

Low dose interleukin-2 selectively expands circulating regulatory T cells but fails to promote liver allograft tolerance in humans

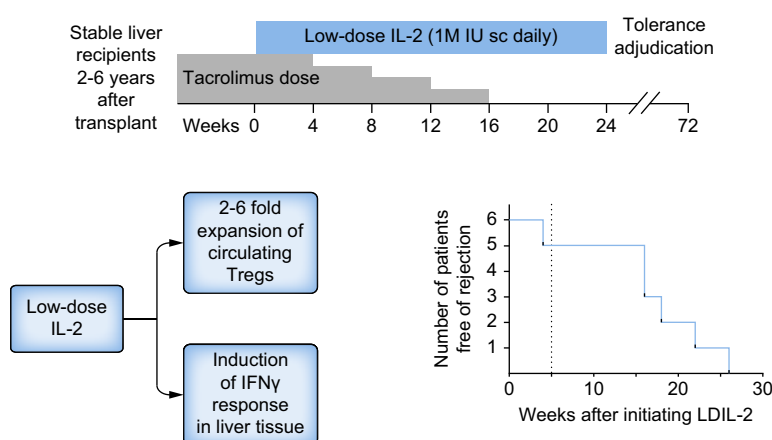
Authors

Tiong Y. Lim, Elena Perpiñán, Maria-Carlota Londoño, ..., Marc Martínez-Llordella, Tim Tree, Alberto Sánchez-Fueyo

Correspondence

sanchez_fueyo@kcl.ac.uk (A. Sánchez-Fueyo).

Graphical abstract



Highlights

- We aimed to determine the capacity of low-dose IL-2 to promote allograft tolerance in individuals on tacrolimus monotherapy.
- Low-dose IL-2 expanded circulating Tregs but did not increase donor-specific Tregs or promote their trafficking to the liver.
- Treatment elicited an IFN γ -orchestrated inflammatory response in the liver with accumulation of lymphomonocytic cells in sinusoidal areas.
- Low-dose IL-2 was not conducive to liver allograft tolerance and may have increased the risk of T-cell-mediated rejection.

Impact and implications

The administration of low-dose IL-2 is an effective way of increasing the number of circulating regulatory T cells (Tregs), an immunosuppressive lymphocyte subset that is key for the establishment of immunological tolerance, but its use to promote allograft tolerance in the setting of clinical liver transplantation had not been explored before. In liver transplant recipients on tacrolimus monotherapy, low-dose IL-2 effectively expanded circulating Tregs but did not increase the number of Tregs with donor specificity, nor did it promote their trafficking to the transplanted liver. Low-dose IL-2 did not facilitate the discontinuation of tacrolimus and elicited, as an off-target effect, an IFN γ -orchestrated inflammatory response in the liver that resembled T cell-mediated rejection. These results, supporting an unexpected role for IL-2 in regulating the immunogenicity of the liver, highlight the need to carefully evaluate systemic immunoregulatory strategies with investigations that are not restricted to the blood compartment and involve target tissues such as the liver.

Low dose interleukin-2 selectively expands circulating regulatory T cells but fails to promote liver allograft tolerance in humans

Tiong Y. Lim¹, Elena Perpiñán¹, Maria-Carlota Londoño^{1,2}, Rosa Miquel^{1,3}, Paula Ruiz¹, Ada S. Kurt¹, Elisavet Kodela¹, Amy R. Cross⁴, Claudia Berlin⁴, Joanna Hester⁴, Fadi Issa⁴, Abdel Douiri⁵, Felix H. Volmer⁶, Richard Taubert⁶, Evangelia Williams⁷, Anthony J. Demetris⁸, Andrew Lesniak⁸, Gilbert Bensimon^{9,10}, Juan José Lozano¹¹, Marc Martínez-Llordella^{1,†}, Tim Tree⁷, Alberto Sánchez-Fueyo^{1,*}

Journal of Hepatology 2023. vol. 78 | 153–164



Background & Aims: CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) are essential to maintain immunological tolerance and have been shown to promote liver allograft tolerance in both rodents and humans. Low-dose IL-2 (LDIL-2) can expand human endogenous circulating Tregs in vivo, but its role in suppressing antigen-specific responses and promoting Treg trafficking to the sites of inflammation is unknown. Likewise, whether LDIL-2 facilitates the induction of allograft tolerance has not been investigated in humans.

Methods: We conducted a clinical trial in stable liver transplant recipients 2–6 years post-transplant to determine the capacity of LDIL-2 to suppress allospecific immune responses and allow for the complete discontinuation of maintenance immunosuppression (ClinicalTrials.gov NCT02949492). One month after LDIL-2 was initiated, those exhibiting at least a 2-fold increase in circulating Tregs gradually discontinued immunosuppression over a 4-month period while continuing LDIL-2 for a total treatment duration of 6 months.

Results: All participants achieved a marked and sustained increase in circulating Tregs. However, this was not associated with the preferential expansion of donor-reactive Tregs and did not promote the accumulation of intrahepatic Tregs. Furthermore, LDIL-2 induced a marked IFN γ -orchestrated transcriptional response in the liver even before immunosuppression weaning was initiated. The trial was terminated after the first 6 participants failed to reach the primary endpoint owing to rejection requiring reinstitution of immunosuppression.

Conclusions: The expansion of circulating Tregs in response to LDIL-2 is not sufficient to control alloimmunity and to promote liver allograft tolerance, due, at least in part, to off-target effects that increase liver immunogenicity. Our trial provides unique insight into the mechanisms of action of immunomodulatory therapies such as LDIL-2 and their limitations in promoting alloantigen-specific effects and immunological tolerance.

Clinical Trials Registration: The study is registered at ClinicalTrials.gov (NCT02949492).

© 2022 The Authors. Published by Elsevier B.V. on behalf of European Association for the Study of the Liver. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Introduction

CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) can suppress cytopathic immune responses such as transplant rejection and are essential for the establishment and maintenance of allograft tolerance. Thus, the spontaneous development of liver transplant tolerance observed in mice does not take place in the absence of Tregs,¹ whereas in rats, adoptive transfer of Tregs promotes prolonged liver allograft survival.² In humans, several years after liver transplantation, a small proportion of recipients spontaneously become operationally tolerant and can safely discontinue all immunosuppressive medications.³ This is associated with a transient accumulation of Tregs in the liver.⁴ Furthermore, operational tolerance has been intentionally

induced shortly after liver transplantation by administering an autologous cell product enriched in Tregs in combination with lymphodepletion and splenectomy.⁵

IL-2 signalling through STAT5 is essential for the stability, function, and survival of Tregs. CD25, the IL-2 receptor (IL-2R) alpha chain, is highly expressed in Tregs, which, together with an amplified endogenous IL-2 signalling system,⁶ allows them to respond to very low concentrations of IL-2. Low doses of recombinant IL-2 (LDIL-2), ranging between 0.5 and 3 million IU/day, have been administered to humans to expand the pool of endogenous circulating Tregs. This has been shown to be safe and to exert clinical benefits in chronic graft vs. host disease (cGvHD) and a variety of different autoimmune disorders.⁷

Keywords: Liver transplantation; Regulatory T cells; Rejection; Transplant immunology.

Received 22 March 2022; received in revised form 11 August 2022; accepted 17 August 2022; available online 7 September 2022

* Corresponding author. Address: Institute of Liver Studies, James Black Centre, King's College London, Denmark Hill, London SE5 9NU, UK. Tel.: +44-2032-994015. Fax: +44-2032-993167.

E-mail address: sanchez_fueyo@kcl.ac.uk (A. Sánchez-Fueyo).

[†] Present address: Quell Therapeutics Ltd, Translation & Innovation Hub, 84 Wood Lane, London W12 0BZ, UK.

<https://doi.org/10.1016/j.jhep.2022.08.035>



ELSEVIER

Calcineurin inhibitors such as tacrolimus, which are the mainstay immunosuppressive agents in transplantation, have a negative impact on Treg homeostasis by reducing IL-2 availability and increasing Treg apoptosis.⁸ In murine models, this effect can be counteracted by the administration of exogenous IL-2, which preferentially promotes the expansion and intragraft accumulation of donor-specific Tregs⁹ and synergises with tacrolimus in prolonging allograft survival.⁸ We hypothesised that the administration of LDIL-2 to stable liver transplant recipients in whom effector T-cell responses are adequately controlled by tacrolimus treatment, would selectively expand circulating Tregs, restore their IL-2-dependent homeostasis, and promote liver allograft tolerance, allowing for the complete discontinuation of tacrolimus without triggering rejection.

Materials and methods

Trial design

'LITE' (LDIL-2 to expand endogenous regulatory T cells and achieve tolerance in liver transplantation; [ClinicalTrials.gov](#) identification: NCT02949492) was an open-label, activity, safety and efficacy, prospective, single-arm clinical trial in which stable adult liver recipients on calcineurin inhibitor-based immunosuppression initiated LDIL-2 treatment and attempted to discontinue all immunosuppressive agents if a >2-fold expansion of circulating Tregs was observed after 4 weeks of treatment. The primary endpoint was successful immunosuppression withdrawal, defined by the absence of rejection and a rejection-free liver biopsy at 1 year following

discontinuation of immunosuppression. Secondary endpoints were as follows: incidence of rejection, graft loss, patient survival, IL-2-related adverse events, effect of LDIL-2 on blood and liver Treg numbers, sequential immunological changes in blood and liver tissue, development of serum anti-human leucocyte antigen (HLA) antibodies. Eligibility criteria are outlined in [Table S6](#). An ethics committee approved the study protocol (REC number 14/LO/1014), and all participants provided written informed consent.

Trial procedures and LDIL-2 dosing

IL-2 (Proleukin, Novartis, Basel, Switzerland) was initiated at 1 million IU/day s.c. Each vial of Proleukin was reconstituted in 5% dextrose water, and individual 1-ml sterile syringes filled with 0.3 ml of 3.38 MIU/ml Proleukin were given to trial participants for home administration. To maximise patient retention, dosing was flexible (0.5–2 million IU/day) based on IL-2 tolerability and its effects on circulating Treg numbers. Following 4 weeks of LDIL-2 treatment, participants who showed >2-fold increase in peripheral blood absolute Treg numbers and stable liver function underwent a liver biopsy to exclude sub-clinical graft damage and subsequently initiated immunosuppression withdrawal. Immunosuppression was withdrawn in 6 steps lasting 2 weeks each. LDIL-2 was maintained for a total of 6 months or until the development of allograft rejection ([Fig. 1A](#)). Circulating Tregs and liver tests were monitored weekly during the first 4 weeks and every 2 weeks thereafter until the discontinuation of IL-2 treatment. In individuals tolerating immunosuppression withdrawal, a final protocol biopsy was required 18 months after enrolment.

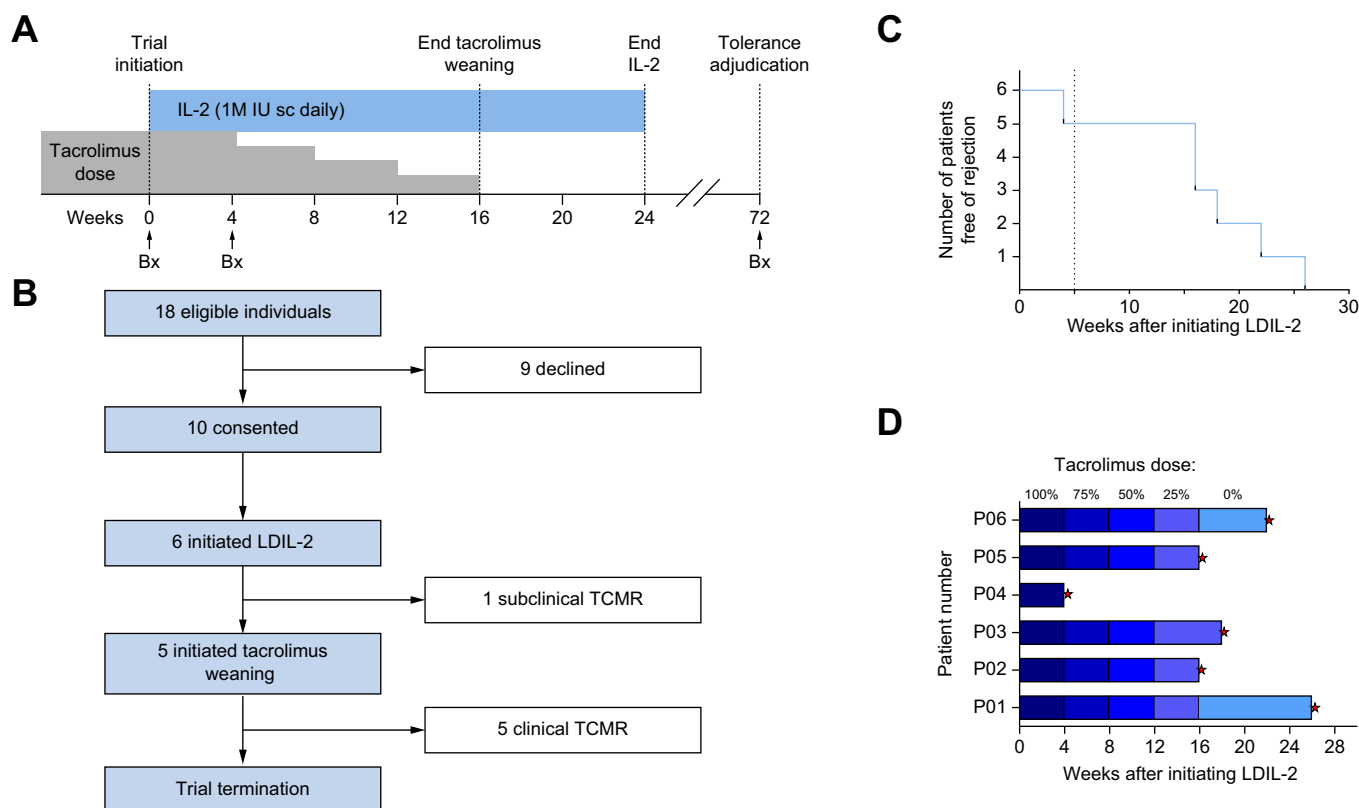


Fig. 1. Trial design and participant flow. (A) IL-2 dosing and immunosuppression withdrawal scheme. (B) Enrolment diagram. (C) Time to rejection. Dotted line denotes initiation of tacrolimus withdrawal. (D) Timing of rejection episodes (star) in relation to the tacrolimus withdrawal steps (bar segments). LDIL-2, low-dose IL-2; TCMR, T-cell-mediated rejection.

Adverse events were graded according to the Common Terminology Criteria for Adverse Events (version 4.03) and assessed for their seriousness and relatedness to trial procedures. All severe adverse events and rejection episodes were reviewed by the Data Safety Monitoring Board (DSMB). Urgent safety measures approved by the DSMB were instituted immediately after P02's severe rejection episode, consisting in the following: (i) halting further participant recruitment; (ii) reducing IL-2 dose to 0.5 M IU/day for the 4 individuals undergoing immunosuppression withdrawal; and (iii) monitoring liver tests weekly throughout the duration of the trial. The overall IL-2 doses administered per individual are shown in Fig. S6.

Rationale for study design and sample size considerations

Recipient age and time post-transplantation were specified to define a trial population with a very low likelihood of spontaneous operational tolerance.^{3,11} Immunosuppression withdrawal was not initiated until after 4 weeks of LDIL-2 treatment to provide enough time to assess clinical and histological stability and ensure all participants had achieved maximum Treg expansion. To demonstrate the superiority of a clinically meaningful immunosuppression withdrawal success rate $\geq 40\%$ as compared with $<15\%$ predicted based on historical data,^{3,11} we estimated the need to enrol 19 individuals undergoing immunosuppression withdrawal.

Statistical analysis and detailed experimental methods

The details are provided as supplementary data.

Results

Trial population and outcome of immunosuppression withdrawal

Eighteen liver transplant recipients were approached to take part, 10 agreed to participate, and 6 initiated LDIL-2 treatment (Fig. 1A and B). Patient demographics are summarised in Table 1. At trial entry, all 6 participants were on tacrolimus monotherapy immunosuppression. In response to LDIL-2, circulating Tregs showed a progressive increase and achieved >2 -fold expansion by Week 1 in 5/6 participants and in all 6 participants by Week 4 (range 2- to 6.7-fold; Table S1). Participant P04 did not initiate tacrolimus withdrawal owing to the detection of sub-clinical moderate T-cell-mediated rejection (TCMR) in the 4-week protocol liver biopsy, which spontaneously improved histologically following the discontinuation of LDIL-2 without modifications to the participant's immunosuppressive regimen (Table 2). The remaining 5 participants were considered suitable for initiating tacrolimus withdrawal. Four participants succeeded in reducing their tacrolimus doses by 75%, and 2 completely discontinued their immunosuppression, but all of them experienced allograft dysfunction within 6 months of the trial initiation, which was because of TCMR and required immunosuppression reinstitution. As a result, none reached the trial's primary endpoint (Fig. 1C and D). Among the 5 participants who initiated tacrolimus weaning, rejection episodes were mild or moderate in 4 and rapidly resolved after reinstitution of immunosuppression without the need for high-dose steroids, but participant P02 developed severe TCMR that did not respond to steroid boluses or thy-moglobulin, progressed to chronic ductopenic rejection, and

Table 1. Clinical and demographic characteristics of trial participants.

Participant	Age (yr)	Sex	Time since transplant (mo)	Baseline tacrolimus levels (ng/ml)	Tacrolimus levels at Week 4 (ng/ml)	Indication for transplant	BSA (m ²)	eGFR (ml/min)	LFTs at baseline (IU/L)				Pre-IL-2 anti-HLA antibodies		Post-IL-2 anti-HLA antibodies		DSA detected
									AST	ALT	ALP	GGT	Class I	Class II	Class I	Class II	
P01	43	M	38	3.4	7.4	Hepatitis C	2.08	102	18	25	110	30	-ve	-ve	-ve	-ve	No
P02	33	F	31	7.0	4.6	Wilson's	1.56	54	23	15	81	8	+ve	+ve	+ve	+ve	No
P03	29	F	37	5.0	5.6	Biliary atresia	2.01	90	22	10	58	10	-ve	+ve	-ve	-ve	No
P04	35	M	40	4.2	4.2	Seronegative ALF	1.81	90	22	19	85	36	-ve	-ve	-ve	-ve	No
P05	22	M	45	5.6	8.3	Hepatic adenomatosis	2.20	135	24	27	64	12	-ve	+ve	-ve	+ve	MFI >10,000
P06	39	F	39	4.4	8.6	Seronegative ALF	1.84	77	26	19	70	70	-ve	-ve	-ve	-ve	No

DSA was measured before initiating weaning and at the time of rejection.

ALF, acute liver failure; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSA, body surface area; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate; GGT, gamma-glutamyl transferase; LFT, liver function test; MFI, mean fluorescent intensity.

required retransplantation (P02 follow-up after retransplantation was uneventful with no further episodes of rejection). Following review by the independent DSMB, recruitment was halted, and the trial was subsequently terminated once rejection was confirmed in all 6 participants. A list of all adverse events is provided in [Table S2](#).

Effects of LDIL-2 on the immunophenotype, epigenetic, and gene expression profile of circulating immune cells

LDIL-2 resulted in significant increases in circulating eosinophils, basophils, CD56^{bright} natural killer (NK) cells, and Tregs and a reduction in the absolute numbers of circulating B cells ([Fig. 2A](#)). In contrast, we observed no changes in the overall numbers of non-regulatory CD4⁺ and CD8⁺ T cells. We conducted a more in-depth analysis of CD4⁺ and CD8⁺ T cells during the first 4 weeks of LDIL-2 treatment by using a 25-marker spectral flow cytometry panel and using unsupervised hierarchical clustering to overcome the limitations of manual gating. The only CD4⁺ T-cell subpopulations influenced by LDIL-2 were those exhibiting a Treg phenotype (as defined by co-expression of Foxp3 and Helios), with a significant increase of activated/memory CD25^{high} Treg subsets ([Fig. 2B](#)). In these cells, LDIL-2 resulted in increased expression of markers associated with activation/suppression potency ([Fig. 2C](#)). In contrast, among CD8⁺ T cells, LDIL-2 induced the increase of a small subset of terminally differentiated CCR7⁺ TIM-3⁺CD45RA⁺ T cells, which at most reached 0.6% of the total CD8⁺ T-cell population ([Fig. 2D](#)). The number of circulating Tregs as assessed by flow cytometry correlated with the proportion of CD4⁺ T cells exhibiting a demethylated Treg-specific demethylated region (TSDR). Furthermore, the ratio between circulating Tregs and TSDR-demethylated CD4⁺ T cells increased as a result of LDIL-2 ([Fig. 3](#)). Although the suppressive capacity of expanded Tregs was not directly assessed and could have been compromised as a result of tacrolimus exposure, our results suggest that LDIL-2 expanded bona fide Tregs. Whole-blood gene expression profiling experiments performed in parallel revealed a significant increase in transcripts known to be preferentially expressed by Tregs (e.g. CD25, FOXP3, IL34, CTLA4, and IL1RL1) and a decrease in transcripts enriched in B cells (e.g. CD19, CD22, CXCR5, CD79A, BLNK, and TNFRSF13B). The expression of genes involved in cytotoxicity, allograft rejection, or liver inflammation was not influenced by LDIL-2 treatment while tacrolimus doses remained stable, but their transcript levels increased once tacrolimus weaning was initiated and/or at the time of rejection (e.g. SAA1, CXCL9, BIRC5, CL17, CCL22, and TNFRSF12A) ([Fig. 4](#) and [Table S3](#)). The absolute numbers of circulating Tregs and conventional T cell subsets throughout the trial duration are displayed in [Fig. 4](#).

LDIL-2 does not preferentially expand donor-specific tregs in peripheral blood

We used an activation-induced marker assay to determine whether the increase in circulating Tregs was caused by preferential expansion of donor-reactive clones ([Fig. 5A–D](#)). All participants displayed detectable alloantigen-specific Foxp3⁺Helios⁺ Tregs and CD4⁺Foxp3⁺ conventional T cells (Tconv) directed against donor and third-party cells at the 3 time points analysed (baseline and Weeks 1 and 4). However,

despite the significant increase in the total number of circulating Tregs induced by LDIL-2, the proportion of them co-expressing CD137 and GARP following donor alloantigen stimulation did not significantly change over the first 4 weeks of LDIL-2 treatment. In contrast, a significant increase in the proportion of third-party specific Tregs was observed during the same period ([Fig. 5D](#)). However, the proportion of effector T cells (CD4⁺ and CD8⁺ Tconv) with donor or third-party alloantigen specificity was not modified by the 4 weeks of LDIL-2 treatment ([Fig. 5D](#)). We confirmed the negligible effect of LDIL-2 on circulating alloreactive effector immune responses by utilising an assay quantifying donor-specific CD8⁺ T cells, which had previously shown to predict rejection following liver, intestine, or kidney transplantation ([Fig. S1](#)).¹² In addition, we assessed if 4 weeks of LDIL-2 treatment induced changes to the sensitivity of different immune cell subsets to IL-2, by quantifying phosphorylated STAT5 levels in response to increasing IL-2 concentrations. Only Tregs and not cytopathic/effector immune cell subsets acquired increased responsiveness to IL-2 ([Fig. S2](#)). Taken together, these results indicate that during the first 4 weeks of treatment, the effects of LDIL-2 on circulating T cells were almost exclusively circumscribed to Tregs, with the only exception of a small expansion of a CD8⁺ T cell subset that did not influence the overall number of donor-specific effector T cells.

LDIL-2 does not promote the preferential expansion of intrahepatic tregs and primes the liver allograft for rejection

A comparison of the protocol liver biopsies performed before the initiation of LDIL-2 and 4 weeks afterwards revealed a significant increase in mononuclear inflammatory cells, which accumulated as patchy aggregates in the lobular areas ($p < 0.03$; [Table 2](#) and [Fig. S3](#)). With the exception of P04, we did not identify typical histological features of TCMR such as portal inflammation, lymphocytic cholangitis, or endothelialitis. In contrast, these features were apparent in all participants at the time of clinical rejection ([Table 2](#)). We quantified the number of CD4⁺Foxp3⁺, CD8⁺Foxp3⁺ T, and CD4⁺Foxp3⁺ T cells in the same liver biopsies by immunofluorescence. As expected, the number of CD4⁺Foxp3⁺, CD8⁺Foxp3⁺ T, and CD4⁺Foxp3⁺ T cells in the portal tracts significantly increased at the time of rejection. However, although we observed a positive trend in the number of intrahepatic CD4⁺ T cells detected over the first 4 weeks of LDIL-2 treatment, this was not associated with a preferential accumulation of CD4⁺Foxp3⁺ T cells ([Fig. 6A](#)). To validate these results, we used a NanoString GeoMx (Nanostring Technologies, Seattle, USA) platform to evaluate the expression of a set of 46 immune-related proteins in 156 distinct segments selected across the participants' sequential liver biopsies ([Fig. 6B](#)). The portal tract segments exhibited no changes in protein levels in the first 4 weeks of LDIL-2 treatment, whereas a significant increase in T-cell-, macrophage-, and NK cell-related proteins (e.g. CD4, CD8, GZMB, CD56, CD163, and VISTA) was observed at the time of rejection. Analysis of the lobular area segments revealed a decrease in the levels of immunoregulatory proteins typically associated with Tregs (e.g. CD25, FOXP3, TIM3, IDO1, GITR, and LAG3) at Week 4, with an increase in myeloid-related (CD68 and VISTA) and immunoactivatory proteins (CD40, CD44, and HLA-DR) at the time of rejection ([Fig. 6C](#)). Taken together, these results

Table 2. Liver histopathology assessments.

Participant	Portal inflam. (Ishak)	Interface activity (Ishak)	Lobular inflam. (Ishak)*	Banff inflam.	Banff BD	Banff endoth	Banff TCMR RAI	Portal fibrosis (Venturi)	Peri-sinusoidal fibrosis (Venturi)	Central fibrosis (Venturi)	Lobular lymph ^{†,‡}	Steatosis
P01												
Baseline	1	0	0	0	0	0	0	0	0	1	10.9	0
Week 4	1	0	2	0	0	0	0	0	1	1	32.8	0
For cause	2	2	2	2	2	2	6	0	1	0	20.1	0
P02												
Baseline	1	0	0	0	0	0	0	0	0	0	12.5	0
Week 4	1	1	2	1	1	0	2	1	0	0	24.5	0
For cause	3	4	4	3	2	3	8	1	2	1	79.3	0
P03												
Baseline	0	0	1	0	0	0	0	0	0	0	14.5	0
Week 4	1	0	2	1	1	1	3	1	1	0	30.2	0
P04												
Baseline	2	1	1	1	1	1	2	3	0	0	24.8	0
Week 4	2	1	2	3	2	2	7	2	1	1	36.3	0
For cause	2	0	0	1	1	1	2	2	0	0	16.5	0
P05												
Baseline	1	0	1	0	0	0	0	2	1	0	18.6	0
Week 4	1	0	2	0	0	0	0	2	2	0	41	0
For cause	1	1	2	2	1	2	5	2	1	0	26.1	0
P06												
Baseline	0	0	1	0	0	0	0	1	1	0	19.7	0
Week 4	1	0	2	1	0	1	2	1	1	0	26.5	0
For cause	2	1	1	2	2	2	7	1	0	0	25.5	0

For cause biopsies were performed at the time of allograft dysfunction (except for P04, in whom the biopsy was performed 3 months after discontinuing LDIL-2). Paired statistical analyses were performed using the Wilcoxon signed-rank test. Only those comparisons with $p < 0.05$ are noted. No signs of acute or chronic antibody-mediated rejection according to Banff criteria were noted in any of the biopsies analysed.

BD, bile ducts; endoth, endothelialitis; inflam., inflammation; LDIL-2, low-dose IL-2; lymph., lymphocytes; NRH, nodular regenerative hyperplasia; RAI, rejection activity index; TCMR, T-cell-mediated rejection.

* $p < 0.03$ baseline vs. Week 4.

[†]Mean cells/field.

[‡] $p < 0.03$ baseline vs. Week 4.

indicated that, although LDIL-2 modified the immune cell composition of the liver allograft by increasing the number of lymphocytes in the lobular sinusoids, it did not lead to a preferential accumulation of Tregs.

To better characterise the changes induced by LDIL-2 on the liver allograft, we conducted RNA sequencing (RNAseq) experiments on cryopreserved liver tissue specimens obtained at the time of the baseline and Week 4 protocol liver biopsies.

LDIL-2 significantly increased the transcript levels of 29 genes (false discovery rate <0.05) with CXCL9 and CXCL10 being the 2 genes whose expression was most markedly induced (fold changes 4.5 and 4, respectively; Table S4). Functional analysis using Gene Ontology (GO) terms identified immune response, IFN γ -mediated signalling pathway and major histocompatibility complex (MHC) class II protein complex as the most highly enriched pathways (Fig. 5D). In addition, an 11-gene set

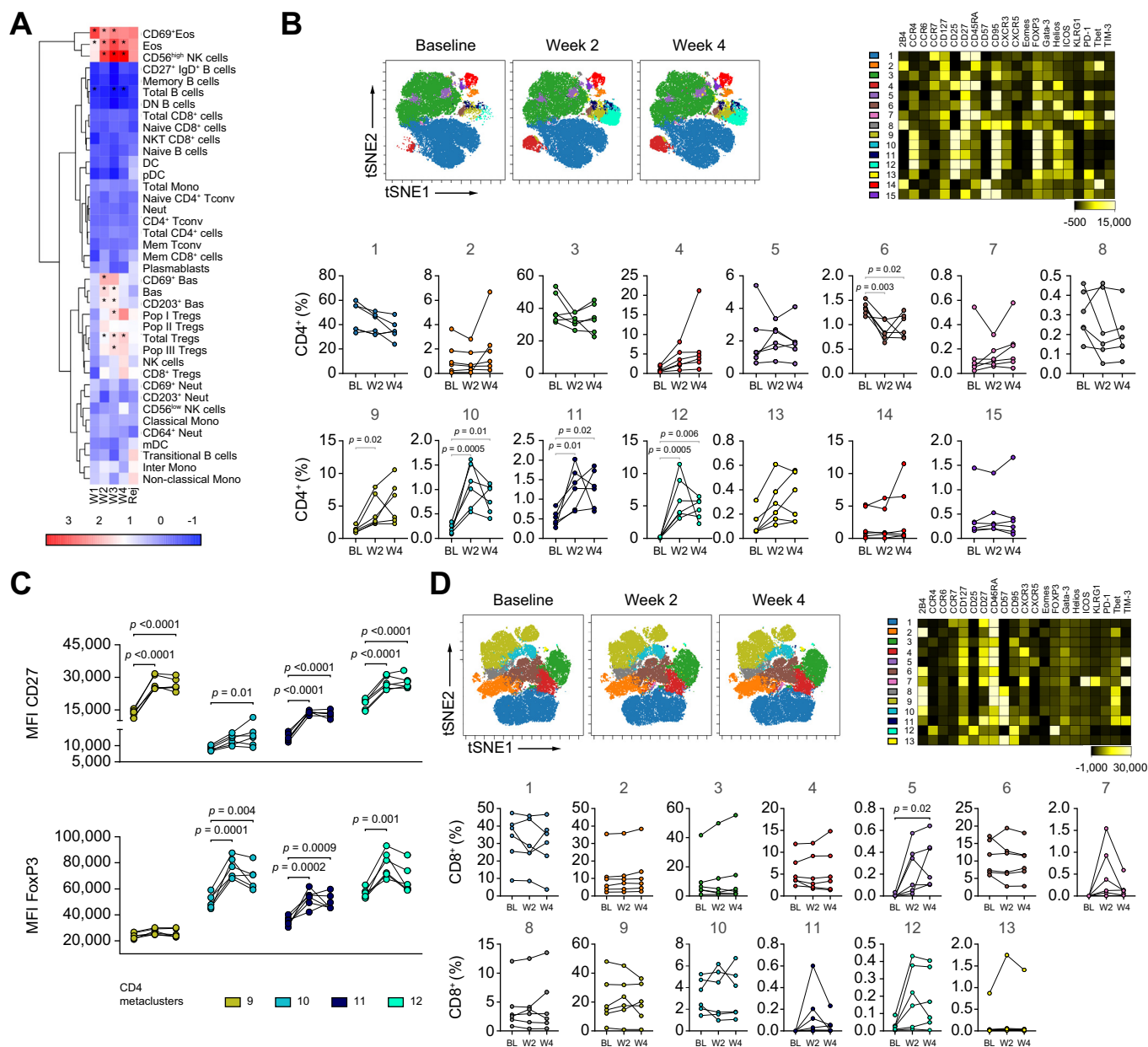


Fig. 2. Sequential changes in circulating immune cell subsets during the first 4 weeks of LDIL-2 treatment. (A) Heatmap displaying the changes in the absolute numbers of predefined circulating immune cell subsets at the indicated time points. The colour scale corresponds to log2 fold changes as compared with the baseline pre-LDIL-2 time point, and the asterisks denote $p < 0.05$. Red pixels correspond to an increased number, whereas blue pixels indicate decreased number. (B) viSNE plots and heatmaps representing different CD4⁺ T-cell metaclusters identified by FlowSOM, with percentages at baseline, Week 2, and Week 4. The colours in the heatmap represent the median expression of markers for each metacluster at baseline, varying from black for lower expression to yellow for higher expression. (C) MFI of CD27 and Foxp3 in CD4⁺ T-cell metaclusters 9, 10, 11, and 12 at baseline, Week 2, and Week 4. Statistical analysis was performed using one-way ANOVA with Tukey's multiple-comparisons tests; if not indicated, p values were not significant. BL, baseline; LDIL-2, low-dose IL-2; MFI, mean fluorescence intensity; NK, natural killer; Rej, rejection; Treg, regulatory T cell; W1, Week 1; W2, Week 2; W3, Week 3; W4, Week 4. (This figure appears in color on the web.)

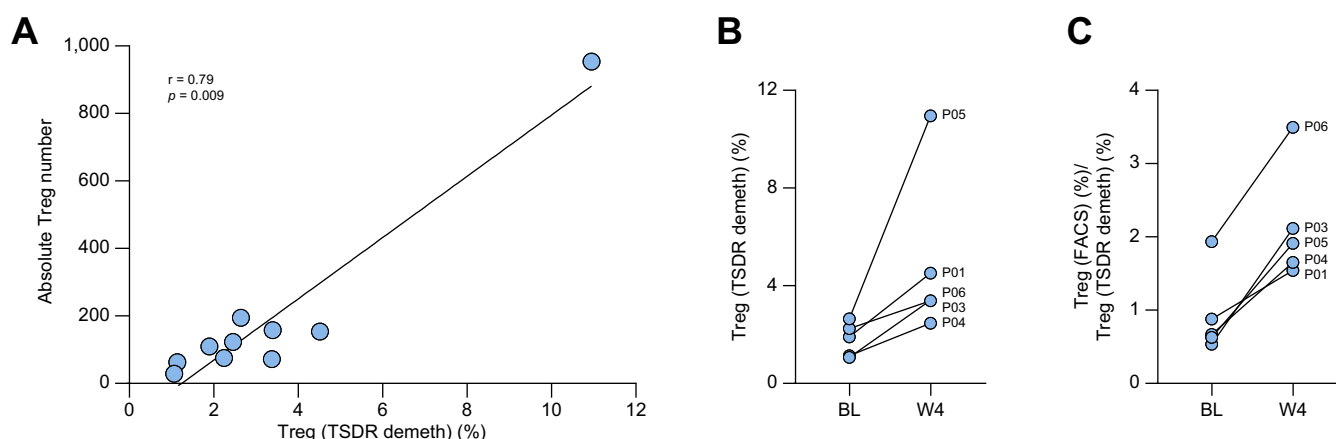


Fig. 3. Sequential changes in the proportion of circulating CD4⁺ with demethylated TSDR as assessed by real-time PCR. (A) Spearman correlation between absolute Treg numbers (as assessed by flow cytometry on fresh blood specimens) and the proportion of CD4⁺ with demethylated TSDR (all 10 specimens available for analysis were included). (B) Proportion of CD4⁺ with demethylated TSDR at baseline and 4 weeks after initiating LDIL-2 treatment. (C) Ratio between the proportion of CD4⁺Foxp3⁺CD127⁺ among CD4⁺ (as assessed by flow cytometry) and the proportion of CD4⁺ with demethylated TSDR. BL, baseline; LDIL-2, low-dose IL-2; Treg, regulatory T cell; TSDR, Treg-specific demethylated region; W4, Week 4.

previously described as being highly specific for liver allograft TCMR was significantly over-represented in the differentially expressed dataset (Fig. 6E). We observed an equally significant enrichment in multiple IFN γ -related gene sets known to be involved in TCMR across a variety of different transplant settings (Table S5). Of note, no enrichment in NK-related transcripts was observed.

LDIL-2 treatment results in a sustained increase in circulating chemokines and pro-inflammatory cytokines

To investigate if the inflammatory response induced by LDIL-2 in the liver tissue was associated with systemic effects, we quantified the serum levels of a set of 30 immune effector proteins. As early as 1 week after initiating LDIL-2 treatment, IL-12, CXCL10, IFN γ , IL-2, IL-5, CCL22, and CCL13 were found to be higher than before treatment. Furthermore, the levels of IL-10, IL-5, CXCL10, IL-12, IFN γ , CCL22, IL-2, IL-4, CCL13, and TNF α were increased in a sustained way throughout the first 6 weeks of LDIL-2 treatment (Fig. S4A). No such changes were observed in the transcript levels of the corresponding genes measured in blood samples collected at the same time points (Table S3). This contrasted with the marked increase in the gene expression of the IFN γ -induced chemokines CXCL10 and CXCL9 observed in the liver tissue (Table S4). To explore the cellular source of these chemokines, we correlated the intra-hepatic protein levels of CXCL9 with markers preferentially expressed by specific cell types as assessed by the NanoString GeoMx platform (Fig. S4B). CXCL9 significantly correlated with the macrophage-monocyte lineage markers CD14 and CD68, whereas we observed no correlation with CD3 (T-cell marker) and CD34 and a negative correlation with the myofibroblast cell marker smooth muscle actin.

Discussion

The expansion of endogenous Tregs through the administration of LDIL-2 or IL-2 immunocomplexes successfully prevents and/or reverts immunopathology in various murine models.^{8,14,13} In

the clinic, LDIL-2 is well tolerated, consistently leads to an increase of circulating Tregs, and has been associated with clinical responses in cGVHD, HCV-induced vasculitis, and systemic lupus erythematosus (SLE).^{10,15,16} However, the extent to which Treg expansion in blood is necessary and sufficient to induce clinical benefits is unclear. Furthermore, whether LDIL-2 results in an increase of Tregs within inflamed tissues has not been thoroughly investigated in humans. Our trial is the first one to explore the use of LDIL-2 in solid-organ transplantation and to use a hard clinical endpoint such as immunological tolerance. The trial population was selected based on previous studies demonstrating <15% likelihood of spontaneous allograft tolerance,^{3,11} which rendered the inclusion of a control group unnecessary as well as ethically unacceptable. To clearly distinguish the effects of LDIL-2 from those attributable to rejection, no changes to immunosuppression were allowed during the first 4 weeks of LDIL-2 treatment. Given the evidence derived from murine transplant models indicating the need to achieve high Treg/Tconv ratios to induce allograft tolerance,¹⁷ we chose an IL-2 dosing regimen capable of ensuring persistently high circulating Treg levels throughout the immunosuppression weaning period while minimising size effects. Our regimen, which included daily doses of 1 million IU IL-2 for up to 6 months, is at odds with what has been utilised in most autoimmune indications but resembles the scheme safely used in cGVHD, in which daily doses of 2 million IU IL-2 have been administered for very prolonged periods and shown to be well tolerated and to reduce disease activity.¹⁶ In consonance with previous studies, we observed a marked and sustained increase in circulating Tregs. However, this was not associated with the preferential expansion of donor-reactive Tregs, did not promote the accumulation of Tregs within the liver allograft, failed to induce tolerance, and primed the allograft for rejection by inducing an IFN γ -orchestrated response even before immunosuppression weaning was initiated.

The rationale for using LDIL-2 in the clinic is based on its preferential effect on Tregs as opposed to Tconv. However, despite the selectivity for Tregs, LDIL-2 influences the

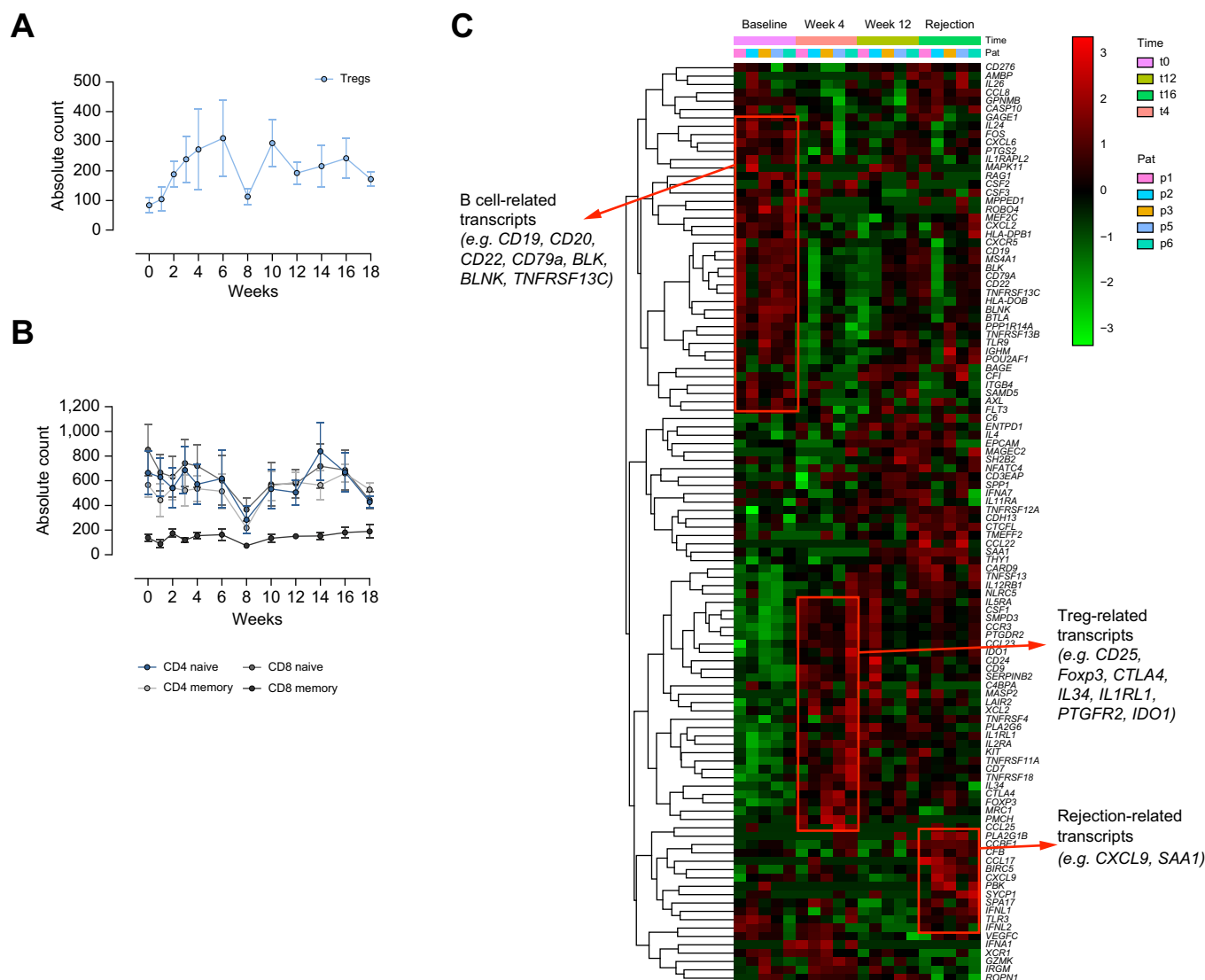


Fig. 4. Sequential changes in T cell subsets and whole-blood gene expression profile throughout the trial duration. (A and B) Absolute numbers of Tregs, CD4⁺CD45RA⁺Foxp3⁺ (naïve), CD4⁺CD45RA⁺Foxp3⁺ (memory), CD8⁺CD45RA⁺ (naïve), and CD8⁺CD45RA⁺ (memory) T cell subsets as assessed by flow cytometry on fresh blood specimens. Data correspond to mean \pm SEM. (C) RNA samples extracted from whole blood specimens collected at baseline, at Weeks 4 and 12 after initiating LDIL-2 treatment, and at the time of clinical rejection were analysed on a NanoString platform. The heatmap displays the 110 genes differentially expressed at p value < 0.05 in at least 1 comparison between time points. Rows represent genes and columns represent samples. The intensity of each colour denotes the standardised ratio between each value and the average expression of each gene across all samples. Red pixels correspond to an increased abundance, whereas green pixels indicate decreased transcript levels. LDIL-2, low-dose IL-2; Treg, regulatory T cell. (This figure appears in color on the web.)

homeostasis of other circulating immune cell subsets as well, in particular CD56^{high} NK cells, eosinophils, T follicular helper cells (TFHs), B cells, and activated non-regulatory T cells expressing CD25.^{18–21} Furthermore, it induces the systemic release of cytokines such as IL-10, TGF β , IL-5, TNF α , and IL-17 and, most notably, the chemoattractant CXCL10.^{22,23} Our findings, and in particular the impact of LDIL-2 on non-Treg immune cell subsets, are therefore in line with previously published reports, which used a variety of different dosing regimens. What had not been previously recognised is that the effects of LDIL-2 on blood immune cell subsets may not be mirrored by what takes place within the target tissues.

Considering that the accumulation of Tregs in tissues is critically dependent on their antigen specificity,^{24–26} the failure of LDIL-2 to promote the expansion of circulating donor-reactive Tregs as well as their trafficking into the liver allografts could be explained by a reduced number of donor-reactive Tregs in liver transplant recipients. This is supported by the results of an adoptive Treg transfer trial conducted using a very similar design and an almost identical cohort of individuals to those enrolled in our study.²⁷ In contrast, the patchy accumulation of lympho-monocytic cells in the lobular areas observed 4 weeks after LDIL-2 treatment, together with the induction of a macrophage-driven IFN γ -orchestrated

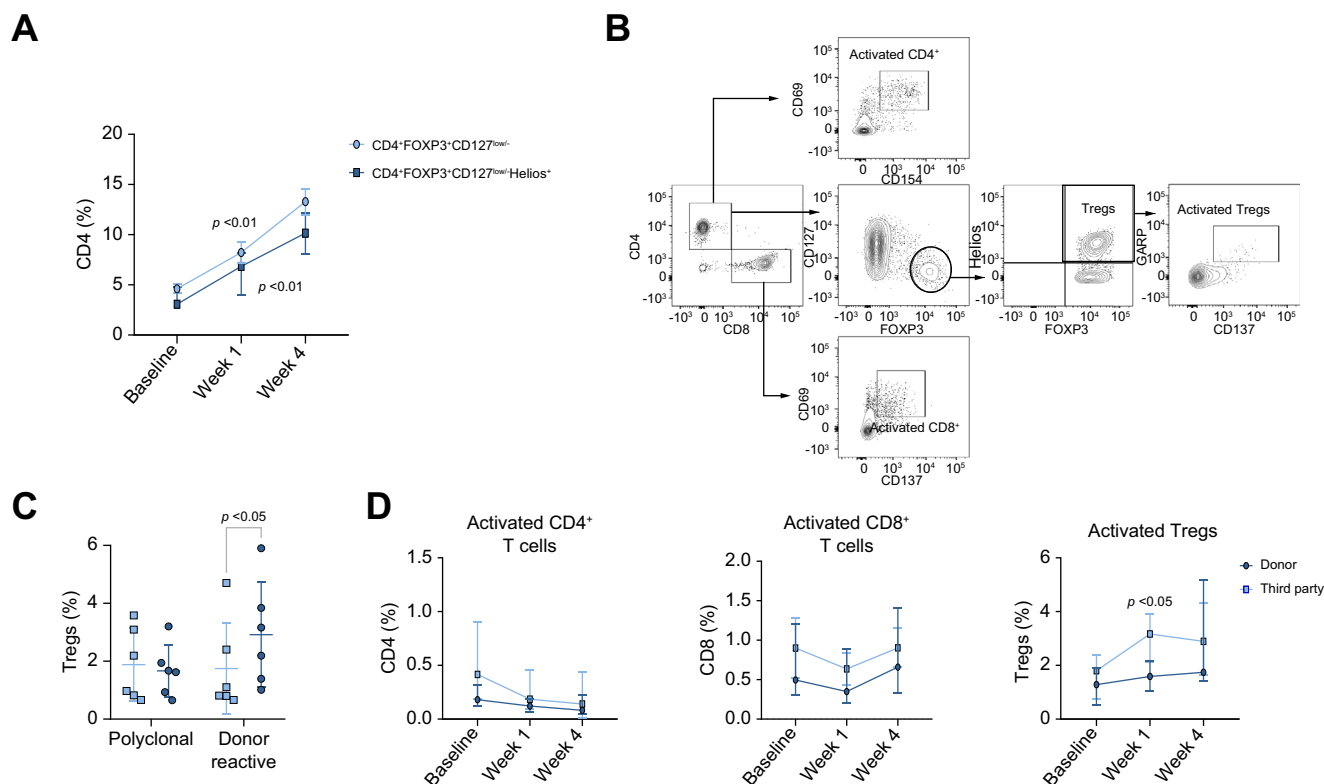


Fig. 5. Effect of LDIL-2 on alloantigen-specific T cell subsets. (A) Proportion of Tregs and Helios⁺ Tregs in the cryopreserved PBMC specimens used for the activation induced marker assays. (B) Gating strategy used to identify alloantigen-specific Tregs and CD4⁺ and CD8⁺ T cells following 16-h culture with surrogate donor or third-party PBMCs in the activation-induced marker assay. (C) To validate the assay, purified Tregs from healthy individuals were expanded *in vitro* in polyclonal conditions or in the presence of allogeneic B cell stimulators to generate cell products with different proportions of donor-reactive Tregs. Alloantigen specificity was assessed as outlined in (A) following 16-h culture with same-donor (red circles) or third-party (blue squares) allogeneic PBMCs. Analysis was performed using 2-way ANOVA with Tukey's multiple-comparison test. Error bars correspond to median and IQR. (D) Frequency of alloantigen-specific Tregs and CD4⁺ and CD8⁺ T cells in PBMCs collected from the trial participants before (baseline), 1 week, and 4 weeks after initiating LDIL-2. Error bars correspond to median and IQR. Statistical analysis was performed using a repeated-measures ANOVA. LDIL-2, low-dose IL-2; PBMC, peripheral blood mononuclear cells; Treg, regulatory T cell.

transcriptional signature, could be explained by a direct effect of IL-2 on Kupffer cells. This was indeed recently described by De Simone et al.²⁸ in a murine model of antigen-specific intrahepatic T cell priming. In this model, IL-2 administration reverted the T-cell dysfunction typically induced by intrahepatic priming and caused a clustered accumulation of activated effector T cells in the liver sinusoids.²⁹ This phenomenon was entirely dependent on sinusoidal Kupffer cells, which, in response to IL-2, acquired a pro-inflammatory profile and the capacity to activate antigen-specific effector T cells.²⁸ Given the striking similarities between the findings of De Simone et al.²⁸ in mice and our own observations, we hypothesise that a similar mechanism caused the clinically silent IFN γ -driven intrahepatic transcriptional response detected in our participants 4 weeks after LDIL-2, and contributed to allograft dysfunction and rejection once tacrolimus was weaned. Of note, the overexpression of CXCL9 or CXCL10 in tissues is well known to promote the preferential trafficking of effector immune cell subsets over Tregs^{30–34} and, as such, might have exacerbated the process described above by tipping the balance between Tregs and effector T cells even further. We believe these mechanisms could explain the development of

refractory rejection requiring retransplantation in P02, an extraordinarily rare event in immunosuppression withdrawal trials in liver transplantation. The contribution to an exacerbated rejection response of other effector immune cell subsets such as NK cells seems unlikely, given the absence of detectable NK markers in the RNASeq and NanoString GeoMx experiments.

Ours is the first report investigating the effect of LDIL-2 on tissue-resident immune cells in humans. Our findings need to be considered with caution given the premature termination of the trial, the small sample size, and the lack of ex vivo evaluations of Treg suppressive capacity. Furthermore, the generalizability of our findings to clinical settings outside of liver transplantation and/or to the use of engineered IL-2 mutants remains to be established. Alloimmune responses directed against organs expressing mismatched MHC molecules constitute a formidable barrier to the induction of tolerance as a result of the large proportion of pre-existing alloreactive T cells.³⁵ Moreover, the liver exhibits unique immunological properties that might not be relevant to other settings.²⁸ However, organs such as kidneys are also capable of inactivating infiltrating effector lymphocytes,³⁶ and Kupffer cells are not the only tissue-resident macrophages in the body involved

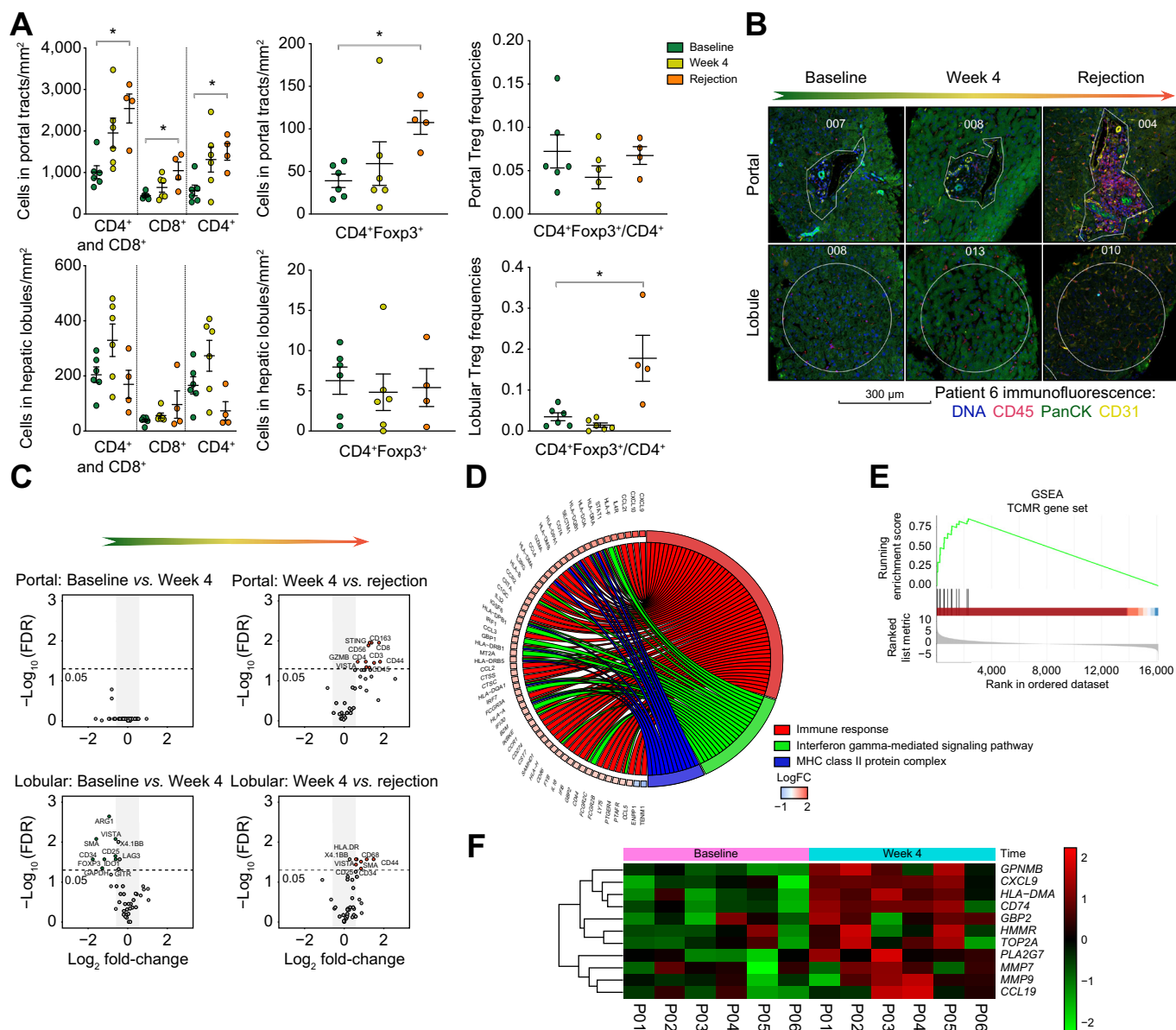


Fig. 6. Impact of LDIL-2 on intrahepatic T cell subsets and liver tissue transcriptional profile. (A) Number of liver-infiltrating CD4⁺CD8⁺, CD8⁺CD4⁺, and Foxp3⁺CD4⁺CD8⁺ T cells in the portal tracts (upper panels) and lobular areas (lower panels) as assessed by immunofluorescence before initiating LDIL-2 4 weeks afterwards and at the time of rejection. (B) Representative immunofluorescence microscopy from portal tract and lobular areas of interest from participant 6 selected for protein expression assessment by GeoMx Digital Spatial Profiling. (C) Differential protein expression analysis comparing portal or lobular structures across time points; proteins with false discovery rate <0.05 are annotated. Statistical analyses were performed using a linear mixed model. (D) Chord diagram containing the 3 top GO pathways significantly enriched in the RNAseq transcriptomic profile of Week 4 liver biopsies as compared with baseline. (E) GSEA plot displaying the enrichment in an 11-gene TCMR gene set in biopsies obtained at Week 4 in comparison with baseline. (F) Heatmap showing the relative expression of the 11-gene TCMR gene set at baseline and Week 4. Rows represent genes, and columns represent samples. The intensity of each colour denotes the standardised ratio between each value and the average expression of each gene across all samples. Red pixels correspond to an increased abundance whereas green pixels indicate decreased transcript levels. GO, gene ontology; GSEA, gene set-enrichment analysis; LDIL-2, low-dose IL-2; TCMR, T-cell-mediated rejection; Treg, regulatory T cell. (This figure appears in color on the web.)

in immunoregulation.³⁷ Furthermore, an increase in serum CXCL10 was described in healthy individuals as soon as 2 days after administration of ultralow-dose IL-2,²³ indicating that the activation of IFN γ -mediated immune responses is likely to be a general outcome of LDIL-2 treatment.

Conclusions

We report for the first time on the use of LDIL-2 to suppress alloimmunity and promote liver allograft tolerance in humans. The effects of LDIL-2 on the liver allograft and on the circulation were drastically different. Thus, LDIL-2 markedly

expanded circulating Tregs but did not promote their trafficking into the liver and rendered the allograft more immunogenic as a result of the activation of an IFN γ -mediated inflammatory response. Overall, LDIL-2 did not promote, and probably hindered, the development of liver allograft

tolerance. Our study illustrates the fine balance existing between tolerance and immunity in human liver transplantation and the need to thoroughly investigate at the tissue level the off-target effects of systemic immunomodulatory therapies such as IL-2.

Affiliations

¹Institute of Liver Studies, King's College Hospital, Medical Research Council (MRC) Centre for Transplantation, School of Immunology & Microbial Sciences, Faculty of Life Sciences and Medicine, King's College London, London, UK; ²Liver Unit, Hospital Clinic Barcelona, IDIBAPS, CIBERehd, Barcelona, Spain; ³Liver Histopathology Laboratory, King's College Hospital, London, UK; ⁴Transplantation Research Immunology Group, Nuffield Department of Surgical Sciences, University of Oxford, John Radcliffe Hospital, Oxford, UK; ⁵School of Population Health and Environmental Sciences, King's College London, London, UK; ⁶Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany; ⁷Department of Immunobiology, School of Immunology & Microbial Sciences (SIMS), King's College London, London, UK; ⁸Department of Pathology, University of Pittsburgh, Pittsburgh, PA, USA; ⁹Département de Pharmacologie Clinique, Hôpital de la Pitié-Salpêtrière et UPMC Pharmacologie, Paris-Sorbonne Université, Paris, France; ¹⁰Laboratoire de Biostatistique, Epidémiologie Clinique, Santé Publique Innovation et Méthodologie (BESPI), CHU-Nîmes, Nîmes, France; ¹¹Bioinformatic Platform, Biomedical Research Center in Hepatic and Digestive Diseases (CIBERehd), Carlos III Health Institute, Barcelona, Spain

Abbreviations

cGvHD, chronic graft vs. host disease; DSMB, Data Safety Monitoring Board; GEO, Gene Expression Omnibus; GO, Gene Ontology; HLA, human leucocyte antigen; IL-2R, IL-2 receptor; LDIL-2, low-dose IL-2; LITE, LDIL-2 to expand endogenous regulatory T cells and achieve tolerance in liver transplantation; MHC, major histocompatibility complex; NK, natural killer; RNAseq, RNA sequencing; SLE, systemic lupus erythematosus; TCMR, T-cell-mediated rejection; Tconv, Foxp3⁺ conventional T cells; TFH, T follicular helper cell; Treg, regulatory T cell; TSDR, Treg-specific demethylated region.

Financial support

The LITE trial was funded by the Medical Research Council (grant number MR/P007694/1), and supported by the Medical Research Council Transplantation Centre (reference J006742/1) and the National Institute for Health Research Biomedical Research Centre based at Guy's and St Thomas' National Health Service Foundation Trust.

Conflicts of interest

AS-F and MM-L are Founders of Quell Therapeutics Ltd, and MM-L is employed by Quell Therapeutics Ltd. GB holds a patent on the use of low-dose IL-2 in autoimmune/autoinflammatory diseases (WO2012123381A1). The remaining authors declare no conflicts of interest relevant to the current manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Obtained the study funding: AS-F. Conceptualised and designed the trial: AS-F, GB, TT. Responsible for sample collection, sample processing, data acquisition, analysis, and interpretation: TYL, EP, PRM, ASK, EK, TT, MM-L, M-CL, RM, AC, CB, RT, FI, JH, AD, FHV, EW, AJD, AL, J-JL, AS-F. Prepared the manuscript: TYL, AS-F. Reviewed and contributed to the final draft: all authors.

Data availability statement

All data are available in the main text or the supplementary materials except for the transcriptional analysis raw data that can be accessed through the Gene Expression Omnibus (GEO) with accession number GSE186580.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2022.08.035>.

References

- Li W, Kuhr CS, Zheng XX, Carper K, Thomson AW, Reyes JD, et al. New insights into mechanisms of spontaneous liver transplant tolerance: the role of Foxp3-expressing CD25⁺CD4⁺ regulatory T cells. *Am J Transpl* 2008;8:1639–1651.
- Pu LY, Wang XH, Zhang F, Li XC, Yao AH, Yu Y, et al. Adoptive transfusion of ex vivo donor alloantigen-stimulated CD4⁺CD25⁺ regulatory T cells ameliorates rejection of DA-to-Lewis rat liver transplantation. *Surgery* 2007;142:67–73.
- Benítez C, Londoño M-C, Miquel R, Manzia T-M, Abalde JG, Lozano J-J, et al. Prospective multicenter clinical trial of immunosuppressive drug withdrawal in stable adult liver transplant recipients. *Hepatology* 2013;58:1824–1835.
- Taubert R, Danger R, Londoño M-C, Christakoudi S, Martinez-Picola M, Rimola A, et al. Hepatic infiltrates in operational tolerant patients after liver transplantation show enrichment of regulatory T cells before proinflammatory genes are downregulated. *Am J Transpl* 2016;16:1285–1293.
- Todo S, Yamashita K, Goto R, Zaitzu M, Nagatsu A, Oura T, et al. A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation. *Hepatology* 2016;64:632–643.
- Yu A, Snowwhite I, Vendrame F, Rosenzweig M, Klatzmman D, Pugliese A, et al. Selective IL-2 responsiveness of regulatory T cells through multiple intrinsic mechanisms supports the use of low-dose IL-2 therapy in type 1 diabetes. *Diabetes* 2015;64:2172–2183.
- Klatzmman D, Abbas AK. The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. *Nat Rev Immunol* 2015;15:283–294.
- Whitehouse G, Gray E, Mastoridis S, Merritt E, Kodala E, Yang JHM, et al. IL-2 therapy restores regulatory T-cell dysfunction induced by calcineurin inhibitors. *Proc Natl Acad Sci* 2017;114:7083–7088.
- Ratnasothy K, Jacob J, Tung S, Boardman D, Lechler RI, Sanchez-Fueyo A, et al. IL-2 therapy preferentially expands adoptively transferred donor-specific Tregs improving skin allograft survival. *Am J Transpl* 2019;19:2092–2100.
- He J, Zhang X, Wei Y, Sun X, Chen Y, Deng J, et al. Low-dose interleukin-2 treatment selectively modulates CD4⁺ T cell subsets in patients with systemic lupus erythematosus. *Nat Med* 2016;22:991–993.
- Shaked A, DesMarais MR, Kopetskie H, Feng S, Punch JD, Levitsky J, et al. Outcomes of immunosuppression minimization and withdrawal early after liver transplantation. *Am J Transpl* 2019;19:1397–1409.
- Ashokkumar C, Soltys K, Mazariegos G, Bond G, Higgs BW, Ningappa M, et al. Predicting cellular rejection with a cell-based assay. *Transplantation* 2017;101:131–140.
- Grinberg-Bleyer Y, Baeyens A, You S, Elhage R, Fourcade G, Gregoire S, et al. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J Exp Med* 2010;207:1871–1878.
- Sheean RK, McKay FC, Cretney E, Bye CR, Perera ND, Tomas D, et al. Association of regulatory T-cell expansion with progression of amyotrophic lateral sclerosis: a study of humans and a transgenic mouse model. *JAMA Neurol* 2018;75:681–689.
- Saadoun D, Rosenzweig M, Joly F, Six A, Carrat F, Thibault V, et al. Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med* 2011;365:2067–2077.
- Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea 3rd EP, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* 2011;365:2055–2066.
- Tang Q, Lee K. Regulatory T-cell therapy for transplantation: how many cells do we need? *Curr Opin Organ Transpl* 2012;17:349–354.
- Hartemann A, Bensimon G, Payan CA, Jacqueminet S, Bourron O, Nicolas N, et al. Low-dose interleukin 2 in patients with type 1 diabetes: a phase 1/2 randomised, double-blind, placebo-controlled trial. *Lancet Diabetes Endocrinol* 2013;1:295–305.
- Camu W, Mickunas M, Veyrune J-L, Payan C, Garlanda C, Locati M, et al. Repeated 5-day cycles of low dose aldesleukin in amyotrophic lateral sclerosis (IMODALS): a phase 2a randomised, double-blind, placebo-controlled trial. *EBioMedicine* 2020;59:1–12.

- [20] Rosenzweig M, Lorenzon R, Cacoub P, Pham HP, Pitoiset F, El Soufi K, et al. Immunological and clinical effects of low-dose interleukin-2 across 11 autoimmune diseases in a single, open clinical trial. *Ann Rheum Dis* 2019;78:209–217.
- [21] Dong S, Hiam-Galvez KJ, Mowery CT, Herold KC, Gitelman SE, Esensten JH, et al. The effect of low-dose IL-2 and Treg adoptive cell therapy in patients with type 1 diabetes. *JCI Insight* 2021;6:147474.
- [22] Rosenzweig M, Churlaud G, Mallone R, Six A, Dérian N, Chaara W, et al. Low-dose interleukin-2 fosters a dose-dependent regulatory T cell tuned milieu in T1D patients. *J Autoimmun* 2015;58:48–58.
- [23] Ito S, Bollard CM, Carlsten M, Melenhorst JJ, Biancotto A, Wang E, et al. Ultra-low dose interleukin-2 promotes immune-modulating function of regulatory T cells and natural killer cells in healthy volunteers. *Mol Ther* 2014;22:1388–1395.
- [24] Rosenblum MD, Gratz IK, Paw JS, Lee K, Marshak-Rothstein A, Abbas AK. Response to self antigen imprints regulatory memory in tissues. *Nature* 2011;480:538–542.
- [25] Spence A, Purtha W, Tam J, Dong S, Kim Y, Ju C-H, et al. Revealing the specificity of regulatory T cells in murine autoimmune diabetes. *Proc Natl Acad Sci* 2018;115:5265–5270.
- [26] Dawson NAJ, Lamarche C, Hoeppli RE, Bergqvist P, Fung V, McIver E, et al. Systematic testing and specificity mapping of alloantigen-specific chimeric antigen receptors in T regulatory cells. *JCI Insight* 2019;4:e123672.
- [27] Tang Q, Leung J, Peng Y, Sanchez-Fueyo A, Lozano J-J, Alice Lam KL, et al. Autologous donor-alloantigen reactive Treg cell therapy for minimization of immunosuppression after liver transplantation. *Sci Transl Med* 2022. in press.
- [28] De Simone G, Andreatta F, Blieriot C, Fumagalli V, Laura C, Garcia-Manteiga JM, et al. Identification of a Kupffer cell subset capable of reverting the T cell dysfunction induced by hepatocellular priming. *Immunity* 2021;54:2089–2100.e8.
- [29] Bénéchet AP, Simone G De, Lucia P Di, Cilenti F, Barbiera G, Bert N Le, et al. Dynamics and genomic landscape of CD8⁺ T cells undergoing hepatic priming. *Nature* 2019;574:200–205.
- [30] Wendel M, Galani IE, Suri-Payer E, Cerwenka A. Natural killer cell accumulation in tumors is dependent on IFN- γ and CXCR3 ligands. *Cancer Res* 2008;68:8437–8445.
- [31] Yang X, Chu Y, Wang Y, Zhang R, Xiong S. Targeted in vivo expression of IFN- γ -inducible protein 10 induces specific antitumor activity. *J Leukoc Biol* 2006;80:1434–1444.
- [32] Pandey V, Fleming-Martinez A, Bastea L, Doeppler HR, Eisenhauer J, Le T, et al. CXCL10/CXCR3 signaling contributes to an inflammatory microenvironment and its blockade enhances progression of murine pancreatic pre-cancerous lesions. *Elife* 2021;10:e60646.
- [33] Kikuchi N, Ye J, Hirakawa J, Kawashima H. Forced expression of CXCL10 prevents liver metastasis of colon carcinoma cells by the recruitment of natural killer cells. *Biol Pharm Bull* 2019;42:57–65.
- [34] Bronger H, Singer J, Windmüller C, Reuning U, Zech D, Delbridge C, et al. CXCL9 and CXCL10 predict survival and are regulated by cyclooxygenase inhibition in advanced serous ovarian cancer. *Br J Cancer* 2016;115:553–563.
- [35] Lakkis FG, Lechler RI. Origin and biology of the allogeneic response. *Cold Spring Harb Perspect Med* 2013;3:a014993.
- [36] Tilstra JS, Avery L, Menk AV, Gordon RA, Smita S, Kane LP, et al. Kidney-infiltrating T cells in murine lupus nephritis are metabolically and functionally exhausted. *J Clin Invest* 2018;128:4884–4897.
- [37] Amodio G, Cichy J, Conde P, Matteoli G, Moreau A, Ochando J, et al. Role of myeloid regulatory cells (MRCs) in maintaining tissue homeostasis and promoting tolerance in autoimmunity, inflammatory disease and transplantation. *Cancer Immunol Immunother* 2018;68:661–672. 684 2018.