Transplant International

ORIGINAL ARTICLE

Donor helper innate lymphoid cells are replaced earlier than lineage positive cells and persist long-term in human intestinal grafts – a descriptive study

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SUMMARY

Intestinal grafts carry large donor lymphoid load that is replaced by recipient cells. The dynamics of this process may influence the tolerance, rejection or graft-versus-host disease. We analysed distribution and turnover of T and B (Lin+) lymphocytes, natural killer (NK) and helper innate lymphoid cells (hILC) in intestinal epithelium (IEp) and lamina propia (LP) from a long-term cohort of eight intestinal recipients and from a single patient monitored deeply during the first 8 months post-transplant (posTx). Long-term intestinal grafts showed significantly higher %hILC than native bowels in IEp and LP until 10 years posTx and recovery to normal levels was observed afterwards. We also observed an imbalance between hILC subsets in IEp [increase of type 1 (ILC1) and decrease in type 3 (ILC3) innate lymphoid cells] that persisted along posTx time even when %hILC was similar to native bowels. Regarding hILC origin, we still detected the presence of donor cells at 13 years posTx. However, this chimerism was significantly lower than in Lin+ and NK populations. According to these findings, observation from the patient monitored in early posTx period showed that recipient hILC repopulate earlier and faster than Lin+ cells, with increase in ILC1 related to rejection and infection episodes.

Transplant International 2020; 33: 1016–1029

Key words
chimerism, helper innate lymphoid cells, intestinal transplantation, intraepithelial lymphocytes, lamina propria lymphocytes

Received: 18 February 2020; Revision requested: 4 March 2020; Accepted: 25 March 2020;
Published online: 26 April 2020

Introduction

Intestinal transplantation (ITx) is the most immunogenic among solid organ transplants (SOT) because of its large lymphoid load [1,2]. Because of its condition of immunologically reactive graft, ITx shows a higher rate of rejection [3-5] and graft-versus-host disease [6,7]. Other complications such as post-transplant lymphoproliferative disease [8-10] or infections [11-15] are also more frequent in ITx because of the higher levels of immunosuppression used compared with other solid organ transplants.

Innate lymphoid cells (ILC) are recently described as lymphocytes that lack rearranged antigen receptors and lineage (Lin) markers [16]. ILC include cytotoxic ILC

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doi:10.1111/tri.13609

[natural killer (NK) cells] and helper ILC (hILC), the later consisting of three main groups regarding cytokine pattern production and transcription factor expression [17-19]. ILC1 typically express the transcription factor T-bet and produce IFNγ, so they are involved in inflammation and defense against the intracellular pathogens [20]. ILC2 produce IL-4, IL-5, IL-13, and amphiregulin, express the transcription factor GATA3 and are implicated in immunity to helminths and tissue repair [21,22]. ILC3 participate also in tissue repair and play an important role in extracellular bacteria immunity. They produce IL-22 and IL-17A, express RORγt and are the most heterogeneous group with at least three distinct subtypes which include lymphoid tissue inducer (LTi) cells, ILC3 NCR− and ILC3 NCR+ [23,24].

hILC have been identified in various locations such as lymphoid tissue [23], gut [23,25], lung [26], skin [27], adipose tissue [28] as well as in peripheral blood (PB) [17]. In gut, ILC1 and ILC3 are the most represented hILC subsets [29]. Regarding ILC1, two subsets can be distinguished in intestine: intraepithelial ILC1 and lamina propria (LP) ILC1 [30]. Intraepithelial ILC1 are cytotoxic, CD127−/low and express CD103, NKp44, NKp46 and CD56 [25]. In contrast, LP ILC1 express CD127 but not NKp44 and CD56, and produce higher levels of IFNγ [30]. Regarding ILC3, three subsets have been identified in gut. LTi cells are CCR6+ NKp44− and the first hematopoietic cells recruited in the foetal gut (responsible for the formation of Peyer Patches, cryptopatches and isolated lymphoid follicles) [24]. ILC3 NCR− mainly produce IL-17A and are considered ILC3 NCR+ precursors [18] whereas ILC3 NCR+ are the main innate source of IL-22 in mucosa [23].

The studies about ILC in ITx are scant. Our group was the first to describe that during the first two years after transplantation, ILC become the most prominent lymphoid population in intestinal epithelium (IEp) while there is a profound depletion of T cells [31]. Later, the group of Columbia has analysed the hILC turnover in ITx. Firstly, Zuber et al. [32] demonstrated that recipient replacement of T cells (CD45+ CD3+) was slower than that of NK cells or hILC (CD3− CD56+) in IEp and LP of intestinal grafts. Then, Weiner et al. [33] showed that donor-ILC can persist 8 years posTx in the LP.

Considering these data together with the fact that chimerism is crucial for rejection occurrence [5,34], we hypothesized that the identification of changes in ILC distribution and origin could provide new potential biomarkers associated with the graft outcome. In this work, we expand our previous study to LP and blood compartments, making a more accurate characterization of ILC and their donor or recipient origin in a longer observational period. We report that lymphoid distribution remains different from native intestines not only in IEp but also in LP until 10 years post-transplantation (posTx), except intraepithelial ILC1 and ILC3 which remain unbalanced. Moreover, we show that donor hILC persist in intestinal grafts up to 13 years posTx and recipients' hILC infiltrate the graft earlier and faster than Lin+ cells.

Materials and methods

Patients and controls

Twenty-eight adult patients received a small bowel (SBT) or multivisceral transplant (MVT) in our hospital between 2005 and 2018 but only 14 of them were alive in the study period. Among these 14 patients, nine were included in our study as we dispose of the proper antibody combination. Clinical data of the nine studied recipients are summarized in Table 1.

Patients transplanted more than 3 years ago (P1–P8) were monitored by endoscopic and biopsy controls once a year or when clinical events appeared. Number of studied biopsies for each patient, their collection posTx time and their paired histological diagnosis are listed in Table 2.

Patient P9 was monitored by endoscopic and biopsy controls once a week or when clinical events appeared from the first day after transplantation to almost 9 months posTx (Table 3).

As control group, we included ileal biopsies from eight native intestines from deceased donors.

In addition, PB from all patients as well as different 12 healthy controls was analysed.

Experiments were approved by the institutional review board (CEIC 13/370) and written informed consent was obtained from all patients and controls.

Immunosuppression protocol

In patients transplanted more than 3 years ago (P1–P8), the main immunosuppressive drug for maintenance therapy was tacrolimus except in patients P5 and P6 which was everolimus (Table 2).

Immunosuppression protocol of patient P9 is summarized in Table 3.

Biopsy collection and intestinal lymphocytes isolation

Biopsies from ileal mucosa were obtained for each time point at 10, 15 and 20 cm from ileostomy or from

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Table 1. Clinical data from small bowel and multivisceral transplant recipients.

Patient	Underlying disease	Gender	Age at Tx	Organ Tx	PosTx follow-up period (years)	Bx (*n*)
P1	Intestinal pseudo-obstruction syndrome	Female	31	MVT	3.4	1
P2	Desmoid tumour	Male	41	SBT	6.0–6.8	5
P3	Desmoid tumour	Male	44	SBT	6.3–6.7	2
P4	Intestinal obstruction	Female	53	SBT	7.8	1
P5	Acute mesenteric ischemia	Male	65	SBT	8.8–9.4	2
P6	Gardner syndrome	Female	32	MVT	11.3–12.6	3
P7	Crohn's disease	Male	38	SBT	11.2–12.2	2
P8	Acute mesenteric ischemia	Male	45	SBT	12.2	1
P9	Desmoid tumour	Female	60	SBT	0–0.7	27

Bx, number of biopsies obtained and studied during the posTx follow-up period; MVT, multivisceral abdominal transplantation; SBT, small bowel transplantation; Tx, transplantation.

ileocolic anastomosis when the ileostomy was closed. Mucosal fragments were taken in abnormal areas when evident or in random normal areas in case of no apparent injury. A total of 3–6 fragments were obtained from each biopsy procedure. All fragments were placed in saline solution 0.9% at room temperature until their processing.

In a maximum of 15 min after collection, all the biopsy fragments obtained from each patient at a single time point were pooled together in complete medium containing RPMI, 10% FCS, 1% Glutamine, 1% Penicilline/Streptomicine, 1mM EDTA and 1mM DTT for 1 h to obtain intraepithelial lymphocytes (IEL) as previously described [35]. Then, biopsies were disrupted with 1 mg/ml Collagenase D (Sigma-Aldrich, Darmastadt, Germany) for 1 h to obtain LP lymphocytes (LPL) [36].

Flow cytometry

Peripheral blood mononuclear cells (PBMC), IEL and LPL were resuspended in FACS buffer and stained with the antibodies detailed in Table S1. Cells were acquired on BD Canto II (BD Biosciences, San Jose, CA, USA) and analysis was performed with FlowJo software (TreeStar Inc, Ashland, OR, USA).

Lymphocytes were gated according to their complexity (SSC) and CD45 expression. Then, they were divided according to Lin, CD16 and CD62L markers. Lin+ cells included T and B cells, Lin− CD16+ CD62L+ cells included NK cells and Lin− CD16− CD62L− included hILC [17,37,38] (Fig. 1a). The rationale of this panel was to maintain the same gating strategy in all the studied compartments (IEp, LP and PB) as ILC1 in IEp are CD127−/low [25]. hILC subsets were distinguished according to CRTH2 and CD117 as follows: ILC1 (CRTH2− CD117−), ILC2 (CRTH2+ CD117−/+) and ILC3 (CRTH2− CD117+) [17] (Fig. 2a). In PB, hILC CRTH2− CD117+ were considered as ILC precursors (ILCP) which was described by Lim et al. [39]. As the starting material was different between each patient, population data were represented as percentage in order to homogenize the results.

Recipient and/or donor cells were identified by using antibodies against selected mismatched HLA antigens (Table S2 and Fig. S1).

HLA typing

Donor typing was performed by microlymphocytotoxicity assay for HLA-A and B (Lambda Monoclonal Typing Tray Set; One Lambda, Los Angeles, CA, USA) until October 2015 and thereafter by Sequence Specific Primers Polymerase Chain Reaction (SSP-PCR) including also HLA-C typing (Micro SSPTM Generic HLA Class I DNA Typing Tray; One Lambda). HLA-DR and DQ typing was performed by SSP-PCR (Micro SSPTM Generic HLA Class II DNA Typing Tray; One Lambda). Recipient typing was performed by Sequence Specific Oligonucleotide Primed Polymerase Chain Reaction (SSO-PCR) for HLA-A, B and DR (One Lambda).

Statistical analysis

For quantitative variables, Student's *T* or Mann–Whitney *U* tests were used when comparing two variables and ANOVA test or Kruskal–Wallis and Dunn's multiple comparison tests were used when comparing more than two variables. The correlation between two variables was analysed by Spearman test and graphically represented by scatter plot and regression line. *P* values

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Table 2. Donor cells proportions in long-term intestinal grafts (patients P1–P8).

					% Donor cells				
ID	Years posTx	Histological diagnosis	IS	Compartment	Lin+	NK	hILC	ILC1	ILC3
P1	3.4	Minimal changes	FK + MMF	IEp	10	45	<1	<1	3
				LP	5	12	<1	<1	13
P2	6.0	Minimal changes + fungal infection + CMV infection	FK	IEp	4	55	2	2	<1
				LP	9	5	<1	<1	<1
	6.1	Minimal changes + fungal infection + CMV infection	FK	IEp	5	41	4	4	24
				LP	6	12	3	4	27
	6.4	Minimal changes + inflammation	FK	IEp	6	19	5	4	7
				LP	21	20	7	9	8
	6.6	Minimal changes	FK	IEp	1	<1	<1	<1	<1
				LP	<1	1.1	5	4	5
P3	6.3	Minimal changes	FK	IEp	38	4	<1	<1	<1
				LP	31	<1	<1	<1	<1
	6.7	Minimal changes	FK	IEp	40	4	<1	<1	<1
				LP	51	<1	<1	<1	<1
P4	7.8	Lymphoid follicular hyperplasia	FK + MMF	IEp	10	49	3	3	3
				LP	8	28	4	3	4
P5	8.8	Minimal changes	EV	IEp	43	<1	2	<1	<1
				LP	63	58	1	2	10
	9.4	Minimal changes	EV	IEp	62	4	2	<1	3
				LP	69	3	3	2	<1
P6	11.3	Minimal changes	EV	IEp	<1	38	1	1	NA
				LP	<1	23	2	<1	<1
	11.6	Intraepithelial lymphocytosis	EV	IEp	8	6	2	5	4
				LP	2.4	1.2	<1	<1	<1
	12.6	Minimal changes	EV	IEp	<1	30	<1	<1	<1
				LP	<1	1.1	1.1	<1	<1
P7	11.2	Minimal changes	FK + CS	IEp	<1	7	NA	NA	NA
				LP	4.7	28	18	15	<1
	12.2	Minimal changes	FK	IEp	<1	3	2	<1	<1
				LP	<1	7	<1	<1	<1
P8	12.2	Minimal changes	FK	IEp	<1	<1	9	7	NA
				LP	<1	5	11	8	NA

CMV, cytomegalovirus; CS, corticosteroids; EV, everolimus; FK, tacrolimus; IEp, Intestinal epithelium; IS, immunosuppression; LP, lamina propria; MMF, mycophenolate mofetil; NA, chimerism was not assessed due to low number of cells; PosTx, post-transplant.

<0.05 were considered statistically significant. The software package GRAPHPAD PRISM (v 5.02) was used for statistical analysis.

Results

hILC are significantly increased in IEp and LP until 10 years posTx

We studied the distribution of Lin+ cells, NK cells and hILC among IEL, LPL and PBMC in recipients with more than 3 years posTx (P1–P8) and healthy controls (eight for native intestines and 12 for PB). No differences were observed in gender distribution, age neither IEL nor LPL infiltration (defined as percentage of CD45+/SSC cells, data not shown) between the control group and recipients.

Regarding Lin+ population frequency, intestinal grafts showed a significant decrease when compared with native intestines in both IEp and LP. Correlating with this, hILC frequency was significantly higher in both compartments (Fig. 1a,b). NK cells showed also a significant increase in IEp and a trend towards higher frequency in LP in transplanted versus native intestines (Fig. 1b). However, these differences in the distribution of lymphoid populations were restricted to the graft, as no changes were observed in PB despite on the immunosuppression received (Fig. S2a).

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Table 3. Histological diagnosis, immunosuppressive therapy from patient P9.

Days PosTx	Histological diagnosis	Induction/rejection treatment	Maintenance IS
0	–	TMG (4 doses) + BAS (2 doses) + CS + Plasmapheresis	FK + CS
2	Minimal changes	Rituximab (1)	FK + CS
6	Minimal changes		FK + CS
9	Inflammation		FK + CS
13	Minimal changes		FK + CS
16	Minimal changes		FK + CS
20	Minimal changes		FK + CS
23	Minimal changes		FK + CS
27	Indeterminate changes for rejection		FK + CS
30	Indeterminate changes for rejection		FK + CS
41	Indeterminate changes for rejection		FK + CS + EV
48	Indeterminate changes for rejection		FK + CS + EV
55	Minimal changes		FK + CS + EV
62	Indeterminate changes for rejection		FK + CS + EV
76	Acute rejection grade 1		FK + CS + EV
90	Minimal changes		FK + CS + EV
118	Indeterminate changes for rejection		FK + CS + EV
174	Acute rejection grade 1–2	CS bolus	FK + CS + EV
188	Acute rejection grade 2–3		FK + CS + EV
195	Acute rejection grade 2–3		FK + CS + EV
202	Acute rejection grade 3 + CMV infection	BAS (2 doses) + CS bolus	FK + CS + EV
209	Acute rejection grade 3 + CMV infection	TMG (5 doses)	FK + CS + EV
216	Acute rejection grade 2–3		FK + CS + EV
223	Indeterminate changes for rejection		FK + CS + EV
237	Reparative mucosa		FK + CS + MMF
244	Indeterminate changes for rejection		FK + CS + MMF
251	Indeterminate changes for rejection		FK + CS + MMF
258	Indeterminate changes for rejection		FK + CS + MMF

BAS, basiliximab; CMV, cytomegalovirus; CS, corticosteroids; EV, everolimus; FK, tacrolimus; IS, immunosuppression; MMF, mycophenolate mofetil; PosTx, post-transplant; TMG, thymoglobulin.

Interestingly, lymphoid subsets data from recipients seemed to show a bimodal distribution (especially in Lin+ and hILC). Analysing the possible effect of posTx time, we observed that, indeed, it correlated significantly with a progressive increase of Lin+ cells and a decrease of NK cells and hILC frequencies (Fig. 1c). Because the correlation plots also showed two differentiated groups (below and above 10 years posTx), we analysed them separately: <10 years posTx (patients P1–P5) or >10 years posTx (patients P6–P8; Fig. 1d). In this case, we observed that significant differences in Lin+ cells, NK cells and hILC proportions were only observed in patients with <10 years posTx. However, intestine grafts recovered the IEL and LPL subset distribution of native bowels after 10 years.

We also evaluated the frequencies according to the main maintenance immunosuppressive therapy (tacrolimus versus everolimus). Although differences were not significant, we noticed a lower Lin+ cells frequency and higher hILC frequency in tacrolimus treated patients (P1–P4, P7 and P8) compared with patients treated with everolimus (P5 and P6; Fig. S3a).

Imbalance of hILC subsets in IEp of transplanted intestines

When we studied the distribution of hILC subsets, we observed that transplanted intestines showed significantly higher ILC1 and lower ILC3 frequencies than native intestines leading to an imbalance of hILC subsets (ratio ILC1/ILC3) in IEp (Fig. 2a,b). This finding was limited to the epithelial compartment, since no differences were observed in LP and blood (Fig. S2b). Regarding ILC2, similar frequency was observed in transplanted and native intestines in IEL and LPL: low or undetectable as already described (Fig. 2a,b) [29].

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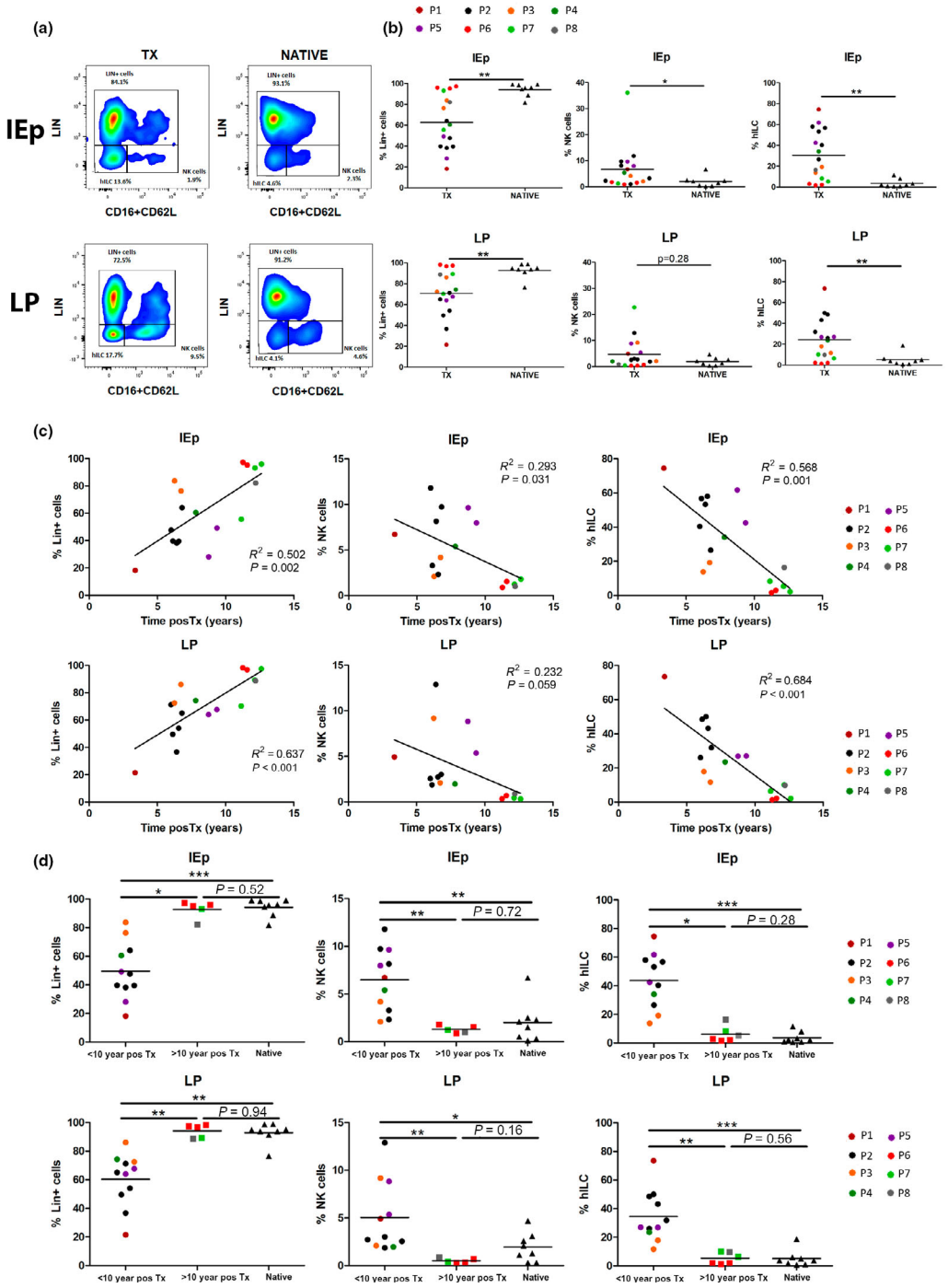

Figure 1 Intraepithelial lymphocytes (IEL) and LP lymphocytes (LPL) subsets distribution is different in transplanted versus native intestines until 10 years posTx. (a) Representative flow cytometry dot plots from Lin+ cells, NK cells and helper innate lymphoid cells (hILC) in intestinal epithelium (IEp) and lamina propia (LP) of transplanted and native intestines. (b) Statistical analysis of Lin+ cells, NK cells and hILC in IEp and LP of transplanted and native intestines. In transplanted intestines, each patient is represented with a different colour (legend). Each dot corresponds to a biopsy procedure in a single time point. (c) Correlation between Lin+ cells, NK cells and hILC frequencies and time posTx (years) in IEp and in LP. In transplanted intestines, each patient is represented with a different colour (legend). Each dot corresponds to a biopsy procedure in a single time point. (d) Statistical analysis of IEL and LPL subsets from patients transplanted <10 years posTx, patients >10 years posTx and native intestines. In transplanted intestines, each patient is represented with a different colour (legend). Each dot corresponds to a biopsy procedure in a single time point. **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001. Bars show means.

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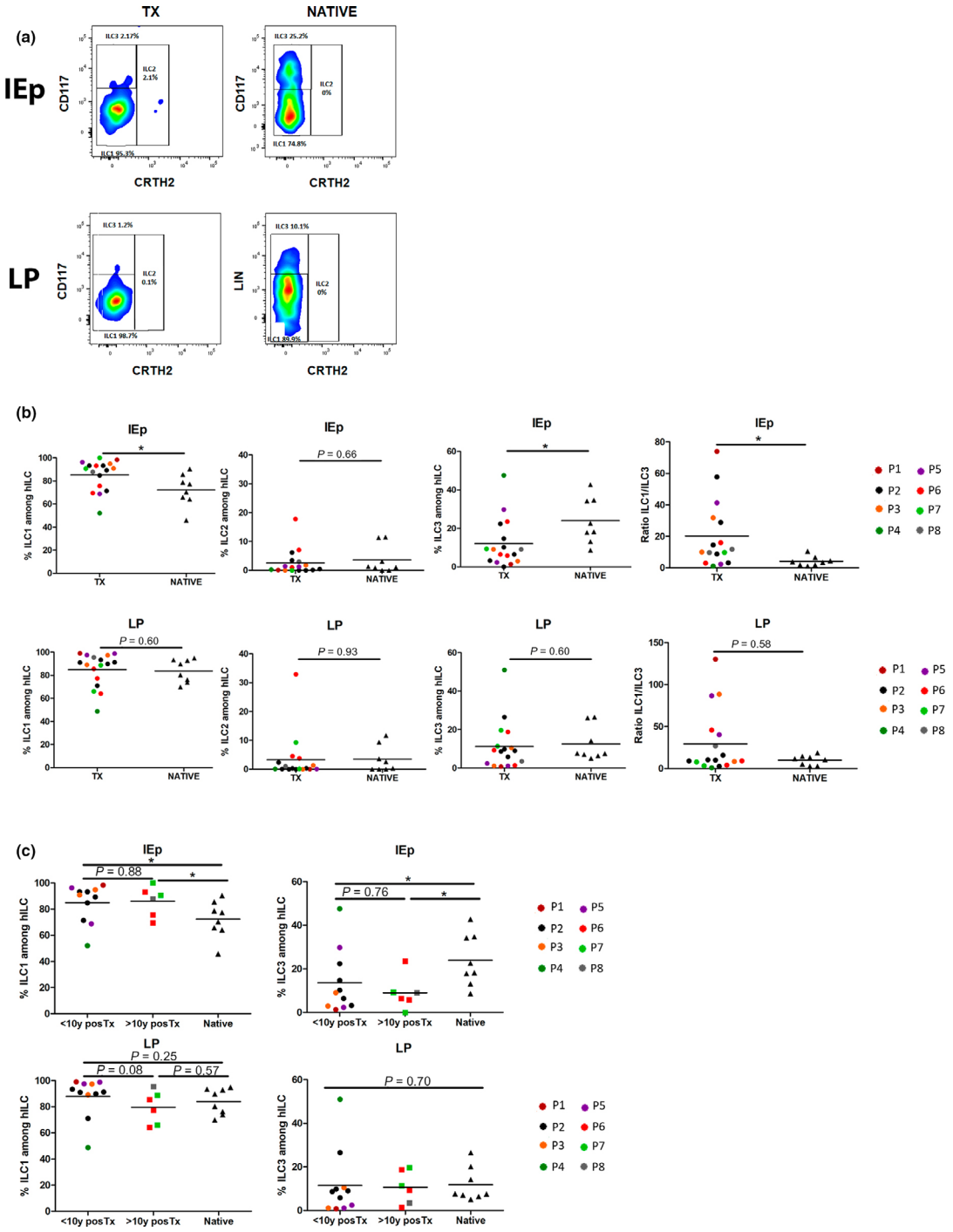

Figure 2 Helper innate lymphoid cells (hILC) subsets are imbalanced in the intestinal epithelium (IEp) of transplanted intestines. (a) Representative dot plot from ILC1, ILC2 and ILC3 gated from hILC in transplanted and native intestines. (b) Statistical analysis of hILC subset frequencies and ratio ILC1/ILC3 in IEp and lamina propia (LP) from transplanted and native intestines. In transplanted intestines, each patient is represented with a different colour (legend). Each dot corresponds to a biopsy procedure in a single time point. (c) Statistical analysis of hILC subsets in IEp and LP from patients transplanted <10 years posTx (patients P1–P5), patients >10 years posTx (patients P6–P8) and native intestines. Each patient is represented with a different colour (legend). Each dot corresponds to a biopsy procedure in a single time point. **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001. Bars show means.

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Analysing recipients according to posTx time, we observed that differences in ILC1 and ILC3 in the IEp persisted beyond 10 years posTx (Fig. 2C). Thus, IEp in long-term grafts did not recover the hILC subset distribution of native bowels even the percentage of total hILC did so.

The type of maintenance immunosuppressive therapy also showed to have no effect on hILC subsets distribution (Fig. S3b).

Donor hILC persist up to 13 years posTx

We further studied the persistence of donor cells among Lin+ cells, NK cells and hILC in IEp, LP and blood of long-term intestinal recipients P1–P8.

Patients transplanted between 3 and 10 years ago (P1–P5) showed persistence of donor Lin+ cells above 1% (average IEp: 6%; average LP: 8%). In contrast, in patients transplanted more than 10 years ago (P6–P8), donor Lin+ cells were detected but in a very low frequency, in most samples below 1% (average IEp and LP: 2%) (Table 2; Fig. 3a).

The persistence of donor NK cells was very variable in IEp and in LP (Fig. 3b), showing high levels in patients P2 (<1–55% in IEp and 1–20% in LP), P4 (49% in IEp and 28% in LP) and P6 (6–38% in IEp and 1–23% in LP; Table 2). These high levels of NK chimerism could be related to CMV and fungal infection (pathogen detected in biopsy) in P2 and lymphoid follicular hyperplasia (LFH) in P4. Interestingly, also P2 and P6 (but not any other patient) showed repeated episodes of LFH along their posTx history.

hILC resulted in the least chimeric population, with the donor frequency being significantly lower than in Lin+ and NK cells (Fig. 3D). Although donor cells were almost absent in three of eight patients (Table 2), they were still detectable up to 13 years posTx in particular patients (Fig. 3C). However, no association with clinical outcome could be determined as the chimerism in hILC was residual.

Owing to the low number of hILC subsets, chimerism in hILC subsets could not be assessed in three of eight patients (P6, P7 and P8). In those patients whose analysis was possible, donor cells represented 1–7% among ILC1 and 3–24% among ILC3 in IEp and 2–9% among ILC1 and 4–27% among ILC3 in LP (Table 2). Although no significant differences were observed between ILC1 and ILC3 in IEp either LP, donor cell frequencies in ILC3 were higher than in ILC1 in three of eight patients (P1, P2, P5; Table 2, Fig. S4).

No correlation was found between Lin+ cells, NK cells or hILC chimerism and histologic diagnosis, immunosuppressive therapy, number of HLA mismatches and type of transplant (MVT versus SBT).

In blood, donor cells frequency was <0.01% in all lymphoid populations (data not shown).

Recipient hILC repopulate earlier and faster than recipient Lin+ cells

To study the repopulation dynamics in detail, we monitored PBMC, IEL and LPL in patient P9 from day 2 to day 258 posTx.

During the first 15 days posTx, Lin+ cells suffered a marked reduction in blood, IEp and LP compartments, probably because of the induction therapy with thymoglobulin, basiliximab and rituximab, whereas hILC persisted and became more represented within the intestine and PB. A new decrease in Lin+ cells was also seen in all compartments since day 209 posTx when thymoglobulin was administered again as treatment for rejection (Fig. 4a).

Considering the histological diagnosis from biopsies (Table 3), we observed that until day 27 posTx biopsies were classified as minimal changes, from day 27 to 118 as indeterminate for rejection (IRx), from day 174 to 216 as acute rejection and CMV infection and from day 223 to 258 as IRx again. During the first month posTx, when biopsies were classified as minimal changes, we observed that ILC3 had an important representation (around 30%) among hILC in IEp and LP. However, this subset decreased substantially when IRx and CMV infection were identified, with ILC1 being the most represented subset in IEp and in LP (≈90% of hILC; Fig. 4b). This ILC1 predominance was mainly because of recipient cells infiltration and it was maintained during acute rejection (Fig. 5b).

Regarding the chimerism, recipient NK cells and hILC started to infiltrate the grafts IEp and LP during the first posTx days, when biopsies were classified as minimal changes. In contrast, recipient Lin+ cells could not be detected until the third week posTx and this infiltration was linked to IRx diagnosis (Fig. 5a). In addition, we observed that recipient hILC represented more than 80% of total hILC from day 90 posTx in IEp and from day 195 posTx in LP. However, in the Lin+ cells, a similar level of recipient cells infiltration was not achieved until day 216 posTx in IEp as well as in LP (Fig. 5a). No differences were observed in recipient infiltration between ILC1 and ILC3 (Fig. 5b).

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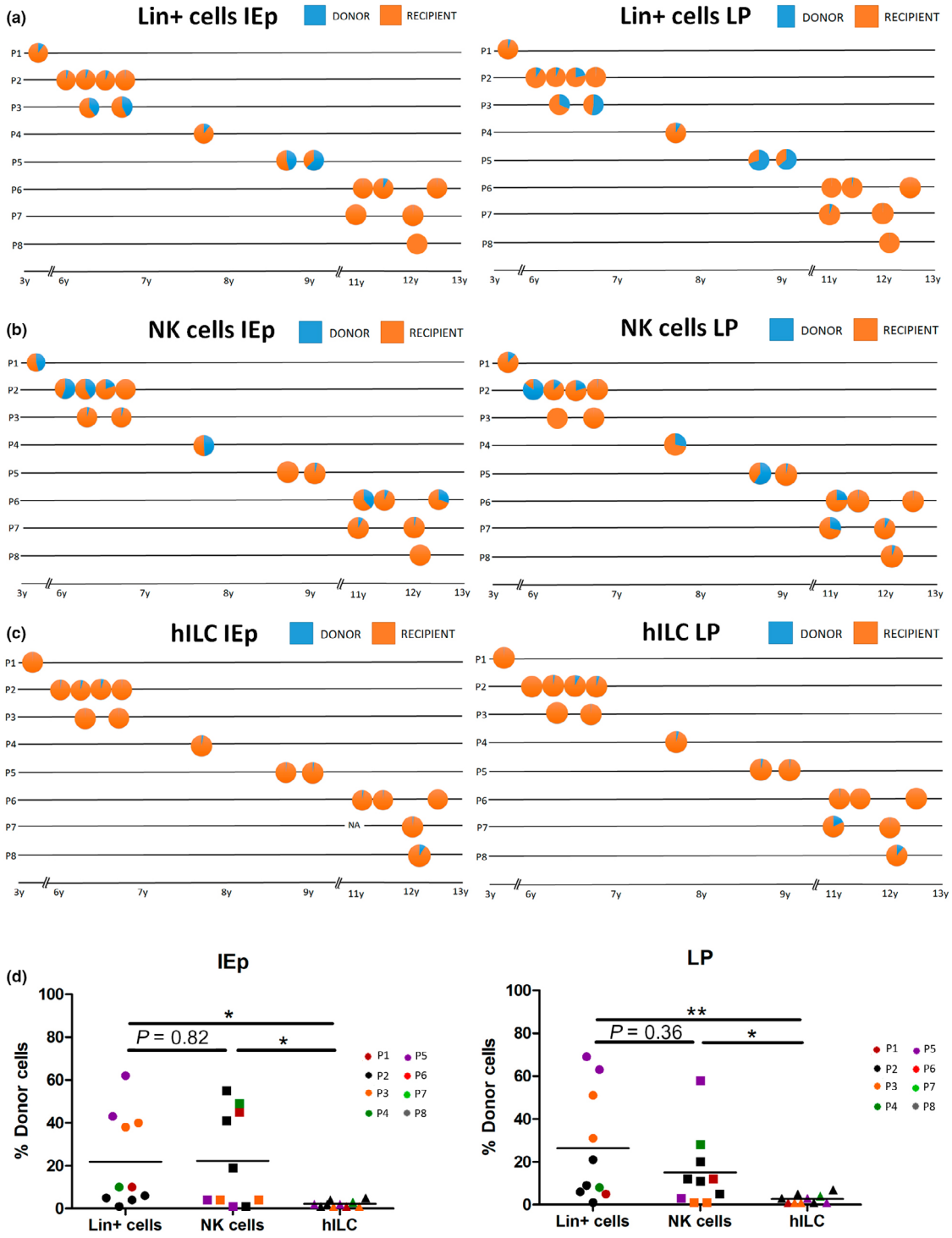

Figure 3 Donor Lin+ cells persist longer than hILC and are detectable until 13 years posTx. (a–c) Frequency of recipient and donor lymphoid populations (Lin+ cells, NK cells and hILC) in IEp and LP from patients P1–P8. (d) Statistical analysis of donor cells frequency in IEL and LPL subsets from patients <10 years posTx (patients P1–P5). Each patient is represented with a different colour (legend). Each dot corresponds to a biopsy procedure in a single time point. NA, Chimerism was not assessed because of low number of cells; ns, not significant. **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001. Bars show means.

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In the analysis of PBMC, macrochimerism (>1% of donor cells) could be identified during the first month posTx. Donor Lin+ cells were detected in all determinations whereas donor NK cells and donor hILC were detected in isolated cases (Fig. S5).

Discussion

The study of lymphoid populations in intestinal grafts and their replacement by recipient cells remains one of the most interesting topics in ITx. However, the technical difficulties to address these studies make the literature scant.

Regarding the study of lymphoid populations, our group was the first to describe the different composition in epithelium of transplanted bowels versus native intestines, showing an ILC increase at least during the first two years posTx [31]. In the present work, we have expanded the ILC study to other compartments (LP and blood) with a more accurate characterization (differentiating among NK cells, hILC and their subsets) in an extended follow-up period (up to 13 years posTx, which is the longest reported). According to the results previously obtained by our group, we have found that not only IEL but also LPL subsets distribution was different in transplanted versus native bowels. Similarly to what we described in IEL, a lower Lin+ cells frequency (which are mainly T cells in IEp and include T and B cells in LP) and a higher proportion of NK cells and hILC (reported in our previous work as CD3− lymphocytes) were observed in the LP. One of the remarkable findings of this work is that these changes in IEL and LPL distribution were progressively recovered in both compartments, reaching native levels from 10 years posTx onwards. Considering that all patients had a well-functioning graft, the progressive normalization of IEL and LPL distribution could be contemplated as a potential biomarker for long-term stable graft function and tolerance.

In the serial analysis of patient P9, we could also observed the reduction of Lin+ cells frequency with the increase of hILC in IEp, LP and blood along posTx time, which is mainly associated with thymoglobulin effect on T and B cells. The reduction of Lin+ cells but not of hILC is supported by our recent observation that number and function of circulatory hILC are not affected by immunosuppressive drugs in kidney and liver transplanted recipients [40]. This resistance of hILC becomes then crucial in intestinal grafts, where a deep depletion of Lin+ cells commits the integrity and functionality of the organ, especially in the early postTx time.

Therefore, the distribution of hILC subsets might be relevant because of the pro-inflammatory and pro-tolerogenic roles of ILC1 and ILC3 respectively. Here, we observed that the long-term transplanted intestines showed significantly higher ILC1 and lower ILC3 frequencies than the native intestines in IEp but this imbalance was not recovered to native distribution. Interestingly, this alteration was maintained even in those recipients who recovered the percentage of total hILC. Considering that all long-term recipients have a well-functioning graft and none of them suffered rejection during the period of the study, the ILC1–ILC3 imbalance could not be linked to a poor outcome or specific clinical events. The fact that this was observed only in IEp but not in LP could be a sign of a specific and local adaptation of this compartment. Some studies have described changes in ileal microbiota in the context of ITx [41-44] that affect directly to IEL subsets and epithelial cells. Thus, the microbiota may elicit signals that promote the upregulation of the different hILC subsets.

In contrast with the long-term cohort, in patient P9 we identified an ILC1 expansion—coincident with the appearance of acute rejection and CMV infection. The role of ILC1 in controlling CMV at sites of initial infection in response to local pro-inflammatory cytokines has been already described [45-48]. Since rejection and CMV infection affect the whole intestinal mucosa, changes in hILC subsets are observed not only in IEp but also in LP. This last observation is remarkable as the localization of the hILC subset imbalance could be used as a biomarker of graft stability, being related to clinical events and poor outcome when is observed also in LP.

Regarding chimerism, only the group of Columbia has addressed this issue [32,33]. However, these works did not allow to have a complete view of the dynamics turnover of all lymphoid populations in parallel in IEp and LP. Thus, the present work is the first one in studying the chimerism in Lin+ cells, NK cells and hILC in IEp, LP and blood in ITx recipients.

In the long-term cohort (P1–P8), we observed that donor lymphoid cells are still detected in IEp and LP up to 13 years posTx, which is the longest period of study reported. In most of the long-term transplanted patients (P1–P7), donor Lin+ cells persisted in higher frequency than donor hILC. This observation is opposed to what is described by Weiner et al. [33], as they reported higher donor frequencies in hILC than in T cells. In addition, we observed that donor hILC

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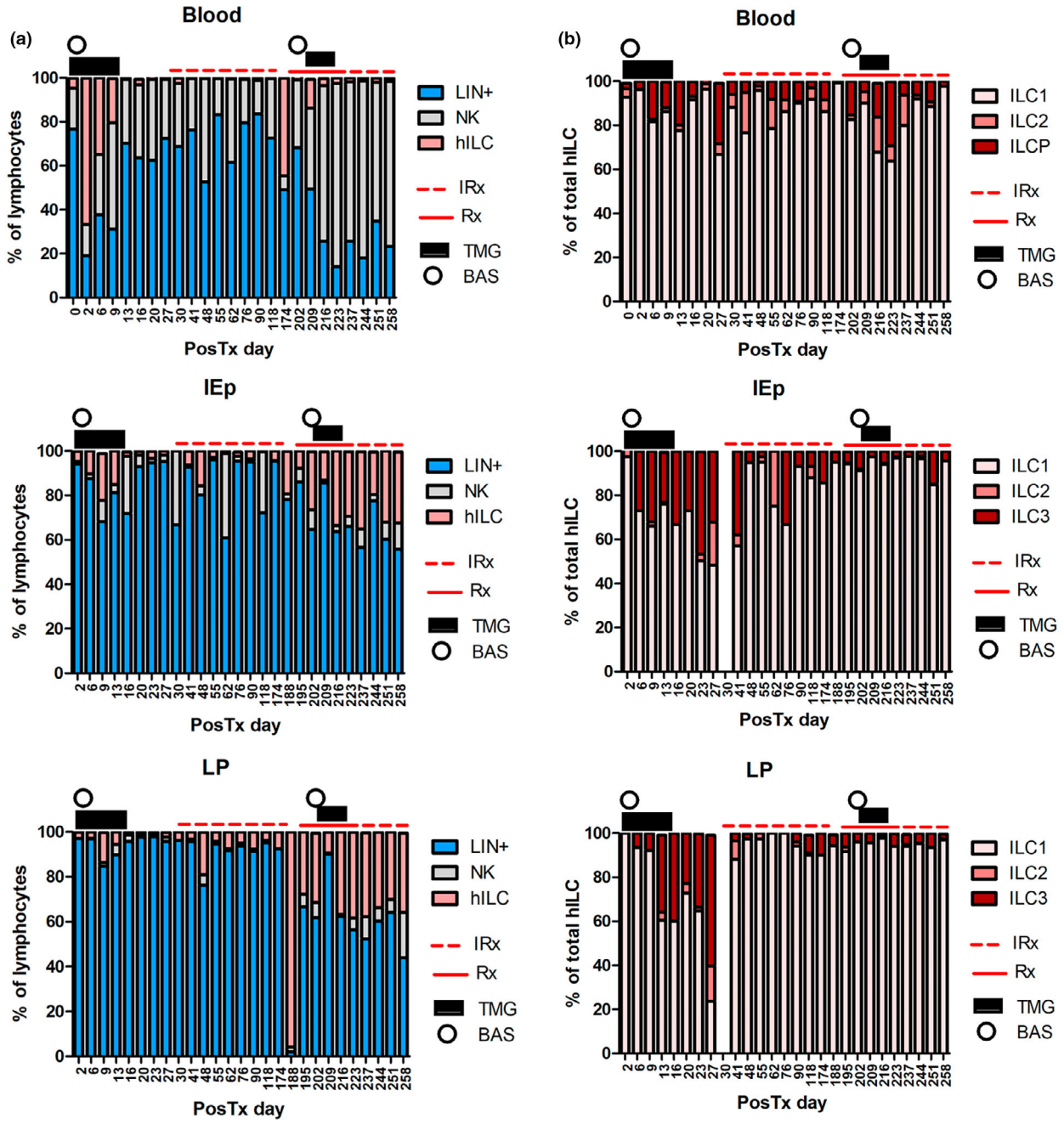

Figure 4 Evolution of the lymphoid populations' distribution in IEp, LP and blood from patient P9. (a) Lin+ cells, NK cells and hILC distribution in blood, IEp and LP. (b) hILC subsets distribution in blood, IEp and LP. BAS, basiliximab; ILCP, innate lymphoid cell precursors; IRx, indeterminate for rejection; Rx, rejection; TMG, thymoglobulin.

persisted longer (almost 13 years posTx) than what Weiner et al. [33] described (8 years posTx).

Another interesting point to consider is whether donor cells from a specific hILC subset could persist longer than another. In this line, our results showed that donor cells frequency in ILC3 was higher than in ILC1 in three of ei patients. Similarly, Weiner et al. [33] described that donor chimerism in CD56-ILC3 was greater than that of the other hILC subsets.

In the short-term transplanted patient (P9), recipient hILC infiltrated earlier and in a more aggressive way than Lin+ cells. Thus, similarly to Zuber et al. [32], we demonstrated that donor hILC are replaced by recipient hILC in the early posTx.

Transplant International 2020; 33: 1016–1029
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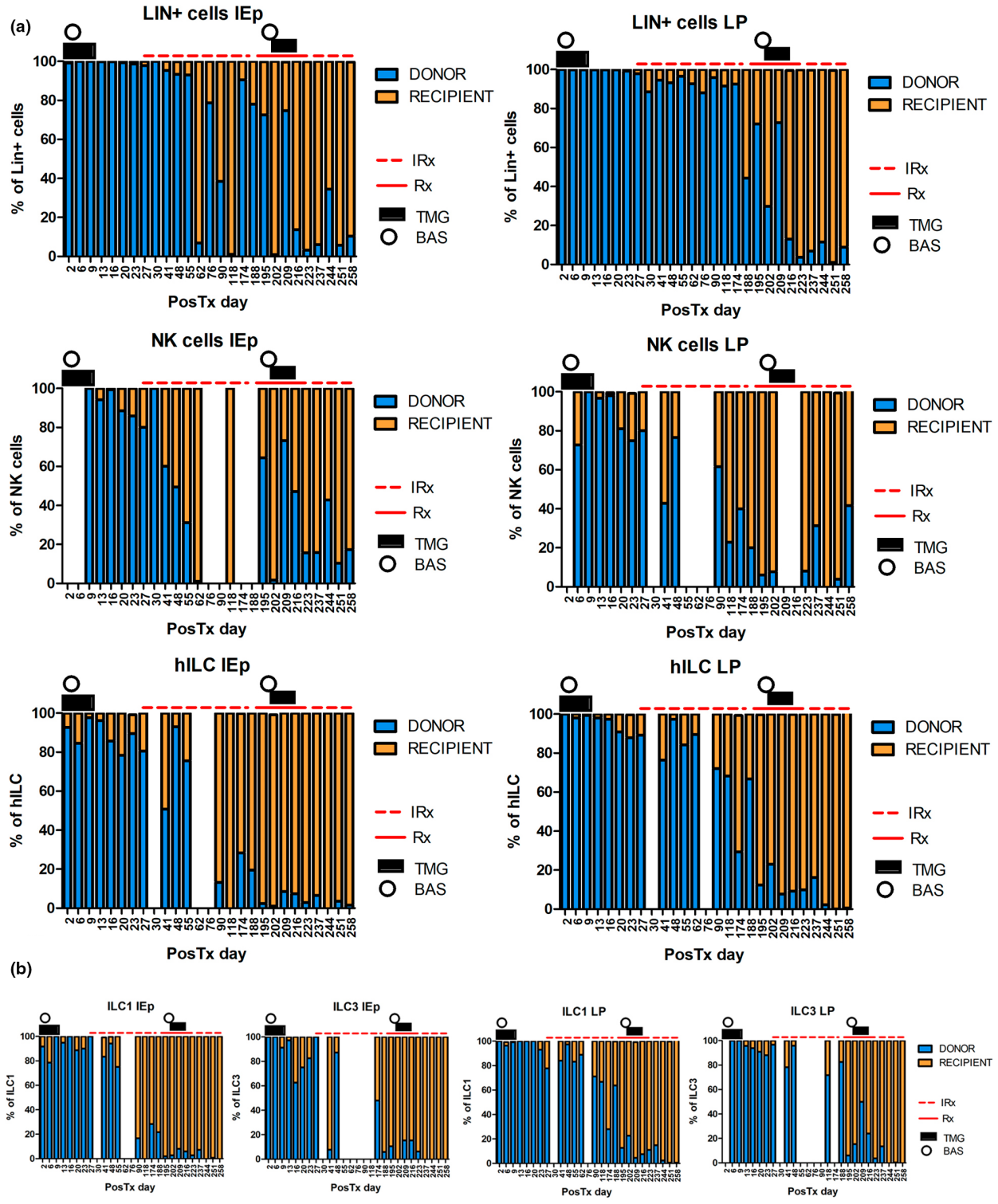

Figure 5 Evolution of the lymphoid populations' chimerism in IEp, LP and blood from patient P9. (a) Chimerism analysis of Lin+ cells, NK cells, hILC in IEp and LP. (b) Chimerism analysis of ILC1 and ILC3 in IEp and LP. BAS, basiliximab; IRx, indeterminate for rejection; Rx, rejection; TMG, thymoglobulin.

The infiltration of the recipient cells occurred in different ways in the three lymphoid populations. Whereas recipient hILC infiltrate the graft early in the posTx, Lin+ cells were not detected until the IRx diagnosis was made. The greater frequency of recipient cells was observed during the acute rejection episode in Lin+

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cells, NK cells and hILC. In this way, it could be interesting to consider vedolizumab (humanized monoclonal antibody anti-α4β7) as an induction treatment or a rejection therapy in ITx since the three lymphoid populations–require the expression of α4β7 to migrate and infiltrate the gut [49].

It is known that the balance between donor and recipient lymphoid cells is a crucial factor for rejection [5,34]. It seems that it is especially relevant for Lin+ cells but not for hILC as we have observed that they infiltrate the graft in the absence of rejection. However, hILC could be implicated in this process through the regulation of the ILC1–ILC3 subsets equilibrium.

Our study has some limitations such as low number of patients, lack of clinical events in the study time and the fact that in our cohort most of patients were transplanted long time ago. In addition, during the period of the study only one patient could have been followed regularly from transplant time. Lastly, the small tissue samples prevented us to perform further functional analysis and the inclusion of additional surface markers in our panel such as NKp44.

In conclusion, this study deepens the understanding of ITx lymphoid dynamics. On one hand, we describe the frequency normalization of Lin+, NK cells and hILC in long-term grafts, which could constitute a potential biomarker of well-functioning. On the other hand, the persistent imbalance between ILC1/ILC3 in IEp is also an interesting finding that could open new windows to future research. Finally, the chimerism analysis reveals different recipient repopulation dynamics: slower and more progressive in Lin+ cells and earlier and deeper in hILC.

Authorship

EG-M: performed the experimental work and analysed the results. ML-L and FJG-E: helped with sample collection and experiments. EU-M: obtained biopsy samples. IJ, CL and JC-P: recruited and followed up the transplanted patients and controls. EP-A and PT: designed the research and wrote the paper.

Funding

Funding for this study was obtained from projects 13/00045 and 13/01407 to E. P-A (Instituto de Salud Carlos III, Spain, cofounded by the European Regional Development Fund).

Conflicts of interest

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

SUPPORTING INFORMATION

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

Table S1. Monoclonal antibodies (MAb) used in the study.

Table S2. HLA typing from recipients and donors.

Figure S1. Representative flow cytometry dot plots for chimerism analysis of intestinal grafts.

Figure S2. Blood lymphoid populations in transplanted patients versus healthy controls.

Figure S3. Statistical analysis of IEL and LPL populations according to immunosuppressive therapy.

Figure S4. Frequency of recipient and donor hILC subsets (ILC1 and ILC3) in IEp and LP from patients P1–P8.

Figure S5. Macrochimerism was detected during the first month posTx in patient P9.

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