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Treg-specific IL-2 therapy can reestablish intrahepatic immune regulation in autoimmune hepatitis

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ABSTRACT

Autoimmune hepatitis (AIH) is a chronic autoimmune inflammatory disease that usually requires life-long immunosuppression. Frequent relapses after discontinuation of therapy indicate that intrahepatic immune regulation is not restored by current therapies. As steroid therapy preferentially depletes intrahepatic regulatory T cell (Tregs), immune regulation might be re-established by increasing and functionally strengthening intrahepatic Tregs. In recent clinical trials with low dose IL-2, the Treg compartment was strengthened in autoimmune diseases.

Therefore, we tested complexed IL-2/anti-IL-2 to increase the selectivity for Tregs. We used our model of experimental murine AIH (emAIH) and treated the mice with complexed IL-2/anti-Il-2 in the late course of the disease.

The mice showed increased intrahepatic and systemic Treg numbers after treatment and a reduction in activated, intrahepatic effector T cells (Teffs). This resulted in a reduction in liver-specific ALT levels and a molecular pattern similar to that of healthy individuals.

In conclusion, complexed IL-2/anti-IL-2 restored the balance between Tregs and Teffs within the liver, thereby improving the course of emAIH. Treg-specific IL-2 augmentation offers new hope for reestablishing immune tolerance in patients with AIH.

1. Introduction

Autoimmune hepatitis (AIH) is a type of chronic autoimmune inflammatory disease of the liver tissue. The treatment for AIH has remained largely unchanged for decades. The first-line treatment is corticosteroids with or without azathioprine [1–3]. The ideal management of nonresponders remains unclear. Treatment approaches in small cohorts have included anti-TNF, anti-CD20, TGF-β and interleukin-2 (IL-2). Most patients require life-long immunosuppression and will relapse after discontinuation of therapy. This indicates current therapies are blocking pathogenic immune responses without reestablishing an immune tolerance. This could be due to the fact that corticosteroid therapy is depleting intrahepatic Tregs to a larger extent than effector Tregs [4]. Therefore, future therapies should aim in restoring intrahepatic immune regulation to enable a discontinuation of immunosuppressive therapy.

Regulatory T cells (Tregs) are immunosuppressive and play a key role in restraining autoreactive effector T lymphocytes and preventing autoimmunity. Due to their constitutive expression of the alpha chain of the IL-2 receptor (CD25), Tregs are highly responsive to IL-2. Open-label clinical trials with low-dose IL-2 (ldIL-2) showed beneficial effects in various autoimmune disorders, e.g. systemic lupus erythematosus (SLE), rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus, psoriasis, Behcet's disease, granulomatosis with polyangiitis,

Abbreviations: AIH, autoimmune hepatitis; emAIH, experimental murine autoimmune hepatitis; Ad, adenovirus; FTCD, formiminotransferase cyclodeaminase; Tregs, regulatory T cells; IHLs, intrahepatic lymphocytes; IL-2, interleukin-2; AST, aspartate aminotransferase; ALT, alanine transaminase; HE, hematoxylin and eosin; mHAI, modified hepatitis activity index.

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Takayasu's disease, Crohn's disease, ulcerative colitis, sclerosing cholangitis, AIH, hepatitis C vasculitis, graft-versus-host disease, and type 1 diabetes (T1D) [5–7]. However, as CD25 is not exclusively expressed by Tregs, other T cells and NK cells might be activated as well, as has recently been shown in clinical trials in type 1 diabetes and after liver transplantation [8–10]. In order to restrict the IL-2 effect to Tregs mutated IL-2 proteins or antibody-complexed IL-2 formulation are being developed as next generation IL-2 therapies.

Here, we used our well-established model of experimental murine AIH (emAIH) [11–13] and determined the effect of complexed IL-2 treatment. In addition to the increase in intrahepatic and systemic Tregs three weeks after treatment, we observed reduced intrahepatic activation of T cells. This led to a significant reduction in liver-specific ALT levels and to a molecular pattern similar to that of healthy individuals.

2. Methods

2.1. Ethics statement

Animal care and experiments were performed in accordance with institutional and national guidelines. All animal experiments were performed according to protocols approved by the Animal Welfare Commission of Hannover Medical School and the local Ethics Animal Review Board (Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany).

2.2. Mice

Animals were maintained under specific pathogen-free conditions at the Central Animal Facility of Hannover Medical School (Hannover, Germany). NOD/Ltj mice were intravenously injected with a total of 4×10^9 infectious particles containing Ad-FTCD in PBS [11–13]. 2.5 μg IL-2 and 25 μg anti-IL-2 or 1 μg IL-2 and 5 μg anti-IL-2 were administered for five consecutive days. All mice were sacrificed 16–18 weeks post infection.

2.3. Adenovirus construction

The generation of Ad-FTCD was previously described before [11–13]. Briefly, FTCD was amplified by PCR from cDNA generated from human liver cells; the sequence was verified by sequencing both DNA strands. The constructs were cloned into the Ad transfer vector pShuttle-CMV (Stratagene). By homologous recombination, this shuttle vector was recombined with pAdEasy-1, which carried deletions in the E1 and E3 regions. The genome of the generated adenovirus could be amplified only within the HEK 293 packaging cell line, which complements the essential regions. The purification of recombinant adenovirus was performed using a cesium chloride gradient, and the adenoviral stocks were quantified using an Adeno-X™ rapid titer kit (Clontech).

2.4. Histology and immunohistology

Murine livers were fixed in formalin and embedded in paraffin or were embedded in Tissue-Tek® O.C.T.™ compound (Sakura) without fixation for cryosectioning (8 μm). Paraffin-embedded sections (5 μm) were prepared for hematoxylin and eosin (HE) staining. After being stained, the sections were examined in a blinded manner by a pathologist using the approved modified hepatitis activity index (mHAI) for autoimmune hepatitis. Immunofluorescence microscopy was performed as previously described [11–13]. Briefly, cryosections were fixed with acetone, rehydrated, blocked, stained with anti-CD4, anti-CD8, anti-Foxp3 and DAPI and analyzed with an AxioImagerM1 using Axiovision software (Zeiss).

2.5. Flow cytometry

Organs were minced, and intrahepatic lymphocytes (IHLs) were separated using a 40%/70% Percoll (GE Healthcare) gradient. Red blood cells in the spleen were lysed, and lymphocytes were subsequently stained with appropriate combinations of anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD45, anti-Ki-67, anti-B220, anti-Foxp3, anti-CD62L, anti–IFN–γ and anti-IL-10. All data were acquired with an LSRII SORP interfaced with FACSDiva software (BD Biosciences).

2.6. Serum analysis

Blood samples were collected via the retro-orbital route. Aspartate aminotransferase (AST) and alanine transaminase (ALT) were measured by photometric enzyme activity assays with an Olympus AU400 chemistry analyzer using serum, as previously described [11–13].

2.7. Protein detection in the serum by olink

Proteins were measured using the Olink® MOUSE EXPLORATORY panel* (Olink Proteomics AB, Uppsala, Sweden) according to the manufacturer's instructions. The proximity extension assay (PEA) technology used for the Olink protocol has been well described [14] and allows for 92 analytes to be analyzed simultaneously. Briefly, pairs of oligonucleotide-labeled antibody probes bind to their targeted protein, and if the two probes are brought in close proximity, the oligonucleotides will hybridize in a pairwise manner. The addition of DNA polymerase leads to a proximity-dependent DNA polymerization event, which generates a unique PCR target sequence. The resulting DNA sequence is subsequently detected and quantified using a microfluidic real-time PCR instrument (Biomark HD, Fluidigm). The data are then quality controlled and normalized using an internal extension control and an interplate control to adjust for intra- and interrun variations. The final assay read-out is presented in Normalized Protein eXpression (NPX) values, which is an arbitrary unit on a log2-scale in which a high value corresponds to high protein expression. All assay validation data (detection limits, intra- and interassay precision data, etc.) are available on the manufacturer's website (www.olink.com).

2.8. Real-time PCR using fluidigm technology

2.8.1. Nucleic acid isolation and cDNA synthesis

Total RNA was isolated from frozen liver samples with an RNeasy mini kit (Qiagen) and was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher). Then, 2 μg of RNA was reverse transcribed into cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche).

2.8.2. Preamplification and quantitative RT-PCR (Fluidigm)

The preamplification of cDNA was performed using Fluidigm® PreAmp master mix and TaqMan® assays according to the manufacturers' guidelines. Quantitative RT-PCR was performed in a 48.48 Dynamic Array IFC using the preamplified samples. IFC priming and loading were performed on a Juno instrument using the prime script Prime 48.48 GE and Load Mix 48.48 GE. RT-PCR data were generated using a BioMark™ HD instrument. The gene TaqMan® assays that were utilized can be found in the corresponding figure.

2.8.3. Bioinformatics analysis

The normalization of the Ct values was performed by subtracting the mean values of the housekeeping genes *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* and *beta actin (Actb)* from the mean values of the genes of interest. Heat map and PCA analyses of the -delta Ct values were plotted via Qlucore software ($p < 0.05$ and $q < 0.2$).

2.9. Statistical analysis

In general, an unpaired, 2-tailed Student's *t*-test was performed using GraphPad Prism version 7.00 for Mac (GraphPad Software, La Jolla California, USA, www.graphpad.com). A Welch's t-test had to be used because equal SDs could not be assumed. Significant differences with $P \leq 0.05$ are indicated by *, and very significant differences ($P \leq 0.01$) are indicated by **. $P > 0.05$ was considered to be not significant (ns).

3. Results

3.1. Treg numbers were profoundly increased after ldIL-2 treatment

IL-2 was originally used to treat melanomas or other cancers by promoting T effector cells. Therefore, it is important to find doses that are still considered ldIL-2 but are high enough to trigger Tregs. In order to increase the selectivity of IL-2 for the high affinity IL-2 receptor we used complexed IL-2. We tested two different therapeutic options at ratios of 1:10 and 1:5. Doses of 2.5 μg IL-2 and 25 μg anti-IL-2 or 1 μg IL-2 and 5 μg anti-IL-2 were administered for five consecutive days, and the T cell populations were analyzed two days later (Fig. 1A). There was a decrease of $CD8^+$ T cells and an increase $CD4^+$ T cells (Fig. 1B–D). However, especially within the CD4 compartment, there was a strong increase in Tregs, which accounted for up to 80% of splenic CD4 T cells and 25–50% of cells in the liver. Due to a larger increase with the higher dose of complexed IL-2, we performed all further experiments with higher dose.

3.2. LdIL-2 treatment ameliorates emAIH

The induction of emAIH takes place over a period of twelve weeks after a single administration of Ad-FTCD [12,13]. Subsequently, IL-2/anti-IL-2 therapy was administered over five days, and the effect was analyzed after another month to investigate if intrahepatic immune tolerance was re-established. Measurable parameters for AIH therapy are histological analysis, especially the analysis of transaminases. We have already shown in previous studies that the mHAI in mice was significantly lower than that in AIH patients. Although there was a trend towards lower mHAI scores, this did not reach statistical significance (Fig. 2A and B). While the level of AST decreased slightly after therapy, there was a significant reduction in the level of liver-specific ALT (Fig. 2C and D).

3.3. LdIL-2 treatment induces long lasting increase of tregs and results in fewer activated IHLs

We observed a slight reduction in CD4 T cells in the spleen, which led to a reduction in total T cells (Fig. 2E and F). However, a detailed analysis of the T cell populations showed that even after three weeks, disproportionately high numbers of Tregs could be found in the liver and spleen (Fig. 2G and H). There were fewer IFN-γ^+ cells in the spleen and a trend towards less IFN-γ^+ cells in the liver (Fig, 2 G,H). There were no major changes in the number of IL-10 positive cells in either spleen or liver (data not shown), while we observed fewer $CD62L^{high}$ naïve $CD4^+$ T cells in the liver.

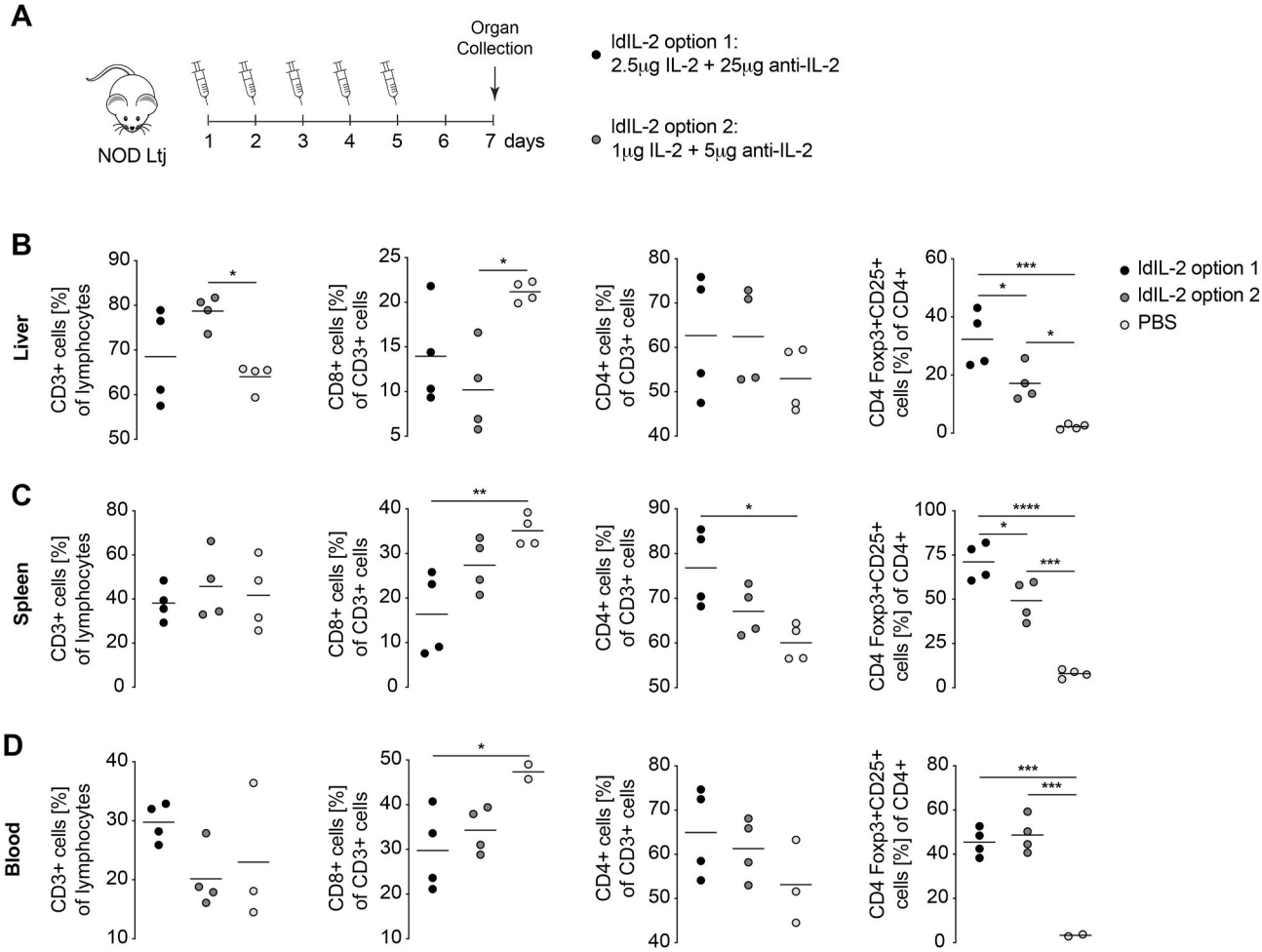

Fig. 1. Treg numbers were profoundly **increased after ldIL-2 treatment.** (A) Scheme of dose finding for ldIL-2. (B) As shown under (A) ldIL-2 was injected either as therapeutic option 1 (black dot) or 2 (grey red) in mice (n = 4). The liver, (C) spleen and (D) blood were analyzed by flow cytometry.

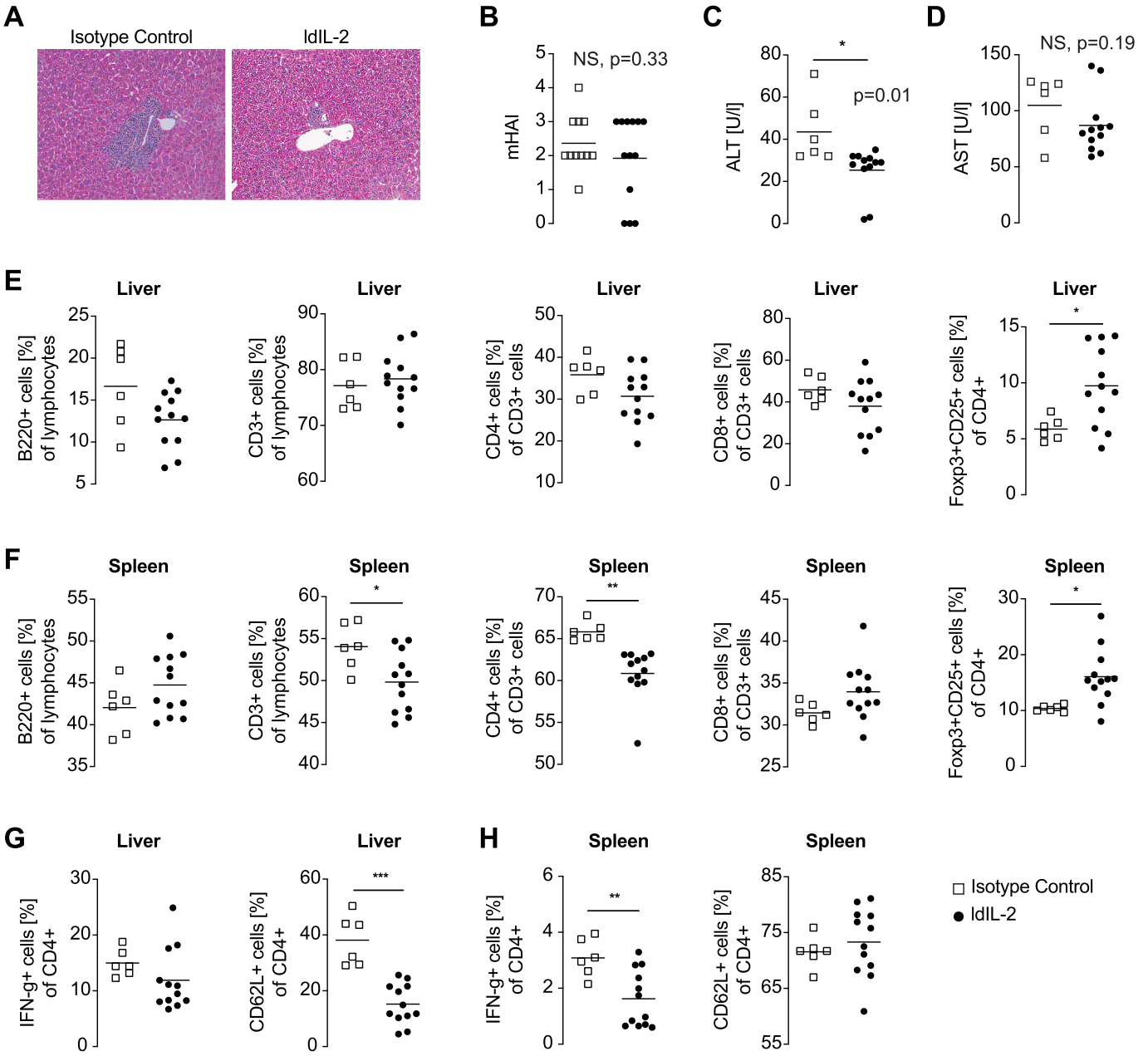

Fig. 2. LdIL-2 treatment reduces serum levels of ALT, induced more Tregs and less activated IHLs. (A) 16 weeks after emAIH induction (control mice, n = 11; white squares) equivalent to 4 weeks after ldIL-2 treatment in treated mice (black circles; n = 13) were analyzed for histology (B) corresponding mHAI, (C) and the serum transaminases ALT and (D) AST. (E) Flowcytometric analyses of IHL and (F) splenic lymphocytes were done regarding B220+, $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells. (G) Intrahepatic and (H) splenic $CD3^+CD4^+CD25$+Foxp3+ Tregs, $CD3^+CD4$+IFN-g+ and $CD3^+CD4^+CD62L$+ were analyzed furthermore.

This effect was even more evident in the histological analysis: the number of Tregs was almost fivefold higher in IL-2-treated animals than in untreated animals (Fig. 3A and B) and the ratio of Tregs to effector T cells also remained at a 3.75-fold increase (Fig. 3D).

Molecular analyses showed strong similarities between IL-2-treated and healthy animals.

The genetic analysis of 21 inflammation associated genes showed differences between untreated, IL-2-treated and healthy animals. The treated and healthy animals were similar. CD36, CCl2, IL1b, ITGAX, TLR4 and TLR9 are all associated with inflammation and were significantly lower in the IL-2-treated emAIH group than in the healthy group or the untreated emAIH group (Fig. 3E).

The effect was similar at the protein biochemical level. Although only 5 out of 92 proteins were differentially regulated, the altered proteins were all related to immunological or regenerative processes (Fig. 3F). The expression of CSF2 (granulocyte-macrophage colony-stimulating factor/GM-CSF) was significantly elevated after IL-2 treatment, while the expression of Igsf3 (immunoglobulin superfamily member 3), Itgb6 (integrin beta-6), Ntf3 (neurotrophin-3) and Plxna4 (plexin-A4) was reduced.

4. Discussion

In our emAIH model, we were able to show that the severity of the disease decreased in response to Treg-specific ldIL-2 treatment. This effect could be shown both by a reduction in ALT, a reduction in activated CD4 T cells, and a transcriptome signature resembling that of animals without emAIH. There was also a very strong increase in Tregs shortly after ldIL-2 administration that was long-lasting and marked.

AIH requires life-long immunosuppression by steroids with or

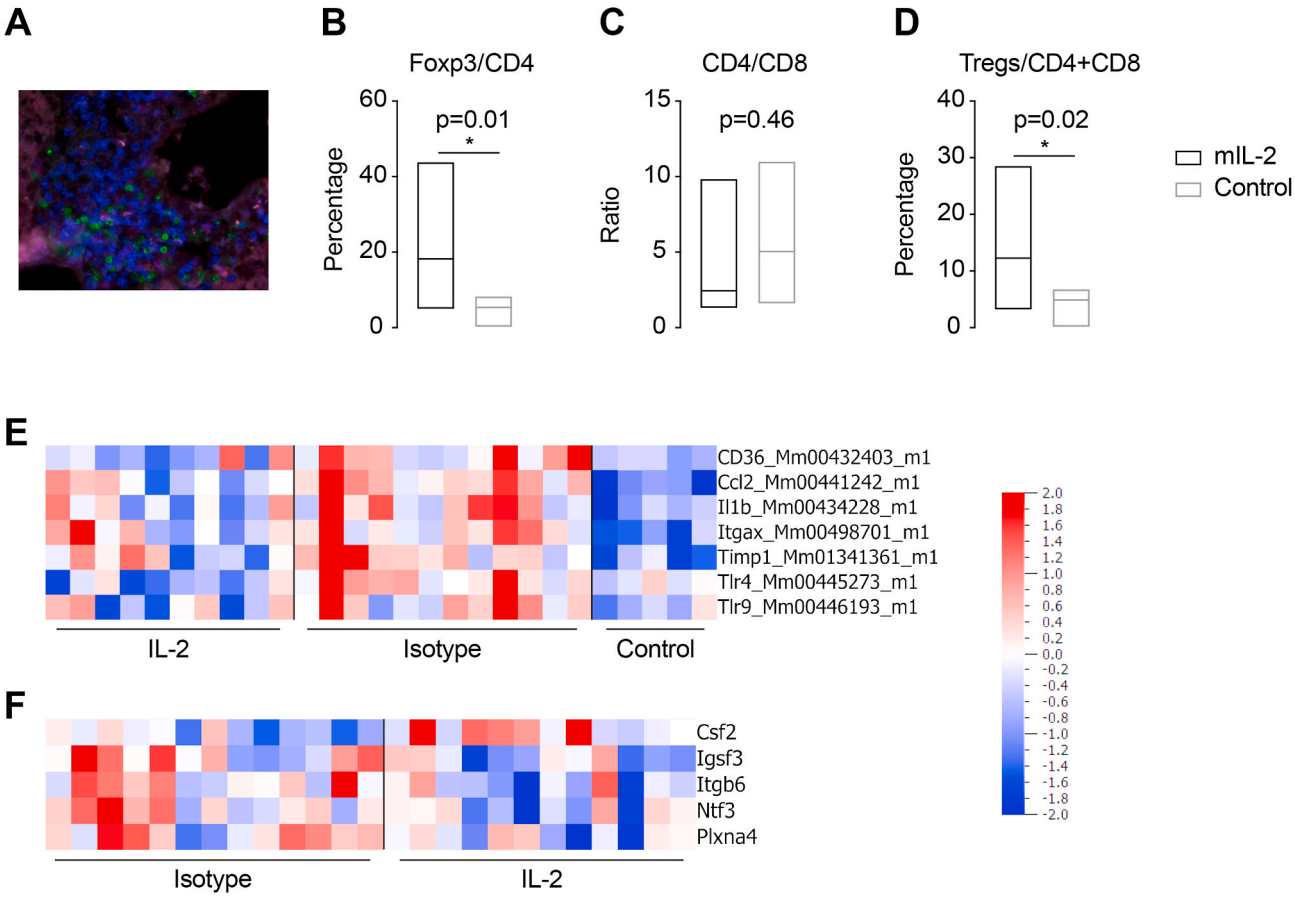

Fig. 3. LdIL-2 induced excessive amount of local Tregs in liver tissue and a transcriptome signature resembling that of animals without emAIH. (A) Cryo-preserved liver sections were immunohistochemically stained for CD4 (blue), CD8 (green) and Foxp3 (red) was performed on liver tissue, (B) quantified and the ratios of Tregs among $CD4^+$, (C) the ratio of $CD4^+$ and $CD8^+$ and (D) Tregs among $CD4^+CD8^+$ were calculated of cells in ldIL-2 treated mice (black boxes) and control mice (grey boxes). (E) The heatmap shows the gene expression quantified by $-\Delta CT$ normalized to *Actb* and *Gapdh* expression in liver tissue. (F) The second heatmap shows serum analyses of LdIL-2 treated and control emAIH NOD/Ltj for 92 proteins by Olink technology. Shown heat-maps taken in account the multiplicity correction after calculating the p (<0.05) and the q values (<0.05) for all 92 proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

without azathioprine in 70–80% of patients. This therapy depletes intrahepatic Tregs more than Teffs, which might explain the high relapse rate of 70–100% after discontinuation of therapy [4]. Therefore, restoring local intrahepatic immune regulation might enable patients to discontinue immune suppression in the future.

IL-2 was originally used as a drug to treat melanomas or other types of cancer [15]. Thus, high-dose IL-2 administration induces the differentiation, survival and proliferation of T cell effectors. The concept of low-dose IL-2 therapy to treat autoimmunity was first suggested by the discovery of Tregs with constitutive expression of the high affinity IL-2 receptor. Both Tregs and IL-2 are essential components for the maintenance of immune tolerance. Instead of inducing or strengthening the immune response by high-dose IL-2, the first studies with ldIL-2 indicated that IL-2 can suppress inflammation, autoimmunity and graft rejection. LdIL-2 increases the number of Tregs and has been successfully used for the treatment of lupus and lupus nephritis [16,17] and is currently being tested in AIH [18,19] and many other autoimmune diseases [5,20].

The important questions here are whether the administration of ldIL-2 strengthens immune regulation under all circumstances and does not affect the immune response, whether this regulation is long-lasting and whether the general immunocompetence of the immune system is maintained.

The fact that small differences in dose have a large influence on the number of Tregs had already been shown by some groups in T1D patients [21–23] and other diseases. However, in T1D, the use of ldIL-2 together with polyclonal Treg infusion exacerbated the expansion of Tregs as well as $GZMB^+$ $CD8^+$ T cells and the NK population in the blood. Thus, the failure to maintain the level of C-peptide may be due to a shift of the immune balance toward activation (personal communication J. Bluestone). Likewise, Sanchez-Fueyo and colleagues attempted to use ldIL-2 to promote the selective expansion of endogenous Tregs in liver transplant recipients in the context of immunosuppression [9,10]. Unfortunately, ldIL-2 alone led to graft rejections in patients after liver transplantation in the corresponding phase IV clinical trial, known as the Lite trial (NCT02949492).

Therefore, future clinical therapeutic approaches will require an improved selectivity of IL-2 for Tregs. As at present, there are two feasible ways of accomplishing this. Mutated IL-2 proteins or complexed with IL-2 will preferentially bind to the high affinity IL-2 receptor. Recently, the Bluestone group described a human complexing antibody that could be used for this purpose [24]. Therefore, we tested this new strategy of complexed IL-2 for the treatment of AIH.

When we used 1 μg with five times more complexing antibody in the dose response experiment, the complexed ldIL-2 led to robust Treg expansion in the spleen and blood but only weak expansion in the liver (Fig. 1). To achieve increased expansion, larger doses were needed. Consequently, a more selective IL-2 approach was needed to expand only Tregs in liver therapy. Therefore, we finally used 2.5 μg with tenfold more complexing anti-Il-2 to achieve more than 30%

intrahepatic Tregs in the CD4 T cell compartment.

This approach was highly effective in ameliorating emAIH and restoring intrahepatic immune regulation and was accompanied by a very strong increase in Tregs shortly after ldIL-2 administration that was also long-lasting and marked. The fact that small differences in dose have a large influence on the degree of Treg expansions had already been shown by some groups in T1D patients [21–23] and other diseases.

While therapies with IL-2 alone have shown activation of non-Treg populations, we had increased systemic and local Tregs (Fig. 2G and H) and a reduction in $CD4^+$ (Fig. 2E and F) and IFN-γ^+ cells (Fig. 2G, F). This led to reduced transaminases (Fig. 2C) and the downregulation of inflammatory genes and proteins in the ldIL-2-treated animals, with an increase in the growth factor GM-CSF that was consistent with this finding (Fig. 3).

The response to therapy was slightly improved and measurable in all individuals in a clinical observation of refractory AIH patients receiving ldIL-2 [18]. While one of the two patients did not respond to therapy, the second patient showed a significant improvement in AST levels. In contrast to this observational study, we found significant accumulation of Tregs in both the spleen and the liver, which lasted for a long time. The reason for this may be either the complexed, Treg-specific IL-2 or the simultaneous administration of prednisolone to the patients. We have shown that steroids have a particularly negative effect on Tregs compared to other T cells [4]. In a clinical study of ldIL-2 treatment in SLE patients who also received prednisolone, 66% of patients were in remission after 24 weeks, compared to 37% in the placebo group [17]. In our study, the transaminases were uniformly reduced (Figs. 2C) and 4/13 animals in the complexed ldIL-2 group no longer exhibited autoimmune hepatitis (mHAI < 2), whereas in the control group, there was only 1/11 of the animals without emAIH (Fig. 2B).

Likewise, the induced immune regulation does not seem to affect general immune competence. While the number of systemic, intrahepatic and splenic Tregs generally doubled at the highest treatment level (Fig. 2G, F), the number of local Tregs increased 3.5–4.5-fold under inflammatory conditions (Fig. 3A, B, D). These Tregs probably recognized their specific antigen in this context. This result was also shown by studies in the allograft transplant model, in which IL-2 preferentially expanded and activated antigen-specific Tregs [25].

In summary, an increase in intrahepatic Tregs requires higher IL-2 levels than those in the blood and spleen. As these increased doses can potentially lead to immune activation, as observed in the LITE trial after liver transplantation, a more Treg-specific activation of the IL-2 receptor is needed. We have nicely shown that complexed IL-2 is able to restore the balance between Tregs and Teffs within the liver, thereby ameliorating emAIH. Treg-specific IL-2 treatment offers new hope for reestablishing immune tolerance in patients with AIH.

Author statement

Conceptualization: MHW and EJ, Methodology: MPM, MHW and EJ, Software: LEBM, Validation: LEBM, Formal analysis: LEBM and MHW, Investigation: LEBM, JP, FN and JS, Resources: MPM, HW, MHW and EJ, Data Curation: LEBM, Writing - Original Draft: LEBM and MHW, Writing - Review & Editing: All authors, Visualization: LEBM, Supervision: MHW and EJ, Project administration: MHW, Funding acquisition: LEBM, MHW and EJ.

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Declaration of competing interest

All authors claim that they have no conflicts of interest based on a desire for financial gain, prominence, professional advancement or a successful outcome.

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