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REGULATORY T CELLS FROM PATIENTS WITH END-STAGE ORGAN DISEASE CAN BE ISOLATED, EXPANDED AND CRYOPRESERVED ACCORDING GOOD MANUFACTURING PRACTICE IMPROVING THEIR FUNCTION

--Manuscript Draft--

Manuscript Number:	JTRM-D-19-00358R1	
Full Title:	REGULATORY T CELLS FROM PATIENTS WITH END-STAGE ORGAN DISEASE CAN BE ISOLATED, EXPANDED AND CRYOPRESERVED ACCORDING GOOD MANUFACTURING PRACTICE IMPROVING THEIR FUNCTION	
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Abstract:	<p>BACKGROUND: Here, we isolated, expanded and functionally characterized regulatory T cells (Tregs) from patients with end stage kidney and liver disease, waiting for kidney/liver transplantation (KT/LT), with the aim to establish a suitable method to obtain large numbers of immunomodulatory cells for adoptive immunotherapy post-transplantation.</p> <p>METHODS: We first established a preclinical protocol for expansion/isolation of Tregs from peripheral blood of LT/KT patients. We then scaled up and optimized such protocol according to Good Manufacturing Practice (GMP) to obtain high numbers of purified Tregs which were phenotypically and functionally characterized in vitro and in vivo in a xenogeneic acute graft-versus-host disease (aGVHD) mouse model. Specifically, immunodepressed mice [NOD-SCID-gamma KO mice] received human effector T cells with or without GMP-produced Tregs to prevent the onset of xenogeneic GVHD.</p> <p>RESULTS: Our small scale Treg isolation/expansion protocol generated functional Tregs. Interestingly, cryopreservation/thawing did not impair phenotype/function and DNA methylation pattern of FOXP3 gene of the expanded Tregs. Fully functional Tregs were also isolated/expanded from KT and LT patients according to GMP. In the mouse model, GMP Tregs from LT or KT patient proved to be safe and show a trend toward reduced lethality of acute GVHD.</p> <p>CONCLUSIONS: These data demonstrate that expanded/thawed GMP-Tregs from patients with end-stage organ disease are fully functional in vitro. Moreover, their infusion is safe and results in a trend toward reduced lethality of acute GVHD in vivo, further supporting Tregs-based adoptive immunotherapy in solid organ transplantation.</p>	
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Response to Reviewers:	<p>Reviewer reports:</p> <p>Reviewer #1: The manuscript submitted by Ulbar et al. describes phenotyping and functional analysis of CD4+ Treg of patients with end-stage liver and kidney disease. There is particular emphasis on the use of GMP-compliant Treg manipulation protocols, implying that the same cells may be used for human therapy. This makes perfect sense as the working hypothesis is that adoptive transfer of such autologous Treg may be used as prophylaxis/therapy to preclude liver/kidney rejection upon solid organ transplantation. The authors demonstrate that their Treg display a stable phenotype, expand and exert Treg-mediated suppression of T cell responses both in vitro and in vivo. The latter is tested in a xenogeneic model of aGvHD.</p> <p>This is a well-written, well-presented manuscript that raises an interesting question. The authors use adequate, seemingly GMP compliant sorting and expansion methodology, acceptable Treg staining panels, in vitro assays, and a state-of-the-art humanized mouse model of aGvHD to test Treg activity in vivo; PBMC-NSG mice have been used by multiple authors to assess Treg function indeed.</p> <p>Major criticisms:</p> <p>1) The key question is whether these cells could be used to prevent kidney/liver rejection upon solid organ transplantation. This reviewer believes that the paper does not provide any clear evidence to support this notion, which diminishes enthusiasm. In order to address this issue, it must be somehow demonstrated that these Treg are fully functional in the kidneys/liver in vivo. At a bare minimum, the authors need to assess Treg-mediated suppression of liver damage in their mice, by histology. NSG mice develop liver aGvHD upon transplantation PMID: 19659776 and this could be used as a surrogate marker to assess Treg-mediated suppression of T-cell mediated "rejection" of the liver. Certainly, a more adequate approach would be to use double-humanized</p>

NSG mice (FRGN) having an MHC mismatched human liver (donor# 1) and human hematopoietic system (donor #2), as described here: PMID: 25310256. The best approach would be testing the same question on NSG mice receiving an actual liver graft from an independent, MHC mismatched human donor.

WE AGREE WITH THE REFEREE THAT DOUBLE-HUMANIZED NSG MICE WOULD BE THE BEST MODEL TO EVALUATE THE EFFECTS OF TREG INFUSIONS. HOWEVER, IT IS IMPORTANT TO POINT OUT THAT IN OUR PAPER WE AIMED TO TEST WHETHER TREGS FROM LT OR KT PATIENTS WERE CAPABLE OF PREVENTING/HALTING GVHD AS A PROOF OF THEIR FUNCTIONAL ACTIVITY. FOR THIS PURPOSE, WE FUNCTIONALLY CHARACTERIZED THE EXPANDED/THAWED TREGS FROM LT AND KT PATIENTS BOTH IN VITRO AND IN VIVO (BY USING A XGVHD MOUSE MODEL). WE ALSO AGREE THAT IT WOULD BE INTERESTING TO ANALYZE WHETHER TREGS FROM LT OR KT MAY PREVENT A GVHD IN THE LIVER. IN OUR MOUSE MODEL, AGVHD WAS VERY AGGRESSIVE AND WEIGHT LOSS WAS A VERY EARLY SIGN OF DISEASE AND RAPIDLY ASSOCIATED WITH DEATH. CONSEQUENTLY, SINCE MOST MICE DIED OVERNIGHT, WE WERE UNABLE TO COLLECT EITHER THE LIVER OR OTHER ORGANS. WE BELIEVE, HOWEVER, THAT OUR DATA, BOTH IN VITRO AND IN VIVO, STRONGLY SUGGEST THAT THE EXPANDED/THAWED TREGS FROM LT AND KT PATIENTS ARE FULLY FUNCTIONAL.

2) It is not clear to me whether the authors expand t/nTreg or p/iTreg cells or a mixture of them, which are quite different in many aspects. Among others, in terms of phenotypic stability, which is a key question here. If the authors were to use circulating Treg from healthy donors, we could call them bona fide tTreg. But as they use diseased patients, Treg origin and status remains an open question, even after methylation assays. I guess the majority of the cells is tTreg, but pTreg contamination may affect the results. E.g. it might explain the limited suppressive capacity observed. Markers of these subsets are a matter of ongoing debate, but the authors may consider using Helios, NRP-1 or other markers of these subsets to provide the reader with some information in this regard.

AS UNDERLINED BY THE REFEREE, FRESHLY ISOLATED CELLS FROM THE DISEASED PATIENTS MAY INCLUDE EITHER T/NTREG OR P/ITREG. HOWEVER, IN THIS PAPER WE AIMED TO CHARACTERIZE NOT THE FRESHLY ISOLATED CELLS BUT THE 21 DAYS EXPANDED TREGS WHICH ARE THE CANDIDATE CELLS TO BE INFUSED. INDEED, EXPANDED TREGS FROM DISEASED PATIENTS HAVE BEEN DEMONSTRATED TO BE PHENOTIPICALLY STABLE AND FUNCTIONALLY ACTIVE.

Minor criticisms:

3) In different EU member countries, GMP compliance is tested by various bodies and organizations. A statement regarding the name of the authority that validated GMP compliance in this study would be required.

THE GMP MANUFACTURING WAS PERFORMED IN THE CELL FACTORY OF FONDAZIONE IRCCS CA' GRANDA OSPEDALE MAGGIORE POLICLINICO IN MILAN, CERTIFIED IN COMPLIANCE WITH THE EUROPEAN GMP REGULATIONS BY THE ITALIAN DRUG AGENCY FROM 2007 (LAST AUTHORIZATION NUMBER Am-51/2018)

4) Please state which Foxp3 region has been studied in methylation assays. Is this CNS2?

AUTHORS CONFIRM THAT CNS2 REGION OF FOXP3 HAS BEEN ANALYZED IN METHYLATION ASSAY. METHODS SECTION HAS BEEN MODIFIED ACCORDINGLY.

5) Page 9 Line 26: I believe that characterizing "CD8-CD25- T cells" as "Teff" is rather problematic. Just state "CD8-CD25- T cells".

AS SUGGESTED, WE MODIFIED THE TEXT ACCORDINGLY

6) Page 7 line 36: Please specify the exact Miltenyi beads used and MACS sorting platform used

THE REQUESTED INFORMATIONS HAS BEEN ADDED IN THE MATERIAL AND METHODS SECTION

7) Some minor typos:

Page 11 Line 56 intraperitoneally transfer

Page 12 Line 24 CD4 is listed twice in the panel. Is it CD127?

Page 18 Line 15 Tregs numbers

Page 18 Line 30 conflict findings

AS SUGGESTED, WE MODIFIED THE TEXT ACCORDINGLY

Taken together, I believe that the manuscript has merit but it has some shortcomings that need to be addressed before publication.

Reviewer #2: Minor points

Are you sure the first and last author equally contributed? It is not rather the first and second? (check the symbols)

FIRST (UF) AND SECOND AUTHOR (MT) CONTRIBUTED EQUALLY AND ARE IDENTIFIED WITH°

LAST (LRM) AND CO-LAST (CL) AUTHOR CONTRIBUTED EQUALLY AND ARE IDENTIFIED WITH §

Please clarify the negative fraction used as effector T cells. Is the fraction CD8-? In that case the GVHD model relies only on CD4 T effector cells? If so, discuss the difference in the GVHD mouse models between regular CD8 T effector and CD4 T effectors in terms of GVHD severity and T reg GVHD rescue capacity.

PBMCS HAVE BEEN PREVIOUSLY USED AS EFFECTOR CELLS IN A XENOGENEIC MODEL OF GVHD AND TREGS HAVE BEEN SHOWN TO REDUCE GVHD LETHALITY IN IMMUNODEFICIENT MICE (HIPPEN KL ET AL. SCI TRANSL MED 2011;3(83):83ra41; HANNON M ET AL. TRANSFUSION 2014;54:353; DEL PAPA B ET AL. BIOL BLOOD MARROW TRANSPLANT 2017;23:1847). DUE TO THE FACT THAT MOST STUDIES USED PBMC AS EFFECTOR CELLS IS VERY DIFFICULT TO COMPARE CD4- VS CD8-BASED GVHD.

HOWEVER, IT HAS BEEN PREVIOUSLY SHOWN THAT TREGS SUPPRESS CD4 T CELL ACTIVATION AND PROLIFERATION BY CONTACT-DEPENDENT AND CONTACT-INDEPENDENT MECHANISMS (SCHMIDT A ET AL. 2012;3:51).

ADDITIONALLY, TREGS CAN ALSO INFLUENCE PROLIFERATION, ACTIVATION AND APOPTOSIS OF CD8+ T CELLS (NIKOLOVA M ET AL. BLOOD (2009) 113:4556-65; BOETTNER T ET AL. J VIROL. (2005) 79:7860-7). BASED ON AN IN

VITRO HUMAN SKIN EXPLANT GVHD MODEL, WANG X ET AL (TRANSPLANTATION. 2009;88:188-97) DEMONSTRATED THAT DONOR-DERIVED TREGS EFFECTIVELY SUPPRESS CD8_ T-CELL-MEDIATED GVH TISSUE

DAMAGE BUT ARE CRITICALLY REQUIRED DURING PRIMING OF EFFECTOR T CELLS. SIMILARLY, IN THIS STUDY WE DEMONSTRATED THAT CD4 ENRICHED CELLS ARE CAPABLE TO INDUCE AN ACUTE GVHD IN NSG MICE AND THAT EARLY INFUSION OF TREGS FROM LT OR KT PATIENTS HAS THE POTENTIAL TO AMELIORATE THE GVHD LETHALITY.

Escalated the protocol please change: scaled-up the protocol
TEXT MODIFIED ACCORDINGLY

Background, page 6 line 3: proved instead of proven

TEXT MODIFIED ACCORDINGLY

Methods, page 6, line 25, please harmonize the exclusion criteria with the data of patient Table I where you included LT patients with HCV/HBV liver cirrhosis. At least specify in the criteria that you allowed viral mediated organ failure, while you had a cutoff on the viremia? Also, please for clarity fill the table with Y N or NA or ND (not done) or Not Available Data instead of "/".

I did not find the table legend.

TABLE I SHOWS CLINICAL DATA OF ALL PATIENTS STUDIED FOR IN VITRO AND GMP IN VIVO EXPERIMENTS. PATIENTS OF THE IN VITRO EXPERIMENTS INCLUDED ALSO VIRUS-RELATED END STAGE ORGAN FAILURE. CONVERSELY, THE CRITERIA OF ENROLMENT IN THE PROTOCOL OF GMP TREG ISOLATION/EXPANSION AND IN VIVO INJECTION EXCLUDED PATIENTS WITH VIRUS-RELATED END STAGE ORGAN FAILURE. THIS SPECIFICATION HAS NOW BEEN ADDED IN THE TEXT.

Page 9, line 10: if bisulphite conversion reaction runs to completion run
TEXT MODIFIED ACCORDINGLY

Results, page 14 line 39: "From the functional point of view" ... please improve the English form

TEXT MODIFIED ACCORDINGLY

Discussion page 18, line 40 : Tregs from LT or KT patients proven proved
TEXT MODIFIED ACCORDINGLY

Discussion page 18, line 48: We also found that in vivo GMP Tregs from LT patients were more effective than KT counterparts in halting GVHD due to the number of samples: 1 and 1 for each group, this comparison is not possible.

WE AGREE WITH THE REFEREE AND WE DELETED THE TEXT ACCORDINGLY

Reviewer #3: The manuscript by Ulbar et al is well-written and addresses the use of expanded Tregs for solid organ transplants and GVHD. However, the impact of Treg infusion for preventing GvHD is quite labile in the manuscript.

I kindly ask the authors to comment and/or clarify the following points:

-There are several publications on Tregs as safe and effective treatment of GVHD and Type 1 diabetes (references 12-17 in the manuscript), while few results have been published on clinical trials testing the efficacy of Tregs in solid organ transplantation. What is the novelty brought by the present manuscript on this regard? The same authors state: "Our data are consistent with previous models of xenogenic GVHD and Treg infusion."

AS UNDERLINED BY THE REFEREE, FEW RESULTS HAVE BEEN PUBLISHED ON CLINICAL TRIALS TESTING THE EFFICACY OF TREGS IN SOLID ORGAN TRANSPLANTATION. OUR DATA FURTHER SUPPORT THE USE OF GMP EXPANDED/THAWED T-REGS FOR ADOPTIVE IMMUNOTHERAPY IN SOLID ORGAN TRANSPLANTATION. IN ADDITION, FOR THE FIRST TIME BOTH KT AND LT TREGS WERE EXPANDED AND FUNCTIONALLY ANALYZED WITH THE SAME ISOLATION/EXPANSION PROTOCOL.

-The authors state that "In the mouse model, GMP Tregs from LT and KT patient proven to be safe and may improve acute GVHD", or "with the potential of alleviating GVHD". However, this has not been demonstrated in the presented set of experiments (no statistical significance between Teff vs Teff-T reg infusions in the mouse model).

FOLLOWING REFEREE'S COMMENT, WE MODIFIED THE TEXT (DISCUSSION SECTION) POINTING OUT THAT IN VIVO INFUSION OF TREGS FROM DISEASED PATIENTS INDUCED ONLY A TREND TOWARD A REDUCTION OF GVHD LETHALITY.

-HBV/HCV are listed as exclusion criteria, however 8 patients with HCV/HBV cirrhosis are reported in table 1.

TABLE I SHOWS CLINICAL DATA OF ALL PATIENTS STUDIED. SPECIFICALLY, IN VITRO ASSAYS HAVE BEEN PERFORMED WITH CELLS DERIVED FROM PATIENTS WITH OR WITHOUT VIRUS-RELATED END STAGE ORGAN FAILURE. CONVERSELY, IN VIVO EXPERIMENTS HAVE BEEN PERFORMED ONLY WITH CELLS FROM PATIENTS WITHOUT VIRUS-RELATED END STAGE ORGAN FAILURE . THE TEXT (MATERIAL AND METHODS SECTION) HAS BEEN MODIFIED ACCORDINGLY.

-It is stated that the purity at isolation after Miltenyi negative selection and enrichment is more than 90%. However, later on in the manuscript it is reported that "the median Treg purity was 72% (range 50-84) and 55% (range 40-70) in LT and KT patients...". We acknowledge that the purity significantly improves after in vitro expansion.

THESE FINDINGS ARE DUE TO THE FACT THAT AFTER ISOLATION, THE PURITY OF CD4+CD25+ T CELLS WAS ALWAYS >90%. THE CD4+CD25+CD127-FOXP3 + T CELLS WERE AS DESCRIBED.

-Teff cells as well as negative fraction are always referred as CD8-CD25- T cells creating confusion along all the paper.

THE NEGATIVE FRACTION CD8-CD25- T CELLS WAS USED TO TEST THE IN VITRO SUPPRESSIVE ACTIVITY OF THE FRESHLY ISOLATED/EXPANDED TREGS AND IN THE IN VIVO ACUTE GVHD MODEL. THE TEXT WAS MODIFIED ACCORDINGLY

-How polyclonality of Tregs was assessed?
WE DID NOT ASSESS THE POLYCLONALITY OF THE EXPANDED TREGS. INDEED, IT HAS BEEN PREVIOUSLY DEMONSTRATED THAT THE STIMULATION WITH CD3/CD28 BEADS ALLOWS THE EXPANSION OF ONLY POLYCLONAL TREGS (SAFINIA N ET AL. ONCOTARGET 2016; DEL PAPA ET AL BIOL BLOOD MARROW TRANSPLANT 2017). THUS, WE ASSUMED THAT EXPANDED TREGS WERE POLYCLONAL. HOWEVER, TO AVOID CONFUSING WORDS , POLYCLONAL HAS NOW BEEN DELETED FROM THE TEXT.

- The very low immunosuppressive activity of Tregs freshly isolated is quite impressive. The authors claim that this could be attributable to the low purity after isolation. WE AGREE WITH THE REFEREE. OUR RESULTS ARE IN CONTRAST WITH PREVIOUS FINDINGS (DEL PAPA B ET AL, 2017; SAFINIA N ET AL . 2018) SHOWING THAT THE SUPPRESSIVE ACTIVITY OF FRESHLY ISOLATED CD8-CD19-CD25+ CELLS (DEL PAPA B ET AL. 2017) OR CD8-CD25+ CELLS (SAFINIA N ET AL. 2018) FROM HEALTHY INDIVIDUALS IS AROUND 60-80% AT 1:1 TREG/TCONS RATIO. WE BELIEVE THAT THE POOR RESULTS OF IN VITRO IMMUNOSUPPRESSIVE ACTIVITY OF FRESHLY ISOLATED TREGS WERE MAINLY DUE TO THE MAXIMUM WORKING RATIO OF 1:2 TREG/CD8-CD25- T CELLS. IN ADDITION, THE PURITY OF OUR FRESHLY ISOLATED CELLS (CD4+CD25+CD127-FOXP3+) FROM HEALTHY DONORS WAS AROUND 65%. OF NOTE, AFTER 21 DAYS OF EXPANSION, THE PURITY INCREASED SIGNIFICANTLY.

-Some references are reported in red.
WE MODIFIED THE REFERENCE SECTION

Manuscript Classifications:	30.010: Adoptive immune therapy
Additional Information:	
Question	Response
Is this study a clinical	No;

trial?

A clinical trial is defined by the World Health Organisation as 'any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes'.

[Click here to view linked References](#)

REGULATORY T CELLS FROM PATIENTS WITH END-STAGE ORGAN DISEASE CAN BE ISOLATED, EXPANDED AND CRYOPRESERVED ACCORDING GOOD MANUFACTURING PRACTICE IMPROVING THEIR FUNCTION

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Running Title: expanded/thawed GMP-regulatory T cells and end-stage organ disease

Abstract

BACKGROUND:

Here, we isolated, expanded and functionally characterized regulatory T cells (Tregs) from patients with end stage kidney and liver disease, waiting for kidney/liver transplantation (KT/LT), with the aim to establish a suitable method to obtain large numbers of immunomodulatory cells for adoptive immunotherapy post-transplantation.

METHODS:

We first established a preclinical protocol for expansion/isolation of Tregs from peripheral blood of LT/KT patients. We then **scaled up** and optimized such protocol according to Good Manufacturing Practice (GMP) to obtain high numbers of purified Tregs which were phenotypically and functionally characterized *in vitro* and *in vivo* in a xenogeneic acute graft-versus-host disease (aGVHD) mouse model. Specifically, immunodepressed mice [NOD-SCID-gamma KO mice] received human effector T cells with or without GMP-produced Tregs to prevent the onset of xenogeneic GVHD.

RESULTS:

Our small scale Treg isolation/expansion protocol generated functional Tregs. Interestingly, cryopreservation/thawing did not impair phenotype/function and DNA methylation pattern of *FOXP3* gene of the expanded Tregs. Fully functional Tregs were also isolated/expanded from KT and LT patients according to GMP. In the mouse model, GMP Tregs from LT or KT patient **proved** to be safe **and show a trend toward reduced lethality of acute GVHD**.

CONCLUSIONS:

These data demonstrate that expanded/thawed GMP-Tregs from patients with end-stage organ disease are fully functional *in vitro*. **Moreover, their infusion is safe and results in a trend toward reduced lethality of acute GVHD *in vivo***, further supporting Tregs-based adoptive immunotherapy in solid organ transplantation.

KEYWORDS:

End Stage Organ Disease; regulatory T cells; safety; murine model

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Abbreviations

1
2 Regulatory T cells (Tregs)

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5 Kidney transplantation (KT)

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7 Liver transplantation (LT)

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10 Good Manufacturing Practice (GMP)

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12 Acute graft-versus-host disease (aGVHD)

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14 Graft-versus-host disease (GVHD)

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17 Immunosuppressive (IS)

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19 Peripheral blood (PB)

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22 White blood cell (WBC)

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24 Ethylenediaminetetraacetic acid (EDTA)

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27 Peripheral blood mononuclear cells (PBMC)

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29 Treg-specific demethylation region (TSDR)

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32 Carboxyfluorescein succinimidyl ester (CFSE)

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34 Roswell Park Memorial Institute (RPMI)

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37 Fetal Bovine Serum (FBS)

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39 Acid citrate dextrose (ACD)

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42 Dimethyl sulfoxide (DMSO)

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45 Human serum albumin (HSA)

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48 NOD scid gamma (NSG)

BACKGROUND

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3 Organ transplantation is the only curative treatment for patients affected by end-stage liver
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5 or kidney disease. Although immunosuppressive (IS) drugs have reduced the incidence of acute
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7 rejection and transplantation-associated mortality, their administration is associated with major side
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9 effects, such as opportunistic infections, damage of transplanted organs and secondary cancer [1,2].
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11 Therefore, new strategies to improve long-term graft survival while reducing/eliminating IS therapy
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13 and, ideally, inducing immunological tolerance are needed.
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17 Cellular therapy can improve the outcome of solid organ transplantation through anti-
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19 inflammatory effects and the induction of immune tolerance. T cell-mediated immunomodulation is
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21 one of the main mechanisms to maintain operational tolerance (withdrawal of immunosuppressive
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23 drugs while maintaining normal graft function and histology) *in vivo*. It is now accepted that
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25 regulatory T cells (Tregs), a subpopulation of T helper lymphocytes, are responsible for this
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27 immunomodulatory activity, as a result of their suppressive effects directly on effector T cells and
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29 on antigen presenting cells [3,4]. Circulating Tregs constitutively express CD25 and FoxP3 and
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31 represent 5–10% of all peripheral CD4+ T cells [5,6]. Tregs, which are fundamental for the
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33 maintenance of immune homeostasis, demonstrated a key role for transplantation tolerance in
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35 animal models by impairing the function of CD8+ T cells [7-10]. In humans, cell therapy with
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37 human Tregs for the induction of transplantation tolerance represents a promising strategy [10,11].
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39 Indeed, clinical trials using this approach have already demonstrated that expanded polyclonal and
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41 antigen-specific Tregs are safe and effective in the treatment of GVHD and Type 1 diabetes [12-
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43 17]. Conversely, very few results have been published on clinical trials testing the efficacy of Tregs
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45 in solid organ transplantation [18,19]. Moreover, isolated/ex vivo expanded Tregs have been only
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47 tested in animal models [20-22].
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55 Here, we first established a preclinical protocol for expansion/isolation of Tregs from
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57 patients with end-stage liver or kidney disease being in the waiting list for liver/kidney
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59 transplantation (LT/KT). We then **scaled up** and optimized such protocol according to Good
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1 Manufacturing Practice (GMP) to obtain high numbers of purified Tregs which were tested *in vitro*
2 and in a xenogeneic acute graft-versus-host disease (GVHD) mouse model.
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10 **Materials and Methods**

11 *Patients*

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Peripheral blood (20-60 mL) was obtained from 14 LT and 9 KT patients. Patients characteristics are outlined in **Table I**. Patient selection for **GMP Tregs isolation/expansion** was based on the following inclusion criteria: 1) Age \geq 18 years; 2) Diagnosis of end-stage kidney disease in waiting list for living donor kidney transplantation or diagnosis of end-stage liver disease in waiting list for liver transplantation. Exclusion criteria included: 1) HIV, HBV, HCV positivity; 2) syphilis antibody positivity; 3) combined transplant; 4) concurrent uncontrolled infection. **Patient selection for *in vitro* experiments were as above described except virus positivity.** Peripheral blood (20-60 mL) and buffy-coat (30 mL) were also obtained from age and sex matched healthy controls. Human studies were conducted in accordance with the Declaration of Helsinki and approved by the local ethical Committee (232/2015/O/Tess by Investigation Drug Service, Azienda Ospedaliero-Universitaria di Bologna). Informed consent was obtained from all the subject prior to enrollment into the study.

52 *Circulating Treg enumeration*

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Enumeration by flow cytometry of circulating Treg (CD4+CD25+CD127-FoxP3+) was carried out in the peripheral blood (PB) of selected KT and LT patients (n=7 and n=10, respectively) and of healthy controls (n=9). The conjugated monoclonal antibodies used are shown in **Supplementary**

Table I. Surface marker staining was performed for 15 min at room temperature. For intracellular staining, anti-human FoxP3 (PCH101) Staining Set PE Kit was used (eBiosciences), according to the manufacturer's instructions. Isotype control rat IgG2 PE was used as a control. Briefly, cells were stained for surface markers CD4, CD25 and CD127, washed once in PBS and then fixed/permeabilized. After washing, cells were incubated with anti-human FoxP3 antibody for 30 minutes at 4 °C in the dark. A lysis buffer (Becton Dickinson) was used in order to lyse red blood cells. The phenotype of Tregs was analyzed by flow cytometry FACSCantoII (Beckton Dickinson). Data were analyzed using the FACSDiva software (Becton Dickinson). The percentage of positive cells was calculated by subtracting the value of the appropriate isotype controls. The absolute number of positive cells per μL was calculated as follows: percentage of positive cells \times white blood cell count (WBC)/ 100.

Tregs isolation and expansion

EDTA-anticoagulated peripheral blood (60 ml) was collected from 4 LT patients, 2 KT patients and buffy-coat (30 ml) from 5 controls. Peripheral blood mononuclear cells (PBMC) were then isolated by Ficoll-Hystopaque density gradient centrifugation.

Isolation: freshly isolated CD8-CD25⁺T cells were purified from PBMC by negative selection of CD8⁺T cells followed by positive selection of CD25⁺T cells using specific Miltenyi-Biotec Beads (CD8 microbeads human and CD25 microbeads II human) with MidiMACS separator and a purity (CD4⁺CD25⁺) of >90% .

Expansion: freshly isolated cells were plated at $1 \times 10^6/\text{ml}$ cells and activated with anti-CD3/CD28 coated beads (Invitrogen, Paisley, UK; Miltenyi Biotec) at a 4:1 bead:cell ratio at day 0 and then 1:1 bead:cell ratio weekly. Cells were expanded in culture media (TECSMacs GMP medium, Miltenyi Biotec) 5% human AB plasma containing rapamycin (100 nM) (Rapamune[®], Wyeth, USA) for 21 days at 37°C and 5% CO₂. IL-2 (1000 IU/ml, Proleukin[®], Novartis, UK) was added at

1 day 4 post-activation and replenished every 2 days. Cells were restimulated with beads every 7
2 days. After 21 days of culture, beads were magnetically removed and the cells washed in
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4 TECSMacs GMP medium. After washings, fresh beads, rapamycin and IL-2 were added. Expanded
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6 cells were used for further analysis at each time of re-stimulation up until day 21 of expansion.
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10 11 12 13 14 *Phenotypic characterization*

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16 Phenotypic characterization were performed on day 0,7,14,21 of cultures for ex vivo expansion and
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18 after cryopreservation/thawing by flow cytometry as above described. Surface marker staining was
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20 performed in order to asses the content of Tregs and contaminant cells (monocytes (CD14+), B
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22 (CD19+) and T cells (CD8+), NK cells (CD56+), Th17 cells (CD196+CD161+)) present at each
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24 time point of culture (Supplementary Table I) .
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34 *FOXP3 promoter demethylation analysis*

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36 The highly conserved Treg-specific demethylation region (TSDR) within the human *FOXP3* gene is
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38 demethylated exclusively on Tregs and not in any other blood-cell types. Specific DNA methylation
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40 of the Treg *FOXP3* is referred to intron 1, as previously identified [23]. DNA methylation of
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42 *FOXP3* gene (**CNS2 region**) of expanded cells (day +21), either freshly isolated or after
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44 cryopreservation/thawing, was evaluated for a total of 6 subjects (3 patients: 1 KT and 2 LT
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46 patients; and 3 controls). Genomic DNA was extracted from purified Tregs using the *AllPrep*
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48 DNA/RNA Mini Kit (QIAGEN, Hilden, Germany). 500ng of DNA was bisulphite-converted using
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50 the EZ DNA Methylation Kit (Zymo Research Corporation, Orange, CA) according to
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52 manufacturer's instructions, except for the thermal conditions of the conversion (21 cycles of 55°C
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54 for 15 min and 95°C for 30s). Bisulphite-treated DNA was eluted in 100ul of water. DNA
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56 methylation analysis of the genomic region chrX:49,117,049-49,117,467 within *FOXP3* gene body
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1 was performed by EpiTYPER assay (Sequenom, San Diego, CA), a quantitative method based on
2 mass spectrometry that allows to evaluate methylation level at single CpG sites/groups of adjacent
3 CpG sites (CpG units). Ten ng of bisulphite-treated DNA were PCR-amplified and processed
4 following manufacture's instructions. The bisulphite specific primers were: *FOXP3* forward,
5 AGGAAGAGAGATTTGTTTGGGGGTAGAGGATTTA; *FOXP3* reverse,
6 CAGTAATACGACTCACTATAGGGAGAAGGCTCAAAAAAAAAACCAAATCTTCAAAACT.
7 For each gene, CpG sites with missing values in more than the 20% of the samples were removed,
8 as well as samples with missing values in more than the 20% of CpG sites. The R package
9 *massArray* was used to test if bisulphite conversion reaction **run** to completion [24]. For all samples
10 analysed bisulphite conversion was from 98.9% to 100%.

11 *Mixed Leukocyte Reaction Assay*

12 The T cells were used as autologous responder cells for *in vitro* suppression assays. The suppressive
13 activity of freshly isolated and expanded cells were tested by co-culturing **CD8-CD25- T cells**,
14 labeled with 5 μ M carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), with serial dilutions
15 of freshly isolated (CD8-CD25+ T cells) and *ex vivo* expanded autologous Tregs in the presence of
16 CD3/CD28 GMP beads at ratio previously shown by us (in house experiments) to be effective in
17 dose-response experiments (1:10 CD8-CD25-T cells to bead ratio) in RPMI-1640 medium
18 containing 10% FBS, 1% penicilline/streptomycine and L-glutamine (1%) for 5 days at 37°C and
19 5% CO². Proliferation was analyzed by flow cytometry. The percentage of proliferative CD8-
20 CD25- T cells in the absence of Tregs was taken as 100% proliferation.

21 *GMP Treg isolation and expansion for in vivo experiments*

1 The GMP manufacturing was performed in the Cell Factory of Fondazione IRCCS Ca' Granda
2 Ospedale Maggiore Policlinico, certified in compliance with European GMP regulations by the
3
4 Italian Drug Agency (authorization number aM-51/2018).
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7 Steady state leukapheresis from 2 patients (1 KT and 1 LT patient) were processed using GMP-
8 compliant devices and reagents (**Supplementary Figure 1A**). The subjects were evaluated for
9
10 venous accesses suitability. Leukapheresis was carried out by the COM.TEC® (Fresenius Kabi AG,
11
12 Bad Homburg, Germany) cell separator. The treatment of two blood volumes was set up as the
13
14 procedure end-point. ACD-A was used for anticoagulation at a ratio of 1:14- 1:13. For prophylaxis
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16 of citrate-related hypocalcemia, calcium gluconate was administered intravenously during the
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18 leukapheresis.
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24 CD8-CD25+ cells were purified using the CliniMACS System (Miltenyi Biotec, Bergisch
25
26 Gladbach, Germany) according to GMP procedures and following manufacturer's instructions.
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29 Briefly, we performed a sequential two-step process based on the magnetic depletion of CD8+ T
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31 cells, followed by a positive selection of CD25+ cells after an over night storage, using monoclonal
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33 antibodies anti-CD8 (Miltenyi Biotec) and anti-CD25 (Miltenyi Biotec), as described in the
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35 CliniMACS user manual for cell preparation, magnetic labelling, and selection.
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38 Forty millions of CD8-CD25+ cells were expanded *in vitro* in gas-permeable culture bags (MACS
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40 GMP Cell Expansion Bags and MACS GMP Cell Differentiation Bags, Miltenyi Biotec) for 3
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42 weeks in complete medium, consisting of TexMACS Medium (Miltenyi Biotec) supplemented with
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44 100 nM rapamycin (Miltenyi Biotec), 5% allogeneic human heat-inactivated AB plasma, 500-1000
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46 UI/mL IL-2 (Proleukin, Novartis Pharmaceuticals Canada Inc, Dorval, Quebec, Canada). On day 0,
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48 7 and 14, anti-CD3/CD28 beads (MACS GMP ExpAct Treg Beads, Miltenyi Biotec) were added at
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50 different beads:cells ratio 4:1, 1:1, 1:1 respectively.
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56 At day 21 all the cultured cells were collected and the beads were removed using the CliniMACS
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58 device, according to manufacturer's instructions. Cells were counted by an automated and validated
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60 method (Nucleocounter System, Chemometec, Allerød, Denmark). The Treg markers were
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1 evaluated by flow cytometry (BD FACSCanto II, BD Bioscience, San Jose, CA, USA) using the
2 following antibodies: CD45 APC-H7 (BD Bioscience), CD4 FITC (BD Bioscience), CD25 APC
3 (BD Bioscience), CD127 PE-Cy7 (BD Bioscience) and FoxP3 PE (eBioscience, San Diego, CA,
4 USA).
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9 Negative fraction (CD8-CD25- T cells) after GMP selection at day 0 and the final product after
10 GMP expansion at day 21, were cryopreserved and thawed as above described.
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19 *Cryopreservation and thawing of freshly isolated and expanded cells*

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21 Freshly isolated fractions (CD8-CD25+ and CD8-CD25- T cells) and expanded cells (after 7, 14
22 and 21 days of expansion) were washed and cryopreserved with 10% DMSO (CRYOSERV, Mylan
23 Institutional, Canonsburg, PA, USA), 10% HSA (HAS; Kedrion, Lucca, Italy) in a saline solution
24 (B. Braun, Melsungen, AG, Germany) using a controlled-rate freezer. The frozen units were
25 transferred and stored immediately to vapor-phase liquid nitrogen into dedicated tanks. All the
26 cellular fractions were thawed at 37°C and the viability, phenotype and suppressive function were
27 assessed as above described.
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44 *Xenogeneic GVHD mouse model*

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46 NOD scid gamma (NSG) female mice (6-7 weeks of age) were purchased from Charles River
47 (Calco, Italy). All animals were housed under specific pathogen-free conditions in compliance with
48 guidelines of the San Raffaele Institutional Animal Care and Use Committee (IACUC number:
49 632). Mice were maintained for at least 5 days in the animal facility for acclimatization before
50 transplantation. To promote the engraftment of transplanted cells **injected intraperitoneally**, all mice
51 were conditioned with total body irradiation (1.75 cGy) on day 1. Of note, day 1 of the study is
52 considered the day of transplant. All groups of mice, transplanted as well as control mice, were
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1 monitored for 7 weeks. Clinical signs of GVHD (e.g., hunched back, fur loss, skin inflammation)
2 were monitored daily. Body weight was monitored throughout the duration of the study. Animals
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4 showing marked clinical signs as of distress, loss of weight equal to or greater than 20% of their
5
6 starting weight were immediately sacrificed.
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9 To establish whether the expanded Tregs could counteract acute GVHD, a xeno-GvHD model was
10 induced by intraperitoneally transfer of human autologous CD8-CD25- T cells from 1 LT and 1 KT
11 patient into mice. Both CD8-CD25- T cells and expanded Tregs were injected within 30 minutes
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13 from thawing (**Supplementary Figure 1 B**). Specifically, human CD8-CD25- T cells (6×10^6) from
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15 LT or KT patients were injected with (simultaneous injection) or without autologous Treg (6×10^6)
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17 **at a Treg: CD8-CD25- T cells ratio of 1:1**. Timing of Treg infusion is crucial. In our model Tregs
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19 were infused at the time of T effector transplantation.
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31 *In vivo engraftment monitoring*

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33 To assess the in vivo persistence of human cells, transplanted mice were bled 4 weeks after
34 transplantation and sacrificed 7 weeks after transplantation. Phenotype analysis of injected cells and
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36 of peripheral blood at 4 weeks (± 3 days) after transplantation and of PB and spleen at 7 weeks (± 3
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38 days) after transplantation was performed (**Supplementary Figure 1C**). Following a Fc blocking
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40 step, cell surface staining was performed with anti-mouse CD45 and anti-human CD4, CD3, CD25,
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42 FoxP3 mAbs at 1:100 dilution in staining buffer (PBS, 2% FCS, 0.1% NaN₃) (list of mAbs is in
43
44 Supporting Information Table). To detect FoxP3, cells were treated and stained with the FoxP3
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46 fixation/permeabilization kit according to the manufacturer's instructions (eBioscience). Samples
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48 were acquired on a BD FACSCanto II flow cytometer. Manual analysis of flow cytometry data was
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50 performed with FCS Express V4 (DeNovo Software, Glendale, CA).
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Statistical analysis

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2 Statistical analyses were performed using GraphPad Prism (GraphPad Prism 5.0 soft-ware,
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4 SanDiego CA, USA), and data are presented as mean \pm standard error of the mean (SEM). For all
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6 experiments involving multiple comparisons, analysis of variance (ANOVA) followed by a
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8 Dunnett's *post hoc* test was used. For those who involved comparisons between two groups,
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10 Student's t-test was used. The level of significance was set at $p \leq 0.05$.
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Results

Enumeration of circulating Treg from LT and KT patients

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29 We firstly enumerated circulating CD4+CD25+CD127-FoxP3+ Tregs in **End-Stage Organ Disease**
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31 patients and controls. The mean absolute numbers of circulating Tregs from LT (19.8 \pm 10/mL) and
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33 KT (18.8 \pm 7/mL) patients was not significantly different from that of healthy controls (17 \pm 4/mL)
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35 (p=NS; **Supplementary Figure 2**).
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Tregs isolation, expansion, cryopreservation and thawing

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47 Starting from 60 ml of PB (patients) and 30 ml of buffy-coat (healthy controls), CD8 depletion with
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49 subsequent CD25+ enrichment of PBMCs yielded a median of 3.7x10⁶ nucleated cells (range 1.28-
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51 8.8x10⁶) in healthy controls, 1.47x10⁶ nucleated cells (range 5.3x10⁵-2.56x10⁶) in LT patients and
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53 1.77x10⁶ nucleated cells (range 8.5x10⁵-2.7x10⁶) in KT patients. The freshly isolated CD8-CD25+
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55 T cells were 2.0 \pm 1.2 , 1.9 \pm 1.3 and 2.6% \pm 0.7 of the PBMCs, respectively, with no significant
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57 differences between patients/controls (P=NS)(data not shown). The **Figure 1A** shows the gating
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1 strategy for identification of CD4+CD25+CD127-FoxP3+ Tregs which represented more than 80%
2 of the cells (range 70-96%) within the CD8-CD25+ population. The median Treg purity
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4 (CD4+CD25+CD127-FoxP3+ cells) was 72% (range 50-84) and 55% (range 40-70) in LT and KT
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6 patients, respectively and 65% (range 54-80) in healthy controls (**Figure 1B**). No significant
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8 difference was observed between patients and controls (p=NS). On day 0, contaminating cells in
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10 healthy controls were CD19+ B cells (16%, range 14.8-17), Th17 cells (4.7%, range 0.7-6.9) and
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12 CD14+ monocytes (6.3%, range 0.8-11.4). In LT and KT patients, contaminating cells were mainly
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14 CD19+ B cells (6.8%, range 1.1-8.4; 5.4%, range 1.9-8.9, respectively) and Th17 cells(7.3%, range
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16 1.4-12.8; 11.8%, range 10.3-13.3, respectively). Natural Killer cells and monocytes content was
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18 always very low (below 1%) (**Figure 1C**). By comparing patients and controls, Th17 cells were
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20 significantly increased in KT cells as compared with LT or healthy controls (p≤0.05vs LT patients;
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22 p≤0.05 vs healthy controls). In addition, CD19+ cells were significantly reduced in both patient
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24 groups as compared with the normal counterparts (p≤0.05 for LT patients; p≤0.05 for KT patients).
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31 After 21 days, Tregs expanded a median of 197-folds (range 26-567) in LT patients, 266-
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33 folds (range 233-300) in KT patients and 559-folds (range 342-826) in healthy controls (**Figure**
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35 **2A**). Differences between patients and controls were not statistically significant. Starting from
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37 freshly isolated 500x10³ cells, the final yield was a median of 255x10⁶ (range 171x10⁶-413x10⁶)
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39 nucleated cells in healthy controls, 48x10⁶ (range 30x10⁶-284 x10⁶) nucleated cells in LT patients
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41 and 133 (range 116x10⁶-150x10⁶) nucleated cells in KT patients (data not shown). Treg purity
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43 (CD4+CD25+CD127-FoxP3+) increased significantly during expansion (**Figure 2B**) in healthy
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45 donors and KT patients (p≤0.05). Contaminating cells, namely B cells, Th17 cells and monocytes
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47 decreased below 1% in healthy controls and LT patients and below 3% in KT patients (**Figure 2C**).
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53 When cryopreserved/thawed 21 days expanded Tregs were analyzed, the mean percentage of
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55 viable cells was always more than 75% with no significant differences between patients and
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57 controls (**Figure 2D**). The mean percentage of CD4+CD25+CD127-FoxP3+ cells was always
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59 >80% (**Figure 2E**).
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When functional assays were performed, freshly isolated cells (CD8-CD25⁺ T cells) from the three groups exerted a limited (healthy controls and LT patients) or poor (KT patients) suppressive effect toward autologous CD8-CD25⁺ T cells proliferation *in vitro* (Figure 3A). After 21 days of *in vitro* expansion, flow cytometry analysis demonstrated that, according to the increased T regulatory phenotype, Tregs from patients/controls had significant suppressive activity (Figure 3B), which was not modified by cryopreservation/thawing (Figure 3C).

All together these data demonstrate that our small scale Treg isolation and expansion protocol from LT and KT patients lead to functional Tregs which are suitable for cell therapy. Moreover, cryopreservation/thawing does not impair phenotype/function of the expanded Tregs.

FOXP3 methylation analysis

Figure 4A shows that in males the locus, which is localized on X chromosome, displayed low DNA methylation values (CpG sites ranging from 0.08 to 0.44). As expected, in females CpG sites had higher methylation values (ranging from 0.24 to 0.73) due to X chromosome inactivation. We evaluated DNA methylation differences at day +21 in freshly isolated and cryopreserved/thawed expanded cells in patients and controls separately using paired t-test. The large majority of CpG sites was not significant, and intra-individual difference between the two groups were generally small, spanning between -0.13 and 0.23. The only exception was the CpG_3 among controls (p-value: 0.002), but, also in this case, the difference between the freshly isolated and the cryopreserved/thawed cells was small, ranging from 0.06 to 0.07 (Figure 4B).

Collectively, these data suggest that the DNA methylation pattern of *FOXP3* gene is substantially conserved after cryopreservation/thawing of ex vivo expanded Tregs from LT and KT patients.

In vivo study

To investigate whether GMP expanded Tregs from 1 KT and 1 LT patient have inhibitory function *in vivo* after thawing, we used a xenograft model of lethal GVHD. Specifically, irradiated NSG mice were **injected** with cryopreserved/thawed CD8-CD25⁻ T cells either alone or in combination with autologous expanded cryopreserved/thawed Tregs at 1:1 ratio to assess their ability to ameliorate GVHD (**Supplementary Figure 1**). The rationale for this design is that early infusion of Tregs allows the cells to prevent T effector activation. After the immunomagnetic selection and expansion of 40 millions of freshly isolated CD8-CD25⁺ cells under GMP condition, we obtained 4.3x10⁹ total cells (107.1- fold expansion) for the KT patient and 4.6x10⁹ total cells (115.2- fold expansion) for the LT patient. Before expansion the percentage of CD45⁺/CD4⁺/CD25⁺ cells was 84.9 % and 53.9% in KT and LT patient whereas , at the end of expansion, the percentage of CD45⁺/CD4⁺/CD25⁺ cells increased up to 98.3% and 89.2% respectively. At day 21 CD45⁺/CD4⁺/CD25⁺/CD127⁻/FoxP3⁺ cells were 79.2% and 84.4% respectively (**data not shown**).

Tregs were then thawed and characterized for the phenotype and in vitro function. As shown in **Figure 5A and B**, the thawed Tregs were more than 80% CD4⁺CD25⁺CD127⁻FoxP3⁺ and highly suppressive.

Mice receiving KT **CD8-CD25⁻ T cells** lost weight and all succumbed to GVHD by day 40. In contrast, mice receiving KT **CD8-CD25⁻ T cells** and autologous Tregs showed delayed disease-associated weight loss and slightly increased survival rate (by day 48 17% of mice receiving CD8-CD25⁻ T cells with Tregs from the KT patient survived vs 0% for mice receiving CD8-CD25⁻ T cells only; p=N.S.) (**Figures 6A and B**). Similar results were obtained with cells from the LT patient: by day 48 58% of mice receiving CD8-CD25⁻ T cells with Tregs from the LT patient survived vs 12,5% for mice receiving CD8-CD25⁻ T cells only (p=N.S.; **Figure 6C and D**)

To assess human cells engraftment in PB, animals were bled after 4/7 weeks and the percentages of human CD45⁺ cells in blood was determined (**Figure 7**). The gating strategy is

1 shown in **Figure 7A**. No human CD45+ cells were identified in the PB of mice after infusion of
2 Tregs alone from KT or LT patients. Conversely, a wide distribution of human CD45+ cells was
3
4 observed in the PB of mice after transplant of CD8-CD25- T cells alone or CD8-CD25- T cells plus
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6 Treg infusion (**Figure 7 B and C**).

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9 To assess the biodistribution of infused human cells, spleen and PB cells of mice were
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11 stained with anti-human CD45, CD3, CD4, FoxP3 monoclonal antibodies after 7 weeks from
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13 transplant. The gating strategy is shown in **Figure 8A**. By comparing PB and spleen, a higher mean
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15 percentage of human CD45+ cells was observed in the spleen vs PB of the mice after receiving
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17 CD8-CD25- T cells plus Treg of LT patients ($p < 0.003$; **Figure 8B**). Of note, the majority of the
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19 human CD45+ cells were CD3+CD4+ in the blood/spleen of mice after receiving KT or LT cells
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21 (**Figure 8C**). Human FoxP3+ Tregs were identified in the CD3+CD4+ fraction of PB and spleen of
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23 the two groups of mice without differences between KT and LT cells (**Figure 8D**).

34 35 36 **DISCUSSION**

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39 The use of Tregs for the treatment of GVHD and for inducing operational tolerance after solid
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41 organ transplantation is a promising approach.

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44 Here, we isolated, expanded and functionally characterized Tregs from patients with end
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46 stage kidney and liver disease, with the aim to establish a suitable method to obtain
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48 immunomodulatory cells for adoptive immunotherapy post-transplantation.

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51 We demonstrated that fully functional Tregs from patients in waiting list for solid organ
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53 transplantation can be isolated/expanded for clinical studies. These cellular products show
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55 homogenous regulatory T cells characteristics with very low percentage of contaminating cells.
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57 Therefore, unwanted immune side effects are minimized. Of note, based on phenotype/function, the
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1 isolated/expanded Tregs from KT and LT patients are similar. Interestingly, our
2 cryopreservation/thawing procedure allowed cell suspensions with phenotype/function and
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4 hypomethylation of the *FOXP3* gene superimposable to that of the freshly isolated counterparts.
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6 This finding challenges the notion that cryopreservation of Tregs products is detrimental for their
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8 function [26-27].
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12 Our freshly isolated cells from healthy donors and LT/KT patients showed limited
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14 suppressive activity. This is in contrast with previous findings demonstrating 60-80% of
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16 suppressive activity by freshly isolated healthy donors cells, which were CD8-CD19-CD25⁺ cells
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18 [22] or CD8-CD25⁺ cells [25]. However, our results may be due 1) to the low purity of our freshly
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20 isolated cell suspension (CD8-CD25⁺ cells) and 2) to the fact that while our suppressive activity
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22 results were based on a 1:2 Treg/CD8-CD25- T cells working ratio, their ratio was 1:1 Treg/Teff.
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24 Whether this results is also due to the fact that our freshly isolated healthy donors cells derive from
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26 24 hours aged buffy-coats remains a matter of speculation.
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32 Safinia et al firstly proposed a GMP production protocol to expand CD25⁺-enriched cells
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34 from PB in the presence of IL-2 and rapamycin to induce tolerance after liver transplantation [10].
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36 In their 36 days expansion protocol, multiple round of in vitro Treg stimulation were necessary to
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38 achieve clinically relevant Tregs number. In our protocol indeed, we obtained fully functional Tregs
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40 after 21 days of expansion containing less than 1% of contaminating CD8⁺ effector cells.
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42 Expanded, cryopreserved and thawed Tregs remain hypomethylated at intron 1 of the *FOXP3* locus,
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44 confirming their epigenetic stability. Functionally, Tregs showed suppressive function against
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46 autologous CD8-CD25- T cells.
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52 Several human Treg products have been tested in animal models. However, previous data of
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54 Treg cell infusion in non human primates report conflicts regarding the ability of the infused cells to
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56 induce transplant tolerance [28-30]. Thus, we decided to use a mouse model of GVHD in
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58 immunodepressed mice [i.e. NOD-SCID-gamma KO (NSG) mice] receiving human effector T cells
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1 with or without GMP Tregs to prevent the onset of xenogeneic GVHD. In our mouse model, GMP
2 Tregs from LT or KT patients **proved** to be safe and **showed a trend toward reduced lethality of**
3 **acute GVHD *in vivo***. Interestingly, GMP Tregs from LT and KT patients did not induce xenogenic
4 GVHD and did not expand as documented by the absence of Tregs in the PB and spleen of mice
5 after 4 and 7 weeks from transplantation. This is probably due to the lack of human IL-2.
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12 Nevertheless, our data are consistent with previous models of xenogeneic GVHD and Treg
13 infusion. The addition of CD25 expressing cells to human PBMC showed the amelioration of
14 xenogeneic GVHD, whereas the depletion of all CD25+ cells led to the development of lethal
15 xenogeneic GVHD [31]. Also, *in-vitro* expanded Tregs from human peripheral blood or cord blood
16 were able to ameliorate or suppress xenogeneic GVHD [32,33]. More recently, it has been reported
17 that the infusion of polyclonal human Tregs improved murine xenogeneic chronic GVHD [34]. In
18 addition, Del Papa et al. used a similar methodology (immune-magnetic Treg isolation and
19 polyclonal expansion in the presence of CD3/CD28 beads, IL-2, and rapamycin for 19 days)
20 achieving a median of 8.5 fold expansion and maintaining FoxP3 expression over the culture
21 period. NSG mice that received human leukemic cells and expanded Tregs with conventional T
22 cells were rescued from leukemia and survived without GVHD. Mice that received leukemic cells
23 plus conventional T cells died of severe GVHD within 70 days [22].
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44 **CONCLUSION**

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46 In summary, we demonstrated that expanded/thawed GMP Tregs from two different groups
47 of patients with end-stage organ failure were fully functional *in vitro* and their infusion was safe **and**
48 **resulted in a trend toward reduced lethality of acute GVHD *in vivo***. These data further support the
49 expanded/thawed Tregs-based adoptive immunotherapy in solid organ transplantation.
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Ethics approval and consent to participate

Patients/controls provided written informed consent and the study was approved approved by the local ethical Committee (232/2015/O/Tess by Investigation Drug Service, Azienda Ospedaliero-Universitaria di Bologna).

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Consent for publication

Not applicable

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Availability of data and material

All data generated or analysed during this study are included in this article and its additional files

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Competing interests

The authors declare no competing financial interests.

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Authors' contributions

UF and MT contributed equally to this work (first and second Author). CL and RML contributed equally to this work (last and co-last Author). UF and MT design of the study, collection and analysis of data, writing the manuscript ; CM, BMG, PC, GP, FC, methylation data collection and analysis, and critical revision of the manuscript; JT, MA, BM animal model data generation and analysis and critical revision of the manuscript; MT, LC, VM, BS, GR, data collection and analysis in the GMP facility and critical revision of the manuscript; GV apheresis procedure and critical revision of the manuscript; SD, CA and AM, data analysis, and interpretation; CG, BV, LMG, CM, PAD, providing samples from patients/controls and critical revision of the manuscript; CL and RML, design of the study, writing the manuscript, and providing funding.

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Acknowledgments

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Legend to figures

Fig 1. Phenotype of the freshly isolated Tregs. **A.** The gating strategy of Tregs identification after CD8 depletion and CD25 enrichment is shown. Dot plots depict the expression of CD25, CD127 and FoxP3. Interestingly, the majority of the freshly isolated cells expressed CD25. Dot plots are representative of 11 independent experiments from patients/controls. **B.** Graph shows Treg purity, as percentage of CD4+CD25+CD127-FoxP3+ cells after isolation from PB of CTR, LT and KT patients. No significant difference was observed between patients and controls ($p=NS$). **C.** Graph displays the percentages of contaminant cells in the freshly isolated product from CTR, LT and KT patients. Data are represented as mean \pm SEM. ($*p<0.05$)

Fig 2. Phenotype of the *in vitro* expanded Tregs. **A.** Tregs from CTR, LT and KT patients were expanded up to 21 days with IL-2 and Rapamycin. Fold expansion of day 7, 14 and 21 was calculated as compared to day 0 seeded cells. **B.** Graph shows the percentage of Treg (CD4+CD25+CD127-FoxP3+) at each time point stimulation. Treg purity increased significantly during expansion in CTR and KT patients. **C.** The graph shows the percentages of contaminant cells during the expansion period. The contaminant cells decreased below 1% in CTR and in LT patients and below 3% in KT patients at the end of expansion. **D.** Viability of cryopreserved/thawed day 21 Tregs is shown. No significant difference was observed between patients and CTR ($p=NS$). **E.** Phenotype of the cryopreserved/thawed day 21 Tregs is shown as mean percentages of CD4+CD25+CD127-FoxP3+ cells. The thawed Tregs were almost all FoxP3 positive in patients/CTR. No significant difference was observed between patients and controls ($p=NS$). Data are represented as mean \pm SEM. ($*p<0.05$)

Fig 3. Suppressive function of the *in vitro* expanded Tregs. The graphs show the mean percentages of proliferation of the CFSE-labelled autologous CD8-CD25- T cells in the presence of CD3/CD28 GMP beads and Tregs at different ratios. **A.** MLR with freshly isolated Tregs (day 0). Freshly isolated Tregs from the three groups were limited (CTR and LT patients) or very poorly (KT patients) suppressive. **B.** MLR with freshly isolated expanded Tregs (day 21). Expanded Tregs from patients/CTR had significant suppressive activity at 1:2 and 1:4 (Treg:CD8-CD25- T cells) ratio. **C.** MLR with expanded and cryopreserved/thawed day 21 Tregs. Cryopreservation/thawing did not significantly affect the suppressive activity of day 21 expanded Tregs of patients/CTR. Data are represented as mean \pm SEM. ($*p<0.05$, $**p<0.01$, $***p<0.001$)

Fig 4. DNA methylation analysis of FOXP3 intron 1. **A.** DNA methylation values of *FOXP3* intron 1 in day +21 freshly isolated GMP expanded Tregs from patients/healthy controls. **B.** Differences in DNA methylation values of *FOXP3* locus between day +21 freshly isolated and day +21 cryopreserved/thawed GMP expanded Tregs. In both the panels blue and pink lines correspond to male and female subjects respectively, while continuous and dashed lines correspond to healthy controls and patients respectively.

Fig 5. GMP Treg expansion for the *in vivo* study: phenotype and function of cryopreserved/thawed day 21 expanded Tregs. **A.** Graph shows the percentages of CD4+CD25+CD127-FoxP3+ Tregs of the LT and KT patient after thawing. Almost all cells were FoxP3 positive. **B.** MLR assay is shown. Expanded Tregs from the LT or KT patient had significant suppressive activity at 1:2 and 1:4 ratios. ($**p<0.01$)

1 **Fig 6. *In vivo* study: injection of cryopreserved/thawed GMP day 21 Tregs from 1 KT and 1**
2 **LT patient in a xenogeneic GVHD murine model.** **A** and **C** show the percentage of body weight
3 variation of mice receiving CD8-CD25- T cells only, GMP Tregs only or GMP Tregs plus CD8-
4 CD25- T cells from KT and LT patients, respectively. Data are presented as means (KT/LT: CD8-
5 CD25- T cells n=7/8 mice; GMP Tregs n=6/6 mice; GMP Tregs plus KT CD8-CD25- T cells
6 n=12/12 mice). **B** and **D** show the survival rate of mice receiving CD8-CD25- T cells only, GMP
7 Tregs only or GMP Tregs plus KT CD8-CD25- T cells from KT and LT patients, respectively.
8 (survival of mice receiving KT cells: statistically significant differences were observed only for
9 Tregs vs CD8-CD25+ cells (P<0.001) and Tregs vs CD8-CD25+ cells + Tregs (P<0.01); survival
10 of mice receiving LT cells: statistically significant differences were observed only for Tregs vs
11 CD8-CD25+ cells (P<0.05)). Overall, mice receiving GMP Tregs plus CD8-CD25- T cells showed
12 a trend toward a time delay in body weight loss and survival as compared with mice injected with
13 CD8-CD25- T cells only.
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18 **Fig 7. *In vivo* study: human CD45+ cells engraftment in the xenogeneic GVHD murine model.**
19 Animals were bled after 4/7 weeks and the percentages of human CD45+ cells in blood was
20 determined by flow cytometry. **A.** Representative dot plots of human CD45+ cell engraftment at 4
21 weeks after transplant are shown. **B** and **C.** No human CD45+ cells were identified in the PB of
22 mice after infusion of Tregs alone from the KT or LT patient. Conversely, a wide distribution of
23 human CD45+ cells were observed in the PB of mice after transplant of KT/LT CD8-CD25- T cells
24 alone or CD8-CD25- T cells plus Treg infusion. In addition to individual data, mean values and
25 SEM are shown.
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29 **Fig 8. *In vivo* study: biodistribution of the infused GMP Tregs.** Suspensions of spleen and PB
30 after 7 weeks from transplant were stained with anti-human CD45, CD3, CD4, FoxP3 monoclonal
31 antibodies and flow cytometry analysis was performed. Panel **A** depicts the gating strategy to
32 identify the selected subpopulations. Panels **B, C** and **D** show the percentages of human CD45+,
33 CD3+CD4+ and FoxP3+ Treg cells in the blood and spleen of mice after transplant of KT/LT
34 CD8-CD25- T cells plus Treg. In addition to individual data, mean values and SEM are shown.
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39 **Supplementary Fig 1. Design of the *in vivo* study.** **A.** Steady state leukapheresis from 2 patients
40 (1 KT and 1 LT patient) were processed using GMP-compliant devices and reagents. Tregs positive
41 fraction (CD8-CD25+ cells) was purified using the CliniMACS System. Forty millions of CD8-
42 CD25+ cells were expanded *in vitro* for 3 weeks. At day 21 all the cultured cells were collected
43 and the beads were removed using the CliniMACS device, according to manufacturer's instructions.
44 Negative fraction (CD8-CD25- T cells) after GMP selection at day 0 and the final product after
45 GMP expansion at day 21 were cryopreserved and thawed as described in Material and Methods
46 section. **B.** Irradiated NSG mice were infused with the KT or the LT CD8-CD25- T cells, either
47 alone or in combination with autologous expanded Tregs at 1:1 ratio, to assess their ability to
48 ameliorate GVHD. **C.** Mice were bled 4/7 weeks after transplantation and sacrificed 7 weeks after
49 transplantation. FACS analysis of the injected cells (day 1), of PB (4 weeks ± 3 days after
50 transplantation) and of PB and spleen (7 weeks ± 3 days after transplantation) was performed.
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54 **Supplementary Fig 2. Circulating Tregs in KT and LT patients.** Mean absolute number of
55 circulating CD4+CD25+CD127-FoxP3+ Tregs from healthy controls and selected LT and KT
56 patients (p=NS).
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Table I. Patients characteristics

	KT	LT	CTR
Total number	9	14	10
Sex (male/females)	3/6	8/6	5/5
Age (years; mean±SD)	53±13	61±10	58±12
Disease			
HCV/HBV liver cirrhosis	N=0	N=8	NA
Alcoholic liver cirrhosis	N=0	N=3	NA
Dysmetabolic liver cirrhosis	N=0	N=2	NA
Cryptogenetic liver cirrhosis	N=0	N=1	NA
Epatorenal polycystic disease	N=4	N=0	NA
End stage kidney disease	N=4	N=0	NA
Drug-induced tubulointerstitial nephritis	N=1	N=0	NA

Legend: NA (not applicable); LT (Liver Transplant); KT (Kidney Transplant); CTR (Healthy Control)

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Supplementary Table 1.

Antibodies for flow cytometry

Treg (CD4+CD25+CD127-CD45+Foxp3+)	CD4-FITC (SK3), CD25-PeCy7 (2A3), CD127-PE/APC (hIL-/R-M21), CD45-PerCpCy5.5 (2D1), from BD-Biosciences; CD25-Biotin (4E325) and Biotin-APC from Miltenyi; FoxP3-PE (PCH101) from eBioscience.
Contaminant cells (T cells, B cells, NK cells, Th17 cells, monocytes)	CD19-FITC (4G7), CD14-PeCy7 (MoP9), CD56-APC (NCAM16.2), CD8-PE (SK1) and CD196-PE (11A9) from BD-Biosciences; CD161-FITC (191B8) from Miltenyi.
In vivo experiments: Human and mouse CD45+ cells Human CD3+CD4+ Human FoxP3+ Treg	Anti-human CD45-Brilliant Violet510 (HI30), CD4 Pacific Blue (RPA-T4), FoxP3 Alexa Fluor 488 (259D) from Biolegend. Anti-human CD3-APC-Vio770(BW264/56) and anti-mouse CD45 Percp from Miltenyi Biotech. CD25 APC (2A3) from BD-Biosciences

Table I. Patients characteristics

	KT	LT	CTR
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Disease			
HCV/HBV liver cirrhosis	N=0	N=8	NA
Alcoholic liver cirrhosis	N=0	N=3	NA
Dysmetabolic liver cirrhosis	N=0	N=2	NA
Cryptogenetic liver cirrhosis	N=0	N=1	NA
Epatorenal polycystic disease	N=4	N=0	NA
End stage kidney disease	N=4	N=0	NA
Drug-induced tubulointerstitial nephritis	N=1	N=0	NA

Legend: NA (not applicable); LT (Liver Transplant); KT (Kidney Transplant); CTR (Healthy Control)

Figure 1

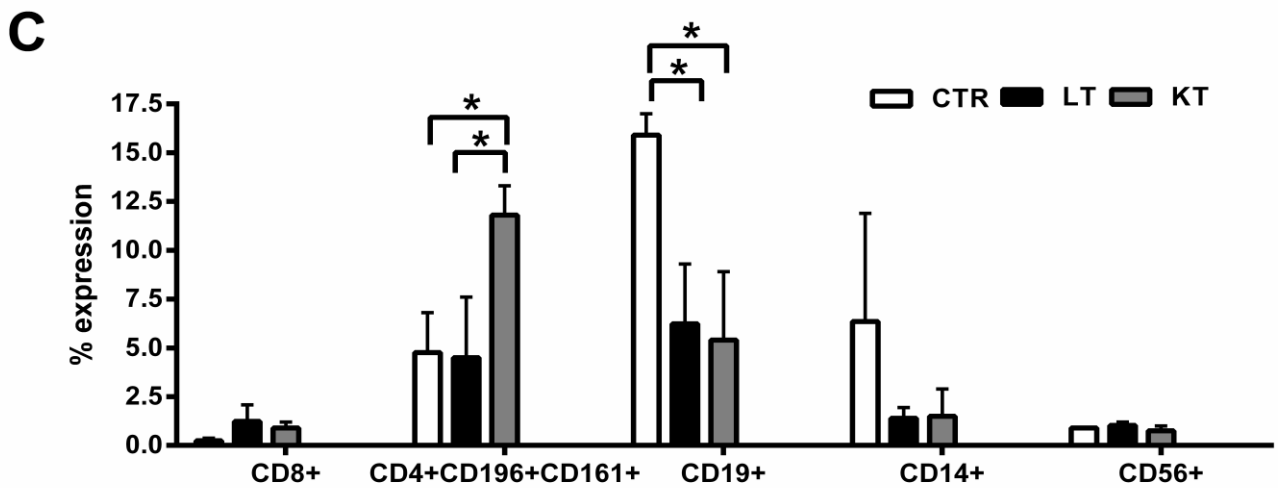
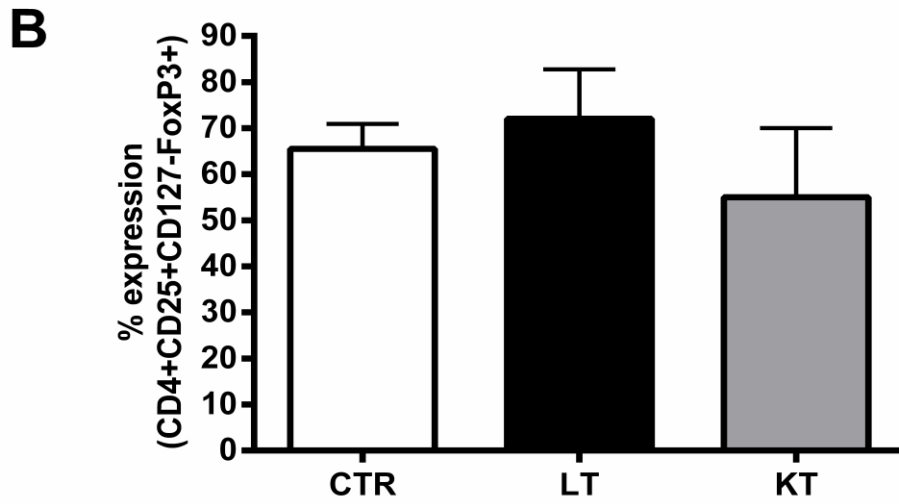
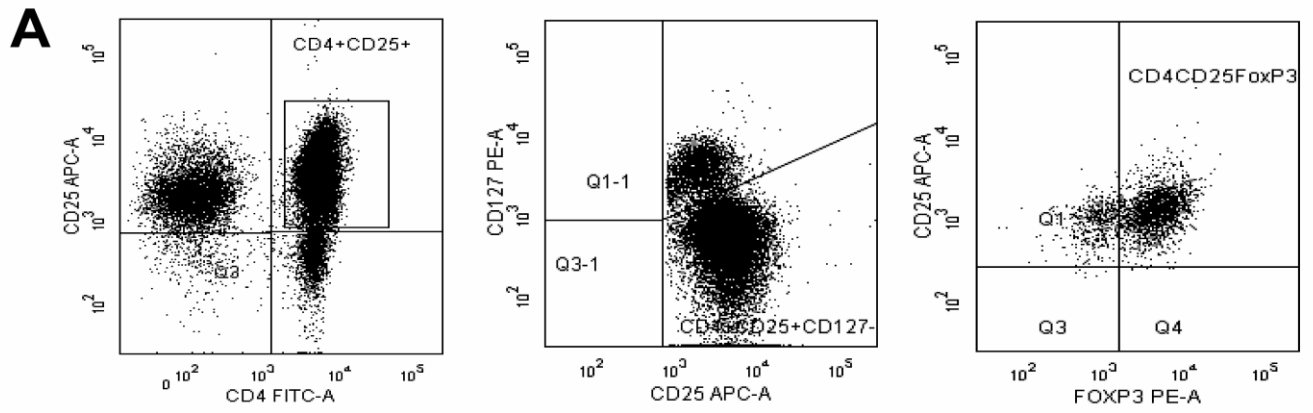


Figure 2

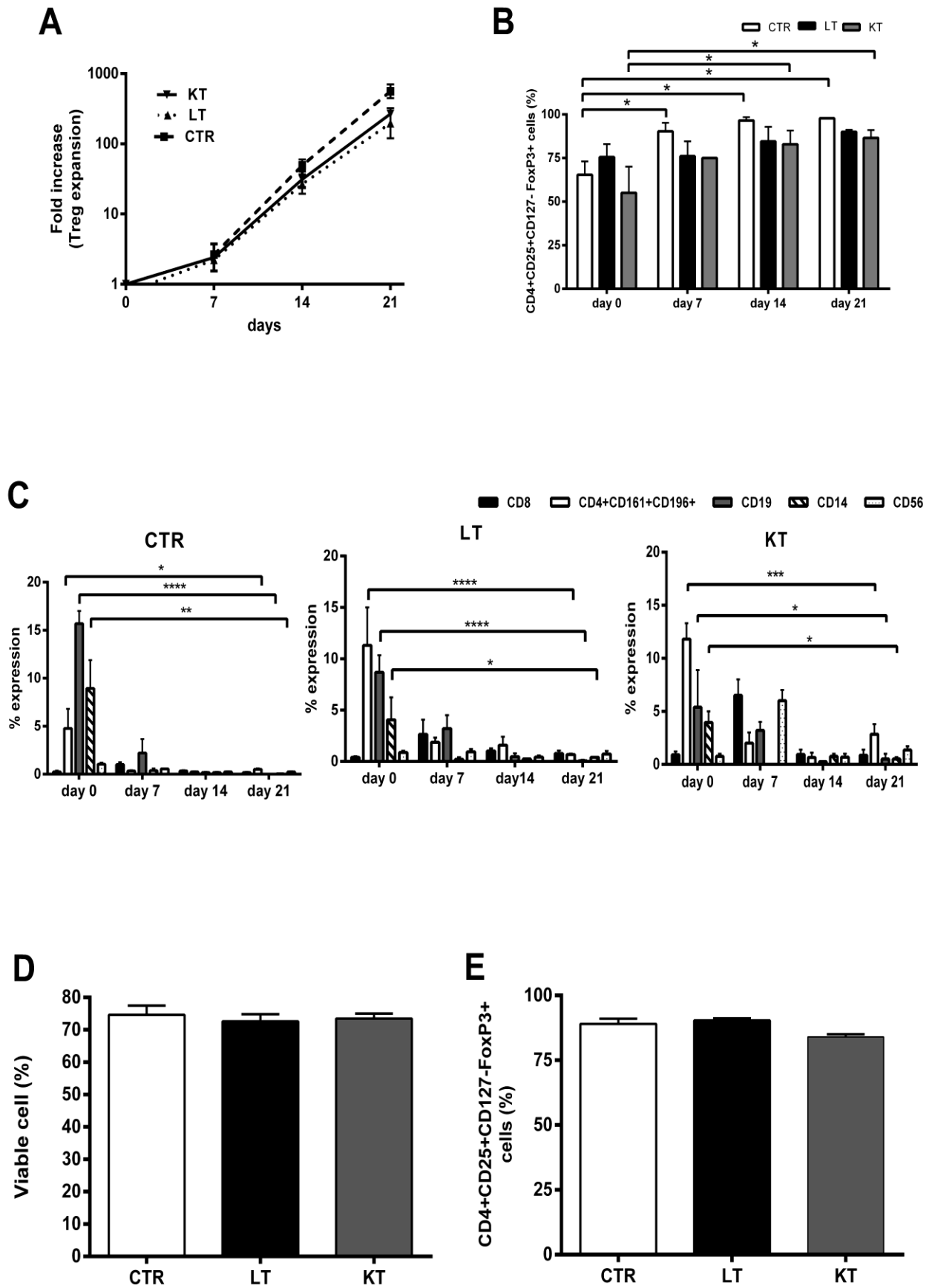


Figure 3

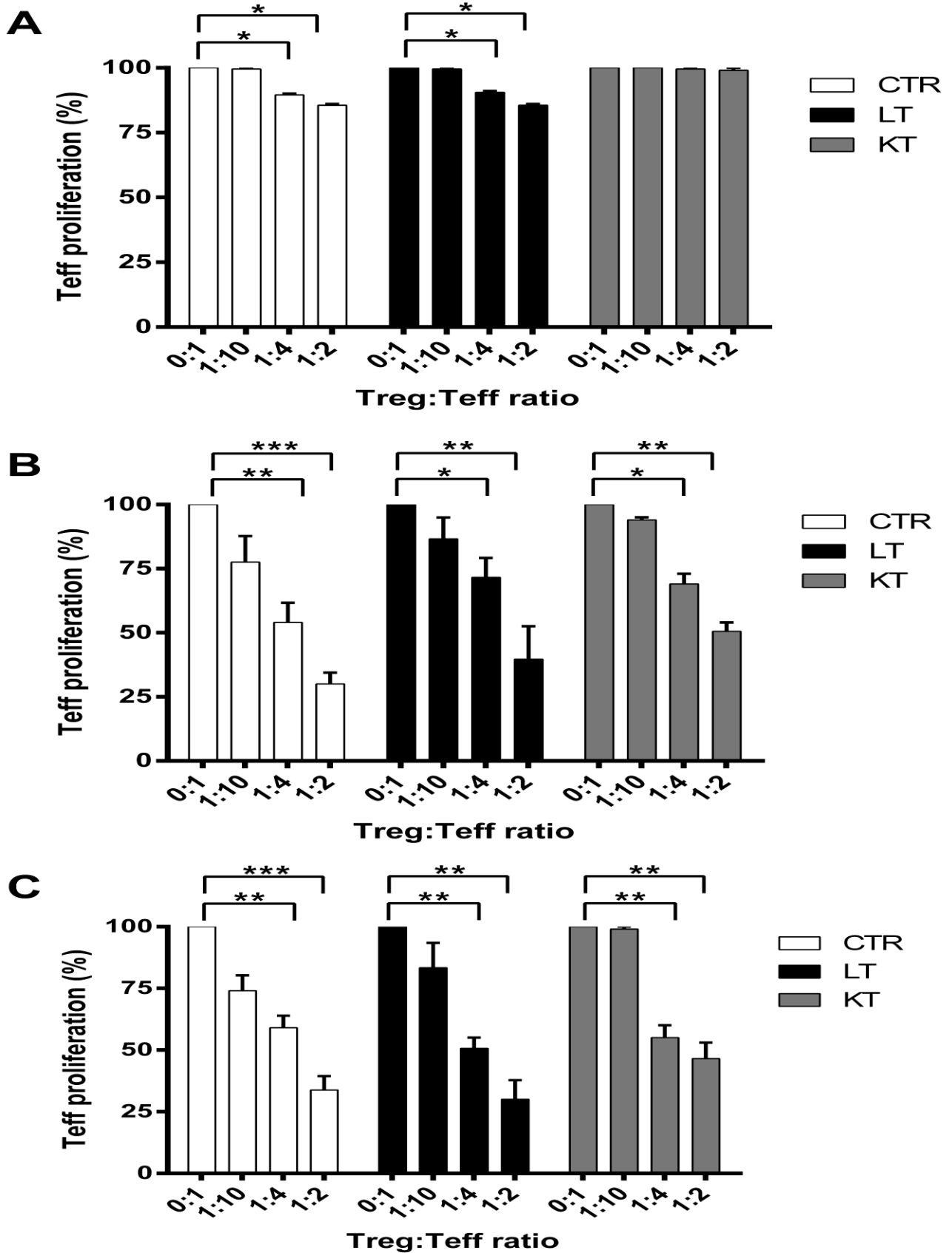


Figure 4

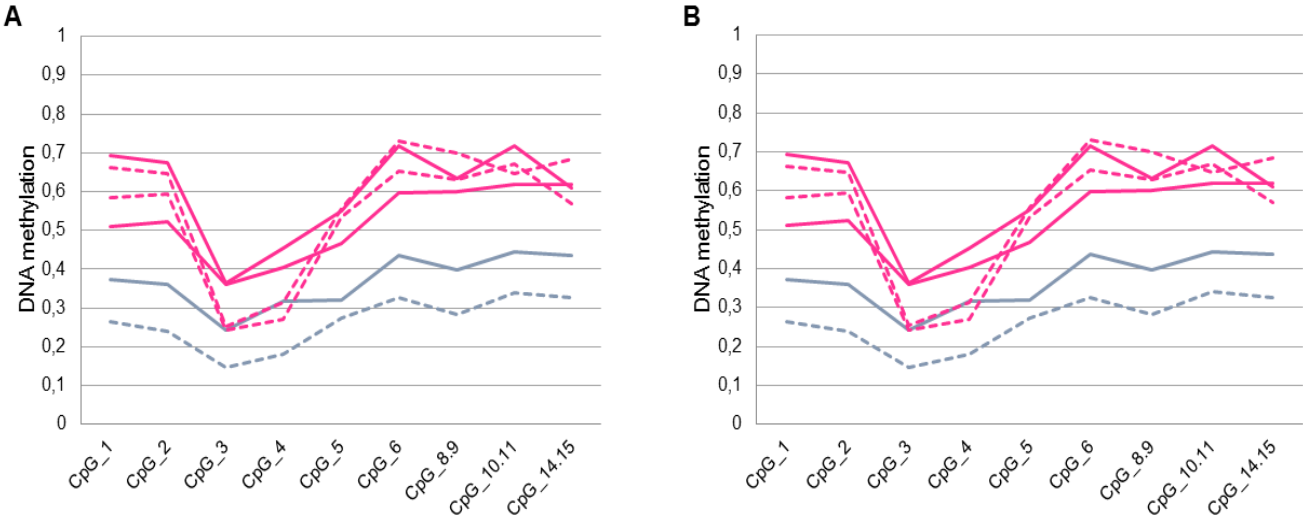


Figure 5

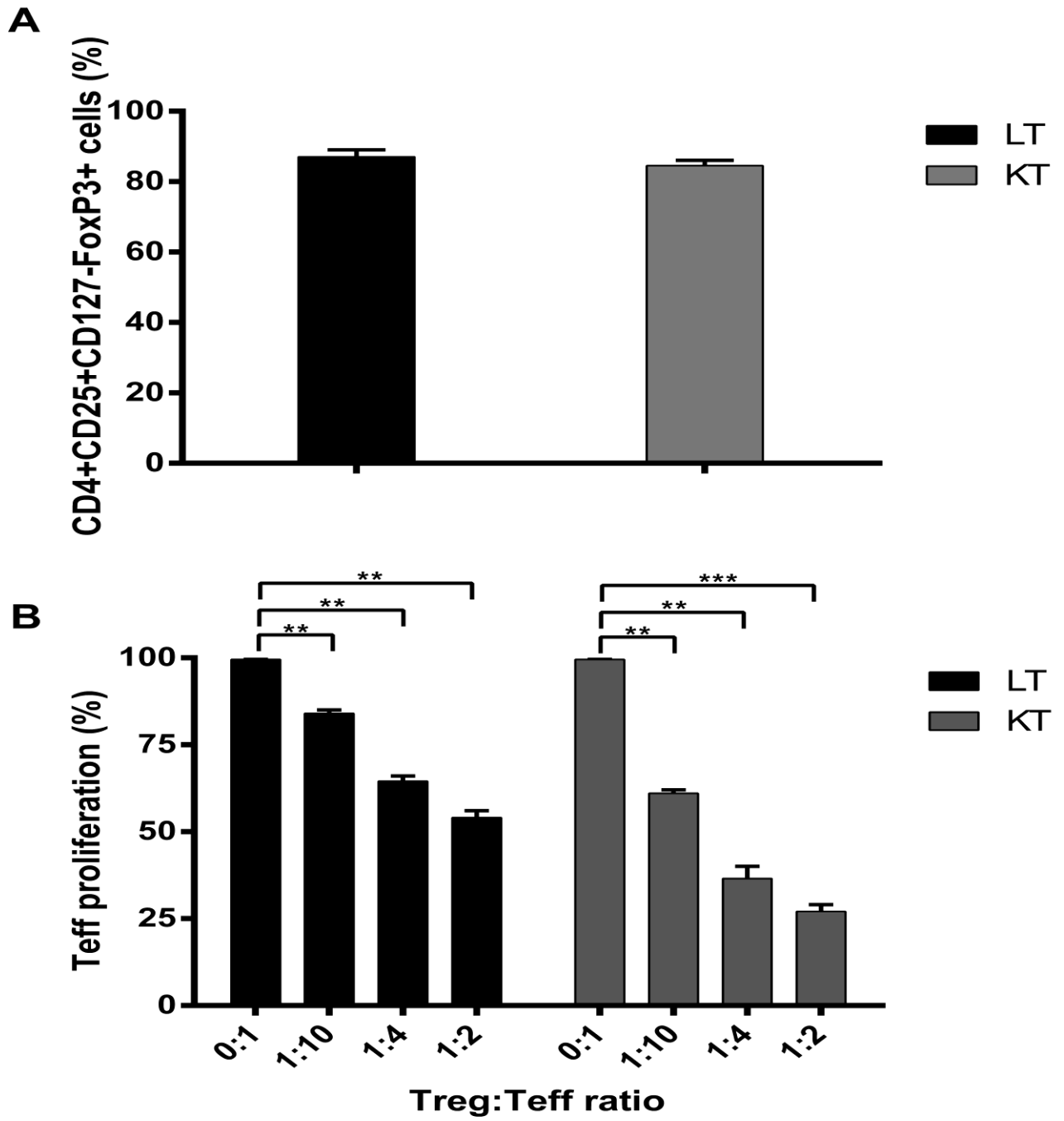


Figure 6

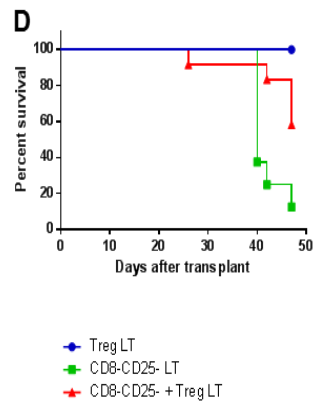
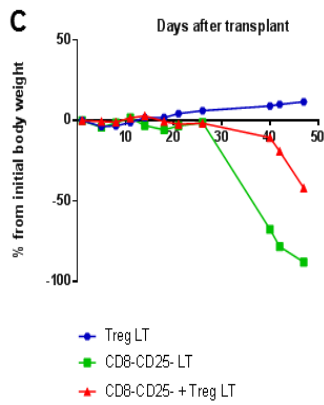
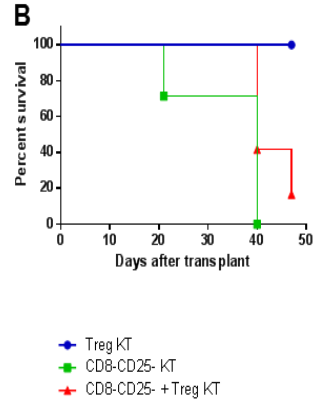
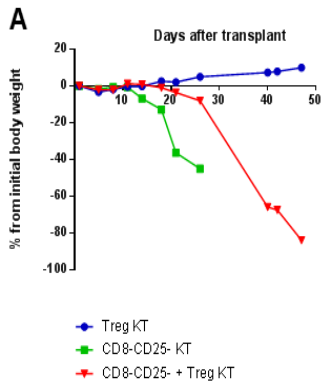


Figure 7

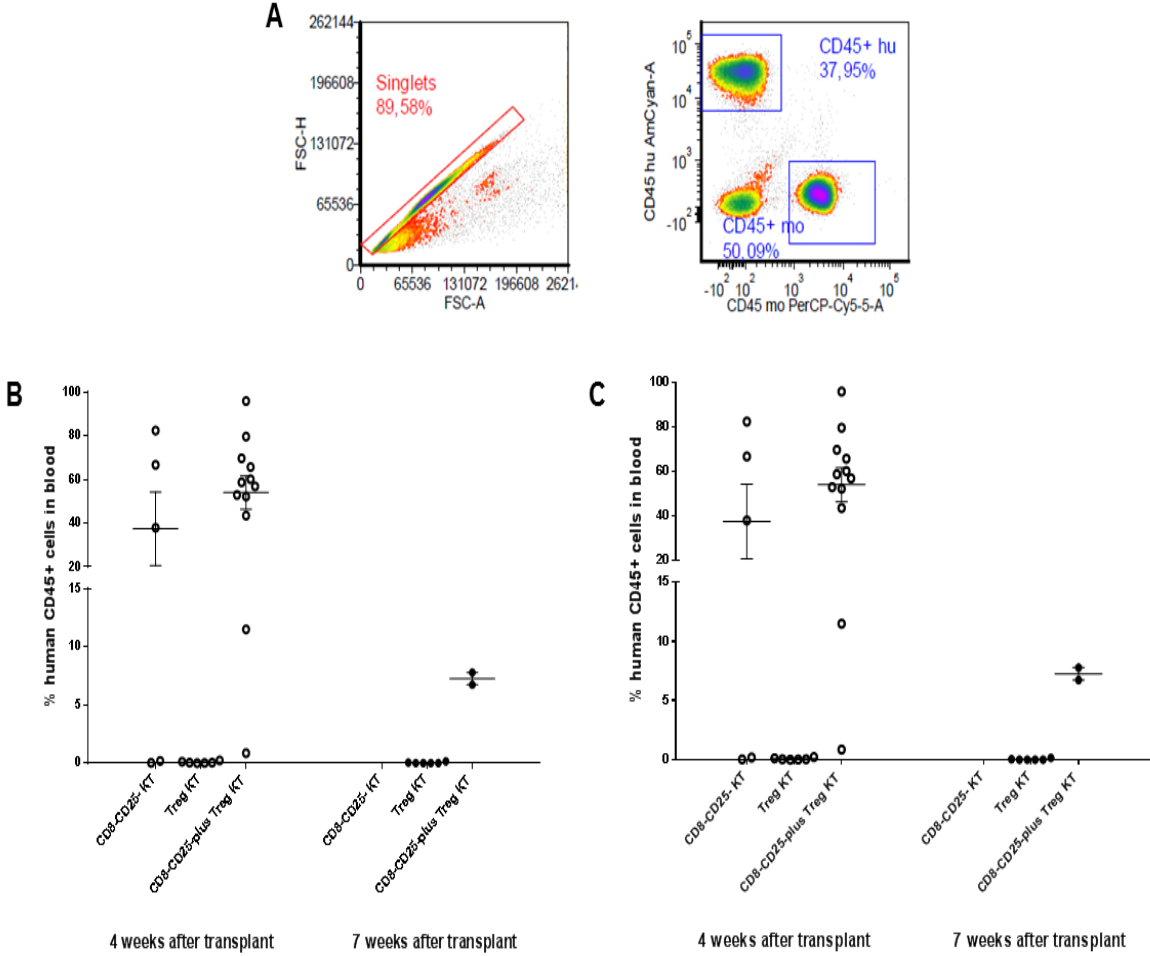
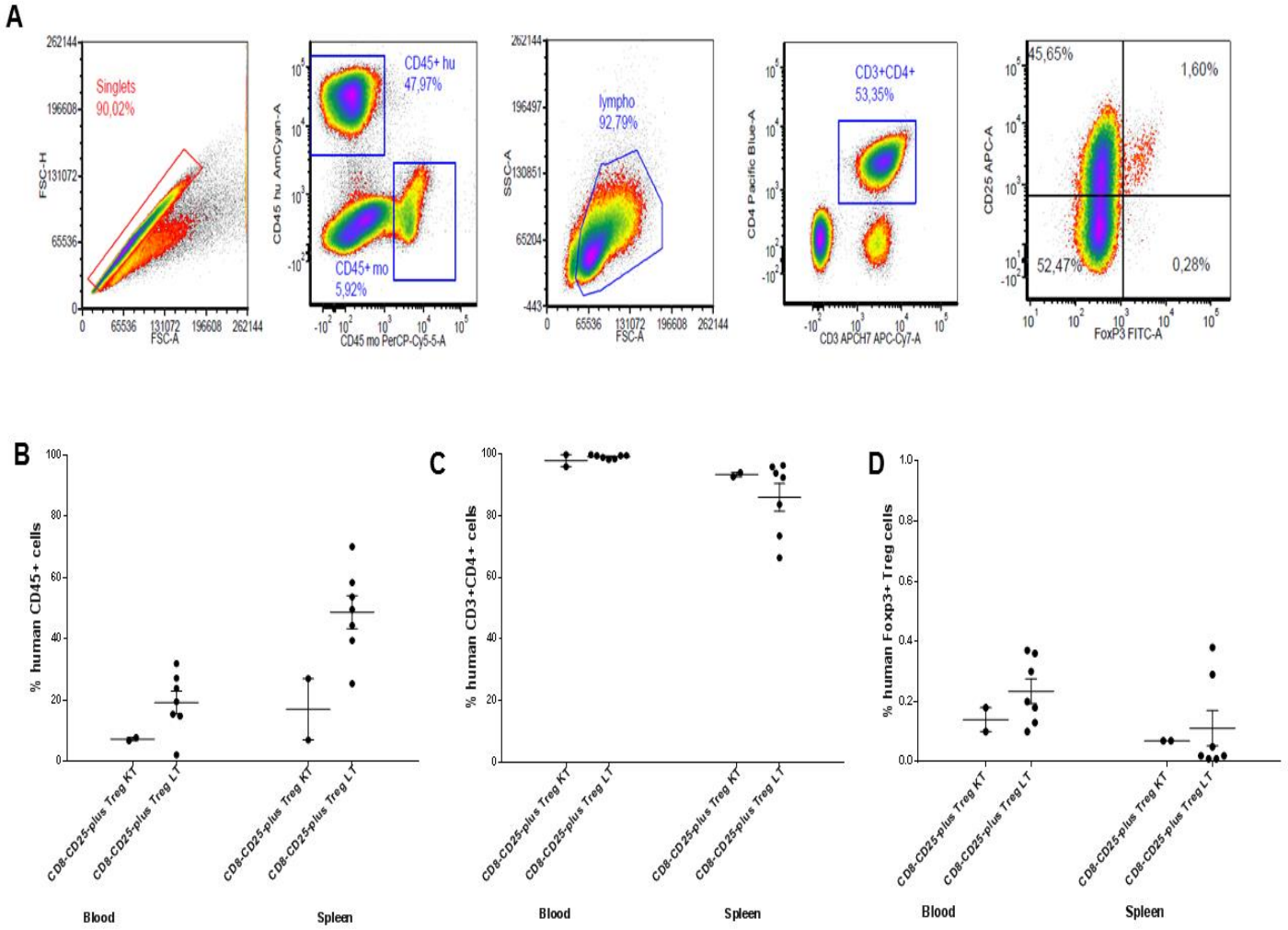


Figure 8





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Supplementary Material

Manuscript Ulbar F et al. Suppl. data.doc



Dear Editor,

please find enclosed our **revised** manuscript, entitled “**REGULATORY T CELLS FROM PATIENTS WITH END-STAGE ORGAN DISEASE CAN BE ISOLATED, EXPANDED AND CRYOPRESERVED ACCORDING GOOD MANUFACTURING PRACTICE IMPROVING THEIR FUNCTION**” (Ref. Number: JTRM-D-19-00358) by Ulbar et al.

We would like to thank the reviewers for the very helpful comments. We have now modified the manuscript following the suggestions provided. Changes to the manuscript are identified by colored text (red). Below please find included a point-by-point reply to the Reviewer’s comment .

Thank you for attention and consideration

Sincerely,

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