Liver homing of clinical grade Treg after therapeutic infusion in patients with autoimmune hepatitis

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Supplementary methods

GMP-grade Treg isolation

Depletion of CD8 T cells and CD19 B cells

Cell collections were sampled for initial flow cytometric analysis then processed immediately in a Grade B clean room with a Grade A Biological Safety cabinet. Cells were washed twice on a Cobe 2991 cell processor (Gambro, Illinois, USA) with wash buffer comprised of CliniMACS Phosphate Buffered Saline (PBS) /EDTA (1mM) supplemented with 0.5% human albumin solution (HAS) and the volume reduced to 87.5mL. Cells were then incubated with one vial each (15mL total) of CD8 and CD19 antibodies conjugated to magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) under continuous agitation at room temperature for 30 minutes. Following incubation, cells were washed in wash buffer and centrifuged at 300g for 15mins without brake to a final volume of 200mL and concentration of 8.75-144 x 10^6/mL. Cells were filtered into a transfer bag prior to then being processed on the fully automated CliniMACS Plus device (Miltenyi) equipped with LS separation column using program “Depletion 2.1” according to manufacturer’s instructions.

Enrichment of T regs ($CD4^{positive}$ $CD25^{high}$ cells)

Cells collected from the depletion run were adjusted to a volume of 380mL in chilled wash buffer, and incubated with 1 vial (7.5ml) of chilled CD25 antibody conjugated to magnetic microbeads under continuous agitation for 15mins, surrounded by chilled cool packs to retain optimal binding conditions. Following incubation cells were washed with chilled wash buffer at 300g for 15 minutes in a refrigerated centrifuge at 4^{o}C and re-suspended in 100mL of chilled wash buffer to get a final concentration of

14.2 - 160 x 10^6/ml. Cells were processed on the CliniMACS Plus equipped with a TS separation column and using program “Enrichment 3.1”.

Pre and Post procedure flow cytometric analysis

The following antibodies were used to analyse pre and post process samples from all stages of the enrichment process: Anti-CD45 – FITC (BC), Anti CD3 – APC - Cy7 (BD), Anti-CD4 PE-Cy7 (BD), Anti-CD8 PE-Cy7 (BD), Anti-CD25 PE (Miltenyi Biotec), Anti-CD20 APC (BD), Anti-CD16-PE (BD), Anti-CD56-PE (BD), Anti-CD127 APC (eBioscience). 7AAD was included to assess cell viability. A lyse no wash assay was developed using Trucount tubes (Becton Dickinson) for single platform analysis to provide absolute cell numbers. FACSDiva software was used for gating with Treg cells identified by the phenotype $CD4^{positive}CD25^{high}CD127^{negative}$.

Analysis of Treg & T effector cells in peripheral blood of AIH patients

Blood samples were collected in EDTA and peripheral blood mononuclear cells (PBMC) were isolated immediately using Lympholyte (VH Bio Ltd) according to standard protocols. Viability was confirmed by incubating with e506 viability dye (Thermofisher) diluted 1/1000 in PBS for 20minutes at 4°C. After washing in 2% foetal bovine serum (Sigma Aldrich) diluted in PBS (2%FBS) cells were resuspended in 2%FBS allowing a volume of 50μl/stain and aliquoted to 5ml polypropylene round bottom Falcon tubes cells were incubated with antibodies to surface antigens for 30 minutes, washed and fixed by 10-minute incubation at room temperature in 500μl 3% formadehyde solution. Cells analysed for expression of intracellular proteins were fixed and stained with fluorophore conjugated antibodies to the intracellular markers

using the Foxp3/Transcription factor staining set (eBioscience) according to manufacturer's instructions. This included 40minutes fixation in the fixative (diluted 1 in 4 according to manufacturer's instructions) followed by 2ml wash with PBS and 1ml wash with the permeabilisation buffer diluted 1 in 10 in distilled H_20). Intracellular antibodies were added to the cells in a 50μl staining volume of permeabilisation buffer, the cells were pulse vortexed to mix and stained for 30 minutes at 4°C. Excess antibodies were removed by washing with 1ml permabilisation buffer followed by 2ml PBS and resuspended in 300μl PBS for analysis. Data were acquired using a CyAN ADP flow cytometer. Single-fluorophore-labelled anti-mouse IgGκ/negative control (FBS) compensation particles (BD Biosciences) were used for compensation. Data were analysed offline using FlowJo software (Tree Star Inc., Ashland, OR). Expression frequencies (percentage of cells presenting with staining greater than that of an isotype matched control) are presented.

Using a panel of antibodies to the surface receptors CD3, CD4, CD8, CD56, CD19, CD25, CD127, CD45RA, and CCR7 we were able to define cell subsets including $CD3^{pos}CD4^{pos}$, $CD3^{pos}CD8^{pos}$, T cells, $CD3^{pos}CD4^{pos}CD25^{pos}CD127^{neg}$ Treg, $CD3^{neg}CD19^{pos}$ B cells, $CD3^{pos}CD56^{pos}$ Natural Killer T cells ("NKT") and $CD3^{neg}CD56^{bright}$ (NK^{bright}), and $CD3^{neg}CD56^{dim}$ (NK^{dim}) cells and divide the CD4, CD8 and Treg cells into memory and naïve subsets according to CCR7 vs. CD45RA expression. Treg subsets were also gated according to CD45RA vs. CD25 expression (Supplementary figure 1).

Analysis of intracellular cytokine expression profiles by peripheral blood Treg, CD4 non-Treg and CD8 cells.

After isolation PBMC were resuspended in RPMI culture medium supplemented with 10% FBS at density $10x10^6$cells/ml and stimulated with Cytostim reagent (Miltenyibiotec) at dose 20μl Cytostim reagent/ml cells for 7hours. During the final 4hours, the release of cytokines was blocked by addition of Brefeldin A to the culture at 0.5μg/ml final concentration. Cells were transferred to 5ml polypropylene round bottom tubes (Falcon) tubes and washed with PBS. Pellets were resuspended in e506 viability dye and stained at 4°C for 20 minutes. Following washing with 2%FCS, cells were fixed with 3% formaldehyde for 10 minutes. Fixative was removed by 2ml wash with PBS and cells were permeabilised by washing with 1ml 0.1% Saponin solution (prepared in PBS). Antibodies to intracellular cytokines IL-2, IL-10, IL-17 and IFN-γ were added in 50μl saponin solution together with antibodies to the T cell subset-defining markers CD3, CD4, CD8, CD25 and CD127 and cells stained for 30minutes at room temperature. Excess antibody was removed by 1 x 1ml wash with 0.1% saponin followed by 2ml with PBS and cells resuspended in 300μl PBS for analysis on the CyAN ADP flow cytometer.

Treg suppression of T responder cells assay

CD4+ T cells were enriched from prescreen PBMC using the Mojo human CD4 enrichment kit (BioLegend) according to manufacturer's instructions. Isolated cells were suspended in 2% FCS solution and stained for 30 minutes on ice with flurochrome conjugated antibodies including anti-CD127 FITC, anti-CD25 PE, anti-CD3 APC and anti-CD4 PerCPCy5.5. After washing with 2% FCS, cells were resuspended in 0.05% Bovine serum albumin solution containing 100mM EDTA for sorting. $CD3^+CD4^+CD25^{neg}$CD127+/- non Treg and $CD3^+CD4^+CD25^+CD127^{neg}$-Treg were collected in separate sterile 5ml propylene tubes cells containing 2ml 10%FCS

RPMI (culture medium).

Treg were centrifuged and suspended at 0.5 x 10^6cells/ml. The sort yielded multiple tubes containing non-Treg, thus non-Treg were pooled and washed twice with PBS to remove all serum before staining cells with cell trace violet dye (Invitrogen) at 1.2μl dye / 5 x 10^6 cells in 1ml PBS. Cells were incubated with the dye at 37°C for 20minutes in a 15ml conical tube. 13ml 10%FCS RPMI was then added to quench the dye. Cells were incubated for a further 5 minutes at 37°C before pelleting at 200rpm and washing twice with 15ml 10%FCS RPMI. Finally the nonTreg (responders) were resuspended in 10% FCS RPMI at density 1 x 10^6 cells/ml. 200μl cultures of Treg and non-Treg were set up at nonTreg:Treg ratios of 1:0, 1:1, 2:1, 4:1 8:1 and 0:1 and activated with Treg Inspector Beads (Mitlenyi Biotec) using 1μl beads/20,000cells. The division of responder nonTreg cells was examined at 5 days using a CyAN ADP flow cytometer. The fluorescence range of division was determined based on the fluorescence peak of a control culture of violet trace labeled but non-activated non-Treg cells.

Supplementary Figures and Figure legends

Fig. S1. Gating strategy for immune cell subsets

Representative flow cytometry plots showing the immune cell purity of the GMP-Treg product. Treg were isolated from the patients' peripheral blood mononuclear cells by leukapheresis and selected using a CliniMACS immunomagnetic cell separation system involving CD19 and CD8 cell depletions followed by $CD25^{high}$ cell selection. GMP-grade Tregs were stained with viability dye followed by antibodies to CD19 (B-cells), CD56 (Natural Killer cells and Natural Killer T cells), CD3, CD4 and CD8 ($CD4^+$ T cells and $CD8^+$ T cells respectively), CD127, CD25 (to identify $CD127^{neg}CD25^{high}$ Tregs from $CD127^+$ $CD25^{neg/mid}$ Non-Treg) and CD45RA and CCR7 to identify the memory and naïve T cell subsets. Purity of the GCLP-Treg product as $CD127^{neg}CD25^{high}$ cells was thereby assessed and the memory/naïve phenotype of the isolate determined.

S1

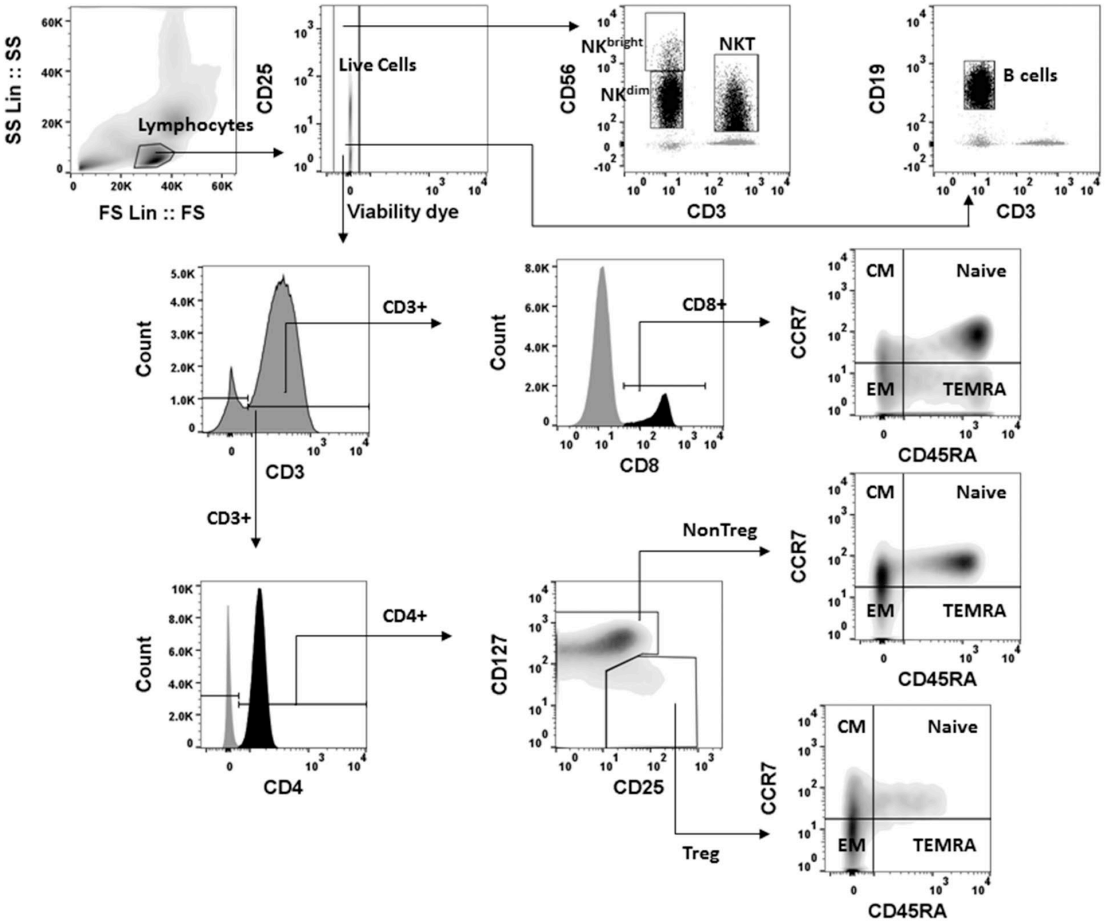
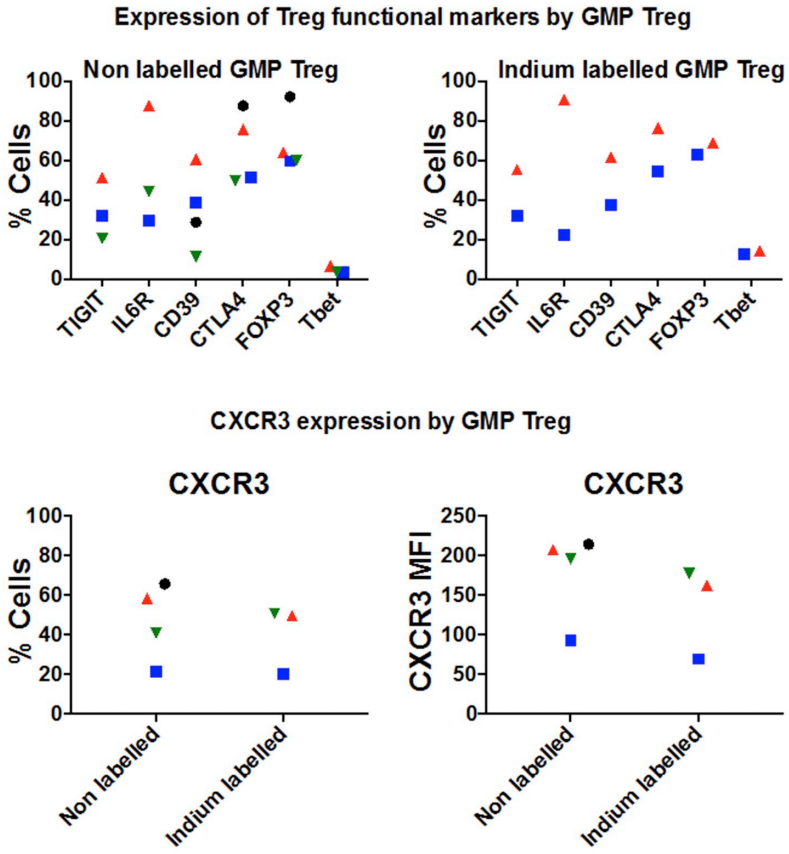
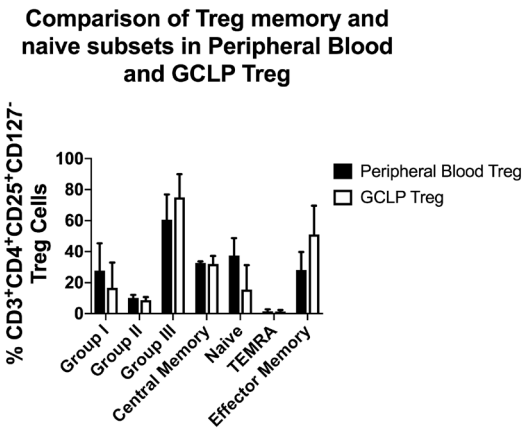

SS Lin :: SS
FS Lin :: FS
Lymphocytes
CD25
Live Cells
Viability dye
CD56
NK^bright
NK^dim
NKT
CD3
CD19
B cells
CD3
Count
CD3+
CD3
CD3+
CD4+
CD4
Count
CD8+
CD8
CCR7
CM
Naive
EM
TEMRA
CD45RA
NonTreg
CD127
CD25
Treg
CCR7
CM
Naive
EM
TEMRA
CD45RA
CCR7
CM
Naive
EM
TEMRA
CD45RA

Fig. S2. Expression of Treg functional markers and chemokine receptors on the GMP-grade Treg before and post indium labeling

(A) Expression of regulatory markers (CTLA-4, CD39, IL-6R, TIGIT), transcription factors (FoxP3 and T-bet) and **(B)** Expression of CXCR3 by the GMP-Treg was assessed by surface or intracellular flow cytometry on GMP-Treg before indium labeling (left) or that were indium labelled and surplus to infusion requirements (right). Each symbol represents different patients. Black circle = Patient 2; Red triangle = Patient 3; Blue square = Patient 4 and Green triangle = Patient 5. Where phenotypic analysis is missing for a patient it was because insufficient cells were received for the full panel of markers to be analysed.**(C)** Comparison of Treg subsets (Group I, II, III and naïve, central memory, effector memory and tissue resident effector memory (TEMRA) and their surface markers expression of peripheral and good clinical laboratory practice (GCLP) clinical grade Treg.

S2


Expression of Treg functional markers by GMP Treg
Non labelled GMP Treg
Indium labelled GMP Treg
% Cells
TIGIT
IL6R
CD39
CTLA4
FOXP3
Tbet
CXCR3 expression by GMP Treg
CXCR3
CXCR3 MFI
Non labelled
Indium labelled


Comparison of Treg memory and naive subsets in Peripheral Blood and GCLP Treg
% CD3+CD4+CD25+CD127- Treg Cells
Peripheral Blood Treg
GCLP Treg
Group I
Group II
Group III
Central Memory
Naive
TEMRA
Effector Memory

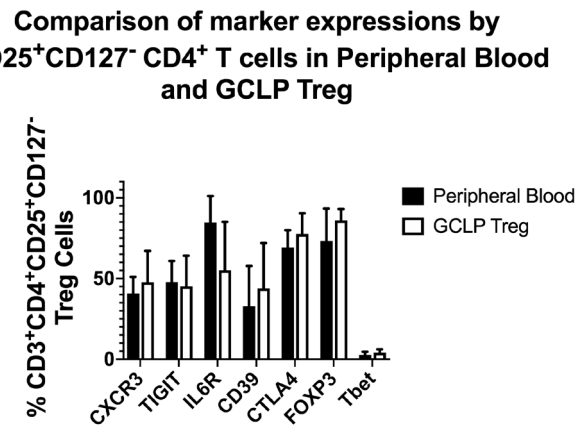
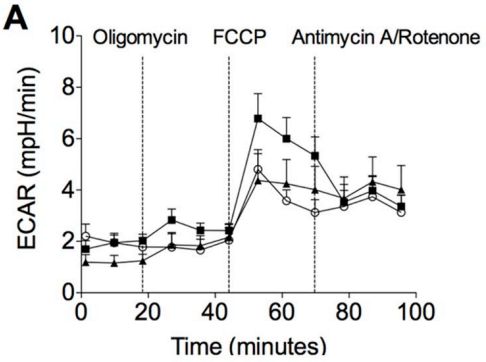

Comparison of marker expressions by CD25+CD127- CD4+ T cells in Peripheral Blood and GCLP Treg
% CD3+CD4+CD25+CD127- Treg Cells
Peripheral Blood
GCLP Treg
CXCR3
TIGIT
IL6R
CD39
CTLA4
FOXP3
Tbet

Fig. S3. The glycolytic profile of CD4 Treg, CD4 non-Treg and CD8 T cells

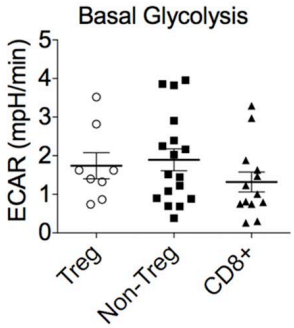
Glycolytic profiles of cell subsets (A) were measured by the extracellular acidification rate (ECAR) using injections of oligomycin (1 μM), FCCP (carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone; 1μM) and antimycin A and rotenone (1 μM). Glycolytic parameters **(B)** basal glycolysis and **(C)** maximal glycolysis were determined. Data are representative of three independent experiments with technical repeats and median and interquartile range shown. Differences between subsets were tested by Kruskall Wallis test but no significant differences were observed. **(D)** Defining CD8 Tcells, non-Tregs and Tregs subsets. Subsets were defined as $CD45RA^{+}CCR7^{+}$ (naïve), $CD45RA^{neg}CCR7^{+}$ (central memory), $CD45RA^{neg}CCR7^{neg}$ (effector memory) $CD45RA^{+}CCR7^{neg}$ (tissue resident effector memory RA-positive). **(E)** non-Treg, Treg and CD8 cell subsets TMRE (mitochondrial membrane potential) and Mitotracker (mitochondrial content) defined by flow cytometry.

S3

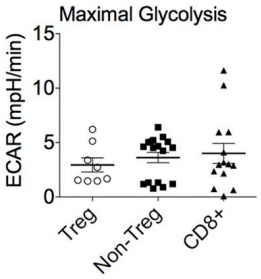
A


Oligomycin
FCCP
Antimycin A/Rotenone
ECAR (mpH/min)
Time (minutes)

B


Basal Glycolysis
ECAR (mpH/min)
Treg
Non-Treg
CD8+

C


Maximal Glycolysis
ECAR (mpH/min)
Treg
Non-Treg
CD8+

D

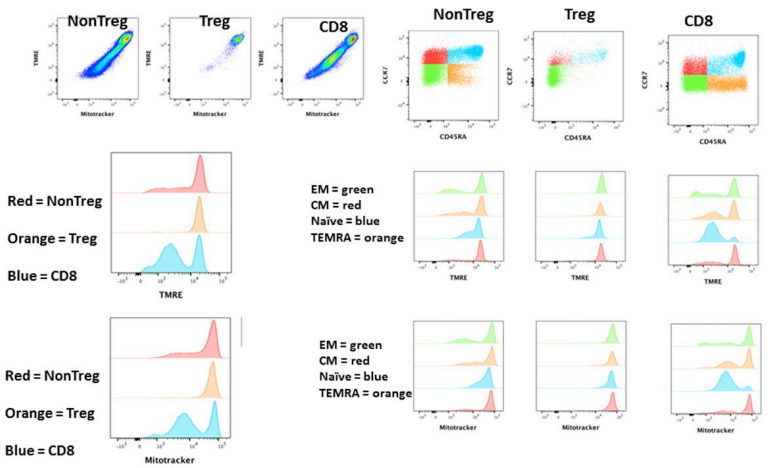
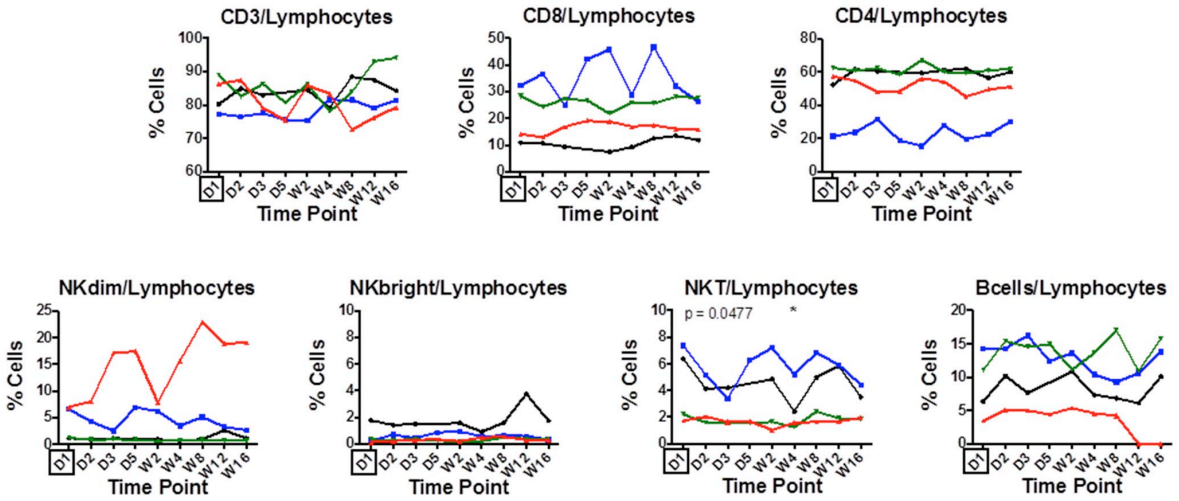

NonTreg
Treg
CD8
TMRE
Mitotracker
CCR7
CD45RA
Red = NonTreg
Orange = Treg
Blue = CD8
EM = green
CM = red
Naïve = blue
TEMRA = orange

Fig. S4. Temporal changes in the frequencies of the main immune cell populations in the peripheral blood during autologous regulatory T cell therapy and 4-month follow-up in autoimmune hepatitis patients.

Frequencies of **(A)** the main immune cell populations (CD3, CD8, CD4 T cells) in the peripheral bloods of patients expressed as a proportion of total live lymphocytes and of **(B)** T cell subsets expressed as proportions of total T cells or CD4+ T cells were assessed by flow cytometry at each time point. PBMC were freshly isolated at each time point and stained with viability dye followed by antibodies to identify the CD3, CD4, CD8, (T cells) CD56 (Natural Killer (NK) and Natural Killer T (NKT)) cells and CD19 (B cells). Cell populations were identified according to the gating strategy in Supplementary Figure 3D. Each patient's profile is shown by a different line: Black = Patient 2; Red = Patient 3; Blue = Patient 4 and Green = Patient 5. Statistically significant changes in frequency were assessed using Friedman's test with Dunn's Multiple Comparisons Post hoc analysis comparing to baseline (Day 1 (D1) pre-leukapheresis (indicated by the box)), $p \leq 0.05$.

S 4A


CD3/Lymphocytes
CD8/Lymphocytes
CD4/Lymphocytes
NKdim/Lymphocytes
NKbright/Lymphocytes
NKT/Lymphocytes
p = 0.0477
*
Bcells/Lymphocytes
% Cells
Time Point
D1 D2 D3 D5 W2 W4 W8 W12 W16

4B

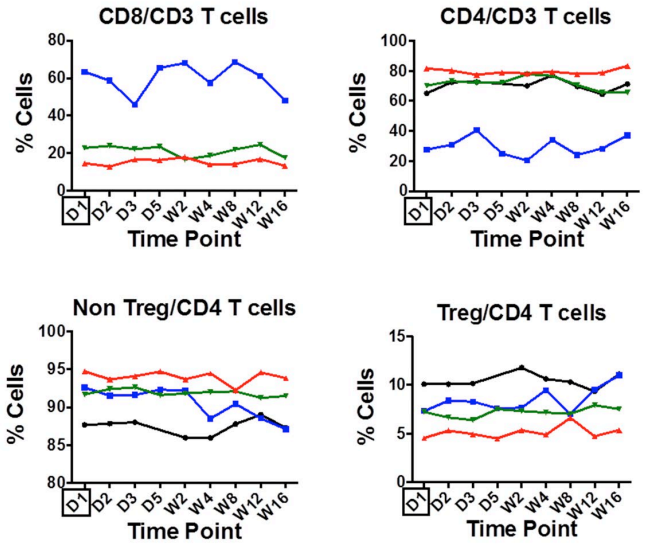

CD8/CD3 T cells
CD4/CD3 T cells
Non Treg/CD4 T cells
Treg/CD4 T cells
% Cells
Time Point
D1 D2 D3 D5 W2 W4 W8 W12 W16

Fig. S5. Changes in the concentrations of inflammatory and regulatory cytokines and chemokines in the sera during autologous regulatory T cell therapy and up to 8 weeks post infusion.

Peripheral blood serum samples were collected on days 1, 3, 5 and at weeks 2, 4 and 8 of the study and were analysed by luminex for concentrations of chemokines and inflammatory and regulatory cytokines. Data are shown for each patient individually. Each patient's profile is shown by a different coloured line: Black = Patient 2; Red = Patient 3; Blue = Patient 4 and Green = Patient 5. Serum samples for patient 2 were diluted 1:4 for analysis of cytokines according to manufacturer's recommendation by using the sera for patients 3, 4 and 5 without dilution. Statistically significant changes in the concentrations of the analytes were assessed using Friedman's test with Dunn's Multiple Comparisons Post hoc analysis comparing to baseline Day 1 (D1) pre-leukapheresis (indicated by the box)). Values of zero indicate no detection at the limit of the assay rather than absolute zero. Where data points are missing, no sample was taken. This precluded inclusion of patient 2 data in the statistical assessment of temporal change compared to baseline.

S5

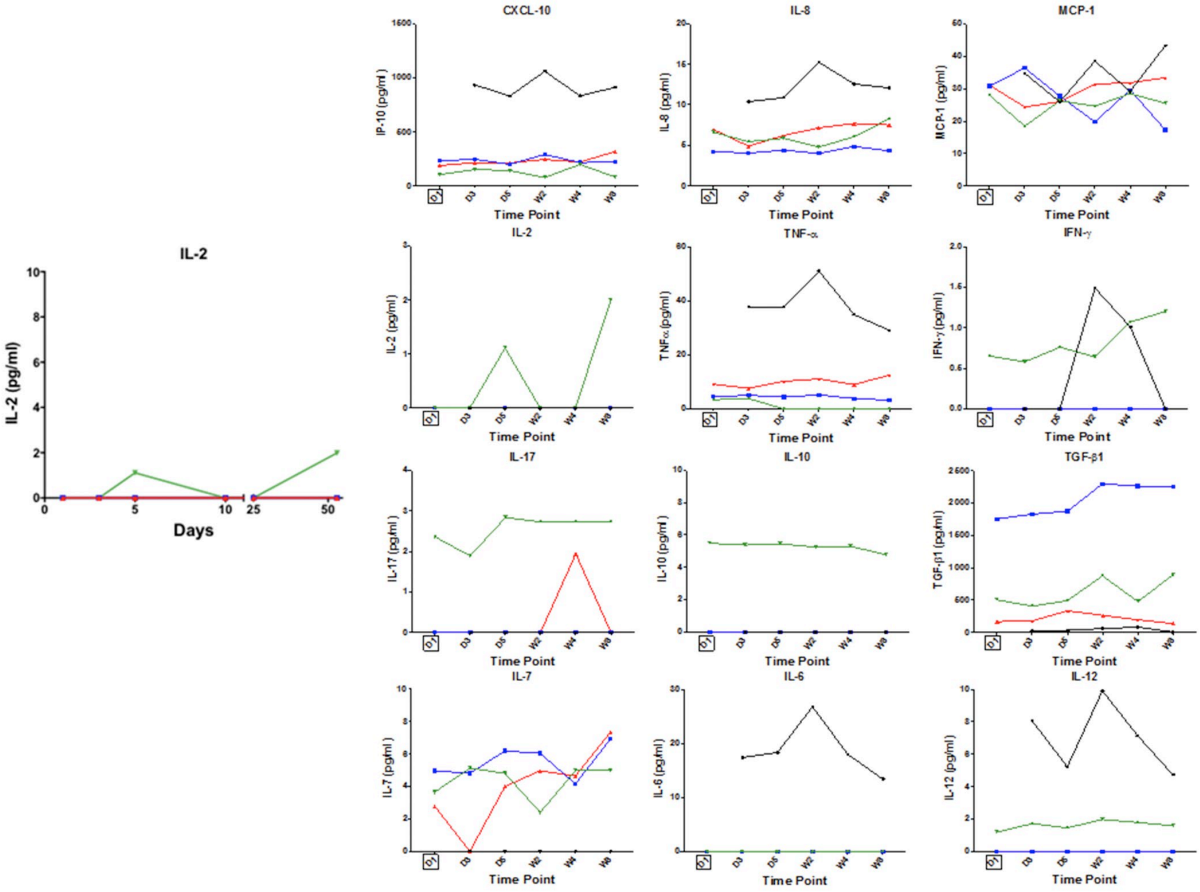

IL-2
IL-2 (pg/ml)
Days
CXCL-10
IP-10 (pg/ml)
IL-8
IL-8 (pg/ml)
MCP-1
MCP-1 (pg/ml)
IL-2
IL-2 (pg/ml)
TNF-α
TNFα (pg/ml)
IFN-γ
IFN-γ (pg/ml)
IL-17
IL-17 (pg/ml)
IL-10
IL-10 (pg/ml)
TGF-β1
TGF-β1 (pg/ml)
IL-7
IL-7 (pg/ml)
IL-6
IL-6 (pg/ml)
IL-12
IL-12 (pg/ml)
Time Point
D3
D5
W2
W4
W8

Fig. S6. Serial Gamma camera scanning of indium labelled GMP-Treg after infusion

Serial Gamma camera imaging performed at 4 hours **(i)**, 24 hours **(ii)** and 72 hours **(iii)** post GMP-Treg infusion demonstrates the presence of indium tropolonate labelled GMP-Treg in the liver, spleen and bone marrow. GMP-Tregs are present and remained in the liver for up to 72 hours **(iii)**. (Anterior = Gamma camera imaging scan from the anterior view; posterior = Gamma camera imaging scan from posterior view. Patient 3 has only a rudimentary splenic remnant.

Individual patient Gamma camera image scan following GMP Treg therapy

A=Patient 2

B=Patient 3

C=Patient 4

D=Patient 5

S6 A

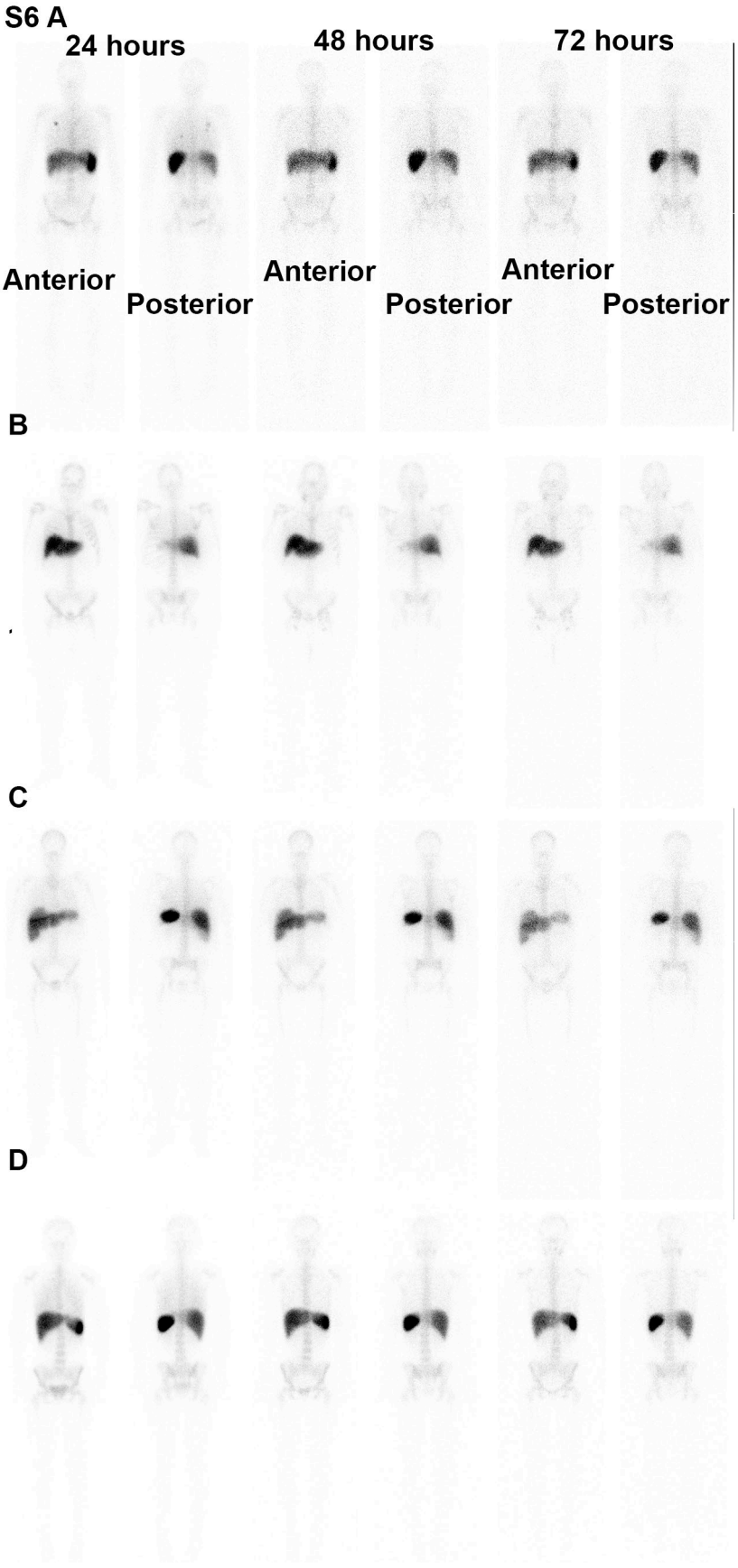

24 hours
48 hours
72 hours
Anterior
Posterior
Anterior
Posterior
Anterior
Posterior
B
C
D

Figure. S7 Individual patient' SPECT CT scan following GMP Treg therapy

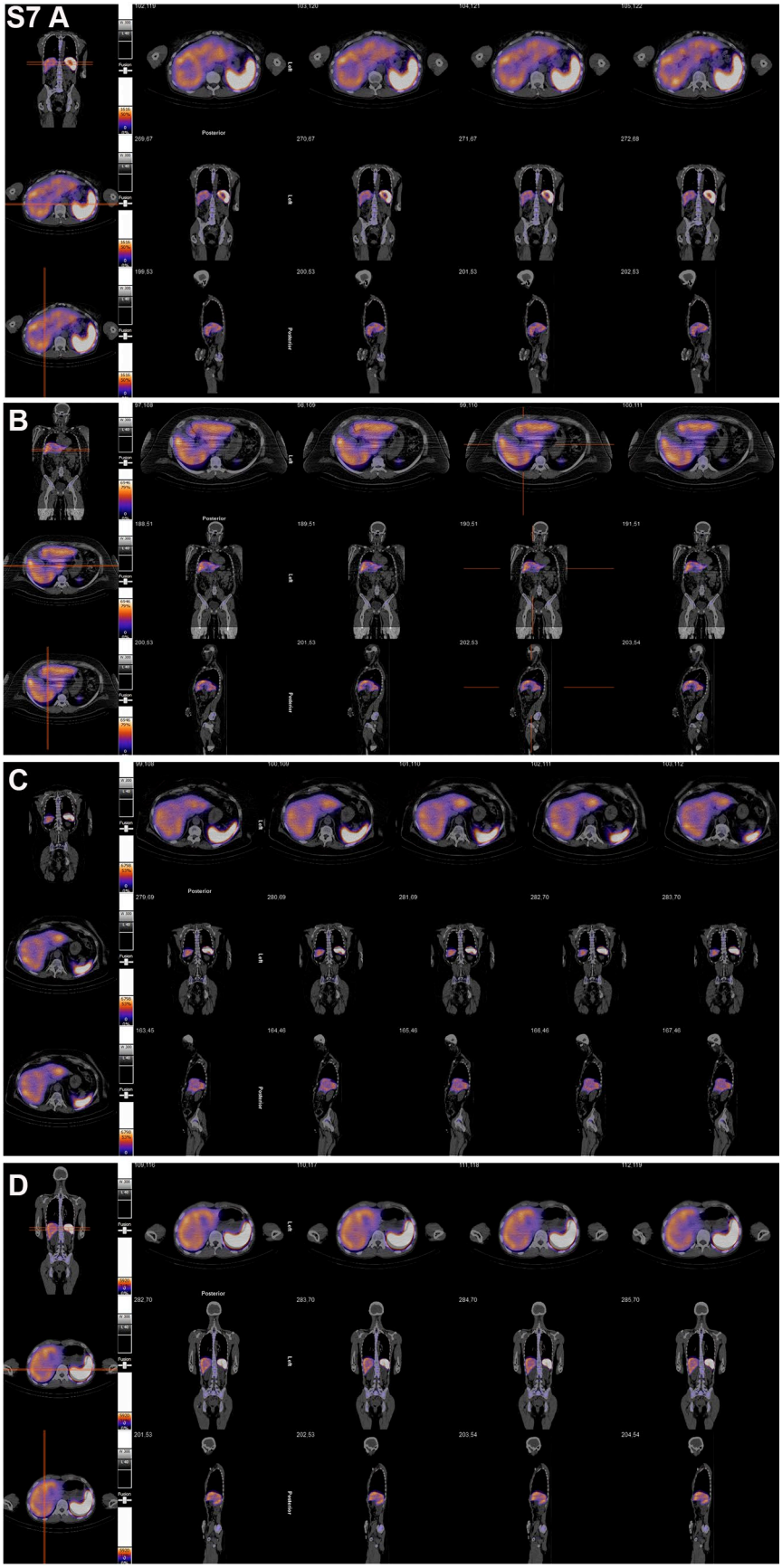
SPEC-CT imaging at 24 hours showing coronal reconstruction from anterior to posterior (Anterior images-liver) (Posterior images – spleen). Patient 3 has only a rudimentary splenic remnant.

A=Patient 2

B=Patient 3

C=Patient 4

D=Patient 5


S7 A
B
C
D

Supplementary Tables.

Table S1. Biochemical, Immunology, ultrasound and liver fibroscan results before and after GMP-grade Treg infusion.

	Patient 2		Patient 3		Patient 4		Patient 5	
ALT	24	31	16	44	15	16	19	21
Bilirubin	6	9	4	7	4	7	15	9
IgG	21.9	21.3	15	11.3	13.3	13.2	10.4	11.7
Ultrasound liver	Coarse, liver	Coarse, liver	Coarse liver	Coarse liver	Coarse liver	Coarse liver	NL	NL
Fibroscan	10.7	11	12.2	11	6.8	4.4	3.3	3.7
Child Pugh Score	A	A	A	A	A	A	NL	NL
UKELD	46	46	45	45	44	44	43	44

ALT = Alanine transaminase, IgG = Immunoglobulin G, NL = normal liver, Child Pugh Score = End stage liver disease score, UKELD = United Kingdom end stage liver disease score.

Table S2. Time points of blood sampling during the study

Day of Study	Blood product sampled defined with respect to leukapheresis	Patient 2, 3, 4, 5
1	Leukapheresis	FACS
2	post leukapheresis, pre GMP-Treg infusion	FACS
3	1 days post GMP-Treg infusion	FACS
5	3 days post GMP-Treg infusion	FACS
11	9 days post GMP-Treg infusion	FACS
25	4th week of study	FACS
53	8th week of study	FACS
81	12th week of study	FACS
109	16th week of study	FACS

Time points of blood samples taken during the AUTUMN trial with respect to the interventions of leukapheresis and Treg infusion.

The methods of immunophenotyping by which each were analysed are stated.

Acknowledgments:

The authors gratefully acknowledge clinical members of the Queen Elizabeth University Hospital Liver and Hepatobiliary Surgical Unit and colleagues from Centre of Liver Research, National Institute of Health Research Birmingham Biomedical Research Centre (NIHR Birmingham BRC). We ackowledge Sister Joanne Bird and all the staff from Haematology Department for Leukapheresis; NHS Blood and Transplant staff for GMP Treg isolation; Nuclear Medicine department staff for imaging and data analysis; NIHR Wellcome Trust Clinical Research Facility staff for overnight stay monitoring; NIHR Liver BRU for trial team support and Cancer Research Centre Trial Unit for trial managment. We also like to thank Kathryn McQuillan, Angela Sanzo, Jane Birtwistle and Siobhan Young for their help with the trial.