Safe and Successful Treatment of Acute Cellular Rejection of an Intestine and Abdominal Wall Transplant with Vedolizumab.

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Publication status.

Published in Transplantation Direct in January 2020. *Transplant Direct 2020, 20:286–294*.

Disclosure.

This study was partially sponsored by an Investigator Initiated Research Grant from TAKEDA Pharmaceutical Company Ltd.

List of abbreviations.

ABC	Apoptotic bodies in crypts.
ACR	Acute cellular rejection.
ATG	Anti-thymocyte globulin.
AWTx	Abdominal wall transplant(ation).
BSA	Bovine serum albumin.
CIRTA	Congress of the Intestinal Rehabilitation and Transplantation Association.
GCSF	Granulocyte colony-stimulating factor.
GvHD	Graft-versus-host disease.
H&E	Haematoxylin-eosin.
HPF	High-power field.
IBD	Inflammatory bowel disease.
ITx	Intestinal transplant(ation).
IV	Intravenous.
MAdCAM-1	Mucosal addressin cell adhesion molecule-1.
MMF	Mycophenolate mofetil.
PBS	Phosphate-buffered solution.
Treg	Regulatory T-cell.

Abstract.

BACKGROUND. Graft survival rates after intestinal transplantation (ITx) are still the lowest in comparison to other solid-organ transplants. One of the main reasons is the frequent occurrence of acute cellular rejection (ACR). Vedolizumab is an antibody against $\alpha4\beta7^+$ integrin involved in gut homing T-cells which has been approved for inflammatory bowel diseases. We report its off-label use to treat ACR after ITx.

METHODS. Following abdominal wall (AWTx) and ITx, clinical course was followed biochemically. Sequential small intestinal biopsies were taken preceding, during and after ACR treatment with vedolizumab, following the standard therapy regime for inflammatory bowel diseases. Rejection was diagnosed histologically, and pro-inflammatory ($\alpha4\beta7^+$, IL-17^+) and regulatory ($FoxP3^+$) T-cells were analysed by immunohistochemistry.

RESULTS. ACR in both the ITx and AWTx resolved upon vedolizumab treatment, which was safe, evidenced by clearing an astrovirus and primary CMV infection. Only a slight reduction of $\alpha4\beta7^+$ cells in the mucosa was observed, and $\alpha4\beta7^+$ and regulatory T-cells could still move into the lamina propria upon infection.

CONCLUSION. Vedolizumab is a safe treatment option for ACR after intestinal transplantation, but its mechanism is probably not only based on inhibition of gut selective T-cell homing.

Background.

Intestinal transplantations (ITx) have been performed for already 30 years, but its graft-survival rates after five years have plateaued at approximately 50% in the last decade[47]. One of the main causes of graft loss is acute cellular rejection (ACR)[174], characterized by gut-homing of inflammatory cells after priming with donor-derived antigens[105,175]. This results in a mixed inflammatory infiltrate in the lamina propria consisting mostly of mononuclear cells accompanied by apoptosis of crypt epithelial cells and epithelial cell damage[176].

Gut-homing of inflammatory cells is one of the main features in ITx that also occurs in other intestinal diseases, such as intestinal graft-versus-host disease (GvHD) and inflammatory bowel disease (IBD)[177,178]. It requires a set of signalling molecules that are responsible for trafficking of leucocytes specifically to the intestine, including α4β7 integrin[179]. This integrin is highly expressed by pro-inflammatory gut T- and B-cells and eosinophils[179]. Its ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), is overexpressed in endothelial cells of venules in the gut's lymphoid organs and mucosa during inflammation[180]. Vedolizumab, a humanized mouse anti-α4β7 monoclonal antibody (Entyvio; Takeda Pharmaceutical Company, Tokyo, Japan) shows therapeutic efficacy in IBD[181], as well as in other immune-mediated intestinal diseases, such as collagenous colitis and eosinophilic gastroenteritis[182-185]. It is believed to be gut-specific due to its exclusive interaction with the heterodimer of the integrin, thereby blocking the influx of inflammatory cells into the gut[186]. More recent data suggests that vedolizumab might not necessarily work on the acquired immune system but also on the innate system[44].

Current treatment of ACR is focused on suppressing systemic T-cell proliferation and/or depletion, but often this is not successful and the rejecting graft needs to be removed[187]. Thus, alternative pharmacological approaches are urgently needed to treat ACR after ITx and, considering its mechanism of action, vedolizumab could be a promising option.

Here, we describe the intra-intestinal cellular dynamics of a combined ITx and abdominal wall (AWTx) transplant patient with ACR of both grafts who did not respond to regular immunosuppressive therapy and was subsequently safely and successfully treated with vedolizumab.

Methods.

Ethical Approval.

Treatment and follow-up studies were fully understood and accepted by the patient and approved by the Ethical Committee of the University Medical Center Groningen (study number M14.163082).

Immunohistochemistry.

Hematoxylin-eosin (H&E) slides were used according to a standard protocol to diagnose graft rejection. Paraffin-embedded tissue sections of the intestinal biopsies gathered by endoscopy were cut (4 μm) from routine diagnostic blocks, coated on Starfrost slides (3054-1, Klinipath, VWR, Breda, The Netherlands), dried, deparaffinised in xylene and rehydrated in alcohol. Endogenous peroxidase was blocked with 0.3% H_2O_2 in phosphate-buffered solution (PBS) for 30 minutes. The slides were then blocked for 30 minutes with 1% bovine serum albumin (BSA)/PBS before being incubated for one hour at room temperature with a primary antibody against FoxP3 (1:100 Abcam [22510], Cambridge, UK) and IL-17 (1:200 R&D Systems [AF-317-NA], Minneapolis, MN, United States of America). The secondary and tertiary steps were performed with horseradish peroxidase-labelled antibodies (1:50, Dako, Agilent, Santa Clara, CA, USA; rabbit anti-mouse and goat anti-rabbit, respectively) in 1% BSA/PBS supplemented with 1% human serum, incubated for 30 minutes. Binding was detected by 3,3-diaminobenzidine and counterstained with haematoxylin.

The Act-1 (anti-α4β7) antibody was used (1:50 Takeda Pharma A/S, Taastrup, Denmark) for staining of vedolizumab-targeted cells. Frozen intestinal tissue embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) were cut, dried, and fixed with 4% paraformaldehyde for ten minutes. Endogenous peroxidase was blocked with 0.075% H_2O_2 in PBS for 30 minutes, followed by 30 minutes blocking in 1% PBS/BSA, one-hour incubation with the primary antibody, followed by the secondary (rabbit anti-mouse peroxidase-labelled) and tertiary (goat anti-rabbit peroxidase-labelled) antibodies (1:50, Dako, Agilent, Santa Clara, CA, USA) for 30 minutes each. Binding was detected by 3-amino-9-ethylcarbazole and counterstained with haematoxylin.

Definitions.

ACR is defined as the presence of ≥ 6 apoptotic bodies per ten consecutive crypts (ABC) accompanied by crypt epithelial cell destruction and the presence of inflammatory cells in the

lamina propria[176]. Areas where there are higher numbers of cells of interest and ABC selected at lower magnification are herewith referred to as hotspots. All biopsies with different types of staining were scanned first for hotspots. If none was found, a random area was chosen (at least five per slide). A high-power field (HPF) is defined at 40x magnification, with an area of 0.24 mm^2.

Cell counting.

Rejection was identified for diagnostic purposes and reported here in that manner, according to the guidelines for each organ[176,188]. Cell-counting was performed independently by a researcher and a pathologist, and a consensus was reached when discrepancies emerged. All stained cells were counted individually per HPF. The presence of cytoplasmic staining using the antibodies directed against α4β7, IL-17 and FoxP3 was considered positive for the antibody. The average of all available fields was taken for analyses. Primary data were analysed using Microsoft Excel (Microsoft, Redmont, Washington, United States of America), GraphPad Prism 5.0 (GraphPad Software, San Diego, California, United States of America) and SPSS 25 (IBM, Armonk, New York, United States of America). Measurements were then grouped into clinically relevant periods and then presented as the median and range up to twelve months post-transplant.

Case description and Results.

A nineteen-year-old female underwent a subtotal colectomy because of ulcerative colitis. Her diagnosis was changed to Crohn's disease after she presented ulcerations in her oesophagus, stomach, and small bowel, complicated by severe perforations. This led to small bowel resection and consequently an ultra-short bowel syndrome with loss of abdominal domain. She had a distal duodenogastrostomy, drained by a percutaneous gastrostomy[189]. After ten months on home total parenteral nutrition, the patient was screened for ITx due to liver function impairment and jaundice. During the following two years she suffered from several episodes of line infections, malnourishment, and poor quality of life, and was put on the waiting list for a combined ITx with AWTx.

Intestinal and Abdominal Wall Transplantation.

In March 2015, the patient successfully underwent ITx in combination with full-thickness AWTx (the surgical description has been published elsewhere[190]). Crossmatch was negative. A list of clinically relevant episodes, immunosuppression and trough levels are listed in Table 6.1.

The induction of immunosuppression consisted of methylprednisolone (500 mg) and anti-thymocyte globulin (ATG; 9 mg/kg). The maintenance plan initially followed a standard scheme consisting of tacrolimus (8 mg/day, trough levels 13-17 mg/L), mycophenolate mofetil (MMF; 2 g/day, trough levels 2-4 mg/l) and prednisolone. The latter drug is administered in the following manner: 2 mg/kg/day intravenous (IV) for days 1-3 post-ITx; 1 mg/kg/day IV or oral for days 4-8; 0.3 mg/kg/day oral for days 9-30; 0.2 mg/kg/day oral for months 2-3; and 0.1 mg/kg/day oral for months 4-6. Standard treatment of ACR is performed in the department in a stepwise manner; firstly, by increasing tacrolimus dosage; secondly by giving a three-day boost of intravenous methylprednisolone; and thirdly by adding a T-cell depleting agent such as ATG.

On day six post-transplantation, the ileum biopsy revealed signs of grade 1 ACR, treated temporarily with an increased dose of tacrolimus to reach trough levels between 15-20 μg/L. On day fourteen, despite adequate trough levels of tacrolimus (20.6 μg/L), ACR returned together with fever and pancytopenia, the latter requiring cessation of MMF treatment. Granulocyte colony-stimulating factor (GCSF) was administered on day 20 (30 million units), resulting in an increase in blood leucocyte count. The transplanted abdominal wall showed no signs of rejection until day 21 (grade 1). ACR persisted and treatment with methylprednisolone (3 days 1000 mg intravenous) suppressed this for eleven days. ACR (grade 2) returned on day 81. Having considered the previous development of pancytopenia under MMF treatment and limited alternative options, we decided to use vedolizumab because of her history of IBD and its safe and potentially promising mechanism of action.

We treated the patient with 300 mg vedolizumab on weeks 0, 2 and 6 (induction), and every 8 weeks thereafter (maintenance, eight infusions during the period of this study), with biopsy controls. Immunosuppression with tacrolimus continued alongside this treatment with trough levels between 17.8-24.5 μg/L (normal-high) during induction and 6.5-19.5 μg/L (normal) during maintenance.

Table 6.1. **Clinically relevant episodes and immunomodulatory treatment within twelve months post-transplant**. Intestinal and abdominal wall events: (0-2), ITx ACR scores ("0", no rejection, "Ind", indeterminate, "1", grade 1); (N-II), Banff score for AWTx ("N", no rejection, "I", grade 1, "II" grade 2); CMV, primary cytomegalovirus infection. Immunomodulation: T, tacrolimus; MMF, mycophenolate mofetil; S, prednisolone; GCSF, granulocyte colony-stimulating factor; M, metylprednisolone; (*) induction of vedolizumab therapy. Trough levels: (**) closest measurement to the specified date.

Day post-transplant	Events			Immunomodulation	Trough levels	
	Clinical	Intestine	Abdominal wall		T (µg/L)	MMF (mg/L)
5		(1)	(N)	T + MMF + S		<0.2
8		(0)	(N)	T↑ + MMF↑ + S	13.4	0.2**
13	Pancytopenia	(1)		T↑ + ~~MMF~~ + S	20.6	0.4
20		(1)	(I)	T + S + GCSF	6.8	<0.2**
24		(0)		T + S	14.8	
25			(I)	T + S	11.9	
32			(I-II)	T + S	13.6	
33		(1)		T + S	12.3	
40		(1)		T + S + M	18.4	
42			(I)	T + S	18	
52		(0)	(I)	T + S	15.2	
61		(0)		T + S	22.8	
62			(N)	T + S	25.7	
67		(Ind)	(I)	T + S	17.1	
81		(1)		T + S	21.1**	

Day post-transplant	Events			Immunomodulation	Trough levels	
	Clinical	Intestine	Abdominal wall		T (µg/L)	MMF (mg/L)
90				T + S + Vedolizumab*	17.8	
95		(1)	(I)	T + S		
104				T + S + Vedolizumab*	24.5	
109		(1)		T + S	20.6	
129				T + S + Vedolizumab*	24.1	
134		(0)	(N)	T + S	18.7**	
186				T + S + Vedolizumab	19.5	
189		(0)	(I)	T + S		
241				T + S + Vedolizumab	17	
254	Astrovirus			T + S		
258		(0)		T + S + Vedolizumab	8.4**	
316	CMV	(0)		T + S	6.5**	
330	CMV			T + S	7.3**	
336	CMV	(0)		T + S + Vedolizumab	8.8	
375		(0)		T + S	5.5**	

Immunosuppression with vedolizumab.

Signs of rejection in the ITx and AWTx grafts disappeared during the induction period and her clinical status steadily improved during the period of this study (one year follow-up). This was accompanied by a slight reduction of vedolizumab-targeted cells in the intestinal graft and an increase in IL-17$^+$ Th17 cells (Figure 6.1) and eosinophils (Figure S6.1). Treatment was safe, since the patient could clear an astrovirus infection on day 259 post-ITx, diagnosed by RNA analysis from faecal samples. Remarkably, also a primary CMV infection (between days 316 and 337 post-ITx, IgM and DNA-positive in PCR) during valgancyclovir prophylaxis cleared without any clinical symptoms. This was accompanied by an increase of $\alpha4\beta7^+$ (pro-inflammatory) and FoxP3$^+$ (regulatory T-cells, Tregs) cells in the graft as well (Figure 6.1). Maintenance therapy continued with tacrolimus (trough levels 5-7 μg/L) and prednisone (10 mg/day). Both cleared infectious episodes occurred during vedolizumab treatment (between the sixth and seventh infusions of vedolizumab).

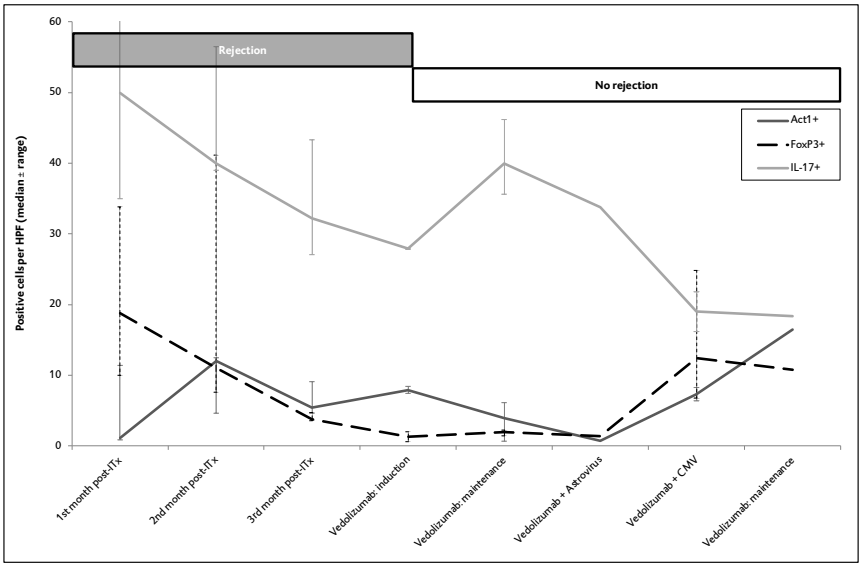

Figure 6.1. **Vedolizumab is safe to use in intestinal transplant recipients to treat ACR**. Timeline showing the presence of proinflammatory cells (Act-1$^+$, IL-17$^+$) and Treg (FoxP3$^+$) in the intestinal graft related to the most clinically relevant events during the first year post-ITx. Results are herewith presented in periods as the median and range of positive cells per HPF (see Methods section for more information). The pre-vedolizumab period is represented per month, and the vedolizumab treatment period is divided into induction (three infusions within two months), maintenance without comorbidities (four months), primary astrovirus (one biopsy), and CMV infections (20 days), and the last biopsy before the end of the first year. More details within each period can be found in Table 6.1. ACR, acute cellular rejection; CMV, cytomegalovirus infection; HPF, high-power field; IL-17, interleukin-17; ITx, intestinal transplantation.

Discussion.

This report presents the first patient with acute cellular rejection after a combined intestinal and abdominal wall transplantation who was safely and successfully treated with vedolizumab. Astrovirus and primary CMV infections were uneventfully cleared, and there were no episodes of ACR after the therapy started. This case allowed us to study immune-cell dynamics surrounding episodes of infection and rejection of an intestinal and abdominal wall graft treated with an integrin-specific antibody.

This patient had a background of Crohn's Disease but there were no signs of recurrent disease. Rejection could not be controlled by the standard treatment options with tacrolimus, MMF or methylprednisolone. Considering her experience with infliximab and the proposed mechanism of action of vedolizumab, it was chosen as the most promising treatment option. ACR disappeared during induction period alongside a decrease of the drug's target cells, which then reappeared under maintenance. This suggests that the therapeutic effect of this drug is not solely based on blocking the entry of $\alpha4\beta7^+$ integrin cells in the intestinal mucosa, as was proposed by others[44,191].

The dynamics of $\alpha4\beta7^+$ cells in the graft may indicate that auto reactive leukocytes with increased $\alpha4\beta7^+$ on their surface that initiated the rejection episode were blocked or downregulated during induction[192]. Studies in a large IBD population have shown that there are increased levels of expression of pro-inflammatory markers such as that of α4 subunit and regulatory molecules for Th17 cells[193]. Patients with IBD also have impaired functions of Th17 helper cells, which maintain homeostasis between the intestinal mucosa and the microbiota[44,193]. Interestingly, our study showed an increase in the presence of this cell type during induction therapy with vedolizumab, alongside the improvement of her clinical picture. On the other hand, during the infection periods there are $\alpha4\beta7^+$ cells migrating to the gut and in the maintenance phase an influx of $\alpha4\beta7^+$ was observed that was not accompanied with rejection (Figure S6.2). An explanation could be that α4β7 upregulation on leucocytes is less prominent in these situations and that these $\alpha4\beta7^+$ cells use alternative routes for migration into the gut. Furthermore, in IBD patients it has been shown that vedolizumab also changes the transcriptional signatures of the innate immune system[44]. Additionally, a Crohn's Disease patient with a grade 3 ACR episode and an infliximab-resistant, refractory rejection was also given vedolizumab in another centre. This patient's inflammatory signs (ACR, inflammatory stenosis) also resolved after induction

therapy, and in this patient maintenance therapy was not needed (Dr A. Pascher, 2015, written personal communication).

Other cases of ITx ACR treated with vedolizumab were presented at the XV Congress of the Intestinal Rehabilitation and Transplantation Association (CIRTA, 2017; Beduschi *et al.* [abstract 320.3] and Norsa *et al.* [abstract 1a.131]). Four paediatric patients with a background of microvillus inclusion disease were transplanted and suffered from ACR. Induction with vedolizumab was successful, but maintenance treatment was not effective (L. Norsa, poster presentation, CIRTA, 2017, abstract 1a.131). The underlying pathophysiology of graft rejection may differ in patients with such disease, as they do not suffer from an impaired immune response that is typical for IBD patients.

Our patient suffered from viral infections during this treatment, which resolved without complications, indicating that this drug is safe. Other recent evidence supports this notion[194]. CMV affects primarily the endothelial cells of the intestine[195]. The union between the integrin α4β7 and the endothelial cell-expressed receptor MAdCAM-1 is blocked with vedolizumab. The asymptomatic resolution of this primary CMV infection could be therefore associated to a milder, yet effective inflammatory response in the vessel walls[44].

One could speculate that the influx of protective T-regs should be compromised by this treatment. However, FoxP3$^+$ T-regs were still present in the graft during vedolizumab treatment and infiltrated the graft during infections (Figure 6.1). This may support other studies that show α4β7 levels are relatively low in a specific subset of FoxP3$^+$ T-regs[192], and thus may depend on different gut-homing mechanisms[44].

Notably, the transplanted abdominal wall of this patient was not a sentinel marker for rejection of the intestinal graft, as observed by others[196]. Rejection of the skin started two weeks after it was detected in the intestine and resolved after three infusions of vedolizumab, later than the intestinal graft. Although vedolizumab is proposed to be gut-selective, we also observed the resolution of rejection on the transplanted abdominal wall. Transplanted skin has an inflammatory microenvironment that might differ from normal skin immune reactions. Unfortunately, technical limitations prevented us from analysing dynamics of vedolizumab target cells in the transplanted abdominal wall as only formalin-fixed paraffin-embedded tissue was available, while the currently available antibody against α4β7 only works on frozen tissue. There is clinical evidence in psoriasis and GvHD of the skin that points to an indirect systemic effect of vedolizumab that might help explain the effects observed on the AWTx[197,198].

Although these hypotheses cannot be substantiated within this case report, new studies in vedolizumab-treated patients should be focussed on $\alpha4\beta7^+$ upregulation in different cell activation processes (alloreactivity, autoimmunity, infection), the use of alternative cell migration routes in these situations and alternative mechanisms of action as suggested in IBD patients[193]. Furthermore, vedolizumab should be studied in more patients undergoing intestinal transplantation without a history of inflammatory disease[199] (also in CIRTA, 2017, Norsa *et a*l. [abstract 1a.131]).

In conclusion, we present an observational study of a unique case with successful treatment of acute cellular rejection of an intestinal and abdominal wall graft which was safe and did not hamper the clearance of an astrovirus and primary CMV infections. Our analyses on the dynamics vedolizumab targets and Tregs suggest that $\alpha4\beta7^+$ cells do play a role in ACR but that cell migration to the gut can also use alternative routes and/or that vedolizumab has additional mechanisms of action. This unique case taught us that vedolizumab can be considered as safe treatment option for treating ACR in patients who failed conventional treatment thereby adding a treatment option improving graft survival in intestinal transplantation.

Acknowledgements.

TAKEDA Pharmaceutical Company Ltd. supplied the Act-1 antibody for immunohistochemistry.

We would like to thank Dr. Andreas Pascher for providing us with valuable information on his patient from the Charité – Universitätsmedizin hospital, Berlin.

Supplementary material.

Supplementary results.

Histopathological analysis of high-power fields shows influx and efflux of vedolizumab-targeted cells (eosinophils and IL-17^{+} cells in Figure S6.1 and T-cells in Figure S6.2).

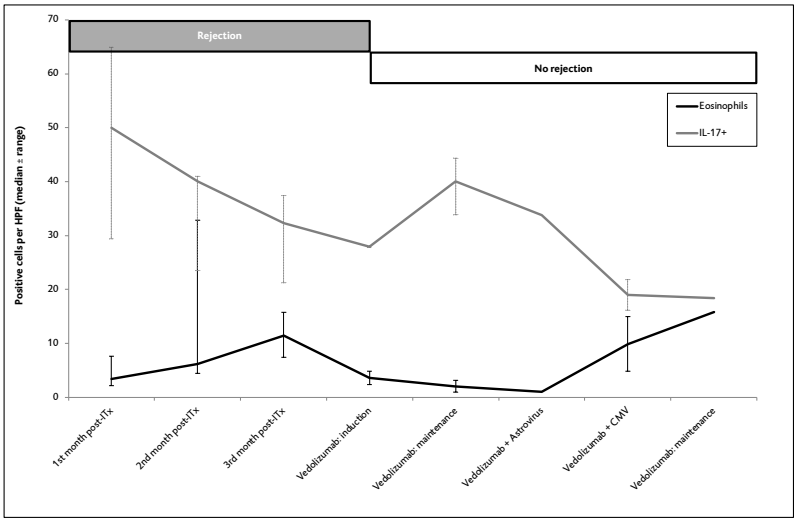

Figure S6.1. **Vedolizumab-targeted cells are actively infiltrating the graft during treatment**. Timeline shows the presence of T-helper cells (IL-17^{+}) and eosinophils in the intestinal graft related to the most clinically relevant events during the first year post-ITx.

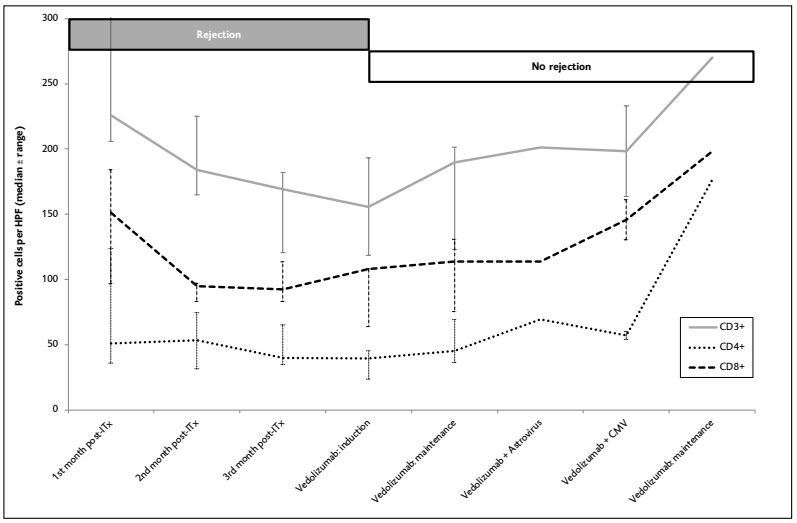

Figure S6.2. **Vedolizumab is safe to use in intestinal transplant recipients to treat acute cellular rejection**. Timeline showing the presence of pro-inflammatory cells (Act-1+) and Tregs (FoxP3^{+}) in the intestinal graft in relationship with the most clinically relevant events during the first year post-ITx.

"Do, or do not. There is no try."

George Lucas.