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

Clinical trial registration: Clinicaltrials.gov NCT02949492

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**Data and materials availability**

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.
**Lay Summary:** The administration of low-dose interleukin-2 to liver transplant patients increased the number of circulating lymphocytes with immunosuppressive properties (regulatory T cells), but did not promote their trafficking into the transplanted liver and failed to induce transplantation tolerance.

**Abstract:**

**BACKGROUND & AIMS:** CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) are essential to maintain immunological tolerance and have been shown to promote liver allograft tolerance both in rodents and in humans. Low dose interleukin-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 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 initiating LDIL-2, patients 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 patients 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 intra-hepatic Tregs. Furthermore, LDIL-2 induced a marked interferon-gamma orchestrated transcriptional response in the liver even before immunosuppression weaning was initiated. The trial was terminated after the first six participants failed to reach the primary endpoint due to rejection requiring immunosuppression reinstitution.
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 the 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.
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\(^1\), while 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.\(^5\)

Interleukin (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 0.5-3 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 versus host disease (cGvHD) and a variety of different autoimmune disorders.\(^7\)

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

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\(^1\) Reference 1
\(^2\) Reference 2
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\(^4\) Reference 4
\(^5\) Reference 5
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\(^8\) Reference 8
intra-graft accumulation of donor-specific Tregs\textsuperscript{9} and synergizes with Tacrolimus in prolonging allograft survival.\textsuperscript{8} We hypothesized 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’ (Low-dose IL-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 low-dose IL-2 (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: 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-HLA antibodies. Eligibility criteria are outlined in Supplementary Table 6. An Ethics Committee approved the study protocol (REC number 14/LO/1014) and all patients provided written informed consent.

Trial procedures and low-dose IL-2 dosing

IL-2 (Proleukin, Novartis) was initiated at 1 million IU/day subcutaneously. 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 maximize 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 that 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 six steps lasting 2 weeks each. Low-dose IL-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 patients tolerating immunosuppression withdrawal a final protocol biopsy was required 18 months after enrolment. 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 DSMB. Urgent safety measures approved by the DSMB were instituted immediately after P02’s severe rejection episode, consisting in: i) halting further participant recruitment; ii) reducing IL-2 dose to 0.5M IU/day for the 4 patients undergoing immunosuppression withdrawal; ii) monitoring liver tests weekly throughout the duration of the trial. The overall IL-2 doses administered per patient are shown in Supp_Fig.6.

Rationale for study design and sample size considerations

Recipient age and time post-transplantation were specified in order to define a trial population with a very low likelihood of spontaneous operational tolerance.\textsuperscript{3,16} 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 patients had achieved maximum Treg expansion. To demonstrate the superiority of a clinically-meaningful immunosuppression withdrawal success rate $\geq 40\%$ as compared to $<15\%$ predicted based on historical data\textsuperscript{3,16}, we estimated the need to enrol 19 subjects undergoing immunosuppression withdrawal.

Statistical Analysis and Detailed Experimental Methods: 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,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 2-6.7 fold; Supp_Table 1). Patient P04 did not initiate Tacrolimus withdrawal due 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 patient’s immunosuppressive regimen (Table 2). The remaining 5 patients were considered suitable for initiating Tacrolimus withdrawal. Four patients succeeded in reducing their Tacrolimus doses by 75% and 2 completely discontinued their immunosuppression, but all of them experienced allograft dysfunction within six months of the trial initiation, which was due to TCMR and required immunosuppression re-institution. As a result, none reached the trial’s primary endpoint (Fig. 1c,d). Among the 5 patients 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 patient P02 developed severe TCMR that did not respond to steroid boluses or thymoglobulin, progressed to chronic ductopenic rejection and required re-transplantation (P02 follow-up after re-transplantation was uneventful with no further episodes of rejection). Following review by the independent data safety monitoring board (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 Supp_Table 2.
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} 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 employing 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, IL1RL1) and a decrease in transcripts enriched in B cells (e.g. CD19, CD22, CXCR5, CD79A, BLNK, TNFRSF13B). The expression of genes involved in cytotoxicity, allograft rejection or liver inflammation were 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, TNFRSF12A*) (Fig.4 and Supp_Table 3). 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 employed an activation-induced marker assay to determine whether the increase in circulating Tregs was due to preferential expansion of donor-reactive clones (Fig.5a-d). All patients displayed detectable alloantigen-specific Foxp3+Helios+ Tregs and CD4+Foxp3+ 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 of time (Fig.5d). On the other hand, 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 (Supp_Fig.1).10 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 pSTAT5 levels in response to increasing IL-2 concentrations. Only Tregs and not cytopathic/effecter immune cell subsets acquired increased responsiveness to IL-2 (Supp_Fig.2). 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 intra-hepatic Tregs and primes the liver allograft for rejection.**

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 \( (p<0.03; \text{Table 2 and Supp_Fig.3}) \). 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 patients 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, while we observed a positive trend in the number of intra-hepatic 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 employed a Nanostring GeoMx platform to evaluate the expression of a set of 46 immune-related proteins in 156 distinct segments selected across the patients’ sequential liver biopsies (Fig.6b). The portal tract segments exhibited no changes in protein levels in the first 4 weeks of LDIL-2 treatment, while a significant increase in T cell, macrophage and NK cell related proteins (e.g. CD4, CD8, GZMB, CD56, CD163, 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, LAG3) at week 4, with an increase in myeloid-related (CD68, VISTA) and immunoactivatory proteins (CD40, CD44, HLA-DR) at the time of rejection (Fig. 6c). Taken together, these results 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 characterize the changes induced by LDIL-2 on the liver allograft, we conducted 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 (FDR<0.05) with CXCL9 and CXCL10 being the 2 genes whose expression was most markedly induced (fold change 4.5 and 4, respectively; Supplementary Table 4). Functional analysis employing Gene Ontology (GO) terms identified immune response, interferon-gamma (IFNg)-mediated signalling pathway and MHC class-II protein complex as the most highly enriched pathways (Fig.5d). In addition, a 11-gene set 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 IFNg-related gene sets known to be involved in TCMR across a variety of different transplant settings (Supp_Table 5). 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,
IFNγ, 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, IFNγ, CCL22, IL-2, IL-4, CCL13, and TNFα were increased in a sustained way throughout the first 6 weeks of LDIL-2 treatment (Supp_Fig.4a). No such changes were observed in the transcript levels of the corresponding genes measured in blood samples collected at the same time points (Supp_Table 3). This contrasted with the marked increase in the gene expression of the IFNγ-induced chemokines CXCL10 and CXCL9 observed in the liver tissue (Supp_Table 4). 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 (Supp_Fig.4b). CXCL9 significantly correlated with the macrophage-monocyte lineage markers CD14 and CD68, while we observed no correlation with CD3 (T cell marker) and CD34, and a negative correlation with the myofibroblast cell marker SMA.
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. 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, hepatitis C virus (HCV)-induced vasculitis, and systemic lupus erythematosus (SLE). 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 investigated in humans. Our trial is the first one to explore the use of LDIL-2 in solid organ transplantation and to employ a hard clinical endpoint such as immunological tolerance. The trial population was selected based on previous studies demonstrating <15% likelihood of spontaneous allograft tolerance, 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 evidences derived from murine transplant models indicating the need to achieve high Treg/Tconv ratios to induce allograft tolerance, we chose an IL-2 dosing regimen capable of ensuring persistently high circulating Treg levels throughout the immunosuppression weaning period while minimizing 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 utilized in most autoimmune indications, but resembles the scheme safely employed in cGVHD, in which daily doses of 2 million IU IL-2 have been administered for very prolonged periods of time and shown to be well tolerated and to reduce disease activity. 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 IFNγ-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 homeostasis of other circulating immune cell subsets as well, in particular CD56^{high} NK cells, eosinophils, T follicular helper cells (TFH), B cells and activated non-regulatory T cells expressing CD25.\textsuperscript{18–21} Furthermore, it induces the systemic release of cytokines such as IL-10, TGFβ, IL-5, TNFα and IL-17, and most notably, the chemoattractant CXCL10.\textsuperscript{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 employed a variety of different dosing regimens. What had not been previously recognized 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\textsuperscript{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 patients to those enrolled in our study.\textsuperscript{27} On the other hand, 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 IFNγ-orchestrated 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\textsuperscript{28} in a murine model of antigen-specific
intra-hepatic T cell priming. In this model, IL-2 administration reverted the T cell
dysfunction typically induced by intra-hepatic priming and caused a clustered accumulation
of activated effector T cells in the liver sinusoids. 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. Given
the striking similarities between De Simone et al.’s findings in mice and our own
observations, we hypothesize that a similar mechanism caused the clinically-silent IFNγ-
driven intra-hepatic transcriptional response detected in our patients 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, 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 re-transplantation in P02, an extraordinarily rare event in
immunosuppression withdrawal trials in liver transplantation. The contribution of other
effector immune cell subsets such as NK cells to an exacerbated rejection response 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 muteins 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. Moreover, the liver exhibits unique immunological properties that might not be relevant to other settings. Yet, organs such as kidneys are also capable of inactivating infiltrating effector lymphocytes, and Kupffer cells are not the only tissue-resident macrophages in the body involved in immunoregulation. Furthermore, an increase in serum CXCL10 was described in healthy individuals as soon as 2 days after administration of ultra-low dose IL-2, indicating that the activation of IFNγ-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 IFNγ-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.
Figure Legends

**Figure 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).

**Figure 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 pre-defined circulating immune cell subsets at the indicated time points. The color scale corresponds to log2 fold-changes as compared to 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 colors 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) Mean fluorescence intensity (MFI) of CD27 and Foxp3 in CD4 metaclusters 9, 10, 11 and 12 at baseline, week 2 and week 4. D) viSNE plots and heatmaps representing different CD8+ T cell metaclusters identified by FlowSOM, with percentages at baseline, week 2 and week 4. Statistical analysis was performed by One-way ANOVA with Tukey’s multiple comparisons tests; if not indicated, p-values were not significant. BL, baseline; W1, week 1; W2, week 2; W3, week3; W4, week 4; Rej, rejection.

**Figure 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.

**Figure 4: Sequential changes in T cell subsets and whole blood gene expression profile throughout the trial duration.** A & 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 +/- 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 analyzed on a Nanostring platform. The heatmap displays the 110 genes differentially expressed at P-value <0.05 in at least one comparison between time points. Rows represent genes and columns represent samples. The intensity of each color denotes the standardized 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.

**Figure 5: Effect of LDIL-2 on alloantigen-specific T cell subsets.** A) Proportion of Tregs and Helios+ Tregs in the cryopreserved PBMC specimens employed for the activation induced marker assays. B) Gating strategy employed to identify alloantigen-specific Tregs, CD4+ and CD8+ T cells following 16h culture with surrogate donor or third-party PBMCs in the activation induced marker assay. C) In order 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 16h 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 interquartile range (IQR). D) Frequency of alloantigen-specific Tregs, 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 interquartile range. Statistical analysis was performed employing a repeated measures ANOVA.

**Figure 6: Impact of LDIL-2 on intra-hepatic 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 patient 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 FDR<0.05 are annotated. Statistical analyses were performed employed linear mixed model. D) Chord diagram containing the 3 top Gene Ontology (GO) pathways significantly enriched in the RNASeq transcriptomic profile of week 4 liver biopsies as compared to baseline. E) Gene set enrichment analysis (GSEA) plot displaying the enrichment in an 11-gene T cell mediated rejection (TCMR) gene set in biopsies obtained at week 4 in comparison to 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 color denotes the standardized 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.
References


Table 1: Clinical and demographic characteristics of trial participants

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<th>Age (years)</th>
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Abbreviations: BSA (body surface area), eGFR (estimated glomerular filtration rate), LFTs (liver function tests), DSA (donor specific antibodies; measured before initiating weaning and at the time of rejection), ALF (acute liver failure), MFI (mean fluorescent intensity).
Table 2: Liver histopathology assessments

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1 mean cells/field; 2 p<0.03 baseline versus week 4; 3 p<0.03 baseline versus week 4.

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 employing 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.

Abbreviations: pt (patient); inflam. (inflammation); lymph. (lymphocytes); RAI (rejection activity index); TCMR (T cell mediated rejection); NRH (nodular regenerative hyperplasia); BD (bile ducts); endoth (endothelialitis)
Figure 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).
Figure 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 pre-defined circulating immune cell subsets at the indicated time points. The color scale corresponds to log2 fold-changes as compared to 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 colors 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) Mean fluorescence intensity (MFI) of CD27 and Foxp3 in CD4 metaclusters 9, 10, 11 and 12 at baseline, week 2 and week 4.

d) viSNE plots and heatmaps representing different CD8+ T cell metaclusters identified by FlowSOM, with percentages at baseline, week 2 and week 4. Statistical analysis was performed by One-way ANOVA with Tukey’s multiple comparisons tests; if not indicated, p-values were not significant. BL, baseline; W1, week 1; W2, week 2; W3, week 3; W4, week 4; Rej, rejection.
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 including all 10 specimens available for analysis. 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, at baseline and 4 weeks after initiating LDIL-2. BL, baseline; W4, week 4; TSDR, Treg-specific demethylated region.
Fig. 4: Sequential changes in T cell subsets and whole blood gene expression profile throughout the trial duration. 

a/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 of fresh blood specimens. Data correspond to mean +/- SEM. 

c) RNA samples extracted from whole blood specimens collected at baseline, at weeks 4 and 12 treatment after initiating LDIL-2 treatment, and at the time of clinical rejection, were analyzed on a Nanostring platform. The heatmap displays the 110 genes differentially expressed at P-value <0.05 in at least one comparison between time points. Rows represent genes and columns represent samples. The intensity of each color denotes the standardized 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.
Figure 5: Effect of LDIL-2 on alloantigen-specific T cell subsets. 

a) Frequency of Tregs and Helios+ Tregs in cryopreserved PBMC specimens in the absence of alloantigen stimulation. Error bars correspond to median and interquartile range. Statistical analysis was performed employing a repeated measures ANOVA. 

b) Gating strategy employed to identify alloantigen-specific Tregs, CD4+ and CD8+ T cells following 16h culture with surrogate donor or third-party PBMCs. 

c) In order 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 (b) following 16h 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 interquartile range (IQR). 

d) Frequency of alloantigen-specific Tregs, CD4+ and CD8+ T cells in PBMCs collected from the trial participants before, 1 week and 4 weeks after initiating LDIL-2. Error bars correspond to median and interquartile range. Statistical analysis was performed employing a repeated measures ANOVA.
Figure 6: Impact of LDIL-2 on intra-hepatic 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 patient 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 FDR<0.05 are annotated. Statistical analyses were performed employing linear mixed model. d) Chord diagram containing the 3 top Gene Ontology (GO) pathways significantly enriched in the RNASeq transcriptomic profile of week 4 liver biopsies as compared to baseline. e) Gene set enrichment analysis (GSEA) plot displaying the enrichment in an 11-gene T cell mediated rejection (TCMR) gene set in biopsies obtained at week 4 in comparison to 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 color denotes the standardized 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.
Graphical Abstract

Stable liver recipients 2-6 years after transplant

Low-dose IL-2

- 2-6 fold expansion of circulating Tregs
- Induction of IFNg response in liver tissue

Tacrolimus dose

Low-dose IL-2 (1M IU sc daily)

Tolerance adjudication

Weeks

0  4  8  12  16  20  24  72

Number of patients free of rejection

0  1  2  3  4  5  6

Weeks after initiating LDIL-2

0  10  20  30