ORIGINAL ARTICLE

Number and function of circulatory helper innate lymphoid cells are unaffected by immunosuppressive drugs used in solid organ recipients – a single centre cohort study

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SUMMARY

In transplanted intestines, depletion of T cells together with long-term persistence of ILC is observed, suggesting ILC insensitivity to immunosuppressive drugs. To further analyze helper ILC (hILC) apparent resistance to therapy, cytotoxic ILC (NK cells), hILC subsets (ILC1, ILC2, and ILC precursors (ILCP)), and their signature cytokines (IFNγ, IL4 + IL13, and IL22) were analyzed in peripheral blood of kidney and liver transplant recipients. Early after transplantation (posTx), transplanted patients showed significantly lower Lin + and NK cells, whereas total hILC, ILC1, ILC2, and ILCP numbers were similar in patients and controls. Between paired pre- and posTx samples, Lin + cell and NK cell counts significantly decreased, whereas all three hILC counts and their cytokine production remained similar. ILC1, ILC2, and ILCP numbers were also similar in patients under thymoglobulin or basiliximab (BAS), patients without induction (only maintenance therapy) and controls. hILC showed lower TMG binding comparing to Lin + cells, reduced expression of CD25 (BAS target), and diminished calcineurin activity with undetectable calcineurin and FKBP12 (tacrolimus target). hILC counts were not related to delayed graft function or biopsy-proven acute rejection. Thus, hILC remain stable early after transplantation and seem unaffected by immunosuppressors, which may be related to reduced targets expression and low calcineurin activity.

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Introduction

Long-term immune rejection remains to limit graft survival in solid organ transplant (SOT) recipients, and this outcome is not modified by the presently used therapeutic immunosuppression [1,2]. Current immunosuppressive protocols are mostly tacrolimus-based and focused to constrain the T-cell response by blocking the activation signal from the T-cell receptor through the calcineurin pathway. This scenario suggests

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that immune response compartments other than the adaptive alloresponse may contribute to deterioration of the graft.

Within innate immunity, innate lymphoid cells (ILC) are recently discovered cells which lack rearranged antigen-specific receptors or lineage (Lin) markers. ILC consist of cytotoxic ILC, represented by natural killer (NK) cells, and helper ILC (hILC), including ILC1, ILC2, and ILC3. hILC are mostly mucosal-resident cells. Recently, multi-potent and uni-potent ILC precursors (ILCP) have been described in peripheral blood [3]. By responding to cytokines from the microenvironment, hILC control the tissular homeostasis during infection, inflammation, and cancer [4,5].

We and others have found that ILC are present in transplanted intestines and survive for long periods of time [6,7]. Moreover, during the two first years after transplantation, ILC become the most prominent lymphoid population in intestinal mucosa while a profound depletion of T cells is observed [5]. Whereas the drop of gut T cells may be a consequence of tacrolimus-based therapy, as it has been recorded in FK-506-treated mice [8], the presence and stability of ILC may suggest that these cells are unaffected by the standard immunosuppression. Small amounts of ILC1, ILC2, and ILCP circulate in peripheral blood [3,9] providing the opportunity to analyze this hypothesis in other SOT recipients.

By studying kidney and liver transplant recipients (KTR and LTR), we demonstrate here that, differently from T, B, and NK cells, peripheral blood hILC numbers and function remain unchanged in the early posTx period and seem unaffected by thymoglobulin (TMG), basiliximab (BAS), and tacrolimus. Relative to other cells, hILC show reduced targets for these immunosuppressive drugs.

Materials and methods

Patients and controls

We studied three cohorts of 101 kidney transplant recipients (KTR), 20 liver transplant recipients (LTR), and 77 age and sex-paired control subjects (CS). All patients were transplanted in Hospital 12 de Octubre between 2015 and 2019. Table 1 summarizes demographic and clinical data of the three cohorts. After transplantation, all KTR were under triple therapy (tacrolimus, mycophenolate mofetil (MMF), and corticosteroids) and LTR received triple or double therapy (tacrolimus and corticosteroids) as maintenance. Patients treated with TMG as induction therapy received five to six doses before and during the first week post-transplantation (posTx). Patients treated with BAS as induction therapy received two doses (just before transplant and at day 4 posTx).

Additionally, human tonsils and peripheral blood samples from other 4 and 2 CS, respectively, were obtained for functional experiments related to immunosuppression targets expression and calcineurin activity (see below).

This study was performed under informed consent from all patients and controls and was approved by the institutional review board (PI13/0045).

ILC phenotyping

Fresh peripheral blood mononuclear cells (PBMC) were obtained from KTR, LTR, and CS and stained to analyze the different ILC populations by flow cytometry (FC) (FACSCanto II; BD Biosciences, San Diego, CA, USA), according to previous literature [3,5,9]. The following monoclonal antibodies were obtained from BD Biosciences (San Diego, CA, USA): FITC-conjugated anti-Lin3 (CD3, CD14, CD19, CD20) (clones SK7, L27, SJ25C1, MφP9), PE-Cy7-conjugated anti-CD127 (clone HIL-7R-M21), PerCP-Cy™5.5 Anti-Human CD294 (CRTH2) (clone BM16), APC-conjugated anti-CD94 (clone HP-3D9), APC-Cy7-conjugated anti-CD45 (clone 2D1), BV421-conjugated anti-CD117 (clone YB5.B8); and from eBioscience™ (San Diego, CA, USA): PE-conjugated anti-Id2 (clone ILCID2). Data were processed using FACS Diva (BD Bioscience) and analyzed using Flowjo software (TreeStar Inc., Ashland, OR, USA).

Lymphocytes were gated according to size (FSC) and complexity (SSC) as well as CD45 positivity. T and B cells were identified as CD45 + CD94− Lin+, NK cells as CD45 + CD94+, and hILC as CD45 + CD94− Lin- CD127+. hILC were positive for Id2, an ILC signature transcription factor (Fig. S1). The hILC subsets were distinguished according to CD117 and CRTH2: ILC1 (CRTH2−CD117−), ILC2 CD117− (CRTH2 + CD 117−), ILC2 CD117+ (CRTH2 + CD117+), and ILCP (CRTH2− CD117+) (Fig. 1a) [10].

Absolute numbers of all cell types were assessed using the following formula: lymphocytes/μl × % cell type among lymphocytes.

Intracellular cytokine detection on hILC

Intracellular cytokine detection on hILC was also determined in 10 KTR and four LTR at pretransplant (preTx) and at day 14 posTx, as well as in 16 CS.

Table 1. Demographic and clinical characteristics of kidney and liver transplant cohorts.

Characteristics	Kidney cohort (KTR)	Liver cohort (LTR)	Control subjects (CS)
Recipients	*n = 101*	*n = 20*	*n = 77*
Gender, *n* (%)			
Male	76 (75.2)	15 (75.0)	51 (66.2)
Female	25 (24.8)	5 (25.0)	26 (33.8)
Recipient age at transplant, years (mean ± SD)	56.9 ± 15.8	52.2 ± 12.2	51.1 ± 9.1
Underlying disease, *n* (%) (Kidney)			
Diabetic nephropathy	24 (23.8)		
Glomerulonephritis	20 (21.8)		
Cystic nephropathy	18 (17.8)		
Nephroangiosclerosis	12 (11.9)		
Tubulointerstitial nephritis	2 (1.9)		
Lupus nephropathy	2 (1.9)		
Others	14 (13.9)		
NA	9 (8.9)		
Underlying disease, *n* (%) (Liver)			
AC		4 (20.0)	
HCV-C + HCC		2 (10.0)	
HCV-C		2 (10.0)	
AC + HCV-C + HCC		2 (10.0)	
HCC		1 (5.0)	
AC + HCV-C		1 (5.0)	
AC + HCC		1 (5.0)	
HCV-B + HCC		1 (5.0)	
Others		6 (30.0)	
Number of transplants, *n* (%)			
1	83 (82.2)	18 (90.0)	
>1	18 (17.8)	2 (10.0)	
Type of allograft, *n* (%)			
Kidney	97 (96.0)		
Kidney–Pancreas	4 (3.9)		
Liver		19 (95.0)	
Liver–Heart		1 (5.0)	
Induction therapy, *n* (%)			
No induction	14 (13.8)	17 (85.0)	
Thymoglobulin	48 (47.5)		
Basiliximab	39 (38.6)	3 (15.0)	
Maintenance therapy			
FK + CE	-	7 (35.0)	
FK + CE+MMF	101 (100.0)	13 (65.0)	
BPAR within 12 months posTx, *n* (%)			
No	94 (93.1)	17 (85.0)	
Yes	7 (6.9)	3 (15.0)	
Delayed graft function, *n* (%)*	57 (56.4)		

AC, alcoholic cirrhosis; BPAR, biopsy-proven acute rejection; CE, corticosteroids; FK, tacrolimus; HCV-C, hepatitis C-related cirrhosis; HCC, hepatocellular carcinoma; MMF, mycophenolate mofetil; NA, not assessed; PosTx, post-transplant.

*Dialysis requirement in first two postoperative weeks.

Among these 14 transplanted recipients, there were patients treated with TMG ($N = 3$) or BAS ($N = 5$) as induction therapy as well as patients without induction, receiving only tacrolimus-based therapy ($N = 6$). PBMCs were stimulated with PMA (phorbol myristate acetate) (40 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) and Ionomycin (1.25 μg/ml; Merck Millipore, Darmstadt, Germany) in presence of Brefeldin A (5 μg/ml; Sigma-Aldrich) during 4 h at 37°C in a 5% CO_2 atmosphere. Cells were stained with the following monoclonal antibodies against surface markers in presence of Brilliant Stain Buffer (BD Biosciences): FITC-conjugated

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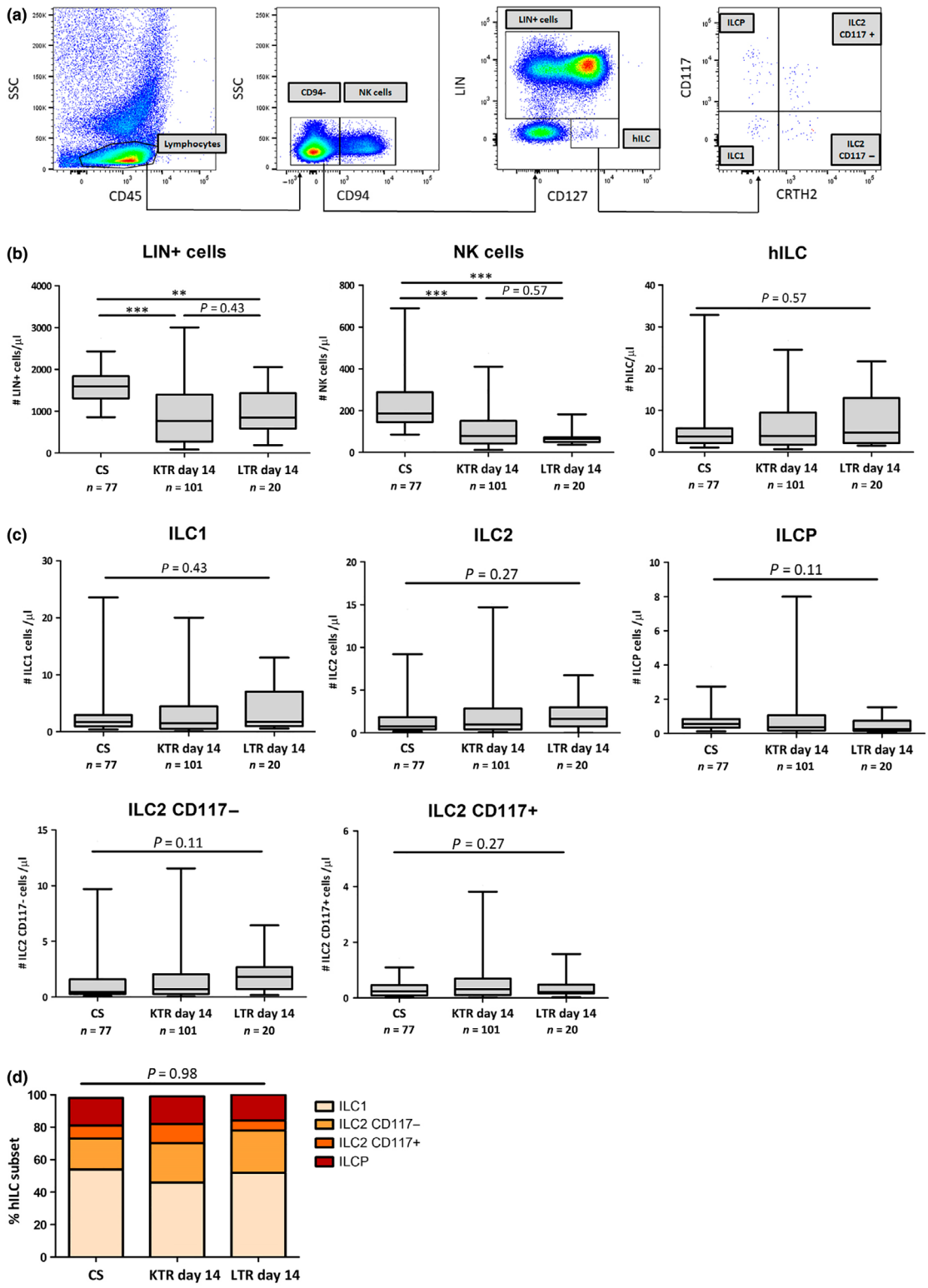

Figure 1 Circulatory hILC remain stable in kidney or liver transplanted recipients compared to control subjects. (a) Gating strategy of ILC subsets (NK cells, ILC1, ILC2 CD117−, ILC2 CD117+, and ILCP). (b) Absolute numbers of circulating Lin + cells, NK cells, and hILC. Bars show medians and whiskers show 10th–90th percentile. (c) Absolute numbers of circulating hILC subsets. Bars show medians and whiskers show 10th–90th percentile. (d) ILC1, ILC2 CD117−, ILC2 CD117+, and ILCP distribution within total hILC. Kruskal–Wallis and Dunn's multiple comparison tests (b and c) or two-way ANOVA test (d) was used for group comparisons. ***P*-value < 0.01; ****P*-value < 0.001. CS: Control subjects/KTR: Kidney transplanted recipients/ LIN: lineage/ LTR: liver transplanted recipients/ SSC: side scatter.

anti-Lin3 (CD3, CD14, CD19, CD20) (clones SK7, L27, SJ25C1, MφP9), FITC-conjugated anti-CD16 (clone 3G8), PE-Cy7-conjugated anti-CD127 (clone HIL-7R-M21), PerCP-Cy™5.5 Anti-Human CD294 (CRTH2) (clone BM16), BV421-conjugated anti-CD117 (clone YB5.B8), and BV510-conjugated anti-CD45 (clone HI30); all from BD Biosciences. Cells were then fixed and permeabilized with Inside Stain Kit (Miltenyi Biotec, Gladbach, Germany) and stained with PE-conjugated anti-interleukin(IL)-4, PE-conjugated anti-IL13 (clone REA1011), APC-conjugated anti-IL22, and APC-Vio770-conjugated anti-IFNγ (clone REA600), all from Miltenyi Biotec. Data were processed using FACS Diva (BD Bioscience) and analyzed using Flowjo software (TreeStar Inc).

TMG binding assay

Fresh PBMC were obtained from CS. PBMC were incubated with TMG (5 mg/ml; Sanofi Genzyme, Cambridge, MA, USA) and stained with the following antibodies: FITC-conjugated anti-Lin3 (CD3, CD14, CD19, CD20) (clones SK7, L27, SJ25C1, MφP9), PE-Cy7-conjugated anti-CD127 (clone HIL-7R-M21), APC-conjugated anti-CD94 (clone HP-3D9), and APC-Cy7-conjugated anti-CD45 (clone 2D1). After a wash with PBS, PE-conjugated anti-rabbit polyclonal antibody was added (eBioscience, San Diego, CA, USA) to detect cell-bound TMG. Data were processed using FACS Diva (BD Bioscience) and analyzed using Flowjo software (TreeStar Inc). Lymphocytes were gated according to size (FSC) and complexity (SSC) as well as CD45 positivity. T and B cells were identified as CD45 + CD94− Lin+, NK cells as CD45 + CD94+, and hILC as CD45 + CD94− Lin- CD127+. The binding of TMG on Lin + cells, NK cells, and hILC was determined by the mean fluorescence intensity (MFI) in PE.

Tonsillar hILC isolation and protein extraction

Mononuclear cells were isolated from human tonsils of CS undergoing amygdalectomy. Tonsils were collected in RPMI and mechanically dissociated using a scalpel, to obtain a cell suspension. hILC were first enriched by negative selection with FITC-conjugated anti-Lin3 (CD3, CD14, CD19, CD20) (clones SK7, L27, SJ25C1, MφP9) and anti-FITC MicroBeads (Miltenyi Biotec, Gladbach, Germany). Then, the enriched cells were stained with FITC-conjugated anti-Lin3 (CD3, CD14, CD19, CD20) and PE-Cy7-conjugated anti-CD127 (clone HIL-7R-M21) and were FC-sorted in Lin + cells and hILC (Lin- CD127+) populations using a FACSAria™ Fusion cell sorter (BD Biosciences) (Fig. S2). Analysis strategy was based on dead cells and doublets exclusion.

Cell lysates from human tonsils FC-sorted cells (Lin + cells and hILC) as well as CS PBMC were prepared in RIPA buffer (Sigma-Aldrich) plus Halt protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA).

Western blot

Protein extracted from PBMCs, isolated Lin + cells, and hILC was loaded on 4–20% precast polyacrylamide gel (Bio-Rad, Hercules, CA, USA) for electrophoresis. Proteins were blotted on a nitrocellulose membrane and probed with rabbit monoclonal anti-calcineurin A (clone EPR1670 (2)) (Abcam, Cambridge, UK), mouse anti-FKBP12 (clone H-5) (Santa Cruz Biotechnology, Heidelberg, Germany), and anti-β-actin horseradish peroxidase (HRP)-conjugated (clone AC-15) (Abcam, Cambridge, UK) antibodies. Anti-mouse-IgGκ HRP-conjugated (Santa Cruz Biotechnology) and anti-rabbit-IgG HRP-conjugated (Cell Signaling Technology, Leiden, The Netherlands) were used as secondary antibodies.

Calcineurin activity assay

Cell lysates were also used to measure their calcineurin activity with Cellular Calcineurin Phosphatase Activity Assay Kit (Abcam, Cambridge, UK). Calcineurin phosphatase activity was measured through the detection of released free phosphate. As positive control, human recombinant calcineurin is included. The contribution of calcineurin activity among other phosphatases was determined using the following formula: (Total phosphatase activity – Phosphatase activity with EGTA buffer)/ Total phosphatase activity). As calcineurin requires calcium for its activity, calcineurin activity is represented by total phosphatase activity minus phosphatase activity using EGTA buffer.

Statistical analysis

Significance of differences was determined by Mann–Whitney U-test when comparing two variables and by Kruskal–Wallis and Dunn's multiple comparison tests when comparing more than two variables. Wilcoxon signed rank test was used for comparison between two paired groups' analysis. Two-way ANOVA was used for comparing the mean differences between two

independent variables. The software packages GraphPad Prism (v 5.02) (GraphPad Software Inc., San Diego, CA, USA) and R (3.4.0 version) (RStudio Inc., Boston, MA, USA) were used for statistical analysis.

Results

Lin + and NK cells decrease while hILC remain stable after kidney or liver transplantation

We first enumerated Lin + cells, NK cells, and hILC subsets in CS and transplant recipients. Because the tacrolimus-based maintenance therapy is administered from day 1, and we had previously observed that two weeks after transplantation most cell subsets were still under the influence of induction therapy, we chose day 14 post-transplant samples to investigate the effect of immunosuppressive drugs. At 14 day posTx, KTR and LTR showed a reduction of Lin + cells and NK cells comparing to CS, whereas hILC absolute values were similar between transplant recipients and controls (Fig. 1b).

The number of ILC1, ILC2, and ILCP was also similar in KTR, LTR, and CS (Fig. 1c). In addition, ILC1, ILC2, and ILCP subsets within total hILC were similarly distributed in transplant recipients *vs* CS (Fig. 1d).

hILC subsets number or function remain unchanged from preTx to 14 day posTx samples

To further delineate the dynamics of these cell populations across transplantation, we assessed their absolute counts and their functionality as determined by cytokine production in paired preTx and day 14 posTx samples. For this analysis, a new, prospective cohort of unselected patients undergoing transplantation was included. Both liver and kidney transplant recipients were analyzed together, as a unique cohort of patients. Figure 2 shows that, for all cell types and all time points, the number of cells was not different between liver (red dots) and kidney (blue dots) patients. Thus, the type of recipient or the different transplanted organs do not affect differently the number of circulating hILC before transplantation and how these cells change during the first 14 post-transplant days.

Regarding the absolute cell numbers, Lin + cells (mean ± SD: preTx = 885.5 cel/µl ± 97.4; posTx = 638.8 cel/µl ± 67.8; *P*-value = 0.02) and NK cells (mean ± SD: preTx = 193.8 cel/µl ± 64.4; posTx = 119.9 cel/µl± 33.8; *P*-value = 0.03) significantly diminished whereas total hILC and the ILC1, ILC2, and ILCP subsets did not change (Fig. 2a). IFNγ, IL4 + IL13, and IL22 were measured as signature cytokines of ILC1, ILC2, and ILCP, respectively. While we detected IFN-γ and IL-4 + IL-13 on peripheral blood hILC of recipients and controls, IL-22 production was barely detected, further confirming that this ILC3 signature cytokine is not produced by ILCP (Fig. 2b). Similarly to that observed in hILC counts, the proportion of cells producing every tested cytokine and the production of every cytokine *per cell* did not change, either between preTx and day 14 posTx samples or when comparing these with CS (Fig. 2c and d). This result suggested that not only hILC numbers but also hILC function remained unaffected by immunosuppressive drugs used around transplantation.

Reduced expression of targets for immunosuppressive drugs in hILC

Because hILC absolute numbers and cytokine production remained unchanged after transplantation, we hypothesized that these cells are resistant to immunosuppressive therapy.

We analyzed the distribution of Lin + cells, NK cells, and hILC in KTR and LTR at day 14 posTx according to the immunosuppressive drug used for induction, either TMG or the anti-CD25 (IL-2 receptor) drug BAS. When compared to CS, day 14 posTx NK cells were significantly lower in recipients with no induction therapy, and the NK reduction was deeper in patients receiving TMG. However, in patients receiving BAS, NK cells were similar to controls despite these patients were also under tacrolimus-based maintenance therapy. Contrarily to observations in NK cells; ILC1, ILC2, and ILCP numbers did not differ among any of the groups (Fig. 3a).

We reasoned that the apparent resistance of hILC to TMG, BAS, and tacrolimus could be related to a low expression or lack of molecular targets for these drugs. TMG includes a variety of antibody specificities mainly against T cells [11]. In fact, after incubating PBMC with TMG, we observed that the binding of TMG to Lin + cells was almost four times higher than the binding to hILC (MFI: 11 164 vs 3604, respectively), this suggesting that targets for TMG antibodies are mostly found in Lin + cells (Fig. 3b). Interestingly, TMG bound to NK cells was intermediate between that of Lin + cells and hILC (MFI: 5930) (data not shown). When analyzing the expression of the BAS target CD25 in hILC from peripheral blood of healthy controls, we observed that only a portion of ILC2 were $CD25^{+}$ cells, whereas most ILC1 and ILCP did not express CD25 in their surface (Fig. 3c).

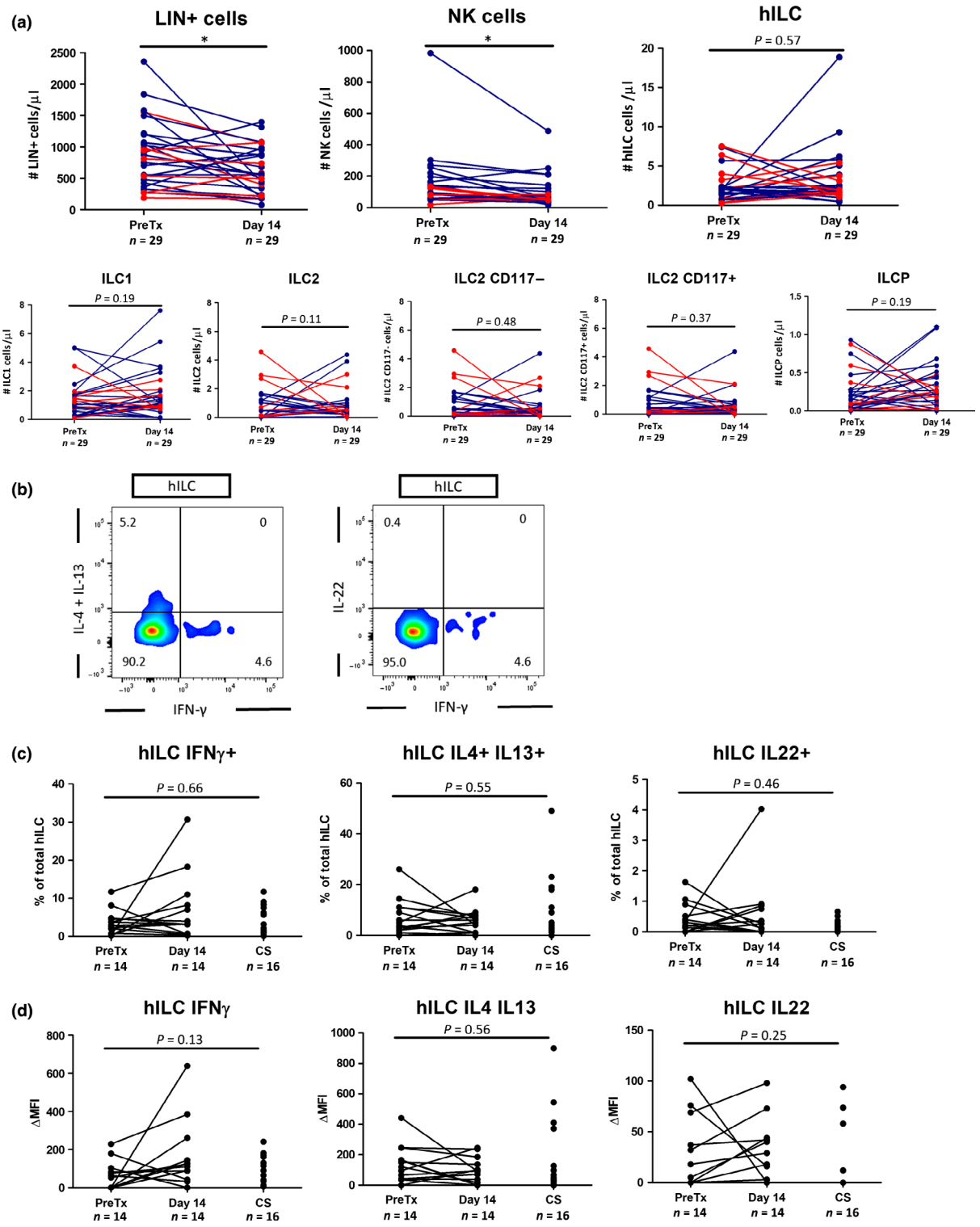

Figure 2 hILC showed no numerical neither functional changes between preTx and 14 day posTx. (a) Comparison of Lin + cells, NK cells, total hILC, and hILC subsets absolute numbers in kidney and liver transplanted recipients at preTx time *vs* 14 days posTx. KTR are represented in blue lines and LTR in red lines. (b) Representative dot plots of IFNγ, IL4, and IL13, and IL22 production by peripheral blood hILC. (c) hILC proportions producing cytokines in transplanted patients at preTx, day 14 posTx, and CS. (d) *Per cell* cytokine production in transplanted patients at preTx, day 14 posTx, and CS. Wilcoxon test was used for two paired groups' comparison and Kruskal–Wallis for more than two groups' comparison. **P*-value < 0.05. CS: Control subjects/ hILC: helper innate lymphoid cells/ KTR: Kidney transplanted recipients/ LTR: Liver transplanted recipients/ preTx: pretransplantation/ ΔMFI: mean fluorescence intensity difference between stimulated and unstimulated cells.

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To investigate the possible resistance to tacrolimus, the calcineurin pathway activity was analyzed in FC-sorted hILC and Lin^+ cells from human tonsils and in PBMC from healthy controls. The calcineurin phosphatase activity was significantly different among the three *ex vivo* tested cell populations. Calcineurin activity was highest in PBMC, intermediate in tonsillar Lin^+ cells, and lower than 10% of the total cellular phosphatase activity in hILC (Fig. 3d). According to these results, both the calcineurin protein and FKBP12 (target of tacrolimus) were strongly detected in PBMC, only FKBP12 but not calcineurin was detectable in tonsillar Lin^+ cells (mostly including B cells) and none of the targets were detected in hILC (Fig. 3e).

hILC counts in the early posTx are not associated with delayed graft function or rejection

Although no differences existed between hILC numbers of transplanted patients and CS, we asked if variations in hILC counts could be associated with renal delayed graft function (DGF) and biopsy-proven acute rejection (BPAR) in KTR and LTR. More than half renal transplant recipients experienced DGF, and BPAR rate in KTR and LTR was 7% and 15%, respectively (Table 1). In KTR, no differences were found in total hILC or hILC subsets counts at 14 days posTx between patients who suffered or did not suffer DGF (Fig. 4a), or between patients with and without BPAR during the first year posTx (Fig. 4b). Taking together KTR and LTR, hILC counts at 14 days posTx were also similar in patients with and without BPAR during the first year posTx (Fig. S3).

Discussion

The present work is the first one to study circulatory hILC in SOT patients, in particular kidney and liver recipients. The phenotype of circulatory hILC subsets has been determined by using antibodies against surface markers; however, the specific transcription factor for each hILC type has not been assessed, this being a limitation of our study. Here, we first recorded that at day 14 after transplantation, Lin + and NK cells were diminished whereas circulatory hILC numbers were similar to CS. This observation is consistent with our previous study in intestinal transplantation where we described that ILC persisted whereas T cells were depleted in the epithelium of intestinal grafts during the first 2 years posTx [6]. Moreover, by comparing circulatory cell counts at preTx and day 14 posTx, here we showed that NK cell numbers decreased while hILC subsets remained unchanged. The reduction of circulating NK cells early after transplantation has been observed in the past and has been correlated either with the depleting effect of TMG used as induction therapy or with the effect of calcineurin inhibitors for maintenance treatment [12–14]. In fact, we observed that patients without induction therapy had a significantly lower number of NK cells than CS, probably reflecting the effect of tacrolimus-based maintenance therapy. Recent works extend these observations by showing that after hematopoietic stem cell transplantation, calcineurin inhibitors used for the treatment of graft-versus-host disease (GVHD) impair the repopulation of NK cells while, importantly, hILC reconstitution is unaffected [15,16]. These results from previous literature are similar to the data obtained in this report.

Also, similar to other works, we observed that patients who received BAS had lower NK numbers than healthy controls [14,17]. However, NK numbers in BAS-treated patients were higher than in patients without induction, despite both, BAS-treated and BAS-untreated patients being under tacrolimus after transplantation. This observation suggests that by blocking the high-affinity IL2 receptor, the anti-CD25 antibody BAS could impair the activation of T cells, thereby increasing IL2 availability which would favor NK cells. Interestingly, NK cells were the first restored group of lymphocytes after treatment with BAS and etanercept in a case of steroid-resistant GVHD [18], and an expansion of $CD65^{bright}$ NK cells has been observed in patients with multiple sclerosis treated with the anti-CD25 antibody daclizumab [19,20].

Regarding hILC function, we observed that the proportions of hILC producing IFNγ, IL4 + IL13, and IL22 did not change at day 14 posTx, either when compared to preTx or to CS. The amount of cytokines synthesized by ILC1, ILC2, and ILCP remained also stable. Thus, hILC are not affected numerically neither functionally around transplantation, when the patients received the highest doses of immunosuppressive drugs.

ILC1, ILC2, and ILCP numbers from recipients at day 14 after transplantation were similar to controls independently of having received TMG, BAS, or no induction. The preferential location of hILC in peripheral tissues could contribute to the resistance of these cells to immunosuppression. However, recent reports demonstrate that hILC migrate and increase in circulation during inflammation and tissue repair [21–23], processes accompanying transplantation. Our observation of conserved hILC numbers and function in peripheral blood of SOT patients, where the

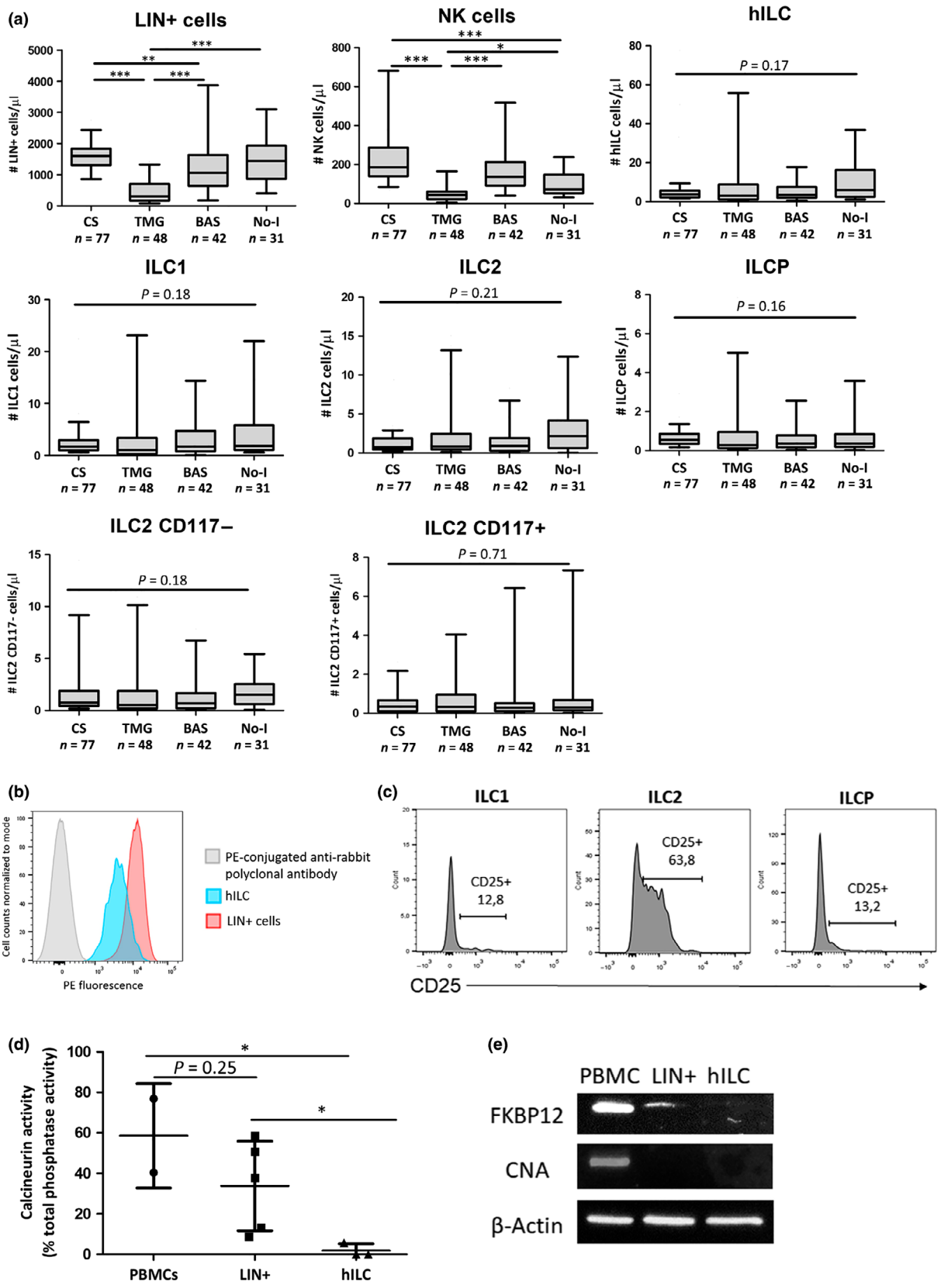

Figure 3 hILC are unaffected by immunosuppressive therapy and they have low levels or lack of targets for drugs. (a) Comparison of Lin + cells, NK cells, total hILC, and hILC subsets absolute numbers according to the induction therapy received (KTR and LTR are analyzed together). Bars show medians and whiskers show 10–90 percentile. Kruskal–Wallis and Dunn's multiple comparison tests were used for group comparisons. (b) Fluorescence graphs representing TMG bound to circulatory Lin + cells and hILC. (c) Representative graphs of CD25 expression in ILC1, ILC2 and ILCP. (d) Calcineurin activity assay in PBMC, FC-sorted tonsillar Lin + cells (T cells and B cells) and FC-sorted tonsillar hILC. Data are represented as mean ± SD. Each point represents a value obtained in an independent experiment. (e) Western blot for FKBP21 and calcineurin A protein in PBMC, FC-sorted tonsillar Lin + cells (T cells and B cells), and FC-sorted tonsillar hILC. Total protein load was measured by staining for β-actin. **P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001. BAS, Basiliximab; CS, control subjects; CAN, calcineurin A; hILC, helper innate lymphoid cells; KTR, kidney transplanted recipients; Lin+, lineage positive cells; LTR, liver transplanted recipients; No-I, no induction; PBMC, peripheral blood mononuclear cells; TMG, thymoglobulin.

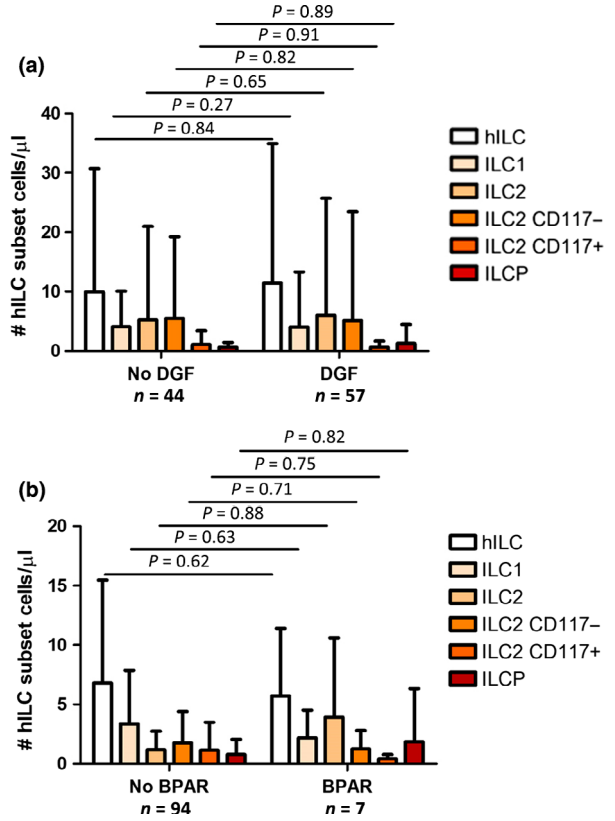

Figure 4 hILC counts are not associated with DGF or BPAR in KTR. (a) Absolute cell numbers of total hILC and hILC subsets are not different between renal recipients with or without DGF. (b) Absolute cell numbers of total hILC and hILC subsets are not different between KTR with or without BPAR. Bars show mean with SD. Mann–Whitney *U*-test was used for group comparisons. BPAR, biopsy-proven acute rejection; DGF, delayed graft function; hILC, helper innate lymphoid cells; KTR, kidney transplanted recipients.

concentration of drugs is high, further suggests intrinsic resistance of hILC to anti-rejection therapy.

We speculated that the seemingly insensitivity of hILC to TMG, BAS, or tacrolimus could be related to low expression or lack of molecular targets for these drugs. TMG antibodies are directed against resting or activated T cells and in a much lesser extent, against B, plasma, or antigen-presenting cells [11]. TMG depleting effect mostly affects T cells, and depletion of B or NK cells only occurs in the presence of very high doses [24]. Currently used TMG is made after immunization of rabbits with pediatric thymocytes, 95% of them being CD3 + cells [25].

We observed that TMG showed a reduced binding to hILC comparing to Lin + cells and NK cells. Thus, the low binding of TMG to hILC could explain the lack of hILC depletion and the maintenance of hILC numbers in patients treated with this drug.

By analyzing the expression of CD25 in hILC, we confirmed previous data showing that only a portion of blood ILC2 are CD25 + cells, whereas most ILC1 and ILCP do not express CD25 in their surface [26]. The lack of CD25 together with the fact that BAS is a non-depleting but a blocking antibody [27] could explain the maintenance of hILC numbers in patients treated with BAS. In addition, development and function of hILC mostly depends on IL7 but not on IL2 [26].

We were not able to detect FKBP12 or calcineurin in isolated hILC. Interestingly, previous experiments have shown a direct correlation between the amount of calcineurin inhibitors targets and sensitivity to immunosuppressors. Overexpression of cyclophilins and FKBPs, receptors for cyclosporine A and tacrolimus, respectively, increased T cells sensitivity to these drugs [28]. The inhibition of immunoglobulin production in T-B cell co-cultures after the addition of calcineurin inhibitors depended on interference of T-helper signals but not on a direct targeting of B cells [29], in agreement with the low FKBP12 and undetectable calcineurin detected by us in tonsillar Lin^+ cells, mostly including B cells [25,30]. Therefore, a low amount or lack of FKBP12 and calcineurin in hILC could explain the resistance of these cells to maintenance immunosuppressors. Isolated hILC showed also very low calcineurin activity, further suggesting that the activation capacity of these cells is more modest than that of T cells and/ or less dependent on the calcineurin pathway.

In this work, we did not address the analysis of the effect of steroids and MMF, also used in maintenance therapy, on ILC. Interestingly, we have observed that the accumulation of ILC in the intestinal grafts mucosa was more prominent in patients taking high steroid doses [5]. Since hILC are particularly quiescent, the anti-proliferative effect of steroids and MMF may show low or no impact on these cells. Knowledge of the effect of immunosuppressors on hILC could be expanded by analyzing *in vitro* cultures of isolated hILC in the presence of the drugs.

Regarding the graft evolution, we did not find a relationship between circulatory hILC and DGF or rejection. The study of larger cohorts could help to confirm these results, particularly given the low rate of BPAR among our patients. On the other hand, the persistence of hILC in SOT recipients could be relevant for the long-term function of transplants. Because long-term allograft survival is still suboptimal despite the chronic immunosuppression, mostly focused to T cells, other immune compartments such as hILC may be contributing to deterioration of the graft. The longitudinal study of posTx circulatory together with graft infiltrating hILC could shed light on that question.

Although hILC are mostly important as tissue-resident cells, data about their role into kidney or liver are still scarce. IL13- and IL5-producing ILC2 are a major renal ILC population in healthy mice and humans [31]. Interestingly, an ILC2-mediated induction of a type 2 response has been shown to protect kidneys from damage both in models of ischemic acute kidney injury or chronic proteinuric disease [31,32]. Intrahepatic hILC are better characterized, and pivotal roles of hILC subsets in liver inflammation, cancer, or fibrosis have been described [33–35]. Among hILC in healthy human livers, NKp44- ILC3 is the most prominent subset, but increased ILC2 have been associated with human liver fibrosis [36,37].

Thus, the distribution of the three hILC subsets in the graft could be relevant in driving the immune response toward a pro-inflammatory state (rejection), an anti-inflammatory state (homeostasis-tolerance), or fibrosis (graft deterioration). If the resistance of hILC to current standard immunosuppression is confirmed, these cells may emerge as important players contributing to either reparation and homeostasis or chronic deterioration of allografts.

Authorship

EG-M, AU-R: performed the experimental work. AA, NP: recruited and followed up the renal transplanted patients. IJ, CJ: recruited and followed up the liver transplanted patients. AL: helped with cell sorting. RL-G, EM, DP: analyzed the results. EP-A, PT: designed the research and wrote the paper.

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Conflicts of Interest

The authors of this manuscript have no conflicts of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Id2 expression in hILC.

Figure S2. hILC and Lin+ isolation by FC-sorting.

Figure S3. hILC counts are not associated with BPAR in KTR plus LTR.

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