Article type : Brief Communication

Paneth and Intestinal Stem Cells Preserve their Functional Integrity during Worsening of Acute Cellular Rejection in Small Bowel Transplantation

M. Pucci Molineris[1,2], V. Gonzalez Polo[1,2], Federico Perez[3], D. Ramisch[2], M. Rumbo[3], G. E. Gondolesi[1,2] and D. Meier[1,2]*

[1]Laboratorio de Investigación Traslacional e Inmunología Asociada al Trasplante, Instituto de Medicina Traslacional, Trasplante y Bioingeniería (IMeTTyB), Universidad Favaloro-CONICET, Buenos Aires, Argentina

[2]Instituto de Trasplante Multiórganico, Hospital Universitario Fundación Favaloro, Buenos Aires, Argentina

[3]Instituto de Estudios Inmunológicos y Fisiopatológicos, UNLP-CONICET, La Plata, Argentina

*Corresponding author:

Dominik Meier, dommeier@gmail.com

Abbreviations: ACR, acute cellular rejection; BZ, bottom zone; CASP3, caspase 3; DEFA5, defensin alpha 5; GvHD, graft versus host disease; ILC3, innate lymphoid cell subpopulation 3; IL22R, interleukin 22 receptor; ISC, intestinal stem cells; ITx, intestinal transplantation; MR, mild ACR; MoR, moderate ACR; ROS, reactive oxygen species; SR, severe ACR; TAC, tacrolimus; TZ, transit-amplifying zone

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ajt.14592

Abstract

Graft survival after small bowel transplantation remains impaired due to acute cellular rejection (ACR), the leading cause of graft loss. Although it was shown that the number of enteroendocrine progenitor cells in intestinal crypts was reduced during mild ACR, no results of Paneth and intestinal stem cells localized at the crypt bottom have been shown so far. Therefore, we wanted to elucidate integrity and functionality of the Paneth and stem cells during different degrees of ACR, and to assess whether these cells are the primary targets of the rejection process. We compared biopsies from ITx patients with no, mild or moderate ACR by immunohistochemistry and quantitative PCR. Our results show that numbers of Paneth and stem cells remain constant in all study groups, whereas the transit-amplifying zone is the most impaired zone during ACR. We detected an unchanged level of antimicrobial peptides in Paneth cells and similar numbers of Ki-67$^+$ IL-22R$^+$ stem cells revealing cell functionality in moderate ACR samples. We conclude that Paneth and stem cells are not primary target cells during ACR. IL-22R$^+$ Ki-67$^+$ stem cells might be an interesting target cell population for protection and regeneration of the epithelial monolayer during/after a severe ACR in ITx patients.

Introduction

Intestinal transplantation (ITx) is the ultimate medical alternative for patients with life-threatening intestinal failure and complications of parenteral nutrition (1,2). Although the incidence of acute cellular rejection (ACR) has decreased in recent years with new immunosuppressive protocols, ACR is still the main cause of graft loss or the recipient's death (3,4). To diagnose ACR in the allograft of ITx patients, a well-accepted grading system has been developed (5,6). The histological criteria for mild ACR is: villus blunting and architectural distortion, mixed but primarily mononuclear inflammatory population including activated lymphocytes, eosinophils, and occasional neutrophils. For moderate ACR: crypt injury at a greater level, mixed but primarily mononuclear inflammatory population including activated lymphocytes, edema, vascular congestion, and villus blunting. For severe ACR:

marked degree of crypt damage, presence of ulceration, marked inflammatory infiltrate with activated lymphocytes, eosinophils, and neutrophils (6). One important feature of this grading schema is the count of apoptotic bodies in crypt epithelium. The crypts of Lieberkühn are composed of two main parts, the transit-amplifying zone (TZ) harboring cells that are in active proliferation until their cell specific differentiation, and the bottom zone (BZ) at the crypt base containing intestinal stem cells (ISC) and Paneth cells (7). Intestinal stem cells are responsible for the constant regeneration of the epithelial layer and reconstitution when injured (8), whereas Paneth cells express their function mainly in producing anti-microbial peptides (9,10). The importance of Paneth cells regulating human gut microflora has been reported in several studies on patients with Crohn's disease (11-13). In these studies, a decrease of defensin alpha 5 (DEFA5) expression resulted in a gut microbiota imbalance generating further inflammatory responses.

Interestingly, it has been shown in mouse models of graft versus host disease (GvHD) that the ISC and Paneth cells are target cells and do not recover during GvHD pathogenesis (14-16). The study by Hanash et al. (15) highlights a protective role of IL-22 for ISC during the intestinal inflammatory damage provoked by GvHD mechanisms. The importance of the IL-22/ IL-22 receptor axis for ISC has further been revealed in a study of patients with GvHD after hematopoietic stem cell transplantation (17). The authors analyzed the IL-22-producing innate lymphoid cell subpopulation 3 (ILC3) in the peripheral blood of these patients and found correlation between a higher number of activated ILC3 and a better protection from GvHD. A fundamental role of IL-22 for the expansion and proliferation of IL-22R positive ISC has also been shown in experiments with mice and human intestinal *ex vivo* organoids (18). So far, no studies about ISC and the IL-22R have been conducted in the ITx field. Fishbein et al. (19) contributed with a study showing diminished number of endocrine cells and their progenitors in crypts of ITx patients with mild ACR. We wanted to go a step further and studied, which are the primary target cells and whether number and functionality of Paneth cells and ISC are affected during worsening of the ACR process.

Materials and Methods

Patients

The present protocol was approved by the Institutional Review Board of Hospital Universitario Fundacion Favaloro (DDI (1125) 511). Patients with intestinal transplantation from 2006 to 2016 were enrolled in this study. Details of ITx surgical procedures, immunosuppressive therapy, and patients' follow-up care were previously reported (20-22). Detailed patients' data are shown in Table 1. Six to 8 biopsies were obtained from the distal ileum and divided randomly, 3-5 biopsies for histological diagnosis and 2-3 biopsies for qPCR analysis. Our study groups were: No rejection (N; without any signs of histopathological and significant clinical symptoms 72 h before endoscopy, such as abdominal pain, self-limited diarrhea/increased ostomy output, respiratory symptoms or episodes of isolated fever), mild ACR (MR; with histological evidence of mild ACR), moderate ACR (MoR), and severe ACR (SR). Our inclusion criteria for N, MR, and MoR biopsies of this retrospective study were: 1. Biopsies unequivocally diagnosed by an experienced pathologist in accordance with the recommendations of the pathology workshop of the VIII Small Bowel Transplantation Symposium (6); 2. At least two previous biopsies and two months without histopathological and clinical events; 3. First appearance of MR and MoR events; 4. Patients without any additional treatment. Biopsies with SR (with ulceration) were only selected based on histopathological diagnosis, as these biopsies were not fulfilling the other stringent inclusion criteria. For gene expression analysis, we included ileum biopsies from healthy volunteers (control group, C), which were endoscopically evaluated for colon cancer, for sample normalization considering changes associated with the complex immunological status of the graft. We did not detect differences between pediatrics and adults; therefore, we pooled both age groups in our analyses.

Immunohistochemistry

Paraffin-embedded intestinal biopsies were used. Tissue sections were deparaffinized following standard procedures as previously described (23). Antibodies enrolled in this study are listed in Table 2.

Procedure of cell counting in small bowel tissue biopsies

The number of Paneth cells, IL22R$^+$ ISC, Ki67$^+$ ISC and apoptotic bodies were determined by examination of immunostained whole tissue specimens from 3-5 biopsies/patient. After the staining procedure, a research group member took pictures of all crypts in the slide and then another scientist, who was unaware of the histopathological conditions of the crypts, performed cell counting in a blind manner. Crypts were divided in two zones. The zone with DEFA5$^+$ Paneth cells was defined as the bottom zone (BZ), whereas the zone localized upstream as transit-amplifying zone (TZ) (Figure S1). Numbers of apoptotic bodies per zone/10 crypts were recorded. We defined IL22R$^+$ or Ki67$^+$ cells, which were interspersed between DEFA5$^+$ Paneth cells at the BZ, as ISC. Numbers of ISC/ 10 Paneth cell$^+$ crypts were determined.

Gene expression analysis

The samples were taken by video endoscopy, immediately embedded in RNA later (Ambion, Austin, USA) and stored at -80°C for total RNA extraction. RNA extraction, cDNA synthesis and qPCR procedure were conducted as previously described (23). Evaluated genes and the corresponding primer pairs are listed in Table 2.

Statistical analysis

Comparisons among groups of data were performed with the Kruskal-Wallis test and the Dunn`s post-test when more than two groups were analyzed, whereas the Mann-Whitney U test was chosen to compare data from two groups. To evaluate the two zones of crypts from the same sample, a paired t test was used. All the statistical analyses were performed using

GraphPad Prism 5.01 software (San Diego, USA). Qualitative variables were analyzed with Chi-square test using IBM SPSS Statistics v23 software.

Results

Paneth cells are intact during episodes of acute cellular rejection in human allograft tissue

To evaluate in the biopsy section whether Paneth cells and ISC are affected by apoptosis or losing their functionality, we first compared the number of $DEFA5^+$ cells by immunohistochemistry between no-rejection, mild, moderate, and severe ACR. We detected $DEFA5^+$ cells in all study groups (Figure 1A-D). The mean number of Paneth cells was minimally lower in MoR and SR than in MR samples. However, this difference was not statistically significant (Figure 1E). The dispersion in the SR group was due to 3 samples with severe exfoliative diagnosis and these samples revealed a reduced number of crypts and Paneth cells/ crypts. In addition, we show here that gene expression levels of two antimicrobial peptides DEFA5 and LYZ in MR and MoR samples are on a comparable level to healthy control samples (Figure 1F). Moreover, the *DEFA5* expression level is slightly higher in MoR samples than in MR samples, whereas no significant difference of *LYZ* expression between both groups was detected.

Transit-amplifying zone is more affected by apoptosis than the bottom of the crypts

In traditional diagnostic method, apoptotic bodies are counted in slides with hematoxylin and eosin staining without distinguishing cell type or location in the crypts to indicate the grade of ACR (Figure 2A-C). We used an anti-CASP3 antibody as an apoptotic marker to determine which crypt zone apoptosis is predominant in and whether $DEFA5^+$ Paneth cells co-express this marker. As we did not detect a reduction of $DEFA5^+$ Paneth cells in our study groups, we used $DEFA5^+$ Paneth cells to distinguish the BZ from TZ (Figure S1). Surprisingly, we could only determine $CASP3^+$ apoptotic bodies and not $CASP3^+$ intact cells (Figure 2E,F).

As we expected, we did not detect CASP3$^+$ Paneth cells either in MR or in MoR samples. The mean number of CASP3$^+$ apoptotic bodies in the BZ is slightly higher in MoR group than in MR and N groups, whereas, we detected a statistically significant difference of the mean number in the TZ from MoR samples than N samples (Figure 2G). In addition, the mean number of CASP3$^+$ apoptotic bodies was significantly higher in the TZ than in the BZ from MoR samples (Figure 2G).

Intestinal stem cells are not affected during early stages of acute cellular rejection

Next, we wanted to determine the status of ISC during the ACR process. As ISC express the IL-22 receptor (IL22R), we used an anti-IL22R antibody to detect ISC and the anti-DEFA5 antibody to distinguish the Paneth cells from the ISC by immunohistology. We could hardly identify a co-localization of IL22R and DEFA5 in Paneth cells, whereas single positive IL22R cells beneath DEFA5$^+$ cells were frequently observed (Figure 3A). We identified IL22R$^+$ ISC in all study groups (Figure 3A-D). There were no statistically significant differences among study groups in term of the number of IL22R$^+$ ISC per Paneth cells positive crypt (Figure 3E). Regarding the gene expression of IL22RA1, no statistically significant difference could be determined between no rejection and MoR groups (Figure 3F). We used three different anti-LGR5 antibodies to identify ISC, but we didn't detect LGR5$^+$ ISC by immunohistochemistry in human small bowel tissue from the distal part (data not shown). Therefore, we measured *LGR5* gene expression level in no rejection and MoR samples and revealed that expression level was comparable and not even reduced in comparison to the normal control group of no Tx samples (Figure 3F).

Proliferation of intestinal stem cells is not hindered during acute cellular rejection

The renewal of intestinal epithelium involves the continuous proliferation of ISC in the crypt base with subsequent migration of these cells to the luminal surface. After intestinal injury, epithelial regeneration is critical for barrier maintenance and organ function. To evaluate this proliferative ability of ISC in the context of ACR, we detected and counted Ki-67$^+$

proliferating cells that were interspersed between Paneth cells. We detected proliferative cells at the BZ in all analyzed biopsies independently of the severity of rejection (Figure 4A-D). Although the number of Ki-67$^+$ ISC per DEFA5$^+$ crypts decreased significantly in SR compared to N and MR groups, there are still Ki-67$^+$ ISC in all residual crypts of these severe cases detectable (Figure 4E).

Discussion

Paneth cells and ISC are affected in several inflammatory GI diseases, such as Crohn's disease and GvHD after hematopoietic stem cell transplantation. In ITx, apoptotic bodies in the crypts have been identified as a key feature for the diagnosis of ACR. However, which cell type in the crypts is affected during worsening of ACR process has not been completely understood. Here, our results indicate that Paneth cells and ISC are not reduced in number, and both cell types retain their functionalities ranging from mild to moderate and severe ACR events in biopsies of ITx patients - suggesting that the bottom of the crypt is not the initial target site of the ACR process. In addition, we show that the TZ is more susceptible to apoptosis than the BZ considering the TZ as the primary target during the ACR process, whereas the BZ might be stimulated for tissue repair after the injury.

Our results showing no reduction of Paneth cells during mild ACR are in line with unpublished results of the study by Fishbein et al. (19). We included two groups with more severe ACR features (more apoptotic bodies in the crypts, more infiltrating cells) in our analysis biopsies than the mild group. In MoR and SR groups, we detected an intrinsically slight reduction of Paneth cell numbers without statistical significance, whereas the level of *DEFA5* gene expression was mildly increased in MoR samples. This increase of *DEFA5* gene expression in the MoR samples might be due to more intensive induction of antimicrobial peptide production compensating the loss of the epithelial integrity. However, the intact DEFA5$^+$ Paneth cells in moderate and severe ACR cleared the way to use DEFA5

as a marker for the BZ in the crypt from biopsy sections. In biopsies with exfoliative diagnosis, a slight decrease in Paneth cells/crypts was detected. Exfoliative rejection is a highly aggressive form of severe ACR where a complete loss of villi and a critical loss of the integrity of whole crypts is one of its characteristics. However, the residual crypts might be responsible for the complete tissue regeneration and mucosal recovery. It would be interesting to investigate these crypts in more details to identify genes involved in tissue regeneration after ACR process.

We used anti-IL-22R antibody to identify ISC localized at the crypt bottom and beneath Paneth cells. No reduction of ISC was detected in the mild and moderate ACR compared to the no-rejection group. Several studies in mice suggested that in the crypt bottom, two different types of intestinal stem cells might be present, a quiescent and a proliferation type (24,25). We confirmed with the proliferation marker Ki-67 that proliferating ISC are present beneath Paneth cells in the crypt bottom. Strikingly, we could still detect these proliferating ISC in biopsies with moderate and severe ACR suggesting that these cells are not the target cells and are not losing their functionality.

Interestingly, the intact architecture of the crypt bottom during mild, moderate and severe ACR is almost the opposite that what has been reported so far in the scenery of GvHD from patients undergoing hematopoietic stem cell transplantation or GvHD mouse models. In a series of GvHD patients at our institution (data not shown), as well as in these reports, Paneth cells and ISC are the primary target cells and their functionality are highly affected (14,26,27). However, the differences in these studies are first, the morphological site, where in ITx we strictly compared distal ileum biopsies whereas in the human GvHD, duodenal biopsies have been studied (26). Whether the different site has an influence of target cells has still to be evaluated. Second, the high-dose chemoradiotherapy that is used for a pre-transplant conditioning for hematopoietic stem cell transplantation damages the GI tract initially and plays a central role in amplifying GVHD (28). ITx has not such a preconditioning step. Third, although the underlying principle of alloimmunity might be similar in both

transplants, details on cellular and molecular level may differ and even be decisive for the primary target cells.

The classical mechanism of ACR is that cytotoxic T cells recognize alloantigens presented by donor epithelial cells and consequently induce apoptosis in target cells (1). Apoptosis goes through a well-defined mechanism including a cascade of activation of different protease enzymes, mainly caspases. Anti-CASP3 antibodies have been proposed as markers for apoptosis. Talmon et al. (29) showed not only CASP3 positive apoptotic bodies, but also CASP3 positive yet intact cells in the intestinal crypts. In our hands, we detected with the anti-CASP3 antibody only apoptotic bodies and not intact cells, consequently no co-localization with specific Paneth cell and ISC markers was feasible. We also implemented M30, another marker for apoptosis, but we haven't detected $M30^+$ cells in the crypts of ITx biopsies from distal ileum, whereas we observed $M30^+$ cells in colonic control tissues (data not shown). In an ischemia/reperfusion model co-localization of M30 or CASP3 with DEFA5 was successfully documented (30). However, the underlined biological mechanisms are different in this model than in the ITx. We speculate that the whole process of induction and targeting cells during ACR is even more complex and that most probably the loss of the epithelial monolayer is not only due to alloreactive cells targeting crypt cells, but endothelial cells might be primarily targeted.

In summary, our study shows that Paneth cells and ISC located at the bottom of crypts are neither the primary nor the main target site during initial ACR process in ITx patients. Paneth cells and ISC remain functionally active even during an advanced stage of ACR resulting as the driving cells for the repair process, whereas apoptotic cells are mainly localized in the TZ provoking a loss of crypt integrity being the main target site during ACR. Further investigation needs to be conducted in the field of IL22/IL22R axis concerning ITx. Stimulation of $IL22R^+$ ISC might be a novel therapeutic option to accelerate mucosal regeneration after a severe ACR and to consequently protect the allograft of a potential complete loss or even loss of the patient through sepsis.

Acknowledgments

The authors thank the endoscopic team of Gastroenterology Unit at the Favaloro Hospital and Dr. Ana Cabanne and Dr. Juan P. Santilli for the pathological examination of the samples. We also thank to Rosana Valverdi for histological preparation of tissue samples. This work was supported by grants from *Agencia Nacional de Promoción de la Ciencia y la Tecnología ANPCYT* (PICT2530). G.E.G., M.R. is member of Research Career from Argentinean National Research Council (CONICET).

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

Figure Legends

Figure 1: Paneth cells during ACR in ITx. Representative immunofluorescence detection of Paneth cells ($DEFA5^+$, green) in ITx biopsies with (A) non-rejection (N); (B) mild rejection (MR); (C) moderate rejection (MoR); (D) severe rejection (SR). Magnification 400X. (E) Number of Paneth cells per $DEFA5^+$ crypt in N (n = 13), MR (n = 9), MoR (n = 12), and SR (n = 6) groups. (F) Gene expression of *DEFA5* and *LYZ* in N (n = 5) and MoR (n = 6) groups. Gene expression was normalized using b-actin gene as reference. Fold increase was calculated using the $\Delta\Delta$Ct method using the average of the control (non-Tx) group as normalizer. The values are shown as mean ± SD and the groups were compared using Kruskal-Wallis test with Dunn`s post-test or one-tail Mann-Whitney U test as appropriate.

Figure 2: Apoptosis in the different zone of intestinal crypts during ACR. Hematoxylin-eosin staining from ITx biopsies with (A) non-rejection (N); (B) mild rejection (MR); (C) moderate rejection (MoR). Apoptotic bodies are indicated with black arrows. Magnification

400X. Immunofluorescence detection of cleaved caspase-3 (red; indicated by white arrows) and Paneth cells (DEFA5$^+$; green) in ITx biopsies with (D) non-rejection (N); (F) mild rejection (MR); (E) moderate rejection (MoR). Magnification 400X. (G) Apoptosis number per ten crypts in the bottom (BZ) or transit-amplifying (TZ) zone. The values are shown as mean ± SD. The groups were compared using Kruskal-Wallis test with Dunn`s post-test and the analysis of the zones was performed through a paired *t*-test.*$P < 0.05$.

Figure 3: IL22R$^+$ intestinal stem cells (ISC) in an ACR context. Immunohistochemical staining to IL22R (violet) and Paneth cells (brown) in ITx biopsies with (A) no rejection (N); (B) mild rejection (MR); (C) moderate rejection (MoR); (D) severe rejection (SR). Magnification 200X. IL22R$^+$ cells interspersed between Paneth cells were considered as ISC (dotted line). (E) Number of IL22$^+$ ISC per DEFA5$^+$ crypt in N (n = 13), MR (n = 9), MoR (n = 12), and SR (n = 5) groups. (F) Gene expression of *IL22RA* and *LGR5* in N (n = 5) and MoR (n = 6) groups. Gene expression was normalized using b-actin gene as reference. Fold increase was calculated using the ΔΔCt method using the average of the control (non-Tx) group as normalizer. The values are shown as mean ± SD and the groups were compared using Kruskal-Wallis test with Dunn`s post-test or one-tail Mann-Whitney U test as appropriate.

Figure 4: Proliferative capability of intestinal stem cells. Immunofluorescence detection of Ki-67 (red) and Paneth cells (DEFA5$^+$; green) in ITx biopsies with (A) non-rejection (N); (B) mild rejection (MR); (C) moderate rejection (MoR); (D) severe rejection (SR). Magnification 400X. Ki-67$^+$ cells interspersed between Paneth cells were considered as proliferative ISC (white arrows). (E) Number of Ki-67$^+$ ISC per DEFA5$^+$ crypt in N (n = 13), MR (n = 7), MoR (n = 11), and SR (n = 5) groups. The values are shown as mean ± SD and the groups were compared using Kruskal-Wallis test with Dunn`s post-test. *$P < 0.05$.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Intestinal crypt. (A) Graphic representation of intestinal crypt. The bottom (BZ) and transit-amplifying (TZ) zone are morphologically defined. (B) Immunofluorescence images ($DEFA5^{+}$, green; DAPI, blue) of a representative biopsie slide. The different orientations that crypts can adopt in the same section are indicated. Magnification 200X.

References

1. Fishbein TM. Intestinal transplantation. N Engl J Med 2009;361(10):998-1008.
2. Sudan D. The current state of intestine transplantation: indications, techniques, outcomes and challenges. Am J Transplant 2014;14(9):1976-1984.
3. Grant D, Abu-Elmagd K, Reyes J, Tzakis A, Langnas A, Fishbein T et al. 2003 report of the intestine transplant registry: a new era has dawned. Ann Surg 2005;241(4):607-613.
4. Selvaggi G, Gaynor JJ, Moon J, Kato T, Thompson J, Nishida S et al. Analysis of acute cellular rejection episodes in recipients of primary intestinal transplantation: a single center, 11-year experience. Am J Transplant 2007;7(5):1249-1257.
5. Wu T, Abu-Elmagd K, Bond G, Nalesnik MA, Randhawa P, Demetris AJ. A schema for histologic grading of small intestine allograft acute rejection. Transplantation 2003;75(8):1241-1248.
6. Ruiz P, Bagni A, Brown R, Cortina G, Harpaz N, Magid MS et al. Histological criteria for the identification of acute cellular rejection in human small bowel allografts: results of the pathology workshop at the VIII International Small Bowel Transplant Symposium. Transplant Proc 2004;36(2):335-337.
7. Karin M, Clevers H. Reparative inflammation takes charge of tissue regeneration. Nature 2016;529(7586):307-315.
8. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annual review of physiology 2009;71:241-260.
9. Bevins CL, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nature reviews Microbiology 2011;9(5):356-368.
10. Clevers HC, Bevins CL. Paneth cells: maestros of the small intestinal crypts. Annual review of physiology 2013;75:289-311.
11. Wehkamp J, Harder J, Weichenthal M, Schwab M, Schaffeler E, Schlee M et al. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. Gut 2004;53(11):1658-1664.
12. Wehkamp J, Schmid M, Fellermann K, Stange EF. Defensin deficiency, intestinal microbes, and the clinical phenotypes of Crohn's disease. J Leukoc Biol 2005;77(4):460-465.
13. Simms LA, Doecke JD, Walsh MD, Huang N, Fowler EV, Radford-Smith GL. Reduced alpha-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease. Gut 2008;57(7):903-910.
14. Eriguchi Y, Takashima S, Oka H, Shimoji S, Nakamura K, Uryu H et al. Graft-versus-host disease disrupts intestinal microbial ecology by inhibiting Paneth cell production of alpha-defensins. Blood 2012;120(1):223-231.

15. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. Immunity 2012;37(2):339-350.
16. Teshima T, Reddy P, Zeiser R. Acute Graft-versus-Host Disease: Novel Biological Insights. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation 2016;22(1):11-16.
17. Munneke JM, Bjorklund AT, Mjosberg JM, Garming-Legert K, Bernink JH, Blom B et al. Activated innate lymphoid cells are associated with a reduced susceptibility to graft-versus-host disease. Blood 2014;124(5):812-821.
18. Lindemans CA, Calafiore M, Mertelsmann AM, O'Connor MH, Dudakov JA, Jenq RR et al. Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. Nature 2015;528(7583):560-564.
19. Fishbein TM, Novitskiy G, Lough DM, Matsumoto C, Kaufman SS, Shetty K et al. Rejection reversibly alters enteroendocrine cell renewal in the transplanted small intestine. Am J Transplant 2009;9(7):1620-1628.
20. Ramisch D, Rumbo C, Echevarria C, Moulin L, Niveyro S, Orce G et al. Long-Term Outcomes of Intestinal and Multivisceral Transplantation at a Single Center in Argentina. Transplant Proc 2016;48(2):457-462.
21. Meier D, Cagnola H, Ramisch D, Rumbo C, Chirdo F, Docena G et al. Analysis of immune cells draining from the abdominal cavity as a novel tool to study intestinal transplant immunobiology. Clin Exp Immunol 2010;162(1):138-145.
22. Gondolesi G, Fauda M. Technical refinements in small bowel transplantation. Curr Opin Organ Transplant 2008;13(3):259-265.
23. Meier D, Docena GH, Ramisch D, Toscanini U, Berardi G, Gondolesi GE et al. Immunological status of isolated lymphoid follicles after intestinal transplantation. Am J Transplant 2014;14(9):2148-2158.
24. Basak O, van de Born M, Korving J, Beumer J, van der Elst S, van Es JH et al. Mapping early fate determination in Lgr5+ crypt stem cells using a novel Ki67-RFP allele. The EMBO journal 2014;33(18):2057-2068.
25. Li L, Clevers H. Coexistence of quiescent and active adult stem cells in mammals. Science 2010;327(5965):542-545.
26. Levine JE, Huber E, Hammer ST, Harris AC, Greenson JK, Braun TM et al. Low Paneth cell numbers at onset of gastrointestinal graft-versus-host disease identify patients at high risk for nonrelapse mortality. Blood 2013;122(8):1505-1509.
27. Takashima S, Kadowaki M, Aoyama K, Koyama M, Oshima T, Tomizuka K et al. The Wnt agonist R-spondin1 regulates systemic graft-versus-host disease by protecting intestinal stem cells. J Exp Med 2011;208(2):285-294.
28. Ferrara JL, Cooke KR, Teshima T. The pathophysiology of acute graft-versus-host disease. International journal of hematology 2003;78(3):181-187.
29. Talmon GA, Saigh QM, DiMaio D, Wisecarver JL, Radio SJ. Characterization of epithelial apoptosis in biopsies of small-bowel allografts using cleaved caspase 3 immunostaining. International journal of surgical pathology 2010;18(6):482-487.
30. Grootjans J, Hodin CM, de Haan JJ, Derikx JP, Rouschop KM, Verheyen FK et al. Level of activation of the unfolded protein response correlates with Paneth cell apoptosis in human small intestine exposed to ischemia/reperfusion. Gastroenterology 2011;140(2):529-539 e523.

Table 1. Summary of patients included in the study

a) Immunohistological staining samples

Category	Variables	N (n=16)	MR (n=9)	MoR (n=13)	SR (n=6)	*P*-value
Recipient age (Bx time, yr)		21 (2-48)	16 (2-35)	23 (4-49)	17 (2-48)	0.75
Recipient gender	M/F	13/3	8/1	8/5	4/2	0.43
Graft age (Bx time, yr)		17 (0.6-42)	14 (0.5-36)	16 (2-43)	16 (1-42)	0.94
Donor gender	M/F	9/7	7/2	6/7	4/2	0.66
Postoperative day (day)		344 (5-3530)	672 (34-3206)	347 (5-1230)	764 (17-3334)	0.62
Immunosuppression	Tac	1	2	1	0	0.09
	Tac/ steroid	6	2	6	1	
	Tac/ Rapamycin	1	2	0	2	
	Tac/MMF	0	2	1	0	
	Tac/ Rapamycin/steroid	5	0	1	2	
	Tac/MMF/steroid	3	1	4	1*	
Type of ITx	Isolated/ MMVTx/ Combined	15/1/0	9/0/0	13/0/0	5/0/1	0.22
Colon	Native/ Transplanted	13/3	9/0	9/4	5/1	0.33
Apoptotic bodies/ 10 crypts		1 (0-1)	8 (6-10) †	10 (6-20) †	11 (10-12) †	0.00

b) Gene expression analysis samples

Category	Variables	N (n=5)	MoR (n=6)	*P*-value
Recipient age (Bx time, yr)		21 (7-48)	15 (3-48)	0.46
Recipient gender	M/F	4/1	4/2	0.62
Graft age (Bx time, yr)		11(4-20)	7 (1-20)	0.52
Donor gender	M/F	4/1	4/2	0.62
Postoperative day (day)		619 (55-1347)	683 (51-1196)	1.00
Immunosuppression	Tac	0	0	0.52
	Tac/ steroid	2	3	
	Tac/ Rapamycin	1	0	
	Tac/MMF	0	0	
	Tac/ Rapamycin/steroid	0	0	
	Tac/MMF/steroid	2	3	
Type of ITx	Isolated/ MMVTx	5/0	6/0	-
Colon	Native/ Transplanted	5/0	5/1	0.34
Apoptotic bodies/ 10 crypts		1 (0-1)	7 (6-9) †	0.01

Bx, biopsy; Tac, tacrolimus; MMF, mycophenolate; MMVTx, modified multivisceral transplant - †; different from the no rejection group. * Patient treated with timoglobuline during a severe rejection event.

Table 2: Description of used reagents.

a) **Antibodies used for immunohistological techniques.**

Antibody	Clonality	Reactiviy	Host species	Clone	Conjugate	Company	Working dilution
Anti- IL-22RA	Polyclonal	Human	Rabbit	-	-	Biorbyt	1:50
Anti- cleaved-caspase 3	Polyclonal	Human	Rabbit	-	-	Millipore	1:50
Anti- alpha defensin 5	Monoclonal	Human	Mouse	8C8	-	Abcam	1:200
Anti- Ki-67	Monoclonal	Human	Rabbit	SP6	-	Novus Biologicals	1:200
Anti- Fc portion	Polyclonal	Rabbit	Goat	-	Biotinylated	Invitrogen	1:200
Anti- Fc portion	Polyclonal	Mouse	Goat	-	Biotinylated	Invitrogen	1:200

b) **Genes and primers used for qPCR analysis.**

GeneID	Symbol	Gene description	Cell type	Primer sequences 5'→3'	Product length (bp)
NM_021010.2	*DEFA5*	Defensin alpha 5	Paneth cells	GGGAAGACAACCAGGACCTTGCTA GCGGCCACTGATTTCACACACC	155
NM_000239.2	*LYZ*	Lysozyme		GGGAATGGATGGCTACAGGGGAATC TCCCATAATCAGTGCTTCTGTCTCCAG	122
NM_003667.3	*LGR5*	Leucine rich repeat containing G protein-coupled receptor 5	Intestinal stem cells	AAGGTGACAACAGCAGTATGGACGA GGCCTGGGGAAGGTGAACACT	150
NM_021258.3	*IL22RA1*	Interleukin 22 receptor subunit alpha 1	Intestinal stem cells Other epithelial cells	AGACACGGTCTACAGCATCG GTGGCTTGAGGGTAGTGTG	224