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Paneth cell alterations during ischemia-reperfusion, follow-up, and graft rejection after intestinal transplantation

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Authorship

AMK, LJC participated in research design, performed the research and data analysis and wrote the paper. GDH participated in performance of the research. IH, EC, HH, RB, OC, FJ, GDH, GG, CD, SOD, JP and DM participated in research design, and reviewed the paper. KL participated in research design, performance of the research, writing of the paper, and supervised the study.

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Abbreviations

IR, ischemia-reperfusion. ITx, intestinal transplantation. PC, Paneth cell(s). PC/crypt, Paneth cell number per crypt. Rej, rejection

Abstract

Background: Ischemia-reperfusion (IR) injury is inevitable during intestinal transplantation (ITx) and executes a key role in the evolution towards rejection. Paneth cells (PC) are crucial for epithelial immune defense and highly vulnerable to IR injury. We investigated the effect of ITx on PC after reperfusion (T0), during follow-up, and rejection. Moreover, we investigated whether PC loss was associated with impaired graft homeostasis. **Methods**: Endoscopic biopsies, collected according to center-protocol and at rejection episodes, were retrospectively included (n=28 ITx, n=119 biopsies) Biopsies were immunohistochemically co-stained for PC (lysozyme) and apoptosis, and PC/crypt and lysozyme intensity were scored. **Results:** We observed a decrease in PC/crypt and lysozyme intensity in the first week after ITx (W1) compared to T0. There was a tendency towards a larger decline in PC/crypt (p=0.08) and lysozyme intensity (p=0.08) in W1 in patients who later developed rejection compared to patients without rejection. Follow-up biopsies showed that the PC number recovered, whereas lysozyme intensity remained reduced. This persisting innate immune defect may contribute to the well-known vulnerability of the intestine to infection. There was no clear evidence that PC were affected throughout rejection. **Conclusion:** This study revealed a transient fall in PC numbers in the early post-ITx period, but a permanent reduction in lysozyme intensity following ITx. Further research is needed to determine the potential clinical impact of PC impairment after ITx.

1. Introduction

Intestinal transplantation (ITx) is a life-saving treatment for patients suffering from functional or anatomical short bowel syndrome and complicated intestinal failure[1]. Among all solid-organ transplantations, the intestine remains immunologically and biologically the most challenging, and most vulnerable to acute rejection. Despite improved surgical and clinical expertise and improved immunosuppression therapy[2], acute cellular rejection is still a frequent (50 percent) and life-threatening complication after ITx[3].

Ischemia-reperfusion (IR) injury, an inevitable consequence of transplantation, is known to contribute to the cascade leading to allograft rejection. IR injury triggers innate immunity, and may thereby enhance the alloimmune response towards the graft and promote rejection[4]. In colonized organs, IR-induced tissue damage is accompanied by bacterial translocation. Translocation of endotoxin occurs directly after reperfusion of the intestinal graft as well as during rejection. This represents another 'danger' signal stimulating alloreactivity and sensitizing the graft towards rejection[5-9]. In other solid organ transplants, IR injury is associated with an increased number of rejection episodes, and impairs both acute and long-term graft function[10,11].

IR of the human small intestine triggers apoptosis of Paneth cells (PC) in the crypt epithelium[12]. PC are crucial for the maintenance of intestinal bacterial homeostasis and contribute to innate epithelial immunity. PC respond to bacterial presence and secrete antimicrobial products, including lysozyme and alpha-defensin, thereby controlling homeostasis between colonizing microbiota and the host[13,14]. PC loss has been associated with increased bacterial translocation and intestinal inflammation in Crohn's disease and a human model of intestinal IR[12,15]. This might suggest that bacterial translocation after graft reperfusion, and consequently the greater vulnerability to rejection[5], could be (partly) due to IR-induced PC loss. We hypothesized that

IR induces PC apoptosis shortly after ITx, which impairs graft homeostasis in the short and long term.

Next to the supposed effect of IR, PC may also play a role in the process of graft rejection. Several diseases associated with intestinal inflammation, such as Crohn's disease and graft-versus-host disease, show PC loss and reduced production of antimicrobial peptides[16-19]. We hypothesized that PC also play a role in the pathophysiology of graft rejection. In this regard, two previous studies have reported no difference in PC number and antimicrobial peptide expression during rejection in ITx patients compared to healthy volunteers[20] or stable grafts[21]. However, whether PC impairment precedes rejection, indicating a role in initiation of the rejection process, and/or whether PC are targeted by the rejection process, is unknown.

In this study we aimed to investigate 1) the change in PC numbers and lysozyme intensity in the early reperfusion phase after ITx, and at multiple follow-up time points up to 5 years post-ITx; 2) the association between the extent of PC impairment in the early post-ITx period and clinical outcomes; and 3) PC loss and lysozyme intensity throughout acute graft rejection episodes.

2. Materials and methods

2.1 Patients and biopsies

The study retrospectively included 28 pediatric patients who underwent ITx between 2003 and 2012 at the Birmingham Children's Hospital, United Kingdom. The study was approved by the research ethics committee (10/H1207/67) and informed consent was obtained. Patient characteristics and clinical information are presented in Table 1. Induction immunosuppressive therapy was identical for all patients (tacrolimus/steroid/basiliximab), whereas maintenance immunosuppressive therapy varied between patients (Table 1). Rejection episodes were treated with high-dose steroids, increase of maintenance immunosuppression and – if refractory – by Thymoglobulin.

Archived biopsy samples, collected as part of the graft monitoring protocol or when clinically indicated, e.g. during rejection, were used for analysis. After graft reperfusion (up to 30 min), a tissue specimen of the terminal ileum was collected intraoperatively (T0). According to the monitoring protocol, mucosal biopsies from the terminal ileum were obtained regularly early after ITx, and with increasing time intervals (2-3 times in the 1st week, weekly during the 1st month, monthly during the 1st year, and yearly thereafter). In this study, biopsies were grouped as shown in Figure 1A. Additional biopsies were obtained during acute rejection. For evaluation of rejection, samples were grouped in biopsies obtained prior to rejection (Pre-Rej), at diagnosis (Rej1), later during rejection (Rej2, day 2-14), and after recovery of rejection (first biopsy with normal histology, Post-Rej). The Pre-Rej biopsy was included in case a routine biopsy was available up to 9 days before rejection.

2.2 Immunohistochemistry and scoring of PC/crypt and lysozyme intensity

Formalin-fixed paraffin-embedded tissue sections were immunohistochemically stained for the detection of lysozyme (PC) and M30 (apoptosis) as previously described[12]. All samples from one patient were stained in the same batch to prevent batch-dependent variability within one patient. The same control sample of human small intestine was included in every batch for inter-batch difference evaluation in staining intensity. Immuno-stained tissue sections were scored for the number of lysozyme-positive cells per crypt (PC/crypt) and M30-positive lysozyme-positive cells (apoptotic PC) in whole biopsy sections or – for larger tissue specimens – in 10 representative microscopic fields (200x magnification). Samples containing =/< 5 crypts were excluded. In addition, lysozyme staining intensity was graded using a 5-point scale from very weak (1) to very intense (5). All scorings were performed by two independent observers in a blinded way. Inter-observer reliability was good, with an intraclass correlation coefficient of 0.80 (95%; CI: 0.75-0.85) for PC count, and 0.89 (95%; CI: 0.86-0.92) for lysozyme intensity scores.

2.3 Histological grading of IR injury and rejection

T0 and W1 samples were H&E-stained and scored for IR injury according to Park/Chiu by an experienced pathologist (GDH). Park/Chiu scores progression of injury from the villi tips to the crypts and into deeper layers of the intestinal wall in 8 grades, in which a higher grade reflects more damage[22]. Grading of rejection biopsies was based on clinical reporting by a small specialist team of three pathologists. Biopsies were graded using a standard scale: no, mild, moderate, or severe acute cellular rejection[23].

2.4 Statistical analysis

Statistical analysis was performed using IBM SPSS 22.0 and GraphPad Prism 6 software. Intraclass correlation coefficient and 95% confidence intervals were calculated based on a mean-rating (K=2), consistency, 2-way mixed-effects model. Linear mixed model analysis was used to examine statistical significance of changes in PC/crypt and lysozyme intensity over time following ITx (covariance structure: heterogeneous compound symmetry) and throughout rejection (compound symmetry). Wilcoxon matched-paired signed rank test was performed to compare Park-Chiu scores. The relation between Park-Chiu and PC scores was analyzed using Spearman correlation analysis. Mann-Whitney test was used to compare PC scores in clinical outcome groups (rejection versus no rejection). All P-values are two-sided. P-values less than 0.05 were considered statistically significant.

3. Results

3.1 PC are affected after intestinal transplantation

To investigate whether PC were affected after ITx, changes in PC/crypt and lysozyme intensity were evaluated, as well as the presence of apoptotic PC. In T0 samples collected shortly after reperfusion, apoptotic cells were abundant in the villi tips, but no apoptotic PC could be detected (data not shown). Interestingly, both the PC/crypt (Figure 1B) and the lysozyme intensity (Figure 1C) were significantly reduced in the first follow-up biopsy (W1) compared

to T0 ($p<0.001$). Also, a strong positive correlation was observed between PC/crypt and lysozyme intensity score at T0 ($r=0.70$, $p=0.003$) and W1 ($r=0.76$, $p=0.001$). PC/crypt remained reduced at W2 and M1 compared to T0 ($p<0.001$ and $p<0.05$ respectively), but restored thereafter gradually to numbers comparable to T0. In contrast, lysozyme intensity continued to be reduced at all time points after ITx compared to T0 (Figure 1C). Evaluation of consecutive time points shows intra-individual as well as inter-individual variation, especially for lysozyme intensity, as illustrated in Figure 1D.

3.2 PC number and lysozyme intensity do not correlate with histologically graded IR injury

To investigate whether the observed PC and lysozyme loss could be related to IR injury, the extent of IR injury was graded in T0 (Median 2, IQR = 2-5) and W1 (Median 2, IQR = 1-6) samples based on the Park-Chiu scoring. Park Chiu scores did not significantly improve in W1 compared to T0 ($p=0.60$). However, five out of 11 patients showed decreased and thus improved Park-Chiu scores, whereas for the other six patients no improvement was observed at W1. The change in Park-Chiu (ΔW1-T0) did not correlate with a change in PC/crypt and lysozyme intensity (ΔW1-T0). Also, there was no correlation between IR injury and PC/crypt or lysozyme intensity at T0 and W1 (Figure S1, SDC, http://links.lww.com/TP/B922). In addition, no relation could be found between cold or warm ischemia time and PC/crypt or lysozyme intensity at T0, W1 or ΔW1-T0.

3.3 Relation between PC (antimicrobial) loss and clinical outcome

To evaluate whether PC impairment in the early post-ITx period was associated with later occurrence of acute rejection, two groups were compared: patients who later developed rejection (n=8) and patients without graft rejection (n=3). At T0 and W1, the PC/crypt and lysozyme intensity did not differ between the groups (Figure 1E,F). However, there was a tendency towards a larger decline in PC/crypt ($p=0.085$) and lysozyme intensity ($p=0.079$) in the first follow-up biopsy relative to T0 (ΔW1-T0) in the rejection group compared to the no-

rejection group (n=8 and n=3 respectively for paired analysis) (Figure 1E,F). In this patient cohort, no relation could be found between the extent of PC loss or decrease in lysozyme intensity in the first week post-ITx (ΔW1-T0), and other clinical outcomes: ICU time, graft loss, and mortality. In addition, there was no association between PC/crypt or lysozyme intensity at T0 or W1, and these clinical outcomes.

3.4 PC alterations throughout rejection

Independent acute rejection episodes were graded as mild (n=18), moderate (n=4), or severe (n=6). Both early rejections (n=21), defined as episodes occurring within 3 months post-ITx, and late rejection episodes (n=7) were included in the analysis. For overall evaluation of PC impairment throughout rejection (n=28), samples were grouped as shown in Figure 2A. We observed no differences in PC/crypt (Figure 2B) or lysozyme intensity (Figure 2C) between Pre-Re, Rej1, Rej2 and Post-Rej. Although overall no significant differences were detected, examination of individual rejection episodes showed notable differences in PC changes between patients (Figure 2D). These inter-individual differences could not be explained by severity or timing of rejection, as no difference was observed between high- and low-grade rejection, and between early and late episodes.

4. Discussion

IR injury is inevitable during ITx and known to induce apoptosis of PC, key elements in innate epithelial immunity. This study focused on PC loss and antimicrobials after ITx, and the relation between PC impairment in the early post-ITx period and long-term graft homeostasis. In addition, we studied the PC alterations in the course of graft rejection.

In contrast to what we expected based on the study by Grootjans *et al.*, in which significant numbers of apoptotic PC were present after 45 minutes of ischemia followed by short reperfusion in human jejunum[12], no apoptotic PC could be detected shortly after reperfusion of the intestinal graft. This observation is in line with the relatively low Park-Chiu scores in T0

biopsies, and both indicate mild IR injury following ITx. A shorter warm ischemia time in the ITx cohort (33 minutes versus 45 minutes in the human IR model[12]), and the fact that ileum is less susceptible to IR injury than jejunum could explain the mild IR injury and absence of PC apoptosis. In addition, organ preservation solution and topical cooling may have a protective effect on the graft and alleviate IR injury. It is also important to consider we cannot rule out that the apoptotic event might have been missed due to timing of the T0 sample.

Despite absence of PC apoptosis very early after reperfusion, the reduction in PC numbers in the first follow-up biopsy indicates that PC are effectively lost in the subsequent days post-transplantation. Since PC count did not correlate with Park-Chiu scores, it is likely that PC loss was not directly related to IR injury. Other post-operative factors (e.g. ICU stay, immunosuppression) or immune factors may also play a role. The reduced lysozyme intensity and PC number in the first months after ITx might, in part, account for the increased susceptibility of the graft to rejection and infection in this early period. The observation that PC numbers gradually recuperate, suggests a normalization of mucosal homeostasis of the graft in the longer term.

Interestingly, in the current study we also show a permanent reduction in lysozyme intensity after ITx. Deficiencies of antimicrobial proteins have been described in several pathologies associated with intestinal inflammation, such as Crohn's disease, obesity and graft-versus-host-disease[17,18,24]. Furthermore, Fishbein *et al*. have reported a decrease in expression of antimicrobial peptides in the intestinal graft of ITx recipients with NOD2 Crohn's disease-associated polymorphism[25]. The NOD2 status of our patient cohort could not be determined for ethical restrictions. In addition, it is likely that immunosuppressive therapy has an effect on lysozyme expression in ITx patients. Indeed, immunosuppressive drugs (like calcineurin-inhibitors) have been shown to alter gene expression of antimicrobial peptides in mouse ileum, as well as microbiota composition[26].

On the other hand, changes in microbiota composition in ITx patients[27] may also be a consequence of reduced intensity of antimicrobial peptides. We speculate that the observed persistent reduction in lysozyme intensity in ITx patients may facilitate colonization by opportunistic pathogens, and thereby partially account for the well-established susceptibility of the grafted intestine to infectious enteritis. In addition, failure of antimicrobial defense combined with a hostile microbiota stimulates the immune response, and may therefore albeit indirectly increase vulnerability to rejection. Indeed, decreased expression of antimicrobial peptides in ITx patients with NOD2 polymorphisms has been shown to be a significant risk factor for graft rejection[25].

The relation between PC loss shortly after ITx and rejection in the long-term could be investigated only in a limited number of patients because of missing T0 and W1 samples. Although sample size was small and statistical significance was not reached, there was a trend showing a larger decline in PC number and lysozyme intensity (ΔW1-T0) in the patients who developed rejections compared to the no-rejection group. These data suggest that the decline at W1 relative to T0, rather than PC (antimicrobial) scores at T0 or W1, may predict rejection. However, this observation should be confirmed in a larger ITx cohort.

One of the histological hallmarks of acute rejection following ITx, is an increased number of apoptotic bodies in the crypt epithelium[23,28]. Our results showed no PC apoptosis during rejection or PC loss compared to biopsies obtained prior to and after rejection, which suggests that PC are not (further) affected by the rejection process in ITx patients. Likewise, a previous study by Fishbein *et al.* reported no change in PC number before and during early mild-graded rejection[21]. This is in stark contrast to graft-versus-host disease following allogeneic hematopoietic cell transplantation, where PC have been shown to be highly affected, and the PC number correlated with clinical severity[16]. When comparing mild and severe acute rejection in our ITx cohort, no differences in PC number and lysozyme intensity were found, which is

in line with observations by Pucci Molineris *et al.* [20]. The inexplicable variation between rejection events suggests that acute rejection is a complex process, in which the interplay of multiple factors influences the PC and antimicrobial immune defense.

Only pediatric patient samples were examined and it cannot be assumed that results are necessarily applicable to adults. Although PC are immune competent at birth[29], the gut microbiome is still developing in young children[30] which might have an effect on PC function. Nevertheless, Pucci Molineris *et al.* reported no differences between the pediatric and adult cohort when examining PC during rejection after ITx[20].

Due to the relatively small number of ITx cases per center, it remains challenging to perform large ITx studies, especially when using tissue samples. Given the retrospective nature of this study, a considerable amount of biopsies was not available for research. Also, some biopsies were not sufficient to give reliable results and were therefore excluded from the analysis. Small sample size impeded thorough statistical analysis of the association between PC impairment and clinical outcome, as well as in-depth analysis of PC alterations during acute rejection and comparing mild and severe rejections. In addition, infection episodes could not be studies due to the respective nature of the study.

This study provides new insights on PC alterations in the early reperfusion phase following ITx. Analysis of multiple follow-up biopsies in the same patients, gave valuable insight in the biology of the graft. We report a loss of PC and lysozyme intensity in the early post-ITx period, but an association with IR injury could not be demonstrated. While PC numbers gradually restored, there was a permanent decline in lysozyme intensity in ITx patients. Further research in a larger cohort is needed to determine the potential impact of PC impairment on rejection and infection after ITx.

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Figure legends

Figure 1. Paneth cells per crypt and lysozyme staining intensity after ITx. A) Timeline of biopsies collected following monitoring protocol. Biopsies were grouped in time points: T0 (after reperfusion, n=18); W1 (day 3-7, n=15); W2 (day 8-14, n=14); M1 (day 17-46, n=11); M3 (day 60-98, n=12); M5 (day 125-178, n=9); M8 (day 197-277, n=9); Y1 (day 311-446, n=17); Y2+ (2-5 years post-ITx, n=14) B) PC/crypt and C) lysozyme staining intensity in PC in biopsies collected after ITx as part of the monitoring protocol *p<0.05, **p<0.01, ***p<0.001 compared with T0. $^{\#\#\#}$p<0.001 compared to all other time-points. Data are displayed in a Tukey Box-and-whisker plot. Outliers are indicated with a dot. D) Representative lysozyme staining on consecutive post-ITx biopsies from two patients. Scale bar represents 100 µm. E) PC/crypt and F) lysozyme intensity in patient group with rejections (n=8) and patients without rejections (n=3) at T0, W1 (3-5 days post-ITx) and ΔW1-T0. Patients with both T0 and W1 biopsies available were included in analysis. Data are displayed in Tukey Box-and-whisker plot.

Figure 2. Paneth cells per crypt and lysozyme staining intensity throughout rejection episodes. A) Timeline of biopsies collected throughout rejection episodes. Samples were grouped in Pre-Rej (up to 9 days prior to rejection, n=15), Rej1 (at diagnosis of acute rejection, n=19), Rej2 (later during acute rejection, n=18), and Post-Rej (after recovery of rejection, n=24), B) PC/crypt and C) lysozyme staining intensity in PC. Data are displayed in a Tukey Box-and-whisker plot. D) Lysozyme stainings in biopsies prior to, during and after rejection episodes from three different patients. Grade (mild, severe) and timing (early, late) of rejection episodes are indicated. Scale bar represents 100 µm.

Table 1. Patient characteristics and clinical information

		median (range)	n patients
Recipient characteristics			
Age, months		54 (11-194)	
Gender (n)	Male/Female		16/12
Weight, kg		15 (6-52)	
Type of ITx (n)	Isolated/combined/MMV/MV		12/12/3/1
Donor characteristics			
Age, months		96 (12-480)	
Gender (n)	Male/Female		11/15
Weight, kg		24 (11-70)	
Surgical variables			
Cold ischemia time, minutes		380 (221-740)	
Warm ischemia time, minutes		33 (10-53)	
Operating time, minutes		381 (250-739)	
Post-surgery			
ICU stay, days		2.5 (1-145)	
In-hospital stay, days		55.5 (25-115)	
Maintenance Immunosuppression (n)	TAC+ST		20
	TAC+ST+RAPA		3
	TAC+ST+MMF		4
Outcome			
Mortality, n (%)			7 (25%)
Reason of death, n	Infection		3
	Rejection		2
	Thrombosis of graft		1
	Technical complications		1
Graft loss, n (%)			10 (35,7%)
Episodes of rejection, n	0/1/2/3/4		6/11/5/4/2
Early rejection, n (%)			15 (54%)

Combined, small intestine and liver; MVV, modified multivisceral; MV, multivisceral; TAC, tacrolimus; MMF, mycophenolate mofetil; RAPA, rapamycin; ST, steroid

Figure 1

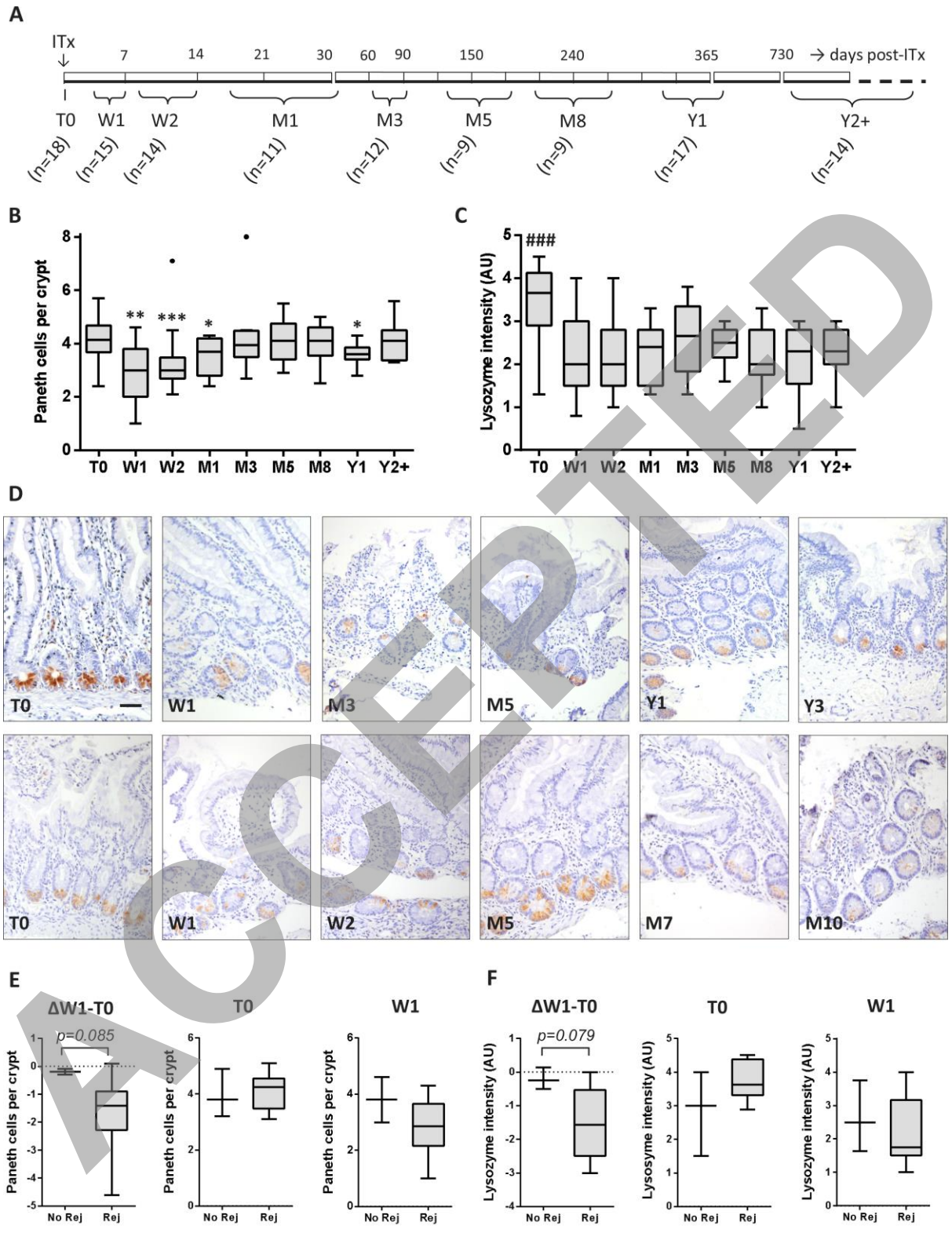

Figure 1. Paneth cells per crypt and lysozyme staining intensity after ITx

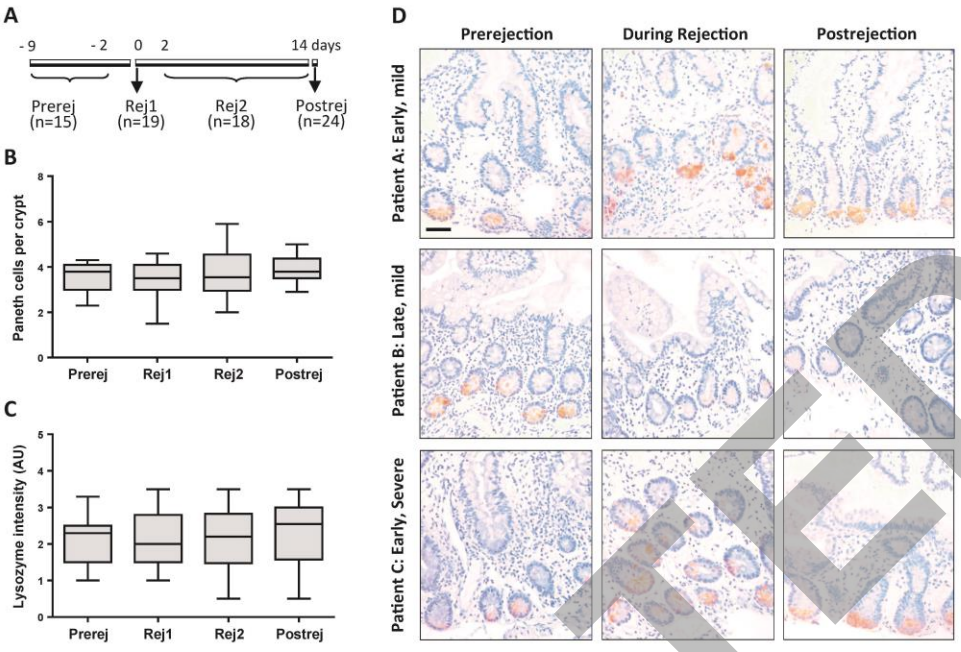

Figure 2. Paneth cells per crypt and lysozyme staining intensity throughout rejection episodes