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Immunosuppressive therapies after intestinal transplant modulate the expression of Th1 signature genes during acute cellular rejection. Implications in the search for rejection biomarkers

Zambernardi A, Chiodetti A, Meier D, Cabanne A, Nachman F, Solar H, Rumbo C, Gondolesi GE, Rumbo M. Immunosuppressive therapies after intestinal transplant modulate the expression of Th1 signature genes during acute cellular rejection. Implications in the search for rejection biomarkers.

Abstract: Background and Aims: Acute cellular rejection (ACR) and infections are leading causes of graft loss and death in intestinal transplant patients. Our aim was to evaluate the impact of maintenance immunosuppressive therapies on the expression of pro-inflammatory mediators in small bowel at ACR diagnosis.
Materials and Methods: We analyzed expression levels of Th1-associated genes, *IFNG, CXCL10,* and *CXCL11* by qPCR in 46 selected graft biopsies unequivocally assigned to mild ACR (n = 14) or normal histopathology and clinical condition (n = 32) from 15 patients receiving two different immunosuppressive (IS) schemes. Double treatment: corticosteroids and tacrolimus (n = 17) and triple treatment: sirolimus or mycophenolate mofetil in addition to the basal therapy (n = 29).
Results: *IFNG, CXCL10,* and *CXCL11* were induced during rejection (p < 0.05; p < 0.005, and p < 0.05, respectively). However, when rejection and control groups were classified according to immunosuppressive treatment, in the rejection group, significant differences of *IFNG, CXCL10,* and *CXCL11* expression (p < 0.001; p < 0.005, and 0.01, respectively) were detected, whereas no differences were observed in the control group.
Conclusion: Gene expression of Th1 response mediators is higher during ACR. Triple IS group showed significantly lower expression of pro-inflammatory Th1 mediators during mild ACR indicating that use of these markers to monitor rejection can be affected by the IS treatment used.

Agustina Zambernardi[a,b], Ana Chiodetti[a], Dominik Meier[b], Ana Cabanne[b], Fabio Nachman[b], Héctor Solar[b], Carolina Rumbo[b], Gabriel E. Gondolesi[b] and Martin Rumbo[a]

[a]Laboratorio de Investigaciones del Sistema Inmune (LISIN), Facultad de Cs. Exactas, Universidad Nacional de La Plata, La Plata and [b]Instituto de Transplante Multiorgánico, Fundación Favaloro, Buenos Aires, Argentina

Corresponding author: Martin Rumbo, Laboratorio de Investigaciones del Sistema Inmune (LISIN), Facultad de Cs. Exactas, Universidad Nacional de La Plata, 47 y 115 (1900) La Plata, Argentina.
Tel.: +54 221 4250497 (ext.45);
fax: +54 221 4254853;
e-mail: martinr@biol.unlp.edu.ar

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At present, intestinal transplantation (ITx) is an accepted therapeutic option for patients with intestinal failure and life threatening complications associated with the chronic use of parenteral nutrition. The evolution of surgical techniques, postoperative management, and immunosuppressive (IS) therapies have led to better patient and graft survival rates comparable to those observed in other transplanted organs. However, acute cellular rejection (ACR) and infections remain the major threats in the early and late postoperative period (1).

The most effective strategy to successfully manage those complications is early intervention to control the rejection or infection process, thus avoiding extensive graft damage (2). This goal can only be achieved by means of strict follow-up protocols. The diagnosis of ACR is based on endoscopy, histopathological analysis of graft biopsies, and presence of clinical symptoms. As most ITx programs have established follow-up programs based on this strategy, important experience has been gained using this approach (3, 4). However, additional tools to help in the diagnostic procedure

are awaited due to the complexity of the differential diagnosis and the limitations of endoscopy procedures to access to the full surface of the total graft, because rejection process can show heterogeneous presentation at different intestinal segments (5).

The major cause of ACR is the allo-reactivity of the recipient's immune cells against the graft. Gene expression (GE) analysis on graft mucosa has been employed to identify the molecular players that participate in ACR and to gain knowledge on the biological rejection process (6–9). These studies have shown that a complex gene network is involved in the ACR process outlining the participation of different immune effectors such as IFNG signaling (8) and different chemokines and inflammatory mediators (9). These results obtained on ITx patients are consistent with similar approaches reported for other solid organ Tx, such as kidney, liver, or heart that have identified core transcriptional responses of the ACR process in these clinical settings (10–12). However, in the ITx field, the impact of different immunosuppressive regimes on pro-inflammatory gene expression has not been assessed so far. In our research, we selected a set of genes involved in different immunological processes that belong to the core immune response activation (12). We studied their expression levels under different immunosuppressive maintenance schemes to determine the potential utility of these candidates as immuno-biomarkers during the follow-up of ITx patients.

Materials and methods

Patients and samples

The present protocol was approved by the Institutional Review Board of Hospital Universitario Fundación Favaloro (DDI [984] 1207). Fifteen patients transplanted from 2007 to 2010 were enrolled in the protocol: 10 pediatric, 14 isolated intestine, and one combined liver/bowel. An overall description of the patients included in the study is shown in Table 1.

In our program, the post-Tx follow-up includes endoscopic guided biopsies twice a week during the first 6 wk post-Tx; then once a week for the following 6 wk; once every other week until 6 months post-Tx; and then once a month until the end of the first year. This scheme is reinitiated if the patient develops an ACR episode.

We retrospectively selected those biopsies that correspond to unequivocally identified diagnosis of ACR in the rejection group (R group) or to complete absence of clinical and histological

Table 1. Summary of patients included in the study. General description of patients, induction immunosuppression, and Tx characteristics are listed

	Adults	Pediatrics
Number of patients included	5	10
Age (yr)	33 ± 16	6 ± 5
Male/Female	5/0	6/4
Type of Tx	5 Isolated	6 Isolated/3 with colon/ 1 combined
Cause of indication of ITx	Atresia/Hirschprung's disease/ischemia/ trauma/post-surgical complications	4 Hirschprung's disease, 4 volvulus, 2 necrotizing enterocolitis
Pre-Tx immunological risk[a]	5 low	7 low/3 high
Induction IS	5 Simulect™	7 Simulect™ 3 Thymoglobulin™
Artery inflow	5 aorta	8 aorta/2 SMA
Venous outflow	5 IVC	7 IVC/2 SMV/1 PV
Venous outflow S/P	Systemic	7 systemic/3 portal
Total ischemia time	05:30 to 10:20	03:50 to 10:00
Warm ischemia time	23–40 min	30–35 min

SMA, superior mesenteric artery, IVC, inferior vena cava, PV, portal vein.
[a]Pre-Tx immunological risk was defined as Low when panel reactive antibody was found negative, and cross-match test was negative. High risk was defined when panel reactive antibody was found positive, or cross-match test was positive for HLAII.

abnormalities in the control group (NC group). Every mild rejection episode at the time of diagnosis, before other IS was administrated, was included in the study. Cases under ACR with comorbidities were excluded from the study. Among them were cases of infection, chronic intestinal graft dysfunction, chronic extra-intestinal pathologies such as renal insufficiency or uncontrolled diabetes. As control samples were included normal biopsies of post-ITx healthy patient. Cases of normal intestinal histology with non-specific clinical symptoms of intestinal or extra-intestinal disease (vomiting in the 72 h previous to endoscopy, abdominal pain, self-limited diarrhea/increased ostomy output, respiratory symptoms, or episodes of isolated fever) were excluded. If a patient underwent a previous rejection or an infectious episode, the immediately subsequent normal biopsy after the episode was not included in the group for analysis. Consequently, all the biopsies included in this group were from patients that had already presented at least one normal biopsy after an infection or rejection episode.

Clinical records, as well as histological and laboratory studies, were used to both select and classify samples appropriately. Thus, under these strict

selection criteria, 46 samples were processed for qPCR gene expression analysis.

Individual variables with a potential impact on gene expression such as pre-Tx immunological risk, induction and maintenance immunosuppressive therapy, ischemia time, tacrolimus level in peripheral blood, side effects related to immunosuppressive therapy (e.g., lower white cell counts) or post-Tx time to event were included in the analysis.

According to our protocols, patients were maintained on corticosteroids and tacrolimus for baseline immunosuppression. After severe ACR or during the first six months after ITx, all the patients were treated additionally with mycophenolate mofetil (MMF) or sirolimus based on their immunological risk (13). Consequently, each sample was classified in two groups, according to the immunosuppressive regimen: two (DT) or three (TT) immunosuppressive drugs. Moreover, the achievement of expected target blood level (ATL) of tacrolimus was considered for each biopsy.

The histological diagnosis of rejection was made according to the recommendations of the Pathology Workshop of the VIII Small Bowel Transplantation Symposium (Miami, 2003) (3).

Gene expression analysis

The samples were taken by video endoscopy, immediately embedded in RNA later (Ambion, Austin, TX, USA) and stored at −80°C for total RNA extraction. The following set of genes was selected and measured from each chosen biopsy sample:

1. *IFNG*, a key immunostimulatory cytokine that participates in Th1 response priming and has played a role in different cases of ACR (10, 11, 14);
2. *CXCL10* and *CXCL11*, two highly inducible chemokines triggered by *IFNG* and responsible for the recruitment of activated T cells involved in experimental models of intestinal transplant rejection (15, 16).

Total RNA was obtained using Illustra RNA-Spin Mini extraction kit (GE Healthcare, Miami, FL, USA) according to the manufacturer's instructions. The concentration and $A_{260\ nm}/A_{280\ nm}$ of total RNA were measured by NanoDrop UV-vis spectrophotometer (Thermo Scientific, CA, USA). Reverse transcription was performed using 500 ng of total RNA per reaction as described (17). The cDNA was amplified by qPCR using the following primers: IFNGamma fwd: CCAACGCAAAGCATACATGA, IFNGamma rev: TTTTTCGCTT

CCCTTTGC. CXCL10 fwd: TCCACGTGTTCAGATCATTGC, CXCL10 rev: TGATGGCCTTCGATTCTGG. CXCL11 fwd: GGGTACATTATGGAGGCTTTCTCA, CXCL11 rev: GAGGACGCTGTCTTTGCATAGG. β-actin fwd: CCTGGCACCCAGCACAAT, β-actin rev: GCCGATCCACACGGAGTACT.

qPCR was performed using iQ TM SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) as previously described (18). Gene expression was normalized using b-actin gene as reference. Fold induction was calculated using the ΔΔCt method (18) using the average of the non-complicated group (NC) as reference.

Statistical analysis

Comparisons between groups of data were performed with the Mann–Whitney test. All the statistical analyses were performed using GraphPad software (San Diego, CA, USA).

Results

Considering that several post-Tx clinical events and complications may have an influence on intestinal gene expression, we selected a set of samples using strict criteria to exclude cases of unclear classification or mixed etiology. Following these criteria, two groups were defined:

Group NC: Normal. This group includes asymptomatic patients with normal biopsies and normal laboratory results (n = 32). All biopsies in this group were taken according to each patient follow-up protocol, and they belong to 12 patients in our study cohort.

Group R: Rejection. This group includes asymptomatic or symptomatic patients with diagnosis of mild ACR as shown by histopathology analysis. The samples (n = 14) in this group belong to 10 patients of our study cohort. In all cases, the immunosuppressive treatment used at the moment of the biopsy was determined by protocol; no samples were processed once IS was adjusted after the diagnosis of ACR.

As expected, the expression of the analyzed genes was induced during rejection episodes (Fig. 1). The expression of *IFNG, CXCL10,* and *CXCL11* was clearly higher in the R group compared to the NC group (p < 0.05; p < 0.005, and p < 0.05, respectively).

To analyze the effects of the immunosuppressive treatment on gene expression, samples of both groups were classified into DT or TT according to
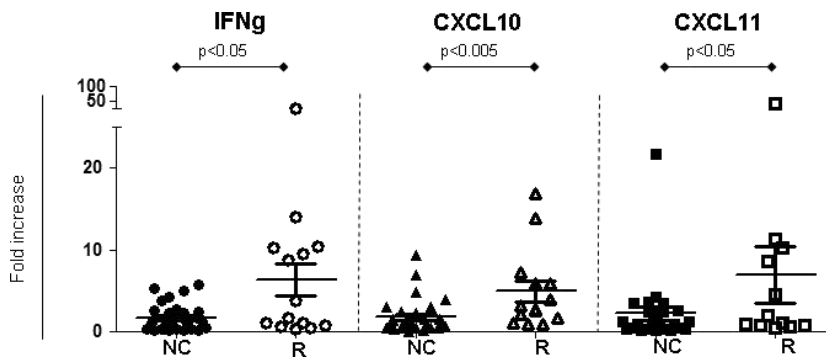
Fig. 1. IFNG, CXCL10, and CXCL11 levels are upregulated in graft mucosa during ACR. Individual sample measurements, mean, and standard error of each group are indicated. NC, no complication group (filled symbols); R, rejection group (open symbols). Statistical level of significance of pairwise comparisons using Mann–Whitney test is indicated.
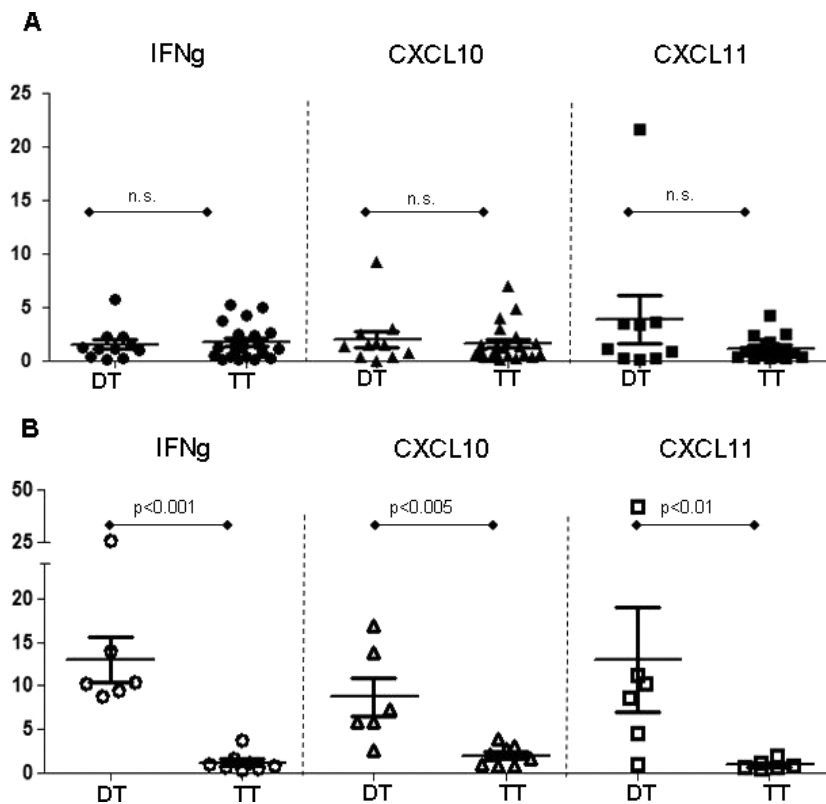

Fig. 2. The immunosuppressive treatment impacts on gene expression during mild ACR episodes. (A) No complication group; (B) Rejection group. The fold increase of gene expression for IFNG (circles), CXCL10 (triangles), and CXCL11 (squares) is depicted. NC, no complication group (filled symbols); R, rejection group (unfilled symbols); DT, double treatment (corticoids + tacrolimus); TT, triple treatment (corticoids + tacrolimus + rapamycin or mycophenolate). Mean and statistical significance according to Mann–Whitney test are shown.

the IS therapy used when the biopsy was taken. No differences of expression levels of the three analyzed genes were found in the NC group (Fig. 2A), whereas expression levels were significantly higher in the R group under DT when compared with the R group under TT (Fig. 2B). Namely, the R group under DT showed a 13-fold increase of *IFNG* expression, whereas the TT group showed a small mean increase below twofold. The difference between DT vs. TT groups was highly significant ($p < 0.001$ Fig. 2B). Similar results were observed for *CXCL10* expression (ninefold increase under DT) and *CXCL11* (13-fold increase under DT).

After the finding of a clear difference in gene expression between patients under TT and DT undergoing ACR, we next reassessed biopsies to determine the degree of histological alterations in all samples of the R group. We confirmed that all the samples of the R group have the same degree of structural damage, and no differences in the number of apoptosis could be found between DT and TT subsets. Typical images of both groups are shown in Fig. 3.

Discussion

ACR is still prevalent and one of the most common cause of graft loss and death after ITx (19). In spite of improved outcomes in the early post-Tx period, delayed ACR is still a major concern (20) and additional diagnostic tools to establish an early diagnosis are awaited. Gene expression

Fig. 3. Histological sections of small bowel allograft with mild acute cellular rejection diagnosis. Characteristically, this level of injury shows preserved global architecture with mild and patchy mixed inflammatory infiltrate and edematous interstitium and increase in crypt epithelial apoptotic bodies (CAB, more than 6 in 10 continuous crypts or multiple CAB in a single one). Panels A and B are from the same patient, who was under double immunosuppressive scheme. Panels C and D are from a patient under triple scheme. H&E staining (magnification of A and C 25×, B and D 400×).
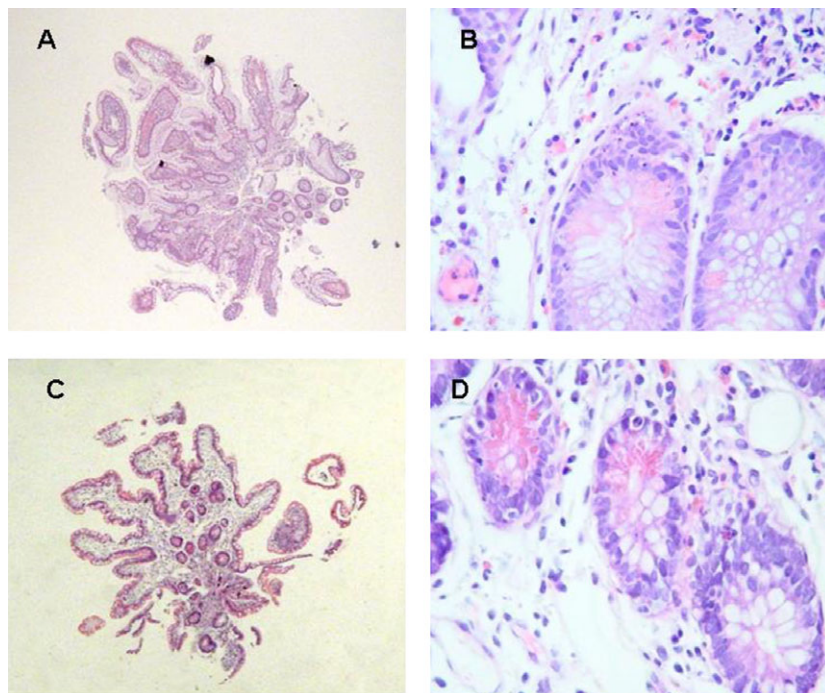
analysis could provide information on ongoing biological processes.

In this study, we measured the expression levels of selected immune related genes in a set of intestinal biopsies from patients undergoing ACR episodes and analyzed the effects of immunosuppressive therapy on gene expression level. We stated a selection criteria used to retrospectively select biopsies to analyze from a tissue bank. The selection of the set of normal samples used as reference to calculate the relative expression of the different genes was particularly stringent to minimize confounders. Previous studies have selected as rejection group for gene assays intestinal ACR with more than grade two in the international grading scheme, corresponding to moderate damage (9). However, we focused on low-grade rejection criteria because these are most frequent situations that imply the most challenging differential diagnostic situations. Mild rejection or grade one is characterized by a patchy mild increase in lamina propria inflammation with increased crypt apoptosis, usually more than six apoptotic bodies in 10 consecutive crypts. Even though crypt apoptosis is the most important feature for diagnosis of rejection, increased number of apoptotic bodies may be noted in other inflammatory and infection processes such as viral enteritis (4). Therefore, the treatment of rejection must be indicated after an accurate differential diagnosis is made.

We focused our analysis on a set of pro-inflammatory genes that are associated to Th1

response, among them *IFNG*, *CXCL10,* and *CXCL11* as they have been reported as important players in ACR in different organs and are candidates to be used as biomarkers of rejection (10, 11). We observed a significant increase in the R group compared to the NC group (Fig. 1). Those findings are consistent with results obtained in animal models describing the critical role of IFN-dependent chemokines in ITx rejection process (15, 16), or in the clinical setting showing the association of *IFNG* production with ITx rejection, either detected by gene expression analysis of the intestinal mucosa (8, 9) or proteomic analysis of intestinal content during ACR episodes (21).

Even though overall results show differences between groups, some variability in the expression levels within each group was observed. This may partly reflect differences in the evolution of each particular process, considering that each biopsy might have been taken at a particular time point of a multistep sequential process. We cannot rule out a bias caused by ACR damage inhomogeneity on the intestinal mucosa, because different individual biopsies were used for gene expression analysis and for histopathology. Because several samples from the same patient at different times of the follow-up were included in the study, we also analyzed any individual factors that may contribute to modify gene expression levels, but we could not detect any special correlation with patient's characteristics considered described in the methods section (not shown).

We further showed that the inclusion of a third drug in the immunosuppressive regime might modulate the expression levels of *IFNG, CXCL10,* and *CXCL11* (Fig. 2). In the case of mild ACR, patients under triple therapy, although developing an ACR episode, show expression levels of the pro-inflammatory genes analyzed that are not different from the normal group. The expression of these genes has been associated to ACR process in different models (12), including small bowel transplant (8, 9). However, these results indicate that small bowel ACR can proceed in spite of subtle changes in the expression of this Th1 signature, according to the IS regime installed. Consequently depending on the type of immunosuppressive treatment used, gene expression levels of IFN, CXCL10, or CXCL11 will be differentially affected, making them unsuitable biomarkers to monitor rejection. No differences could be appreciated related to the specific third drug, MMF or sirolimus (not shown). Although the influence of sirolimus on *IFNG* expression is not clearly established, it is known that *IFNG* signalling may be modulated by mTOR inhibitor molecules (22–24), which may account for the lower expression levels of the *IFNG*-dependent chemokines *CXCL10* and *CXCL11*. On the other hand, it has been reported that MMF may modulate the expression of CXC chemokines on intestinal-derived cell lines (25) and it has been described that the use of MMF in kidney transplant patients downregulates the expression of different cytokines (26). This may also account for the observed reduction in expression of the studied markers in ITx-TT patients.

Several sources of *IFNG* production have been found in the intestinal mucosa, such as NK cells, intra-epithelial lymphocytes, dendritic cells, or lamina propria T lymphocytes. Th1 population during ACR episodes may be an important contributor to the gene expression pattern observed in the intestinal mucosa, although this remains to be proved. Identification of the cellular source may help to define cellular targets for immunotherapy that may improve the management of ACR. Recently, Ruiz and colleagues (9) reported the use of multiplex gene expression profiling by qPCR on FFPE archive biopsies from a tissue bank of follow-up of ITx patients during ACR episodes and in the absence of complications. They have found that different gene expression patterns correspond to different degrees of severity of ACR and confirm their findings by immunohistochemistry. As in the present study, the reported results show important variations among the groups analyzed with significant overlapping among groups. As several immune mediated processes share a common set of effectors and gene pathways that sustain the process (12), the identification of a single marker that is specific for ACR is still elusive. A combination of several markers could constitute a specific determinant for each process. However, as shown here, the type of IS used may have important influence on individual gene expression levels, consequently the effects of these variables should be carefully evaluated before establishing particular cutoff levels for any gene expression analysis. At present in the ITx field, endoscopic and histopathology analyses remain the standard practice to diagnose ACR (27).

Conclusion

Gene expression of *IFNG* and their related chemokines *CXCL10* and *CXCL11* are overexpressed during ACR episodes. The immunosuppressive maintenance scheme influences the expression levels of these genes, indicating that a careful evaluation of these variables should be performed before including these candidates as ACR biomarkers. Further studies are needed to determine clinical implications of these findings.

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