ORIGINAL ARTICLE

# Donor's graft *ex vivo* T-cell depletion with fludarabine reduces graft-versus-host disease signs and improves survival after intestinal transplantation – an experimental study

Maria Vela[1], Pablo Stringa[2], Pablo González-Navarro[1], Mariana Machuca[3], Bárbara Pascual-Miguel[1], Carmen Mestre[1], Nidia M. Arreola[4], Rodrigo Papa-Gobbi[4], Alfonso Navarro-Zapata[1], Sara C. Pires-Lobo[4,5], Ane M. Andrés[4,5], Francisco Hernández-Oliveros[4,5] & Antonio Pérez-Martínez[1,6]

1 Translational Research in Paediatric Oncology, Hematopoietic Transplantation & Cell Therapy, Hospital La Paz Institute for Health Research (IdiPAZ), Madrid, Spain
2 Institute for Immunological and Physiopathological Studies (IIFP-CONICET-UNLP), National University of La Plata, Buenos Aires, Argentine
3 Veterinary Pathology Laboratory, Veterinary Sciences Department, National University of La Plata, Buenos Aires, Argentine
4 Transplant Group – Experimental Surgery, Hospital La Paz Institute for Health Research (IdiPAZ), Madrid, Spain
5 Paediatric Surgery Department, La Paz University Hospital, Madrid, Spain
6 Paediatric Hemato-Oncology Department, La Paz University Hospital, Madrid, Spain

**Correspondence**
Antonio Pérez-Martínez MD, PhD, Pediatric Hemato-Oncology Department, La Paz University Hospital, Paseo de la Castellana, 261, Madrid 28046, Spain.
Tel.: +34917277223;
fax: +34917277042;
e-mail: aperezmartinez@salud.madrid.org

## SUMMARY

Intestinal passenger T leukocytes are responsible for graft-versus-host disease (GvHD) in intestinal transplantation (ITx). We hypothesized that *ex vivo* fludarabine treatment of the bowel graft would diminish the risk of GvHD and improve overall survival post-transplant. We performed isolated heterotopic small bowel transplantations from Lewis (LEW) to Brown Norway (BN) rat strains, which generated GvHD signs from the fourth day post-transplant. These symptoms included rash, weight loss, piloerection, and diarrhea. The grafts of one of the experimental groups were immersed and sealed in cold Celsior preservation solution with 1000 μM fludarabine for 1 h, prior to its implantation into recipient animals. No histological signs of intestinal tissue alterations were observed after fludarabine treatment. Fludarabine-treated bowel recipients showed significantly later and milder clinical signs of GvHD and reduced total donor cell chimerism, as determined by flow cytometry using strain-specific anti-HLA antibodies. Additionally, fludarabine treatment prolonged recipients' overall survival (13.5 days ± 0.3 days vs. 9.2 days ± 0.5). We conclude that active modification of the intestinal leukocyte composition is advantageous in our ITx animal model. Immunosuppression with fludarabine during the surgical procedure, which could be translated directly to the clinic, protects bowel recipients from GvHD and improves overall post-transplant survival.

*Transplant International* 2020; 33: 1302–1311

**Key words**
animal model, chimerism, fludarabine, graft-versus-host disease, intestinal passenger T leukocytes, intestine transplantation

Received: 4 March 2020; Revision requested: 31 March 2020; Accepted: 5 June 2020; Published online: 16 July 2020




doi:10.1111/tri.13672

## Introduction

The principal indications for intestinal transplantation (ITx), as published by the last Intestine Transplant Registry Report, are short gut syndrome [1]. Actuarial survival rates at 1 year are 76%, 56% at 5 years, and only 43% at 10 years. Rates of graft loss have not improved significantly in recent years. Sepsis remains the leading cause of graft loss accounting for over 50% of cases, followed by graft-related causes, including rejection and cardiovascular events. We have observed a significant incidence of graft-versus-host disease (GvHD) after pediatric ITx in our hospital. In accordance with other publications, we reported five cases of GvHD in a series of 52 pediatric ITx [2,3]. The symptoms included the typical GvHD generalized rash, together with diarrhea, respiratory symptoms, and/or severe hematologic disorders. Three of those five patients died within 4 months after the diagnosis. Thus, we conclude GvHD is a devastating complication of ITx, with high mortality associated with severe immunologic dysregulation.

Graft-versus-host disease occurs when immunocompetent donor lymphoid cells damage recipient tissues after allogeneic transplantation. The major targets are epithelial cells of the skin, intestine, and liver. With transplantation of allogenic bone marrow, under conditions of cytoablation, this severe complication can develop in 40–80% of recipients [4]. The occurrence of GvHD after whole organ transplantation has been less common. In ITx, however, the large inoculum of lymphoid cells in a small bowel graft is predicted to increase the likelihood of this disease.

We have developed an animal model to study potential strategies to reduce GvHD incidence after ITx [5–7] and performed isolated heterotopic ITx from Brown Norway (BN) rats into Lewis (LEW) rats, and monitored peripheral blood chimerism as well as GvHD and rejection signs in the recipient rats.

Fludarabine is a chemotherapy medication used for the treatment of leukemia and lymphoma [8] in the purine analog family of medications, which works by interfering with DNA replication. It is a commonly used anti-neoplastic agent with high cytotoxicity specifically against T cells. The drug is effective against both dividing and resting T cells. Our hypothesis was that *ex vivo* perfusion and cold storage of the bowel graft in fludarabine solution for 1 h before its implantation into recipient animals would reduce the number of CD8 T lymphocytes responsible for GvHD.

## Materials and methods

### Rat strains and care

Male LEW ($RT1^l$) and BN ($RT1^n$) rats were purchased from Charles River and were used as donors and recipients, respectively, for heterotopic ITx. Animals were cared for according to the guidelines of the Community of Madrid Animal Protection Area and Ethics Committee (PROEX 140/17).

### Fludarabine-induced apoptosis on Peyer's patch cell subpopulations

Peyer's patches of LEW rats were isolated and disaggregated using a 40-μm cell strainer (Falcon). A total of $2 \times 10^5$ cells were plated in 200 μl of Dulbecco's modified Eagle medium (DMEM; Lonza, Allendale, NJ, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and the indicated concentration of fludarabine (Aurovitas, Madrid, Spain), and cultured overnight at 37 °C, 95% humidity, 5% $CO_2$. The cells were then stained with anti-CD45-FITC (clone OX-1; BD Biosciences, San José, CA, USA), anti-CD4-APC (clone OX-35, eBioscience, San Diego, CA, USA), anti-CD8-PECy7 (clone OX-8; eBioscience), annexin V-PE (BD Biosciences), and 7-aminoactinomycin D (7AAD; BD Biosciences), and analyzed in a Navios flow cytometer (Beckman Coulter, Miami, FL, USA) and FlowJo v10.0.7 software (Tree Star, Ashland, OR, USA).

### Heterotopic intestinal transplantation

The heterotopic small bowel transplantation (Lew -> BN) procedure was performed as previously described [9]. However, we used no calcineurin inhibitors to be able to observe the GvHD signs during the first week post-transplant. During the second week, the main signs correspond to graft rejection. Briefly, the intestinal graft (composed of jejunum and ileum) was removed with the vascular pedicle consisting of the donor superior mesenteric artery and portal vein. In the recipient surgery, the engraftment was performed using two end-to-side vascular anastomoses (donor portal vein with recipient cava vein and donor superior mesenteric artery with recipient abdominal aorta). All procedures were performed under inhaled anesthesia (isoflurane 5% for induction and 2.5% for maintenance). For the fludarabine treatment, each intestine was washed intraluminally and intravascularly with Celsior solution with 1000 μM fludarabine. Subsequently, the grafts were immersed

within the fludarabine solution for 1 h (4 °C) prior to its implantation into the recipients.

### Graft-versus-host disease clinical score

The following parameters were monitored daily in the transplanted rats and were used to establish a GvHD clinical score: piloerection, ocular/nose secretion, antalgic posture, lethargic behavior, feces consistency, weight loss, loss of dorso-lumbar muscle mass. Skin rash and excessive pruritus were considered pathognomonic signs of GvHD. The specific score for each parameter is described in Table S1. Animals were sacrificed when a clinical score of 4 was achieved.

### Detection of chimerism by flow cytometry

We obtained peripheral blood from the recipient rats' tail vein (300 μl) at days 3, 7, and 14 post-transplant. For erythrocyte lysis, 2 ml of 0.22-μm-filtered ammonium chloride 0.85% weight/volume solution was added per 100 μl of blood and incubated for 10 min at room temperature (RT). After centrifugation (600 *g*, 10 min), the supernatant was discarded and cells were resuspended in phosphate-buffered saline (PBS, pH 7.4) with the following antibodies: CD3-PE (clone G4.18, BD Biosciences), CD4-APC (clone OX-35, eBioscience), CD8-PECy7 (clone OX-8, eBioscience), and RT1(a,b,l)-FITC (clone B5, BD Biosciences). After incubation (10 min, 4 °C), cells were acquired in a Navios flow cytometer (Beckman Coulter). FlowJo v10.0.7 software (Tree Star) was used for the data analysis.

### Cytokine determination in plasma

Rat blood samples were collected in ethylenediaminetetraacetic acid vacutainer tubes (BD Biosciences). After centrifugation (600 *g*, 10 min), plasma samples were stored at −80 °C until analysis. IL-2, TNF, and IFN-Ɣ were determined using a Flex Set kit (BD Biosciences). Resulting samples were acquired in a FACSCalibur cytometer (BD Biosciences) and analyzed using FCAP Array v.1.0.1 software (BD Biosciences). Calibration curves were used for quantification. All standards were measured in duplicate. Technical detection limits were as follows: 0.46 pg/ml (IL-2), 27.7 pg/ml (TNFα), and 6.8 pg/ml (IFN-γ).

### Hepatic enzyme activity determination

Plasma aspartate transaminase (AST), alanine transaminase (ALT), urea, and creatinine concentrations were determined spectrophotometrically using an automated analyzer (Dimension Vista 1500).

### Histology

Cut sections of 4 μm were prepared from formalin-fixed paraffin-embedded tissues. Representative sections were stained with hematoxylin–eosin. Histological analysis was performed by a trained pathologist unaware of the treatment protocols. Histological criteria followed for the grading of skin GvHD [10,11] and intestine allograft rejection [12,13] have been previously described.

### Statistical analysis

Comparisons between groups were made using Student's *t*-test. A two-sided value of $P < 0.05$ was considered statistically significant. All analyses were performed using GraphPad Prism (San Diego, CA, USA; version 7.0).

## Results

### Fludarabine treatment specifically eliminates T cells in the bowel graft and induces no appreciable damage

We first tested *in vitro* the effect of fludarabine on intestinal grafts. We analyzed the potential of fludarabine to kill T cells isolated from intestinal Peyer's patches of LEW rats. As shown in Fig. 1a, only high doses of fludarabine, above 100 μM, induce significant apoptosis. The fludarabine-induced apoptosis is specific to both CD4 and CD8 T cells, and maintains those cells that do not express CD3 almost as untreated cells. We further immersed LEW donor rat intestines in 100 and 1000 μM fludarabine solutions. After hematoxylin–eosin staining, we found no histological signs of intestinal tissue alterations in the treated organs (Fig. 1b).

### Mixed chimerism induced after heterotopic LEW -> BN intestinal transplantation

Using an antibody that recognizes rat MHC class I molecules specifically of a, b, or l haplotypes, we followed LEW cell chimerism in BN rats after an ITx. As shown in Fig. S1, this antibody binds specifically to LEW cells, whereas BN cells are negative for this haplotype-specific marker. We also observed, prior to transplantation, a higher percentage of $CD3^+$ cells in LEW peripheral blood than in BN (51% ± 12% vs. 18% ± 5%, respectively). The percentage of CD8 cells



Transplant International 2020; 33: 1302–1311

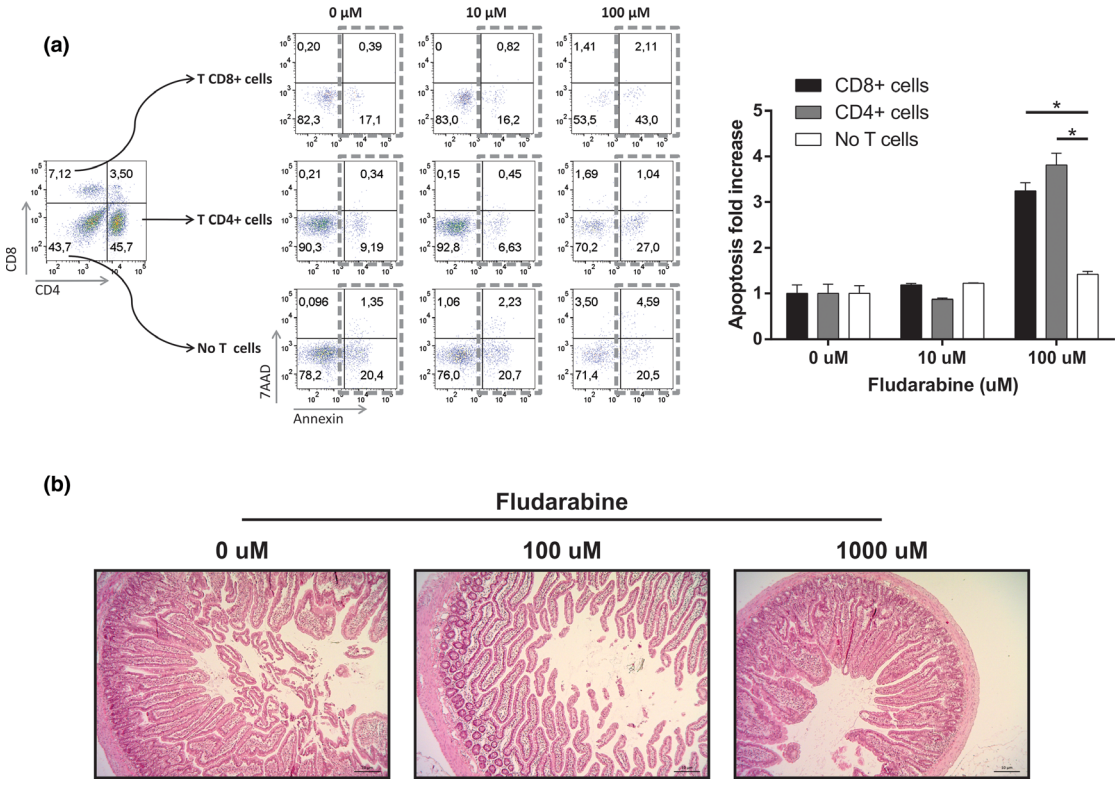


**Figure 1** Effect of fludarabine treatment on intestinal grafts. (a) Peyer's patches of LEW rats were isolated and cultured overnight with the indicated concentration of fludarabine. Using a flow cytometer, different cell subpopulations were identified and fludarabine-induced apoptosis was determined. $*P < 0.05$. $N = 3$. (b) Hematoxylin–eosin staining of Lewis rat intestine treated for 1 h with the indicated concentration of fludarabine. One representative image is shown, $N = 3$, 50× amplification.

among them is higher in the Lewis strain than in the BN strain (36% ± 12% vs. 9% ± 3%, respectively).

We performed seven heterotopic LEW -> BN heterotopic ITx using untreated intestines and monitored the donor cell chimerism. At day 3 postheterotopic ITx, LEW T cells constituted 7.6% ± 1.6% of peripheral blood cells in recipient rats and are almost exclusively constituted by CD4 cells. This chimerism diminishes along time, decreasing to 2.2% ± 1.2% at day 7. Although there were fewer donor cells, the percentage of donor CD8 cells, which are drivers of GvHD symptoms, increased at day 7 (Fig. 2a).

## Skin rash induced after heterotopic LEW -> BN intestinal transplantation

Consistent with the chimerism data, between days 6 and 8 post-transplantation, 33% of transplanted BN rats showed some kind of skin rash, including ears, eyes, snout, and abdomen or general pruritus. Figure 1b shows a rat that has developed representative rash symptoms.

## Bowel graft treatment with fludarabine reduces GvHD clinical signs and reduces T-cell chimerism after heterotopic LEW -> BN intestinal transplantation

We performed seven heterotopic LEW -> BN ITx using fludarabine-conditioned intestines and compared their clinical status to the untreated ITx group. The first group showed significantly later and milder clinical signs of GvHD. The score included rash, weight loss, piloerection, and diarrhea (Fig. 3a).

Fludarabine treatment also reduced the percentage of chimeric T cells at days 3 and 7 post-transplant, as well as the total number of donor cell chimerism at day 7. At endpoint, chimerism was close to zero in both groups (Fig. 3b).

## Inflammatory profile and hepatic function after fludarabine treatment of intestine grafts in heterotopic LEW -> BN transplants

We analyzed inflammatory cytokines IL-2, TNF, and IFN-gamma in plasma samples of the rats prior to the

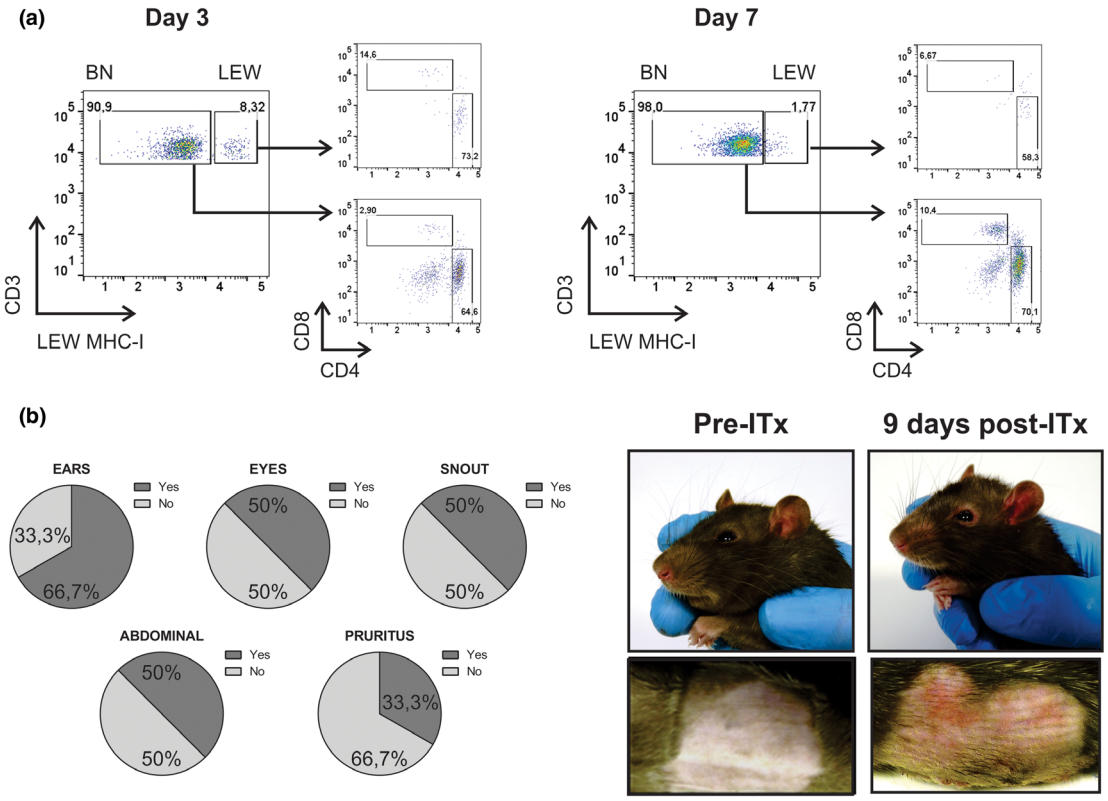


**Figure 2** Mixed chimerism and skin rash after heterotopic LEW -> BN intestinal transplantation. (a) Flow cytometry determination of LEW donor cells in peripheral blood of BN recipients 3 and 7 days postbowel transplant. One representative example is shown. (b) Skin rash and pruritus incidence 6–8 days postintestinal transplantation ($N$ = 7/group).

transplant and 7 days afterward. We observed an increase in all three inflammatory cytokines after heterotopic transplant. Fludarabine graft treatment induced a trend—although statistically not significant—toward a lower inflammatory profile in comparison with untreated graft recipients (Fig. 3c).

Alterations in hepatic function of the transplanted rats were also monitored. We measured AST, ALT, urea, and creatinine levels in rat serum samples. Again, the transplant increased all the biochemical parameters. In this case, the treatment with fludarabine produced no alterations in those levels compared with the untreated graft recipient group (Fig. 3c).

### Bowel graft treatment with fludarabine extends post-transplant survival after heterotopic LEW -> BN intestinal transplantation

The effect of the fludarabine graft treatment on the rats' post-transplant survival is shown in Fig. 4a. Untreated bowel graft recipients died at a median day 9.2 days, whereas the fludarabine-treated graft recipients showed prolonged survival, a median of 13.5 days.

In the histopathological analysis, fludarabine treatment clearly reduced GvHD signs, such as intercellular edema (spongiosis) and separation of dermoepidermal junction (cleft), in recipients' skin samples (Fig. 4b). Although not significant due to a small sample size on each group, we also observed a trend toward reduced damage in the native intestines of the host, which presented also GvHD signs such as edema and mononuclear cell infiltrates (Fig. 4c).

At endpoint, both groups showed similar levels of graft rejection as shown in Fig. 5a. Both untreated and fludarabine-treated organs showed macroscopic (Fig. 5b) and microscopic (Fig. 5c,d) signs of rejection.

## Discussion

Small bowel transplantation is the only life-saving option for patients with intestinal failure who fail total parenteral nutrition or who develop irreversible liver failure [14]. Over the last decade, advances in surgical techniques, immunosuppression protocols, and infection control have substantially improved the benefits of ITx. However, long-term outcomes are not comparable with



Transplant International 2020; 33: 1302–1311

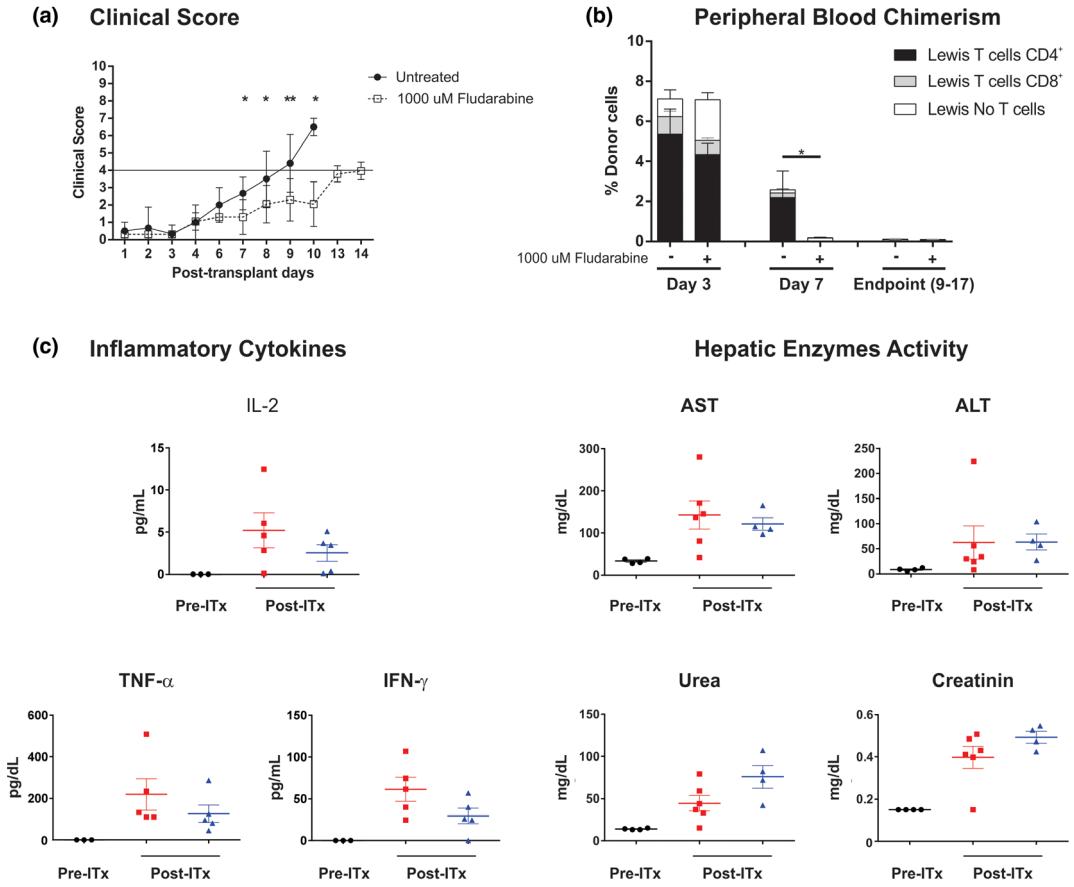


**Figure 3** Clinical signs of GvHD and mixed chimerism after fludarabine treatment of intestinal grafts in heterotopic LEW -> BN transplants. (a) Clinical score of GvHD signs in untreated versus fludarabine-treated (1 h; 1000 μM) recipients ($N$ = 7/group). (b) Peripheral blood donor chimerism in untreated and fludarabine-treated (1 h; 1000 μM) recipients. Blood samples were collected at the indicated timepoints and analyzed by flow cytometry using an antibody specific to MHC class I from LEW strain. (c) Analysis of inflammatory cytokines and hepatic enzyme activity. Rat serum samples were collected prior to ITx (black) and 7 days post-ITx. Animals received an untreated graft (red) or fludarabine-treated grafts (blue). $^{**}P < 0.01$, $^{*}P < 0.05$.

those of other organ transplants due to intestinal allogeneic immune load [15]. Immunosuppressive regimens grant acceptable short-term outcomes, but long-term administration of immunosuppressive agents increases the risk of unfavorable side effects, including hypertension, nephro- and neurotoxicity, post-transplant neoplasias, and metabolic disorders [16]. Thus, new immunosuppressive approaches and strategies to reduce GvHD-derived complications are warranted.

The fludarabine-based approach selectively eliminates T cells present in the intestinal graft that are responsible for the GvHD observed in ITx recipients. Our data show a clear reduction in CD8 lymphocyte chimerism to zero in peripheral blood at day 7 post-transplant. Our strategy is simple and limited in cost, which facilitates its direct translation to the clinic. Our graft immunosuppression technique is performed *ex vivo*, which requires less immunosuppression in the recipient. The feasibility of combined intravascular and intraluminal perfusion is a potential advantage of the intestinal graft, although this approach is not generalized [17]. The beneficial effect to other solid organs without this double perfusion possibility should be considered in future research.

Failure of the chimeric and recipient immunocytes to reach an immunological 'truce' can lead to either rejection of the transplanted whole organ, or to GvHD, and sometimes to both simultaneously [18]. This finding has been particularly well studied after ITx between certain rat strain combinations such as LEW and BN. Murase *et al.* [5–7] had described the ease with which BN (RT1n) multivisceral and intestinal grafts could be transplanted to LEW (RT1l) recipients, as well as the difficulty in controlling intestinal rejection in the opposite strain direction. In our model, with no post-transplant immunosuppression, we observe GvHD signs during the first 7 days, followed during the second week

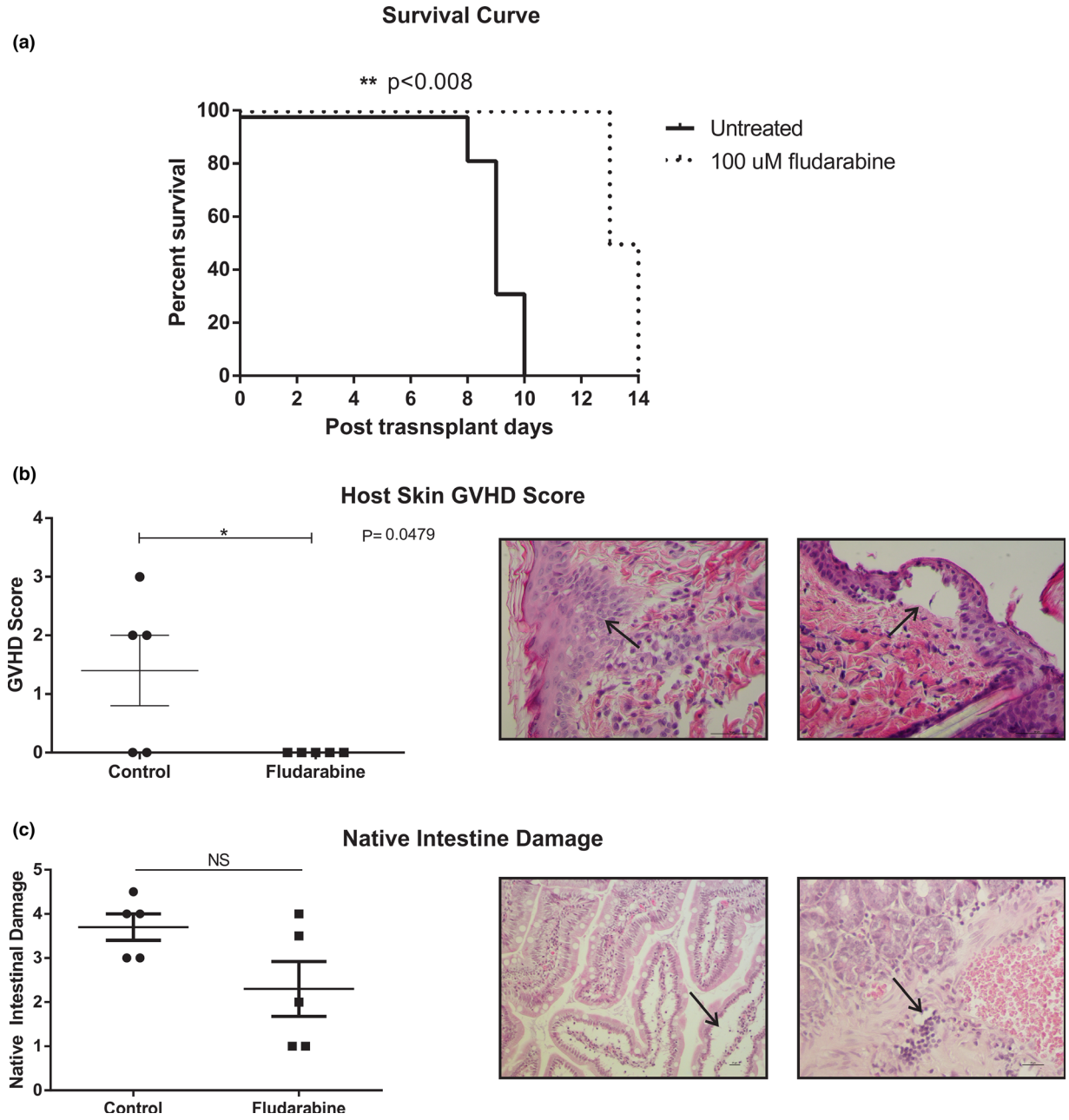


**Figure 4** Survival and histological analysis after fludarabine treatment of intestinal grafts in heterotopic LEW -> BN transplants. (a) Post-transplant survival curve of intestinal graft recipient rats ($N$ = 7/group). (b) Histopathological analysis of host skin at endpoint. Representative images of spongiosis (left) and cleft (right) found in untreated graft recipient skin (40× magnification). (c) Histopathological analysis of native intestines at endpoint where moderate edema (left) and mononuclear cell infiltrates (right) were found, especially in untreated graft recipients (40× magnification). *$P \leq 0.05$, **$P \leq 0.01$.

of rejection signs that lead to reaching of endpoint criteria. We have observed that pretransplant LEW rats have significantly more T lymphocytes and a lower CD4:CD8 ratio than BN rats (Fig. S1). Given CD8 lymphocytes are known triggers of GvHD, this difference could explain the disparity of rejection in LEW -> BN compared with BN -> LEW transplantation [19,20].

The LEW/BN rat strain combination used in our experiments has unusual advantages for the assessment of post-transplant chimerism. The distinction between donor and recipient leukocytes can be made precisely due to the availability of the B5 antibody, which reacts with epitope S of the rat MHC class I antigen, haplotypes RT1[a], RT1[b], and RT1[l] from



Transplant International 2020; 33: 1302–1311

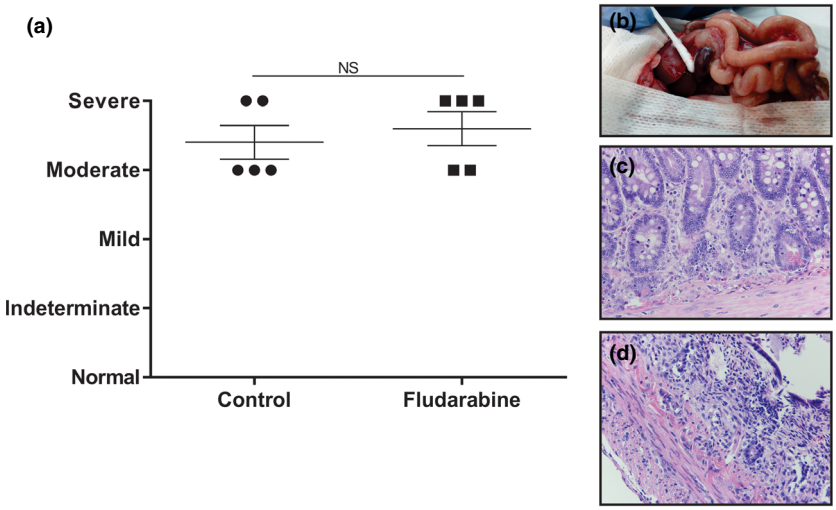


**Figure 5** Analysis of transplanted intestines at endpoint. (a) The histopathological study of the grafts at endpoint showed moderate/severe signs of acute rejection in both experimental groups. (b) Representative macroscopic appearance of the transplanted small bowel at endpoint compatible with graft rejection. (c, d) Hematoxylin–eosin staining of grafts at endpoint showing moderate (c) and severe (d) signs of acute cellular rejection.

most rat strains including LEW, but not those of BN [21].

In the clinic, the interpretation of post-transplant chimerism results remains difficult due to the poor correlation between chimerism values and clinical outcomes. The Pittsburgh group reported the chimerism rate of 44 adults and children after ITx [22]. Those who developed GvHD, which appeared at a median onset of 1.2 months (range 2–96) post-transplant, had a median chimerism of 7.9% (range 0–35.9%), whereas unaffected individuals had a median chimerism of 0.2% (range 0–16%).

According to our data from the animal model, at day 3 post-ITx, LEW T cells constituted 7.6% ± 1.6% of peripheral blood cells in recipient rats, and the percentage of donor CD8 cells, which are drivers of GvHD symptoms, increased over time (Fig. 2a). Fludarabine treatment reduced the percentage of chimeric T cells post-transplant (Fig. 3b). Murase *et al.* [5] had performed LEW -> BN transplantation of various whole organs to address the tolerogenicity of the original primary grafts: Liver was the most tolerogenic and the heart least. Both the GvHD propensity and tolerogenicity in these experiments were closely associated with recipient tissue chimerism 30 and 100 days after the experiments began. The tissue chimerism was invariably multilineage, but the GvHD outcome was associated with T cell over-representation.

A hallmark of GvHD is the development of a so-called 'cytokine storm by graft immunocompetent cells when exposed to host antigens absent in the donor, including IL-2, IL-6, TNF-α, and IFN-γ secretion [3]. Our results confirm an exacerbated immune response post-ITx, with an increased detection of the inflammatory cytokines IL-2, TNF-α, and IFN-γ that is partially averted with the fludarabine pretreatment of the graft (Fig. 3c).

Further confirmation of the GvHD signs can be obtained through a skin biopsy showing lymphocytic infiltrates of the epithelium, apoptotic bodies, lymphocytic exocytosis, and cellular vacuolization similar to that found in GvHD after hematopoietic stem cell transplantation [2]. However, there are no characteristic histological features of GvHD found with a skin biopsy [23]. In our untreated ITx recipients, we observed skin spongiosis and clefts in histological sections (Fig. 4b).

As mentioned, passenger leukocytes of the liver and bone marrow cells, both of which include large numbers of immature leukocytes and cells of myeloid origin, are more tolerogenic and have a lower risk of GvHD than the intestinal passenger leukocytes, which are rich in mature lymphocytes [7]. Elimination of mature lymphoid elements from the bowel allografts by *ex vivo* irradiation is known to eliminate GvHD risk [7,24,25]. These studies have demonstrated that active modification of the intestinal leukocyte composition is advantageous. However, the authors recognize that a further search for methods to achieve this modification other than with *ex vivo* irradiation should be considered, given the possibility of radiation-associated vasculopathy

in the graft. This objective was also met by pretreating the donor with antilymphocyte serum [26], but only when donor animals were pretreated for 48 h before harvesting the intestinal grafts. *Ex vivo* graft infusion with antilymphocyte serum failed to prevent GvHD [27]. This strategy, thus, is difficult to translate into the clinical setting, in which pretreatment of the donor with antilymphocyte serum or similar mAbs for 48 h usually is not possible. In this scenario, our study offers a realistic clinical strategy to reduce the host-reactive passenger leukocytes found in the graft, which might improve organ transplantation.

## Authorship

FH-O and AP-M: proposed research idea and designed the study. MV, PS, PG-N, BP, CM, NMA, RP-G, AN-Z and SCP-L: acquired the data. FH-O, AP-M, MV, PS, MM and AMA: analyzed and interpreted the data. FH-O, AP-M, MV and PS: involved in original draft preparation. AP-M and FH-O: acquired the funding. All authors reviewed the manuscript.

## Funding

This work was supported in part by Hospital La Paz Institute for Health Research (IdiPAZ) (TRASCHILD project), by NUPA (Patients association for parenteral nutrition and intestinal transplantation) grant to Montserrat Arreola, CRIS CANCER Foundation grant to Barbara Pascual-Miguel and by Mutua Madrileña Foundation grant to Francisco Hernández Oliveros.

## Conflicts of interest

The authors of this manuscript have no conflicts of interest to disclose.

## Acknowledgements

This work was supported in part by Hospital La Paz Institute for Health Research (IdiPAZ) (TRASCHILD project) and by Mutua Madrileña Foundation grant to Francisco Hernández Oliveros.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Graft versus host disease score.

**Figure S1.** Flow cytometry analysis of PBMCs from LEW and BN rats. Staining using anti MHC RT1 [a,b,l], anti CD3, anti CD4, and anti CD8 mAbs in LEW and BN peripheral blood cells. A FS/SS plot is shown where the lymphoid population is gated. The CD3 population within that gate is further selected, and the percentage of CD4 + and CD8 + cells within the MHC RT1 [a,b,l]+ gate (LEW cells) and MHC RT1 [a,b,l]- (BN cells) is indicated.

## REFERENCES

1. Grant D, Abu-Elmagd K, Mazariegos G, *et al*. Intestinal transplant registry report: global activity and trends. *Am J Transplant* 2015; **15**: 210.
2. Andres AM, Santamaría ML, Ramos E, *et al*. Graft-vs-host disease after small bowel transplantation in children. *J Pediatr Surg* 2010; **45**: 330.
3. Green T, Hind J. Graft-versus-host disease in paediatric solid organ transplantation: a review of the literature. *Pediatr Transplant* 2016; **20**: 607.
4. Strong Rodrigues K, Oliveira-Ribeiro C, de Abreu Fiuza Gomes S, Knobler R. Cutaneous graft-versus-host disease: diagnosis and treatment. *Am J Clin Dermatol* 2018; **19**: 33.
5. Murase N, Starzl TE, Tanabe M, *et al*. Variable chimerism, graft - versus – host disease, and tolerance after different kinds of cell and whole organ transplantation from lewis to brown Norway rats. *Transplantation* 1995; **60**: 158.
6. Murase N, ÜEmetris AJ, Woo J, *et al*. Graft-versus-host disease after brown Norway-to-lewis and lewis-to-brown Norway rat intestinal transplantation under FK506. *Transplantation* 1993; **55**: 1.
7. Murase N, Ye Q, Nalesnik MA, *et al*. Immunomodulation for Intestinal. *Transplantation* 2010; **70**: 1632.
8. Gamberale R, Galmarini CM, Fernández-Calotti P, *et al*. In vitro susceptibility of CD4+ and CD8+ T cell subsets to fludarabine. *Biochem Pharmacol* 2003; **66**: 2185.
9. Stringa P, Romanin D, Lausada N, *et al*. Gut permeability and glucose absorption are affected at early stages of graft rejection in a small bowel transplant rat model. *Transplant Direct* 2017; **3**: e220.
10. Pintar T, Zorc-Pleskovič R, Alessiani M, Milutinović A, Pleskovič A. Prognostic value of skin histology in GVHD after intestinal transplantation. *Eur J Pediatr Surg* 2007; **17**: 412.
11. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JLM. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood* 1997; **90**: 3204.
12. Wu T, Abu-Elmagd K, Bond G, *et al*. A schema for histologic grading of small intestine allograft acute rejection. *Transplantation* 2003; **75**: 1241.
13. He G, Hart J, Thistlethwaite JR, *et al*. Inhibition of intestinal allograft rejection by an anti-CD8 monoclonal

antibody is not mediated by depletion alone. *Transplant Proc* 1998; **30**: 1069.
14. Yang Y, Song HL, Zhang W, *et al*. Reduction of acute rejection by bone marrow Mesenchymal stem cells during rat small bowel transplantation. *PLoS One* 2014; **9**: e114528.
15. Bharadwaj S, Tandon P, Gohel TD, *et al*. Current status of intestinal and multivisceral transplantation. *Gastroenterol Rep* 2017; **5**: 20.
16. Pondrom S. The AJT Report. Survival rates increase in bowel transplantation: seldom-done procedure comes of age. *Am J Transplant* 2010; **10**: 438.
17. Oltean M, Joshi M, Herlenius G, Olausson M. Improved intestinal preservation using an intraluminal macromolecular solution: evidence from a rat model. *Transplantation* 2010; **89**: 285.
18. Starzl TE, Demetris AJ, Trucco M, *et al*. Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. *Hepatology* 1993; **17**: 1127.
19. Fournié GJ, Cautain B, Xystrakis E, *et al*. Cellular and genetic factors involved in the difference between brown Norway and Lewis rats to develop respectively type-2 and type-1 immune-mediated diseases. *Immunol Rev* 2001; **184**: 145.
20. Nimer SD, Giorgi J, Gajewski JL, *et al*. Selective depletion of CD8+ cells for prevention of graft-versus-host disease after bone marrow transplantation: a randomized controlled trial. *Transplantation* 1994; **57**: 82.
21. Butcher GW. A list of monoclonal antibodies specific for alloantigens of the rat. *Int J Immunogenet* 1987; **14**: 163.
22. Mazariegos GV, Abu-Elmagd K, Jaffe R, *et al*. Graft versus host disease in intestinal transplantation. *Am J Transplant* 2004; **4**: 1459.
23. Feito-Rodríguez M, De Lucas-Laguna R, Gõmez-Fernández C, *et al*. Cutaneous graft versus host disease in pediatric multivisceral transplantation. *Pediatr Dermatol* 2013; **30**: 335.
24. Foster PF, Sankary HN, Kociss K, Xiao F, Williams JW. The interaction of gamma irradiation of the allograft and recipient administration of cyclosporine in rat small bowel transplantation. *Transplant Proc* 1992; **24**: 1175.
25. Grant D, Duff J, Zhong R, Mimeault R, Inch R, Stiller C. Effect of ex vivo allograft irradiation combined with cyclosporine therapy in a pig intestinal transplant model. *Transplant Proc* 1989; **21**: 2879.
26. Shaffer D, Ubhi CS, Simpson MA, *et al*. Preventionof graft-versus-hostdisease following small bowel transplantation with polyclonal and monoclonal antilymphocyte serum. *Transplantation* 1991; **52**: 948.
27. Shaffer D, Maki T, Demichele SJ, *et al*. Studies in small bowel transplantation: prevention of graft-versus-host disease with preservation of allograft function by donor pretreatment with antilymphocyte serum. *Transplantation* 1988; **45**: 262.