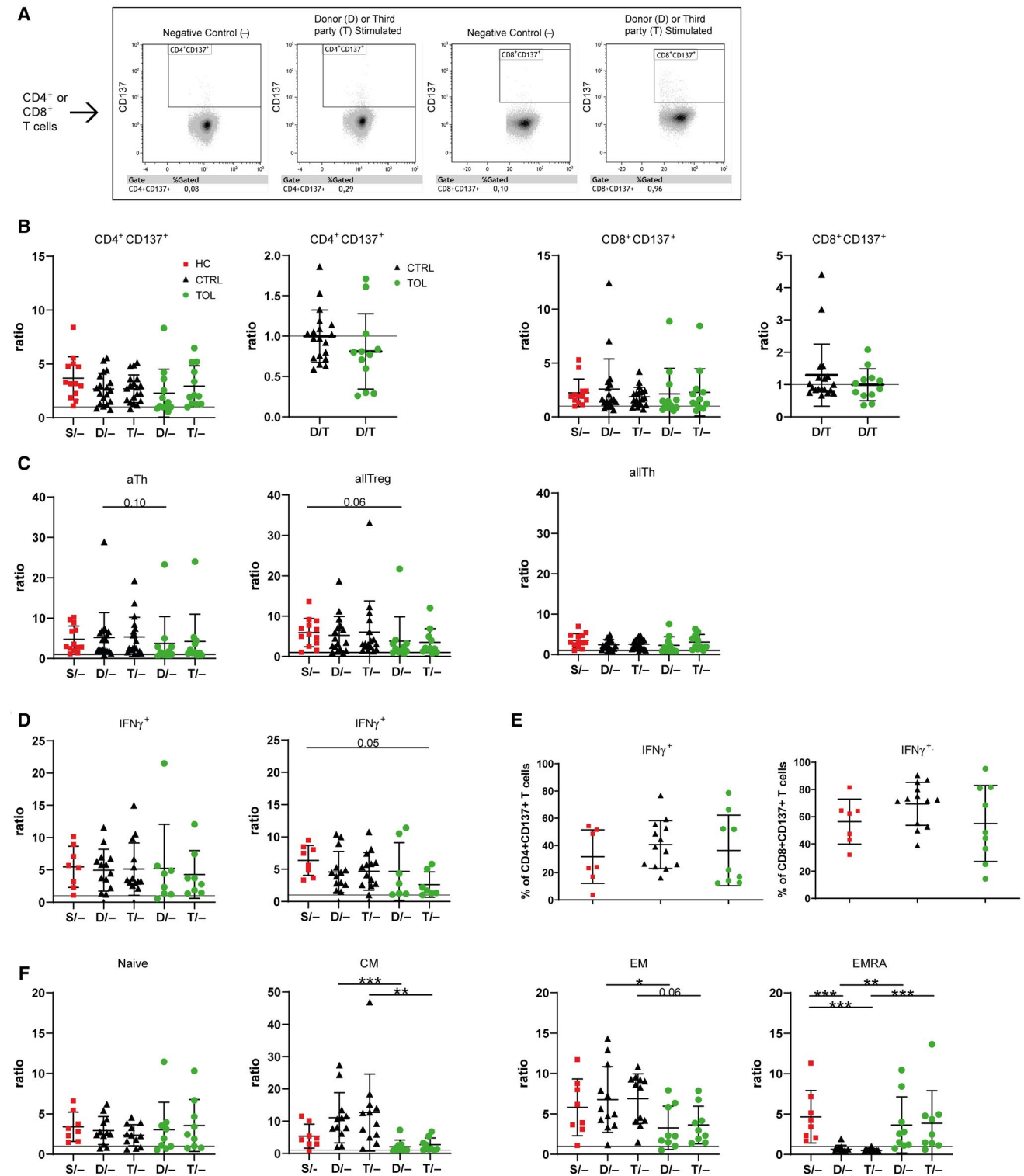
FIG. 3. Alloreactive memory CD4+ T cells are more differentiated in tolerant LT recipients. (A) Representative dot plots indicating CD137 expression in CD4+ and CD8+ T cells cultured in the absence or presence of allogenic splenocytes. Ratios of CD137 expression in T cells stimulated by allogeneic splenocytes (S) for HC, donor splenocytes (D), and third-party splenocytes (T) against unstimulated T cells (S/–, D/–, or T/–) and/or donor against third party (D/T) are presented in B-D and F. Ratios are presented for (B) CD137-expressing CD4+ and CD8+ T cells; (C) CD137-expressing aTh, allTreg, and allTh in CD4+ T cells; (D) CD137-expressing IFNγ-producing CD4+ (left) and CD8+ (right) T cells; and (F) CD137-expressing T cell subsets naïve, CM, EM, and EMRA in CD4+ T cells. A solid line represents a ratio of 1. In (E) percentages of IFNγ-positive cells in PMA/IONO-stimulated CD4+ or CD8+ CD137+ T cells are presented. Panel B/C: HC, n = 13; CTRL, n = 19; TOL, n = 12; Panel D: HC, n = 8; CTRL, n = 13; TOL, n = 8; Panel F: HC, n = 8; CTRL, n = 12; TOL, n = 9. Statistical analyses were performed with 1-way ANOVA, Kruskal-Wallis or Friedman, and posttests. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

subsets of CD4+ T cells and increased relative numbers of circulating aTh within CD4+ T cells may be suited to discriminate TOL from a larger group of LT recipients.

FINDINGS IN TOL AND NON-TOL BEFORE AND DURING IS DRUG WEANING

To investigate whether the immunological characteristics of TOL could be observed during IS drug weaning, we collected blood from 4 LT recipients before, during, and after IS drug weaning. Three of these recipients appeared to be tolerant (Supporting Table 1). The fourth recipient experienced highly elevated liver function values 4 months after IS regimen cessation when blood was withdrawn and was considered non-TOL. After reinstalment of regular IS regimen, the recipient's liver graft function values normalized. Before IS drug weaning, circulating aTh were already enhanced in TOL compared with non-TOL, and this difference remained during the course of complete IS drug weaning (Fig. 5A,B). For aTreg and rTreg no clear differences were observed between TOL and non-TOL during the course of IS drug weaning (Supporting Fig. 5D). These preliminary data suggest that elevations of aTh in TOL may already occur before IS drug weaning. We again observed that CMV-seropositive LT recipients, regardless of their TOL or non-TOL status, have a higher Vδ1:Vδ2 γδT cell ratio compared with CMV-seronegative LT recipients (Fig. 5C). In addition, similar to the data presented in Fig. 3, no clear differences were observed in CD4+ or CD8+ CD137+ T cell responses following donor and third-party stimulation between TOL and non-TOL before or during weaning (Fig. 5D,E). Unfortunately, due to shortage of samples, CD4+ or CD8+ CD137+ alloreactive T cell responses in different differentiation statuses could not be analyzed.

NO SIGNIFICANT DIFFERENCES IN THE DEVELOPMENT OF DSAs BETWEEN TOLERANT AND CONTROL LT RECIPIENTS

DSAs were measured in TOL before LT, before weaning, and after weaning and in CTRL at matching time points. Most of the DSAs that developed were de novo (Fig. 6A). Only 1 CTRL had preformed DSAs (B60; DR11), but these completely disappeared after LT. Just before complete IS drug weaning, 25.0% of TOL had DSAs, and 30.4% of CTRL had ≥1 DSAs at matched time points (Fig. 6B), indicating that TOL cannot be distinguished on basis of DSAs. A total of 2 out of 5 DSA+ CTRL developed >1 DSA, whereas within the TOL group none developed >1 DSA before weaning. Despite their operationally tolerant state, the number of DSA+ TOL doubled after IS drug weaning, whereas it only increased moderately in CTRL at matched time points, although this difference was not statistically significant. For both groups about half of the DSA+ individuals developed >1 DSA after weaning. Most of the de novo DSAs were against HLA Class II DR or DQ. Only 1 LT recipient in the CTRL group developed DSAs against HLA Class I (Fig. 6B). A shift to another DSA across time occurred within both groups (Fig. 6A). No clear differences in cumulative mean fluorescence intensity (MFI) of DSAs were observed among groups before and after weaning (Fig. 6C). These data demonstrate that tolerance develops and is maintained despite development of DSAs.

Discussion

In this study, we characterized circulating T cells subsets that could play a role in the development of or identify operationally tolerant LT recipients. We found that in TOL CM and EM CD4+ T cells
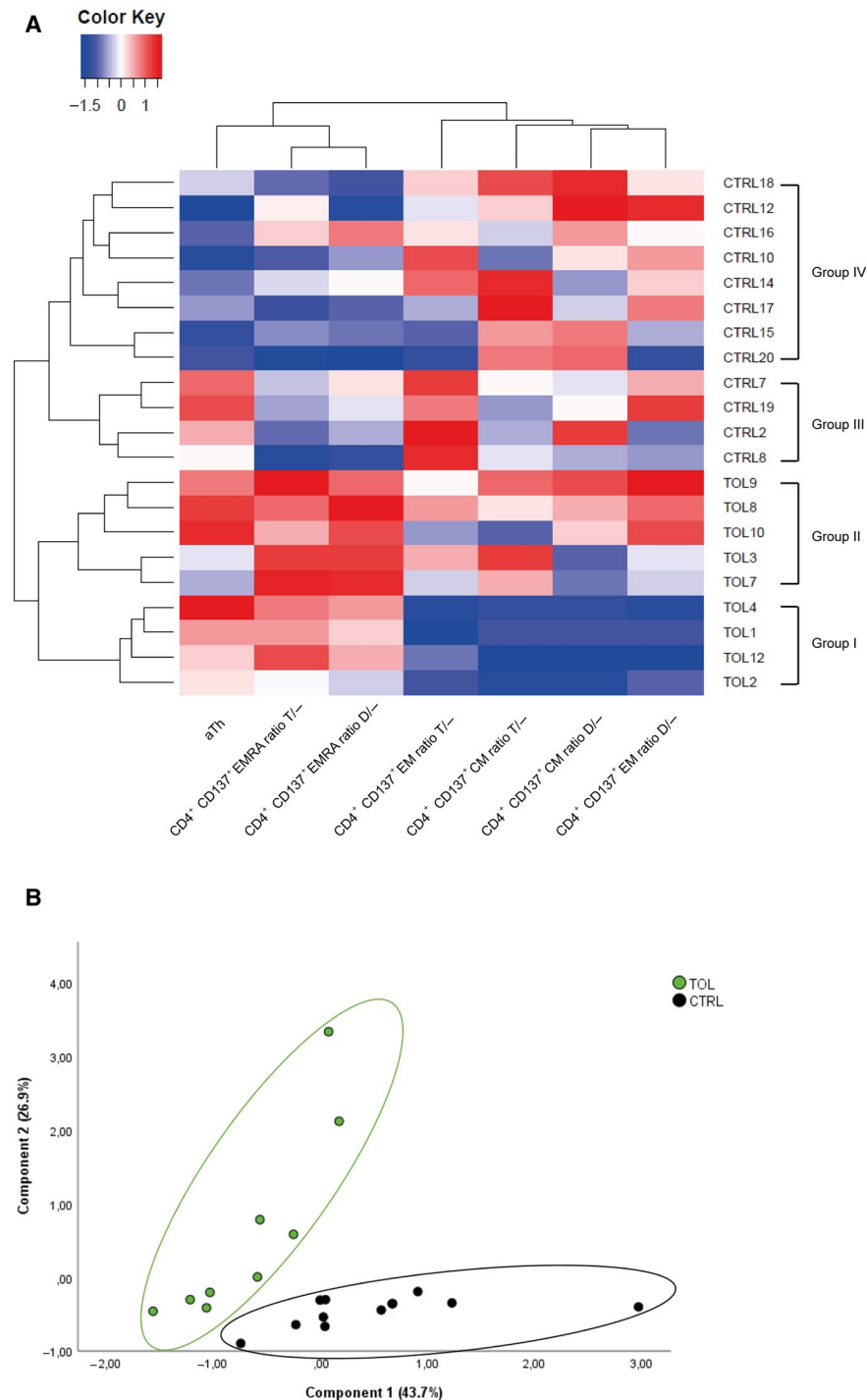
FIG. 4. A tolerance profile discriminating tolerant LT recipients could be established. (A) A heat map with hierarchical clustering analysis is depicted for all LT recipients in whom all significantly different markers between TOL and CTRL were measured in this study. To avoid a selection bias, the LT recipients in whom not all significantly different markers were measured were not included. Analysis was performed with the public Galaxy server, version 3.0.1, R gplots package (R Foundation, Vienna, Austria) with the Euclidean distance method and the complete hierarchical clustering method. Data from each recipient were scaled with a z score according to total data of TOL and CTRL for that marker (color key). (B) Principal component analysis of the significantly different markers between TOL and CTRL is depicted. Rotated component matrix analysis was performed using Varimax with Kaiser normalization. CTRL, n = 12; TOL, n = 9.
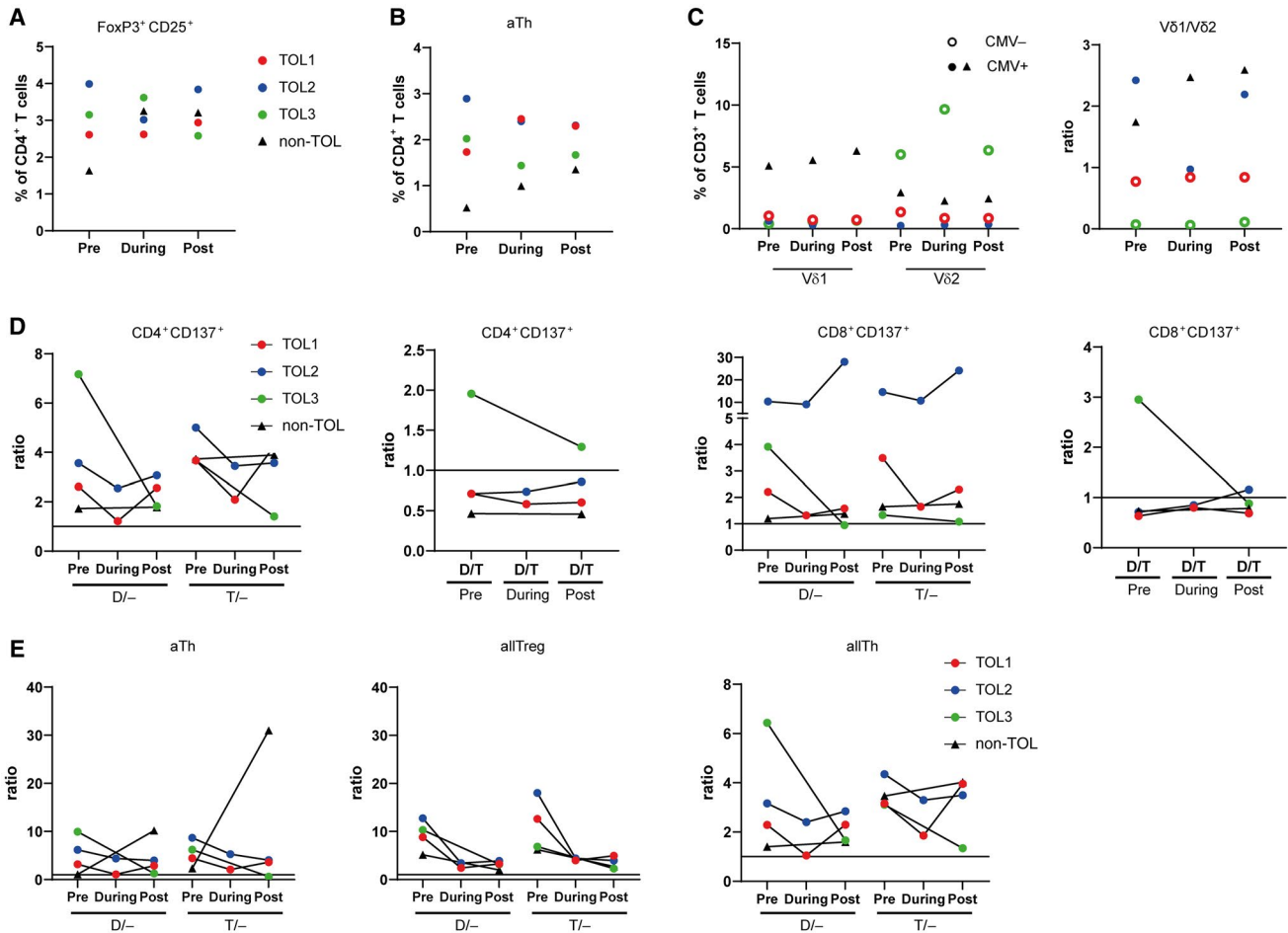
FIG. 5. Findings in a small group of prospectively IS drug-weaned group of LT recipients. Percentages of (A) FoxP3+CD25+ in CD4+ T cells; (B) aTh defined by FoxP3 and CD45RA expression; and (C) percentages and ratios of Vδ1 and Vδ2 γδT cells before, during, and after IS drug weaning are presented. Ratios of T cell responses against donor (D) and third-party (T) splenocytes against unstimulated T cells (D/− or T/−) measured by flow cytometric determination of activation-induced CD137 are presented in D and E. Ratios are depicted for (D) CD4+ and CD8+ CD137+ T cells and (E) CD137-expressing aTh, allTreg, and allTh in CD4+ T cells.

displayed hyporesponsiveness, whereas EMRA CD4+ T cells displayed hyperresponsiveness against donor and third-party stimulation, compared with CTRL. In addition, TOL exhibited an elevated proportion of circulating aTh compared with CTRL. Clustering analysis and principal component analysis revealed that the combination of these CD4+ T cell characteristics accurately discriminated TOL from CTRL. By contrast, no significant differences in alloreactive CD8+ T cells or DSA formation were observed between TOL and CTRL.

As confirmed by other studies,[14,16] a significantly higher proportion of circulating CD4+CD25+FoxP3+ T cells was found in TOL compared with CTRL. However, upon further delineation of these cells

it was found that circulating aTh were elevated in TOL compared with CTRL and HC. Furthermore, in a small cohort we found that these CD4+ T cells were already elevated before weaning in TOL. The aTh subset of healthy and diseased individuals is cytokine-secreting nonsuppressive T cells that transiently express FoxP3.[24] A robust FoxP3 expression requires DNA demethylation of the *FOXP3* gene, as is found for conventional Tregs generated in the thymus. Induced Tregs are generated by specific antigen stimulation in combination with IL2 and transforming growth factor β and have an unstable FoxP3 expression.[25] Unfortunately, we cannot rule out that the elevated aTh subset in TOL is actually induced Tregs. The second novel finding of our study is that allogenic
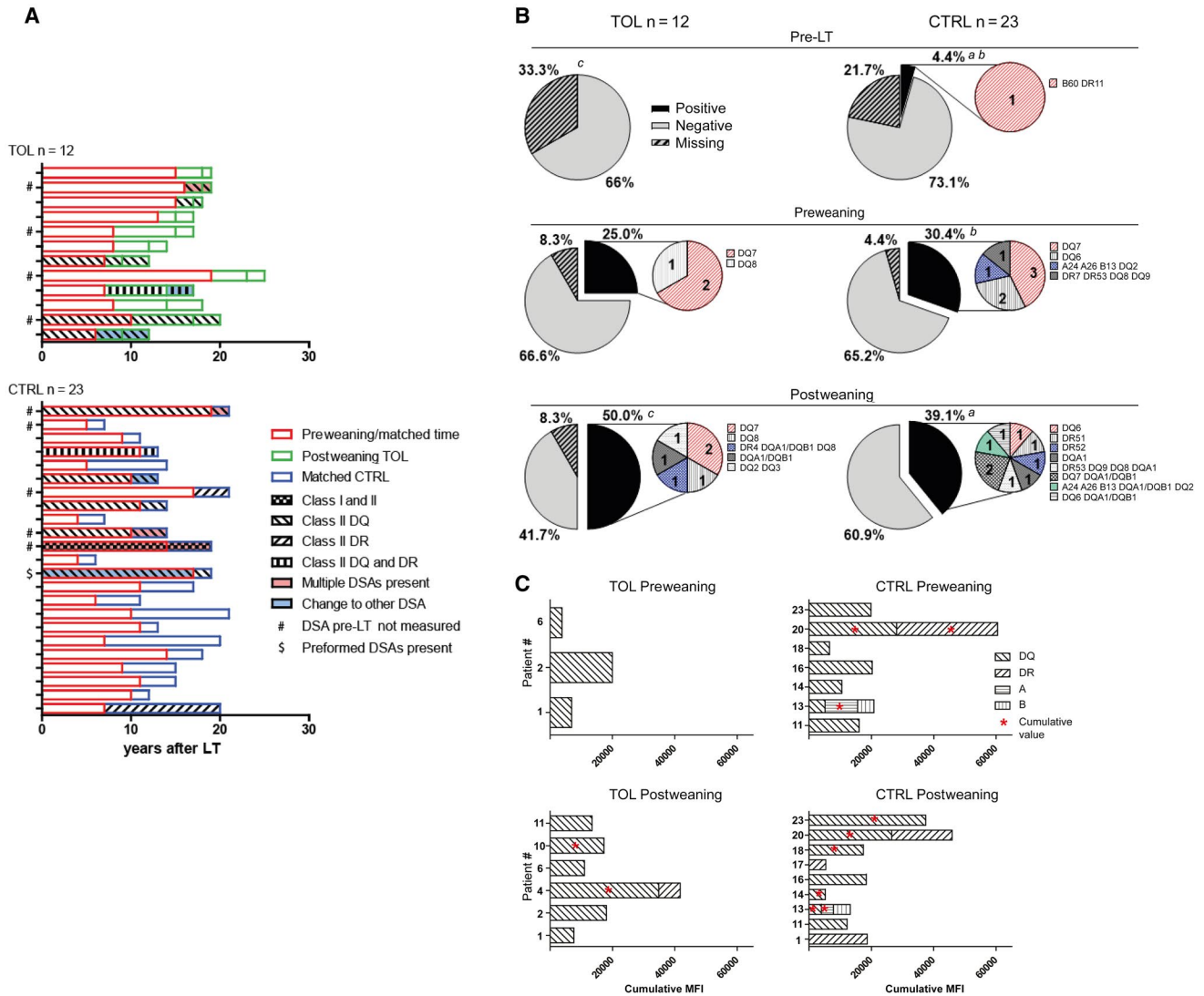
FIG. 6. No significant differences are present in the development of DSAs in tolerant and control LT recipients. Development of DSA formation (A) individually and (B) for the entire group with specific subtypes in TOL before, during, and after IS drug weaning and in CTRL at matching time points are depicted. (C) The cumulative MFI for each DSA+ LT recipient is presented for TOL and CTRL. Statistical analyses were performed with 2-sided Fisher's exact test or Pearson chi-square test. Before versus after weaning: $a = 0.06$, b, $c = 0.13$.

hyporesponsiveness was observed in CM and EM CD4+ T cells, whereas an allogenic hyperresponsiveness was found in the EMRA compartment of TOL. CD4+ CM T cells exhibit a high proliferative capacity and poor effector function, whereas EM T cells exhibit an immediate effector function and only a limited proliferative capacity.[26] Many studies hold Tregs responsible for induction and maintenance of immune tolerance[27] and are investigating the therapeutic potential of Treg therapies in tolerance induction.

Surprisingly, our data suggest that specific T helper subsets might be associated with naturally occurring tolerance. It could be that in TOL alloreactive CM and EM CD4+ T cells are either deleted, anergic, senescent, or inhibited by Tregs.[28] Unfortunately this interaction between T helper cells and Tregs could not be further investigated as this requires large numbers of cells. Until now data on CD4+ EMRA T cells are sparse; nonetheless, it has been suggested that these T cells resemble CD8+ EMRA T cells. They exhibit

cytotoxic potential and can secrete multiple cytokines after activation, but have poor proliferative capacity, and are expanded during chronic viral infections.[29,30] Highly differentiated CD4^{+} T cells were also associated with low proliferative alloreactivity in kidney transplantation.[23] Previous studies have reported a proliferative donor-specific hyporesponsiveness within the total population of CD4^{+} T cells of tolerant LT recipients.[16,20] Our data suggest that this may be due to enrichment of alloreactive CD4^{+} T cells of TOL with EMRA that exhibit poor proliferative responsiveness to alloantigens, explaining their involvement in operational tolerance. However, additional studies have to be performed to investigate the functionality of these alloreactive CD4^{+} EMRA T cells.

Several studies reported that a higher Vδ1:Vδ2 γδT cell ratio could discriminate tolerant from other LT recipients, but without reporting the CMV serostatus. CMV infection influences the composition of immune cell subsets,[18,19] and therefore our groups were matched for the CMV serostatus. Indeed, our results indicate that a positive CMV serostatus is associated with an increased Vδ1:Vδ2 γδT cell ratio due to an increase in circulating Vδ1 in LT recipients, regardless of the tolerance state. This confirms previous studies which indicated that CMV seropositivity is associated with an increased Vδ1:Vδ2 γδT cell ratio in LT recipients.[31] These data also highlight the importance of matching groups on parameters that could influence the markers of interest.

In this study, no significant differences in DSA formation over time, against HLA Class type I or II, or MFI of DSAs were observed between TOL and CTRL, which is confirmed by other studies.[8,11,12] DSAs against donor HLA have been associated with an increased risk of acute and chronic rejection[10] early after LT. Our recipients were included on average 15 years after LT, and hence LT recipients with complications due to DSAs were possibly lost. An increase in DSA formation after complete IS drug weaning in TOL was observed, but this did not lead to clinical complications. This could be explained by the formation of certain less harmful IgG subtypes, their potential weaker complement binding, and possibly a lower HLA antigen density in the liver[32] in TOL. These aspects have to be investigated in the future.

The strength of our study is that we are the first to investigate phenotypic as well as functional features of alloreactive T cells in TOL LT recipients in detail using CD137 as a surrogate marker for the total

alloreactive T cell compartment. Furthermore, we are the first to delineate further the elevated proportion of CD4^{+}CD25^{+}FoxP3^{+} T cells present in TOL. We performed this study with completely matched groups for important clinical parameters, and thereby eliminated potential confounders. Our study also has limitations. Similar to other recent studies,[6,7] in our definition of operational tolerance, liver function tests were used instead of a protocol liver biopsy, due to concerns about possible complications. Subclinical rejection may have been undetected in this way, but in our study biopsies were taken from 5 TOL recipients at some point after complete IS drug weaning and every type of rejection was excluded. We admit that the CTRL LT population could represent a mixed population of tolerant and nontolerant recipients, although, based on available literature,[6-8] we expect that the majority of CTRL recipients are nontolerant. In addition, despite the possibility of a mixed CTRL population, we observed statistically significant differences in relative numbers of circulating aTh and in several alloreactive T helper subsets between TOL and CTRL. We compared a TOL and CTRL group that differ in IS drug usage. Therefore, we included a group of HCs without an IS regimen to compare with TOL. The significant difference of circulating aTh in HC versus TOL, but not in CTRL, indicates that the influence of IS drug is limited on the development on this subset of T cells. Moreover, in a small group of LT recipients we found preliminary evidence that aTh were already increased in TOL before IS drug weaning. If IS drug usage had inhibited alloreactivity of T cells, we could have expected overall T cell hyporesponsiveness in CTRL versus TOL. Instead we observed hyporesponsiveness of alloreactive CM and EM CD4^{+} T cells against both donor and third-party splenocytes in TOL versus CTRL. T cell responses against third-party alloantigen in transplanted recipients can be compared with T cell responses against HLA-mismatched alloantigen in HC. Notably, CM and EM CD4^{+} T cell responses against HLA-mismatched alloantigens in HC were not reduced compared with CTRL, suggesting that the observed hyporesponsiveness of CD4^{+} T cell subsets in TOL versus CTRL cannot be explained by the absence of IS regimen in TOL. In line with this one could argue that the actual difference in functional activity between CTRL and TOL would even have been larger than is observed now if CTRL had not used the IS regimen. We could not investigate unresponsive alloreactive T cells or T cell responses against

indirectly presented alloantigens, as no reliable techniques are available. Finally, our prospective IS drug weaning cohort (n = 4) was too small to draw a robust conclusion on whether enhanced numbers of aTh were discriminative of TOL before IS drug weaning.

In this study we identified enhanced frequencies of aTh and highly differentiated alloreactive CD4+ T cells in blood as new markers associated with operational tolerance after LT. Validating whether these T cell markers can be used to discriminate tolerant from nontolerant LT recipients on IS drug regimen requires a prospective study with a larger independent IS drug weaning cohort. Additional studies have to be performed to investigate the functionality of alloreactive CD4+ CM, EM, and EMRA T cells and their involvement in spontaneous operational tolerance.

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