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Managing Immunological Challenges in Intestinal Transplantation

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CONTENTS

ABBREVIATIONS

ACR	Acute Cellular Rejection
AMR	Antibody-mediated Rejection
AT_1R	Angiotensin II Type 1 Receptor
Anti-AT_1R	Anti-Angiotensin II Type 1 Receptor Antibodies
AWTX	Abdominal Wall Transplantation
CMV	Cytomegalovirus
DSA	Donor-specific Antibodies
DRWR	Donor-to-Recipient Body Weight Ratio
EBV	Epstein-Barr Virus
ET_AR	Endothelin Type A Receptor
Anti-ET_AR	Endothelin Type A Receptor Antibodies
GVHD	Graft Versus Host Disease
HLA	Human Leukocyte Antigen
HLAabs	anti-HLA antibodies
HPN	Home Parenteral Nutrition
IBD	Inflammatory Bowel Disease
I/R	Ischemia/Reperfusion
IRI	Ischemia/Reperfusion Injury
ITX	Intestinal Transplantation
IVC	Inferior Vena Cava
IVIG	Intravenous Immunoglobulin
LBP	Lipopolysaccharide Binding Protein
MHC	Major Histocompatibility
MVTX	Multivisceral Transplantation
mMVTX	Modified Multivisceral Transplantation
non-HLAabs	non-HLA antibodies
PP	Plasmapheresis
PTLD	Post-Transplant Lymphoproliferative Disorder
SMA	Superior Mesenteric Artery
SMV	Superior Mesenteric Vein
TCAIM	T-cell Activation Inhibitor Mitochondrial
TNF-alpha	Tumornecrosis Factor Alpha
VCA	Vascularized Composite Tissue Allotransplantation

I. INTRODUCTION

Intestinal transplantation (ITX) is the only curative therapy for patients suffering from irreversible intestinal failure who fail *home parenteral nutrition* (HPN) and intestinal rehabilitation[1]. Although the general acceptance of ITX has taken much longer than for other solid organ transplantations, the increasing short-term and long-term survival rates have turned ITX from an experimental procedure to the standard of care for eligible patients[2-4]. However, the indication and timing of transplantation, but most of all the surgical and immunological challenges after transplantation are still a matter of international and interdisciplinary discussion.

1. Indication for Intestinal Transplantation

ITX is considered for patients who develop severe complications under HPN, like recurrent central venous catheter infections or cholestatic liver dysfunction, and who therefore face a significantly elevated risk of death on HPN[5,6]. In this context, ITX has become a life-saving procedure.

Recent data suggest that the indications for ITX should be expanded to include its use as a preemptive and rehabilitative procedure[7], which would avoid the occurrence of HPN failure and help recover patient autonomy[1,8].

In addition to isolated ITX, intestine-including transplantation procedures in varying combinations are performed, which are also referred to as *modified multivisceral transplantation* (mMVTX: stomach, duodenum, small intestine, pancreas) or *typical multivisceral transplantation* (MVTX: stomach, duodenum, small intestine, liver, pancreas).

Such transplantation procedures are proposed to patients with intestinal failure and additional liver disease, "surgical abdominal catastrophes", slow-growing semi malignant tumours and complete portomesenteric thrombosis with liver cirrhosis. In some patients the additional transplantation of the large intestine, kidney or abdominal wall can be required, depending on the underlying disease.

2. Surgical Challenges of Intestinal Transplantation

ITX and especially MVTX is a time-consuming and complex surgical procedure. Many ITX- and MVTX-recipients are in a deteriorated state of health by the time of transplantation and have undergone several previous surgical procedures, which have often caused severe adhesions or even a "frozen abdomen". In addition, multiple laparotomies have often caused scarring and restriction of rectus fascia and skin. Due to the mandatory relatively short ischemic time of the intestinal graft (six hours), the complex surgical procedure of diseased organ removal and transplantation of the graft should be carried out in a specialized centre by experienced surgeons, who are confident with the strategy and potential risks of the operation and the difficulties of abdominal wall closure.

2.1 Surgical Procedure of Intestinal and Multivisceral Transplantation

Many different principles and various modifications of the transplantation of intestine containing grafts have been described in the literature[1,4,8,9] and will be summarized briefly.

All surgical procedures include the removal of the native bowel and – in case of MVTX – a native hepatectomy.

The arterial reconstruction in ITX is performed through a small aortic patch and the superior mesenteric artery (SMA) to the recipient's infrarenal aorta[10]. For MVTX, a Carrel's patch (defined as an aortic patch, including the coeliac trunk and the SMA from the donor) is anastomosed to the recipient's infrarenal aorta.

The venous drainage of the donor's superior mesenteric vein (SMV) into the portal system should always be preferred in isolated ITX due to its physiologic and possible immunologic advantages but depends on the technical feasibility of accessing the recipient portomesenteric axis[11]. Otherwise, the SMV may also be drained into the recipient SMV, splenic vein or inferior vena cava (IVC). For MVTX, the venous drainage of the whole organ package is reconstructed through the IVC and the donor liver using the piggyback technique. Sometimes, a preliminary portocaval shunt of the native vessels is performed in MVTX-recipients. The idea is to prevent portal hypertension and bleeding during the sometimes time-consuming dissection and removal of the diseased organs[12]. The shunt can be left permanently or it can be disconnected following graft reperfusion.

The restoration of intestinal continuity in ITX-recipients is performed via proximal end-to-end or side-to-side jejuno-jejunostomy and distal end-to-side ileo-colostomy. In MVTX-candidates, the upper gastro-intestinal continuity is re-established through a proximal anastomosis of the graft stomach to the recipient gastric cuff or abdominal oesophagus. The distal reconstruction is performed in a similar technique to that for ITX-candidates. In all cases, an additional temporary Bishop–Koop ileostomy is constructed for diagnostic purposes, which can be taken down after approximately six months, once an adequate restoration of oral nutrition and stabilization of the immunosuppression therapy has been achieved[10]. Many centres additionally include a part of the colon, mainly the right hemicolon, especially in patients who do not have any remaining native colon or in those where the native colon is severely injured by the underlying disease (e.g. motility disorder, Crohn's disease). Other centres reported to have abandoned this technique due to its potential to increase mortality[13].

2.2 Abdominal Wall Closure after Intestinal and Multivisceral Transplantation

At the end of the surgical procedure of ITX and especially MVTX, a successful primary closure of the abdominal wall is crucial, in order to avoid postoperative abdominal infections, fistulas and resulting mortality. Unfortunately, abdominal closure is a major technical challenge in these patients, due to several reasons[14,15]:

Firstly, the majority of ITX- and MVTX-recipients have extensive abdominal scarring or a so-called 'frozen abdomen' as a consequence of the multiple previous surgical procedures. Subsequently, they suffer from a relevant loss of the abdominal domain and an insufficient elasticity of the abdominal wall[16]. Very often large portions of the abdominal wall itself are scarred and injured from prior laparotomies, enterocutaneous fistulas, reconstructed ostomies or invasive desmoid tumours, so that a concomitant resection of the abdominal wall is often inevitable[14,17].

Secondly, the ideal *donor-to-recipient body weight ratio* (DRWR) is reported to be between 1.1 and 0.76, thus keeping donor and recipient sizes as identical as possible[18]. However, concerning ITX and particularly MVTX, this approach disregards the discrepancy between the intact abdominal cavity of the donor and the inseparable frozen abdomen of the recipient with a severely injured abdominal wall. Neither an optimal donor-to-recipient ratio of BMI nor an optimal DRWR can guarantee the successful primary closure of the abdominal wall. The use of smaller donors would be the best approach but is challenging given the increasing organ shortage. As a consequence, waiting list mortality is also increasing and enforces the acceptance of inadequately size-matched donors[14,19].

Thirdly, a tension-free primary closure is further hindered by the oedema of the graft following reperfusion and of the body trunk due to the intraoperative fluid resuscitation[20]. Forcing primary abdominal closure despite tension can lead to an abdominal compartment syndrome, which does not only risk graft ischaemia and necrosis but was also shown to be detrimental[21]. Although a primary abdominal closure of all abdominal wall layers is the preferred technique, this goal is only achieved in 50-85%. Unfortunately, a secondary wound dehiscence appears in 20-33% of the patients[14,19].

In view of the described technical challenges, the approach of a staged abdominal closure has been introduced from the field of plastic surgery, where it is used for adults with severe abdominal trauma or children with omphaloceles and gastrochisis[14,22].

For the technique of a staged abdominal closure, the use of different materials like absorbable and non-absorbable mesh, acellular dermal matrix or donor allofascia, augmented by vicrylmesh has been described in the literature with varying success[23]. In all these techniques, the crucial step is to achieve a rapid skin closure in order to cover the prosthetic material and prevent wound infection and potential fistula formation. If necessary, split- or full-thickness skin grafts may be considered.

Some centres use regional myocutaneous or fasciocutaneous flaps in a one-stage procedure, to avoid the placement of alloplastic material. This is a reliable method for permanent closure, but results in anatomic changes and is associated with an increased morbidity, particularly in children[14,21]. Therefore, a*bdominal wall transplantation* (AWTX) has come into focus. AWTX as full thickness, vascularized, myocutaneous free flap, was first described by Levi and Selvaggi[24,25]. It allows for primary closure without entailing any permanent anatomical changes and permits early mobilization and rehabilitation due to a higher stability and low infection risk. This tech-

nique may involve a prolonged stay in the operating room with subsequent prolonged anaesthesia and a potentially increased morbidity. However, recent promising data of the Oxford Transplant Centre even suggest immunological advantages despite the additional high immunogenicity of the transplanted skin[26] and could show the beneficial effects of AWTX through improved surgical techniques[14,27,28].

3. Immunological Challenges of Intestinal Transplantation

The correct indication and the surgical procedure of this type of transplantation are certainly demanding. Nevertheless, the major obstacle for a successful outcome after ITX is to overcome the high immunogenicity of the intestine.

Due to the large intrinsic lymphoid mass and immunogenic nature of the intestine, ITX-recipients are susceptible to rejection and require high amounts of immunosuppression. As a consequence, they also suffer from higher infection rates and have a greater risk of *post-transplant lymphoproliferative disorder* (PTLD) or *graft versus host disease* (GVHD) than any other transplant-recipient. These circumstances pose great challenges and may have a high impact on longterm patient and graft survival as their sequelae are often extremely difficult to cope with[29].

3.1 Rejection

Intestinal immunogenicity is distinguished by a constant colonization with microorganisms, an extensive amount of gut-associated lymphoid tissue, large numbers of resident leukocytes and especially the strong expression of histocompatibility antigens. As a consequence, ITX-recipients can mount high cellular and humoral alloimmune responses soon after transplantation.

Despite increasing survival rates, *acute cellular rejection* (ACR) remains to be the major obstacle for longterm patient and graft survival and is the leading cause of intestinal graft loss. The clinical occurrence of ACR is usually observed within the first months after ITX and most patients experience at least one episode. Common clinical features are fever, nausea, vomiting, diarrhoea, abdominal distension and pain. In addition, there is a considerable increase of stomal effluent[30]. During that time, the recipient lymphocytes infiltrate the donor graft's gut-associated lymphoid tissue. The high immunogenicity of the allograft originates from the genotype of the intestinal epithelial cells, which remains that of the donor. The maximum of lymphoid tissue is located in the terminal ileum, which is therefore the region with the highest degree of acute rejection[31].

Due to the constant intense immune stimulation, ITX-recipients require a higher immunosuppression than most other transplant-recipients. Unfortunately, potential non-invasive markers like Citrulline and Calprotectin have not been included in clinical routine because of their low specificity[32,33]. Thus, frequent endoscopies through a diagnostic stoma are crucial in order to detect rejection, but naturally expose recipients to complications like graft ulceration and perforation[34,35]. In order to perform frequent biopsies, especially of the terminal ileum, a diagnostic

ileostomy is placed during the transplantation, which is restored within the first year post-transplant.

On endoscopy, several macroscopic and microscopic patterns are associated with acute rejection including mucosal erythema, congestion, shortening and flattening of the villi, ulcerations and erosions. Unfortunately though, these findings are also observed in infectious graft injury. Furthermore, ACR can be restricted to a specific segment of the intestine and may thus be a focal finding especially in the distal ileum of the graft[31,36]. The exclusive procedure of endoscopy alone has considerable specificity of 93%, but a low sensitivity of only 52%[37]. Thus, the gold standard for diagnosing ACR remains histology.

Whereas the Banff classification has been developed to diagnose rejection in most other solid organ transplantations, no standard grading system exists to evaluate rejection in ITX. Several similar grading schemes are in use, which use the apoptotic body count as a key parameter in diagnosing rejection[38]. The apoptosis of crypt cells is a physiologic and important process for the natural regulation of the intestinal epithelium, however, during rejection, this process is clearly more extensive. Unfortunately, an increased rate of crypt cell apoptosis is also observed in several immunologic or inflammatory diseases like GVHD, bacterial or viral enteritis. In addition, the early clinical signs of intestinal graft rejection like diarrhoea, abdominal distension and pain are unspecific symptoms, which may account for rejection but also for infectious enteritis, drug-induced toxic reactions or even necrotizing enterocolitis[39,40]. Viral infections, especially adenovirus or CMV can clinically and histologically mimic rejection and may account for a high number of misdiagnoses, subsequent unnecessary overimmunosuppression and the aggravation of the clinical course of viral infection can be fatal[41]. Clinically, no reliable way in distinguishing infection from rejection exists, so that the correct diagnosis depends on the pathologist's relevant experience and the time until viral infection declares itself[42], which may delay appropriate therapy. Thus, subacute and subclinical rejections often remain undetected and may progress to severe rejections within days, risking graft and patient loss[43].

3.2 Infection

ITX-recipients are prone to infections, because of the intense immunosuppression they receive, the high bacterial load and the large number of mucosal leukocytes located in the intestine[44].

In the first five years after transplantation, infections account for 48% of the overall mortality and are thus a major reason for mortality in ITX-recipients[45]. Interestingly, patients often suffered from unknown coexisting infections although the diagnosed primary cause of death had not been sepsis. Especially following rejection and subsequent increased immunosuppression, the mucosal barrier can be severely injured, resulting in translocation, peritonitis and bacterial or fungal blood stream infections[46].

Bacterial infections mainly originate from either bacterial translocation disseminating through the portal vein system or else via the lymphatics which contain bacteria and are divided during procurement. Intestinal lymph then leaks into the abdominal cavity, as a potential cause of

peritonitis. Thus typical bacterial infections are gram-negative enterobacteria. According to one study, the infection rate within the first month is 57.5%, with an average of 10.78 ± 8.99 days to the first appearance of infection. Naturally, the prevalent sites of infections were primarily the abdomen as well as pulmonary, wound and urinary tract infections[47].

The most common viral infections are Cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) infections.

Approximately 15-30% of ITX-recipients experience CMV-infection, which can result in graft loss and patient death. Especially CMV-negative patients who receive CMV-positive grafts are at risk, so that this constellation is often avoided. The symptoms of CMV-enteritis include fever, increased stomal output, gastro-intestinal symptoms, leucopenia and flulike symptoms, and are thus very similar to those of ACR. The diagnosis of CMV-infection is made by measuring CMV-antigenemia, as well as by graft endoscopy and mucosal biopsies. Ulcerations within the mucosal layer are typical on endoscopy, but the concrete diagnosis of CMV can only be confirmed by the histopathological findings of inclusion bodies. Antiviral therapy should be initiated with ganciclovir or foscarnet (in case of ganciclovir resistance) immediately after diagnosing CMV-infection. In addition, immunoglobulin can be administered. A reduction of immunosuppression is certainly advisable, but not to the point where it is discontinued, in order to avoid a breakthrough rejection.

Similar to CMV, EBV-negative recipients also run a higher risk of developing EBV, especially if the donor is EBV-positive. Typically, patients suffer from high temperatures, sickness as well as flulike symptoms. An increase in liver enzymes in addition to splenomegaly and lymphadenopathy can be indicative for EBV. Treatment includes an immediate reduction of immunosuppression, but the recurrence rate is about 20%[48].

3.3 Post-Transplant Lymphoproliferative Disorder

The biggest complication of EBV-infections is the development of PTLD. Again, due to the higher immunosuppression, ITX-recipients show an increased incidence of PTLD compared to kidney- or liver transplant-recipients. In fact, the risk of PTLD was also reported to be higher after MVTX than after ITX alone[49].

The first manifestation of PTLD is usually between two weeks and six months post-transplant, but it can also appear at a later time, so that a regular surveillance should be performed using in situ hybridization staining for EBV as well as RNA and EBV polymerase chain reaction.

In order to initially prevent PTLD, different regimens are being used, such as longterm prophylaxis with *ganciclovir*, *valgancyclovir* or IVIG for several months or even one year after transplantation. Another approach is to use prophylaxis of maximal six weeks and start a pre-emptive therapy in case increased EBV replication is observed.

In order to verify the diagnosis of PTLD, biopsies can be taken either from clinically swollen lymph nodes or otherwise from radiologically conspicuous tissue. If, however, PTLD is located

in the intestinal graft itself, the differential diagnosis of PTLD versus CMV or rejection can be challenging. Sometimes, a typical monoclonal or polyclonal immunoglobulin band is shown in the serum, which can help to identify PTLD. B-cell lymphomas are usually more common than T-cell lymphomas, so that a determination of the abnormal lymphocytes sites may be helpful.

A reduction of immunosuppression may sometimes already induce a remission and should thus be the first therapeutic step once the diagnosis of PTLD is affirmed[50]. If, however, there is no evidence of improvement, immunosuppression should entirely be stopped and an additional chemotherapy, including R-CHOP (*rituximab, cyclophosphamide, doxorubicin, vincristine* and *prednisone*) or similar immunotherapy may be initiated. Radiotherapy is not considered as first-line intervention but can exceptionally be used against PTLD in children and adults[49]. Although PTLD is often multifocal and not mainly located in the intestinal graft itself, the intestinal graft may have to be removed as a final option.

3.4 Graft Versus Host Disease

Due to the high immunogenicity of the intestine, ITX-recipients are not only at a higher risk to acquire rejections, infections and malignant diseases but also to develop GVHD. In fact, the intestine's own population of lymphoid cells can raise immunologic response against the host, which manifests as GVHD and occurs in 7-9% of ITX-recipients[51].

GVHD is mainly diagnosed via histology, because it may be subclinical. Patients who present with acute GVHD often show a skin rash and have fever, leukopenia and diarrhoea approximately one to two months following transplantation. In addition, clinical symptoms like malaise, anorexia, arthralgia and abdominal pain have been reported, which again is clinically very similar to acute rejection. However, the typical skin rash, which manifests on the patients' torso soon indicates the diagnosis but has to be confirmed by skin biopsy in order to rule out infectious or drug-induced erythema.

Once the diagnosis of GVHD is histologically confirmed, an immediate treatment with high-dose steroids or T-cell depleting agents like *thymoglobulin* should be administered[52].

II. PRESENTATION OF PAPERS

In this section six clinical and experimental studies will be presented. In the first two publications, we characterized humoral antibody-mediated immune responses after ITX. The following two studies describe our findings of TNF-alpha triggered inflammatory early and late intestinal graft injuries and the last two papers suggest non-invasive rejection markers to prevent and treat allograft rejection at the earliest possible time.

1. Humoral Immune Responses after Intestinal- and Multivisceral Transplantation

In spite of a negative crossmatch prior to transplantation, ITX-recipients experience numerous rejections including humoral immune responses at an early stage after transplantation. Yet, *antibody-mediated rejection* (AMR) in ITX-recipients is not well described, the screening of *donor-specific antibodies* (DSA) against *human leukocyte antigen* (HLA) is not generally accepted and ITX is still performed across a positive crossmatch. The role of antibodies against non-HLA (non-HLAabs) in ITX-recipients has also not yet been recognized, but the high load of immunogenic tissue in the intestine suggests that local self-antigens may represent an additional trigger for humoral immune responses. Since no proper characterization of the typical histological features and clinical consequences of AMR in ITX exist, we performed two clinical studies to investigate the impact of HLA- and non-HLA antibodies in ITX-recipients.

1.1 The Role of Donor-specific HLA Antibodies in Intestinal- and Multivisceral Transplantation

Gerlach UA, Lachmann N, Sawitzki B, Arsenic R, Neuhaus P, Schoenemann C, Pascher A:

"Clinical relevance of the de novo production of anti-HLA antibodies following intestinal and multivisceral transplantation."

(Transpl Int. 2014;27(3):280-289)

AMR is a well described phenomenon in kidney or heart transplantation. The major diagnostic findings to characterize AMR include clinical signs of acute rejection and allograft dysfunction, histological changes indicative of acute injury, deposition of C4d (a split product of complement), and the detection of DSA[53-55].

Only recently, the importance of AMR in ITX or MVTX and its potential impact on longterm graft survival have been published and it is now recognized that the humoral arm of the immune system plays an important role in mediating allograft dysfunction[55,56]. Mounting evidence now suggests an association of pre- and/or post-transplant DSA with rejection and graft loss in ITX- and MVTX-recipients.

The majority of ITX-recipients at our centre had been screened for HLA antibodies (HLAabs) pre- and post-transplant[57]. An adequate treatment with *plasmapheresis* (PP) and *intravenous immunoglobulin* (IVIG) was introduced at the earliest detection of DSA[57]. *Rituximab* (MAB

THERA®, Hoffmann-La Roche, Switzerland) and/or *bortezomib* (Velcade®, Janssen-Cilag, Germany) were additionally applied in case of DSA persistence and/or treatment-refractory rejection[57].

Regarding the results of the presented study, the development of DSA after ITX is clearly associated with rejection. Furthermore, DSA levels could be reduced in the majority of patients by applying the above named therapy. In a subanalysis, we could also show that a high number of antigens and epitope mismatches between donor and recipient significantly affected the formation of *de novo* DSA. Immediate diagnosis and therapy, including B cell depletion and plasma cell inhibition, are therefore crucial to prevent severe graft injury[57].

The described clinical and histological features may contribute to the characterization of AMR as an entity of vascular rejection in ITX-recipients[57].

"Clinical relevance of the de novo production of anti-HLA antibodies following intestinal and multivisceral transplantation."

Gerlach UA, Lachmann N, Sawitzki B, Arsenic R, Neuhaus P, Schoenemann C, Pascher A

Transpl Int. 2014;27(3):280-289

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1.2 Humoral Immune Responses May Be Triggered by Non-HLA Antibodies in Intestinal and Multivisceral Transplantation

Gerlach UA, Lachmann N, Ranucci G, Sawitzki B, Schoenemann C, Pratschke J, Dragun D, Pascher A:

"Non-HLA Antibodies May Accelerate Immune Responses After Intestinal And Multivisceral Transplantation."

(Transplantation. 2016 Aug 5. [Epub ahead of print])

The significant improvement in developing specific immunohistochemical and serologic diagnostic tools has helped immensely to identify humoral immune responses targeting HLA. Nevertheless, antibody-mediated mechanisms continue to deteriorate allograft function and longterm survival in all solid organ transplantations.

Interestingly, acute and chronic rejections have been observed following transplantation in HLA-identical siblings indicating that other humoral immune responses beyond *major histocompatibility* (MHC) antigens might be active.

For example, several studies have shown, that non-HLA-allo- and autoantibodies have a significant impact on the development of allograft rejection in kidney and heart transplantation. These antibodies may be complement or non-complement-fixing and target numerous minor histocompatibility antigens, vascular receptors, adhesion molecules and intermediate filaments. Today, several reliable tests like solid-phase assays, flow-crossmatch techniques and immunofluorescence are capable of precisely detecting non-HLAabs like MICA, angiotensin type 1 receptor (AT_1R), collagen-V, vimentin or antibodies against donor endothelial progenitors and antigens expressed on umbilical vein endothelial cells[58,59].

However, there is still a high variability concerning the impact of a positive test result on allograft function, which not only differs between patients, but also between non-HLA targets. Thus, the functional significance of these antibodies remains to be clarified, as well the question, whether alloimmune responses may also enhance or activate immune responses to self-antigens[60].

In a retrospective analysis we investigated the post-transplant appearance of *anti-Angiotensin II type I receptor antibodies* (anti-AT_1R) and *anti-Endothelin-Type A receptor antibodies* (anti-ET_AR) in relation to immunological events like intestinal allograft rejection or infection[61]. Remarkably, patients, who developed non-HLAabs showed a higher rejection rate, especially of AMR than controls without non-HLAabs. Some patients experienced viral infections at the time of positive non-HLAabs sampling.

Thus we suggest a triggering or accelerating effect of non-HLAabs towards DSA and rejection, which remains to be confirmed by histopathological analysis of the allograft biopsies to prove their pathophysiological relevance.

"Non-HLA Antibodies May Accelerate Immune Responses After Intestinal And Multivisceral Transplantation."
Gerlach UA, Lachmann N, Ranucci G, Sawitzki B, Schoenemann C, Pratschke J, Dragun D, Pascher A
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2. TNF-alpha Inhibitors as Immunomodulators to Reduce Early and Late Immune Responses

The inhibition of *tumornecrosis factor alpha* (TNF-alpha) was previously shown to reduce ischaemia/reperfusion injury (IRI) in the experimental and clinical setting of ITX. Our group has already reported the beneficial effects of a non-depleting anti-CD4 monoclonal antibody and the TNF-alpha inhibitor *Etanercept* in an intestinal allotransplant model[62]. In addition, as one of the first reports of using the TNF-alpha inhibitor *infliximab* in humans, we also described the successful treatment of therapy-refractory ACR in two patients after ITX, who presented persistent ulcerative inflammation of the distal ileal graft. The following two studies describe our experimental and clinical investigations for the further use of TNF-alpha inhibitors to reduce early and late inflammatory graft injury.

2.1 TNF-alpha Inhibitors Like *Infliximab* Attenuate Ischaemia/Reperfusion Injury

> *Gerlach UA, Atanasov G, Wallenta L, Polenz D, Reutzel-Selke A, Kloepfel M, Jurisch A, Marksteiner M, Loddenkemper C, Neuhaus P, Sawitzki B, Pascher A:*
>
> "Short-term TNF-alpha inhibition reduces short-term and long-term inflammatory changes post-ischemia/reperfusion in rat intestinal transplantation."
>
> *(Transplantation. 2014;97(7):732-9)*

IRI is a typical consequence of the temporary interruption of blood flow to the transplanted organ and remains a constant burden in ITX associated with graft motoric dysfunction, inflammatory cascades and eventually loss of structural integrity of the intestinal wall[63].

Molecular and cellular components of the innate immune system are key players in the different processes that trigger further immunological pathways and finally cause graft injury and rejection[64]. Traumatic insults to the intestinal allograft like donor brain death, surgical manipulation and the procedure of transplantation with warm and cold ischaemic phases induce a direct tissue injury through a lack of oxygen and nutrient supply[65]. The following restoration of blood flow to the ischaemic allograft causes oxidative stress which further damages the epithelial integrity, affecting protective Paneth and goblet cells and creating a pathological change to the intraluminal micro-environment[64]. In fact, the loss of architectural integrity with an impaired mucosal barrier and motoric dysfunction results in bacterial translocation, intestinal inflammation, peritonitis and sepsis and thereby additionally challenges patient and graft survival[62].

In an experimental study, we investigated the effects of different TNF-alpha inhibitors (*infliximab, etanercept* and *pentoxifylline*) not only on acute IRI but also on long-term inflammatory responses in ITX. We confirmed that TNF-alpha inhibition generally decreased inflammatory IRI-induced alterations. *Infliximab* however, significantly reduced the histological and immunohistochemical signs of IRI, as well as the numbers of graft-infiltrating cells, not only on the intestinal graft but also in remote organs like the lung[66]. In addition, it significantly improved survival.

Thus, the reduction of TNF-alpha-induced IRI may attenuate or even inhibit the initial mechanisms leading to chronic graft injury and may improve long-term survival after ITX.

"Short-term TNF-alpha inhibition reduces short-term and long-term inflammatory changes post-ischemia/reperfusion in rat intestinal transplantation."

Gerlach UA, Atanasov G, Wallenta L, Polenz D, Reutzel-Selke A, Kloepfel M, Jurisch A, Marksteiner M, Loddenkemper C, Neuhaus P, Sawitzki B, Pascher A

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Web-link to publication: https://dx.doi.org/10.1097/TP.0000000000000032

2.2 *Infliximab* as Salvage Therapy for Treatment-Refractory Rejection and Graft Enteropathy

Gerlach UA, Koch M, Müller HP, Veltzke-Schlieker W, Neuhaus P, Pascher A:

"Tumor necrosis factor alpha inhibitors as immunomodulatory antirejection agents after intestinal transplantation."

(Am J Transplant. 2011;11(5):1041-50)

The TNF-alpha inhibitor *infliximab* has been proven effective in the clinical treatment of Crohn's Disease and in the experimental setting of ITX, where it showed anti-inflammatory and antichemotactic effects especially in the critical early phase after transplantation[67]. In contrast, single isolated *infliximab* treatment could not prevent acute rejection in the experimental setting. Yet, the idea of using immunomodulators as anti-rejection therapy in humans seemed promising.

In order to transfer the strategy of TNF-alpha inhibition into a clinical setting, the effects attributable to the unspecific addition of a human immunoglobulin and the specific binding and inhibition of rat TNF-alpha by *infliximab* were investigated in an ITX rat model, in combination with *tacrolimus* as standard immunosuppressive agent[67-69]. The observed anti-inflammatory and antichemotactic effects identified the combination of *infliximab* and standard immunosuppression as a potent regimen to prevent inflammatory and alloimmunologic responses early after ITX[67,70].

Following these positive results, *infliximab* was successfully initiated as a therapeutic option in the clinical setting of ITX[70]. Based on this experience, experimental data[71], and the knowledge from studies in bone marrow and liver transplantation about the role of TNF-alpha in steroid-resistant rejection[72,73], *infliximab* was more frequently applied in combination with standard immunosuppression.

In a clinical retrospective study, we demonstrated our longterm experience with the successful use of *infliximab* as a salvage therapy. Our results show, that *infliximab* may expand therapeutic options not only for OKT3-refractory rejections and steroid-refractory rejections in order to spare depleting antibodies, but also in patients who presented with *inflammatory bowel disease* (IBD)-like inflammatory alterations of the distal ileal graft, also referred to as allograft enteropathy[74].

"Tumor necrosis factor alpha inhibitors as immunomodulatory antirejection agents after intestinal transplantation."
Gerlach UA, Koch M, Müller HP, Veltzke-Schlieker W, Neuhaus P, Pascher A
Am J Transplant. 2011;11(5):1041-50
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3. Non-invasive Rejection Markers May Detect Intestinal Allograft Injury in Time

More than in any other solid organ transplantation, acute rejection in ITX requires immediate recognition followed by an adequate treatment, because a) it quickly progresses to a severe stage and b) once the mucosal barrier is damaged, there is a high risk of bacterial translocation, peritonitis and sepsis, which immediately increases the patient's morbidity and risk of mortality. The key challenge of gaining adequate control over rejection in ITX-recipients is therefore a better understanding of the pathological pathways in intestinal graft rejection and the identification of early non-invasive rejection markers. Multiple studies have aimed at identifying such eligible markers in order to detect allograft rejection at an early stage or even before histological changes have become evident. The following two papers present two entirely different forms of non-invasive rejection markers, which may indicate upcoming immune reactions in time.

3.1 The Early Detection of Allograft Rejection with Intragraft and Systemic Immune Parameters

Gerlach UA, Klöpfel M, Atanasov G, Polenz D, Vogt K, Ahrlich S, Marksteiner M, Jurisch A, Loddenkemper C, Reutzel-Selke A, Sawitzki B, Pascher A:

"Intragraft and Systemic Immune Parameters Discriminating Between Rejection and Longterm Graft Function in a Preclinical Model of Intestinal Transplantation."

(Transplantation. 2016 accepted for publication Aug 9.)

Whereas treatment strategies for the different forms of rejection following ITX are well established, including the use of depleting antibodies and other biologicals, timely detection of rejection remains a challenge. Furthermore, the pathology and diagnosis of acute and chronic rejection in ITX-recipients remain insufficiently understood. In addition, and unlike in any other field of solid organ transplantation, there is a lack of non-invasive rejection markers in ITX, forcing histological proof of rejection[8,37,75] , which often means a delay in diagnosis and yet another risk of graft injury through frequent endoscopic biopsies[34,35].

As a result, there is a substantial risk that subacute and subclinical rejections remain undetected and may rapidly progress to a severe stage[43]. Several markers in blood and stool have been reported to confirm the histological diagnosis of rejection like *Citrulline*, *Calprotectin*, *Granzyme B* or *Perforin*, but are not specific for allograft rejection, because the same alterations can be observed in other intestinal pathologies[32,76,77].

Another rejection marker studied in experimental ITX is *lipopolysaccharide binding protein* (LBP), which is an acute-phase protein that binds to lipopolysaccharide with a high affinity and increases rapidly in the blood plasma in case of endotoxinemia[78].

Most importantly, Sawitzki et al. have published results of different studies on the association of an early down-regulation of *tolerance associated gene-1* (Toag-1; now named *T-cell Activation Inhibitor Mitochondrial*=TCAIM) transcription level and the development of acute rejection following kidney and heart transplantation[79]. T-cell activation resulted in down-regulation of TCAIM-expression, whereas enforced stable TCAIM-expression in T-cells was prevented T-cell

activation, which resulted in acceptance of skin allografts[80]. Remarkably, the observed changes in TCAIM-expression were not only detectable within the graft but also in the peripheral blood, providing a non-invasive method to detect a developing rejection.

In an experimental ITX rat model we sought to characterize the different states of intestinal allograft rejection (acute severe and chronic moderate/severe intestinal graft rejection) and analysed peripheral and intragraft immune responses. Therefore, we assessed peripheral TCAIM-transcription and plasma LBP-concentration for their potential to differentiate between early rejection and long-term graft acceptance, to better understand the underlying immune processes and subsequently diagnose rejection at the earliest possible stage[81].

The results of our study demonstrate that peripheral and intragraft TCAIM-expression were stable in long-term surviving animals but declined prior to acutely or chronically rejecting animals. In contrast, LBP-levels increased during acute and chronic rejection.

Based on the observation that circulating anti-donor alloantibodies were highly increased we suggest that the reported rejections were mixed cellular and humoral rejections. Thus, the study not only reflects typical clinical findings during rejection, but also highlights the significant benefit of monitoring peripheral TCAIM-expression, LBP-levels and HLAabs for diagnosing rejection.

"Intragraft and Systemic Immune Parameters Discriminating Between Rejection and Longterm Graft Function in a Preclinical Model of Intestinal Transplantation."

Gerlach UA, Klöpfel M, Atanasov G, Polenz D, Vogt K, Ahrlich S, Marksteiner M, Jurisch A, Loddenkemper C, Reutzel-Selke A, Sawitzki B, Pascher A

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3.2 Skin Is an Early Rejection Marker in Combined Intestinal and Abdominal Wall Transplantation

Gerlach UA, Vrakas G, Sawitzki B, Macedo R, Reddy S, Friend PJ, Giele H, Vaidya A:

"Abdominal Wall Transplantation: Skin as a Sentinel Marker for Rejection."

(Am J Transplant. 2016;16(6):1892-900)

Usually, allograft rejection only becomes apparent when graft injury has already set in so that either organ-specific serum parameters increase, or patients suffer from the clinical symptoms of organ dysfunction.

The natural inability of a macroscopic assessment of the visceral organ during rejection processes without using invasive techniques is rendered possible in the setting of composite tissue transplantation, where allograft rejection becomes visible through a rash on the skin component.

The diagnosis of rejection in a skin graft is often diagnosed through the macroscopic aspect of a maculopapular erythematous rash, presenting in different colour intensities. It may be focal, patchy or even diffuse and can be accompanied by a burning pain[82,83]. The microscopic features for the diagnosis of rejection in *vascularized composite tissue allotransplantation* (VCA) were summarized in 2007 in the international Banff classification[84]. Interestingly, the observed clinicopathological changes observed during rejection were similar in all VCA, not only in hand and face allografts[85,86], but also in AWTX[24], suggesting that the histological features of skin rejection manifest in a similar way.

VCA is technically and immunologically a very complex and challenging procedure, but there has been a remarkable progress in this relatively new field of transplantation. However, it has also stimulated controversial discussions over the last decade, because in contrast to other solid organ transplantation, VCA is usually not performed as a lifesaving procedure to restore physiologic function, but rather to assure quality of life.

Whereas these arguments may hold true for limb and face transplantation, AWTX has mainly been used as a method for difficult abdominal closure after ITX in eligible patients[86]. Multiple previous surgical procedures as well as ostomies, enterocutaneous fistulas and desmoid tumours often result in a loss of the abdominal domain and abdominal wall elasticity[21]. Consequently, primary fascial closure is often difficult to achieve and secondary wound dehiscence appears in 20-33% of ITX-recipients[20]. Therefore, the foremost idea of AWTX was to achieve an uncomplicated primary closure to avoid postoperative morbidity and mortality. The surgical feasibility of this procedure has been discussed by several transplant centres[25,28]. The abdominal wall allograft contains peritoneum, muscles, fat and skin, so that two highly immunogenic grafts, intestine and VCA pose an unknown immunological risk for allograft rejection and GVHD[25].

In a retrospective study of patients who underwent combined ITX and AWTX we discovered a beneficial effect of the skin's antigenic nature as an immunological surveillance tool for differential diagnosis of bowel dysfunction following ITX[87].

Although the general patient survival was similar, patients who had undergone an additional AWTX had a better outcome in terms of post-transplant recovery as well as intestinal graft rejection rate and survival. One of the major advantages was that those patients also had lower rate of misdiagnoses, where viral infection was histologically and clinically mistaken for rejection and treated as such[87].

We may suggest, that the skin component of a simultaneously transplanted abdominal wall may serve as a sentinel marker for immunological activity in the host and may be crucial to prevent intestinal graft rejection.

"Abdominal Wall Transplantation: Skin as a Sentinel Marker for Rejection."
Gerlach UA, Vrakas G, Sawitzki B, Macedo R, Reddy S, Friend PJ, Giele H, Vaidya A
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III. DISCUSSION

Over the last two decades ITX has undergone a remarkable progress in volume and outcome in specialized centres worldwide. Nevertheless, longterm data are still rare and given the complicated immunogenic nature of the intestine, several processes in the development of rejection are still not completely understood[9].

Many pathophysiological mechanisms of rejection and corresponding treatment strategies have been studied or were translated from the field of kidney, liver or heart transplantation, but more than in any other solid organ transplantation, longterm graft survival in ITX remains a major challenge. Longterm graft attrition is still a consequence of the extensive immunogenicity of the intestine and the constant risk of rejection, but is also due to the difficult adjustment of an adequate immunosuppression and an overly exposure to infection. The lack of reliable non-invasive rejection markers may aggravate this difficult process of diagnosis, destabilizing the fine line between over- and under-immunosuppression.

In several experimental and clinical studies, we have addressed these challenges by assessing the different forms of rejection and identifying their associated rejection markers. In future studies these results have to be confirmed in larger patient cohorts, so that a risk stratification may be generated for each patient prospectively, in order to establish an individually tailored treatment regimen.

One of the major findings of our studies was the recognition of humoral immune responses in ITX-recipients through HLAabs and non-HLAabs formation. Especially the overall impact of DSA was highlighted: As one of the first studies in this field, we could show a strong correlation between high numbers of antigen and epitope mismatches between donors and recipients and the detected DSA-development, as well as between DSA-development and rejection[57].

Until today, many authors have suggested that due to a high number of pretransplant hospitalizations and the potential necessity for blood transfusions during severe infections, almost 30% of all ITX-and MVTX-recipients have preformed DSA, which were reported to account for a significantly higher risk of rejection[56]. De-novo DSA are detectable in as many as 40% and both, persistent and de-novo DSAs are risk factors for the development of chronic rejection and a reduced longterm survival. In contrast, patients with a liver-containing allograft, showed a better elimination of preformed DSA and a lower rate of *de novo* DSA development, which was associated with a significantly better outcome[55]. The proposed hypothesis of our study was confirmed by other authors[55,56,88], which highlighted the importance of DSA in ITX-recipients and helped to identify AMR as a form of vascular rejection in ITX[89]. The vascular damage is underlined by the generally accepted histopathological features of AMR in solid organ transplantation, which include capillary congestion, submucosal haemorrhage, neutrophilic margination, endothelial hypertrophy, epithelial damage, arteritis, and multiple capillary platelet thrombi[55,90,91].

Similar to the Banff criteria in AMR for kidney transplantation, Cazals-Hatem et al[92] proposed three diagnostic criteria for AMR in ITX: (a) clinical allograft dysfunction, (defined as 20% increase in stoma output or diarrhoea, protein losing enteropathy, and/or endoscopic mucosal injury), (b) increase or appearance of DSAs, and (c) vascular histological lesions. The biopsies of patients who met these criteria showed capillaritis or endotheliitis, capillary congestion and/or blood extravasation, and fibrin and/or hyaline thrombus. According to these findings, vascular graft injury is one of the prominent features in AMR, raising the question, if other mechanisms may be also involved in the vascular inflammatory processes.

Along the same line, some recent studies on alloantibody assessment and outcome prediction after ITX and MVTX reported ongoing rejection even in the absence of circulating antibodies[93,56]. Together with the occurrence of allograft rejections in HLA-identical siblings following kidney transplantation, these findings could signal coexistence of non-HLA or non-donor-specific HLA antibodies and/or non-complement-fixing antibodies which may indicate HLA antibody absorption to the graft[94]. Non-HLA antibodies and their ability to trigger humoral immune responses in solid organ transplantation have increasingly been described, so that the previous diagnostic strategies based exclusively on DSA detection should be revised[58,95].

Mechanisms beyond ABO blood group and MHC class I chain-related gene A and B antigens have been observed in other solid organ transplantations so that we translated the diagnostic and therapeutic approaches to the field of ITX. There certainly exist a variety of antibodies targeting vascular receptors, adhesion molecules, minor histocompatibility antigens, and intermediate filaments, which might be active in the immunocompetent tissue of the intestinal allograft.

According to several studies, the underlying mechanisms that lead to chronic graft injury and rejection may result from immune responses that are not only directed against mismatched MHC antigens but also against self-antigens[96,97]. For example: K-alpha-1 tubulin and collagen V following lung[96], cardiac myosin and vimentin has been detected following cardiac[98], Col III following liver, and AT_1R following kidney transplantation[94,99]. Clinical relevance of antibodies targeting AT_1R is broadly confirmed not only in renal, but also in heart transplantation, where ET_AR-Abs were additionally found[100]. AT_1R-Abs may induce inflammatory processes and thereby enhance allograft rejection by activating NF-kB target genes. Biopsy specimen of patients with rejection associated with AT1R-Abs had evidence of increased tissue factor expression and secondary thrombotic occlusions which were reduced upon treatment with AT_1R blockers like Losartan[101].

In order to further define the mechanisms of vascular graft injury in ITX-recipients, we assessed the potentially hazardous effects of AT_1R and ET_AR-Abs. Interestingly, patients who developed non-HLAabs also had a greater risk of developing a rejection, especially AMR, compared to controls[61]. These findings may reveal an increased humoral immune response, which corresponds to recent reports from kidney and heart transplantation, where the combination of DSA and anti-AT_1R had an increased influence on developing AMR and ACR than either of the antibody alone[102,103].

Following these observations, we assume that some patients are prone to accelerated humoral immune responses. For example, we saw a significant association between an increased number of HLA class II mismatches and the post-transplant development of non-HLAabs. Indeed, we had already detected the same association in our cohort regarding the post-transplant DSA-development[57].

Given the fact, that AMR can often be subclinical and may remain unnoticed, these findings imply that a pre-transplant risk stratification including preformed HLAabs and antigen/epitope mismatches may help to identify and treat patients with a potentially elevated risk of AMR as early as possible before immune activation has reached the stage of rejection[57]. The pharmacologic targeting of non-HLA receptors allows for future studies to focus on the explanation of mechanisms how non-HLAabs may accelerate rejection and affect long-term allograft survival.

In terms of diagnosing AMR in ITX, there is still a lack of consensus in the international discussion regarding the utility of C4d staining for diagnosing AMR in ITX-recipients[55]. Although C4d is an important marker for diagnosing AMR in kidney, heart and lung allografts, its use in identifying AMR in intestinal grafts has not yet been validated. Previous investigations have demonstrated that C4d deposition can be found at similar rates in biopsies with and without rejection[104,105]. Recently, the Pittsburgh group reported on C4d staining in 390 intestinal biopsies where AMR was suspected and could show, that diffuse C4d staining was more common in patients with DSA, especially in those with persistent DSA[56]. Nevertheless, C4d staining is not reliable for the diagnosis of AMR in ITX-recipients, so that DSA detection is the most important indicator of ongoing B cell activation and antibody-mediated graft injury[57]. A better understanding of the interaction of HLA- and non-HLA-associated immune responses and the clear identification of joint effector mechanisms may help to develop individual targeted therapies.

Due to the potential danger of preformed HLAabs, some centres have adopted desensitization strategies to decrease or eliminate preformed HLAabs, but limited evidence exists on their efficacy. Data on treatment strategies for ITX-recipients diagnosed with AMR consists of different strategies with varying success by using different immunosuppressive agents like IVIG, PP, *basiliximab*, *thymoglobulin*, *rituximab* and *bortezomib*[106]. Most treatments were given after transplantation, as part of induction therapy, AMR treatment or DSA removal in a stable patient[107].

Our treatment strategy against the formation of DSA mainly focuses on the combination of PP and IVIG. Although PP is the fastest and most effective way to eliminate circulating DSA, it has failed to show longterm effects and rarely results in complete elimination, so that a DSA rebound is frequently observed. IVIG works through numerous immunomodulatory mechanisms including antiidiotypic antibody networks, blockade of Fc receptors, complement inhibition, and B-cell receptor binding and downregulation[55,108].

Therefore, agents like *rituximab* and/or *bortezomib*, which have long been used for the treatment and prevention of AMR in kidney transplantation have been introduced into the treatment protocol for AMR in our centre. *Rituximab* is a chimeric monoclonal anti-CD20 antibody, directed

against a transmembrane protein expressed on pre-B and mature B-lymphocytes. Thus, *rituximab* results in B-cell depletion via apoptosis and antibody dependent, cell mediated cytotoxicity. It has no effect on CD20 negative plasma cells, which may continue to produce DSA. In contrast, the proteasome inhibitor *bortezomib*, which is well established in the treatment of multiple myeloma, can successfully deplete plasma cells[55]. In a patient with treatment-refractory AMR, we could show, that *bortezomib* may also be effective as salvage therapy in ITX-recipients[109] so that we have used it increasingly and successfully in treatment-refractory AMR.

Another biological agent in the treatment of alloimmune responses after ITX is the TNF-alpha inhibitor *infliximab*. *Infliximab* is an achimeric immunoglobulin G1 monoclonal antibody with an elevated binding affinity to soluble and transmembrane human TNF-alpha. This effect results in a neutralization of TNF-alpha activity, cell lysis and thus, depletion of TNF-alpha producing cells. The investigation of the therapeutic effects of TNF-alpha inhibitors like *infliximab* on the different forms of immune activation was another important focus of our experimental and clinical studies. On the one hand, we could show that *infliximab* can successfully attenuate IRI and on the other hand we also reported its efficacy in the clinical setting of intestinal allograft enteropathy, which has recently been recognized as a late form of allograft inflammation, resembling the manifestations of Crohn's disease.

This exciting twofold potency of *infliximab* may suggest a correlation between the early graft injury through IRI and late manifestations of graft inflammation, which are unresponsive to standard antirejection treatment.

Following the results of our experimental study, we suggest that an induction with TNF-alpha inhibitors may reduce IRI and may induce a better graft-acceptance.

This hypothesis is supported by several authors, who reported that TNF-alpha mRNA expression induces cytotoxicity, apoptosis and dysmotility early after ischaemia/reperfusion (I/R)[68,110]. As a consequence, damage-related and pathogen-associated molecular pathways may synergistically activate the innate immune system. The cellular components of the innate immune system like neutrophils, mast cells, platelets and dendritic cells as well as Toll-like receptors and components of the complement system intensify immune interactions and shape the adaptive T-cell response against the graft, promoting rejection[111]. The recurrence and severity of acute rejection episodes are thought to trigger chronic rejection and eventually graft loss, so that several experimental studies have aimed to reduce IRI and establish effective therapeutic strategies for a better acceptance of the intestinal allograft[69].

Although the impact of TNF-alpha on the initiation of permanent structural changes remains to be further investigated[112], recent experimental ITX studies could show an increase of TNF-alpha mRNA-expression not only during, but also during the manifestation of acute rejection[113]. Furthermore, an induction of apoptosis in T-cells and monocytes has been reported, suggesting a triggering effect of TNF-alpha on the acceleration of rejection[114,115]. Due to this potential correlation between TNF-alpha levels and the severity of rejection, the effects of *infliximab* have been

investigated in experimental studies where its anti-inflammatory and antichemotactic effects were observed in the early post-transplant phase[68].

The occurrence of TNF-alpha-induced molecular changes during IRI and rejection has finally elicited the use of TNF-alpha inhibitors such as *infliximab* to complement standard immunosuppression in the clinical setting[66-69]. With respect to the overall positive reports on the beneficial effects of initial TNF-alpha blockade we have started using *infliximab* (Remicade®, Centocor Inc., ESSEX PHARMA GmbH, Germany) as induction agent in combination with *thymoglobulin* (Thymoglobulin®, Genzyme, USA).

The use of *infliximab* in the treatment of late graft enteropathy is based on the resemblance of this phenomenon to the macroscopic and histological manifestations of IBD. Interestingly, the observed inflammatory ulcerations mainly manifested in the distal ileal graft and resembled a cobblestone pattern of Crohn's disease, so that several experimental ITX studies have attributed the underlying mechanisms to immunological processes similar to IBD, especially Crohn's disease-associated polymorphisms in the *nucleotide-binding oligomerization domain containing 2 (*NOD2) gene[68,116,117]. The histological and clinical resemblance between chronic intestinal graft injury and Crohn's disease has evoked suggestions to designate chronic intestinal allograft inflammation as a third form of IBD[69]. TNF-alpha inhibitors like *infliximab* have been proven effective in IBD with regards to overall patient improvement, decrease in surgical interventions and hospitalizations and have become a cornerstone in the successful therapy of Crohn's disease[118].

The observed chronic inflammatory graft alterations and late-onset rejections were accompanied by an increase of TNF-alpha serum levels. Furthermore, they were refractory to standard anti-rejection therapy but resolve under TNF-alpha inhibition[66], which has led our group to establish *infliximab* as a standard treatment protocol for late graft enteropthy[72].

Ever since, similar reports followed, not only in adult but also in paediatric ITX, where *infliximab* was used as salvage therapy for steroid- and *thymoglobulin*-resistant late acute rejection after ITX[119]. To date, *infliximab* has increasingly been established not only in ITX[120], but also in other fields of transplantation, like treatment-refractory cases of GVHD after liver and bone marrow transplantation[121-124], emphasizing its effect on tissue injury in the context of acute and chronic inflammatory alterations.

The identification of humoral immune responses and the investigation of TNF-alpha-triggered inflammatory processes of the intestinal allograft have certainly supported a broader understanding of the processes that induce immune activation in ITX-recipients. The regular monitoring of HLA- and non-HLA antibodies as well as of serum TNF-alpha-levels has become an important completion to the regular follow-up in our transplant program and may be responsible for an improved longterm survival. However, these parameters can only detect ongoing graft injury but unfortunately are unable to predict a developing immune activation. Other than in parenchymal allografts, rejection of a luminal organ like the intestine not only injures the graft

but also leads to a destruction of the mucosal barrier, entailing bacterial translocation and peritonitis. The rapid progress of rejection may finally lead to graft perforation, accelerating the dramatic process of peritonitis, sepsis and mortality.

The most important requirement for a suitable rejection marker in ITX is therefore not only its ability to detect rejection at an early stage, but rather to predict rejection in the first place so that allograft injury is kept to an absolute minimum. Another feature of an ideal rejection marker would be its non-invasiveness, helping to spare regular graft endoscopies, tissue biopsies and additional graft injury.

Until today, no test was sufficient or reliable enough to replace the gold standard of endoscopic biopsies. Two markers are infrequently used in clinical practice for follow-up: (a) Citrulline, a nonprotein amino acid is a marker of functional enterocyte mass, because the majority of its plasma level derives from glutamine conversion within enterocytes[125-127] and (b) Calprotectin, which is mainly released during the process of cell disruption or death, but can also be secreted extracellularly[128,129]. In addition, epithelial cells in the mucosal layer of the intestine have may express Calprotectin in their cytoplasm. Therefore, the increased calprotectin levels found in stool, may also stem from an elevated shedding of epithelial cells[130]. Falling plasma Citrulline values may correlate with previous mucosal damage, but are rather non-specific for rejection. Furthermore, Citrulline is incapable of predicting a graft-injuring event in advance. In an analogous manner, increasing Calprotectin values in the stool may correlate with the diagnosis of allograft rejection, but do not predict an immunologic event prior to its occurrence.

Therefore, both parameters may be used to exclude rejection if their values are normal, but their broader clinical use is hampered by their large variability and inaccurateness[131]. Thus, the joint weakness of all proposed rejection markers is their lack of specificity and their impracticality for clinical routine[132].

In an experimental study we assessed the different stages of allograft rejection like acute rejection vs. moderate or severe chronic rejection and investigated intragraft and systemic immune parameters like TCAIM, LBP and Perforin. TCAIM-expression appeared to be predictive for rejection and may even allow for a discrimination between acute rejection and allograft acceptance in the early phase after transplantation. The additional assessment of Perforin-expression and LBP-levels may help to confirm the diagnosis and avoid unnecessary endoscopies. Future studies have to clarify, whether the monitoring of TCAIM-expression levels is viable for clinical practice and whether it could be one step towards individual risk stratification, timely detection of ongoing immune responses and an early prediction of clinical outcome[80].

Unintentionally, an entirely different rejection marker was found in the skin component of a simultaneously transplanted abdominal wall graft. While the first successful AWTX was initially intended as an abdominal closure technique in ITX- or MVTX-candidates who suffered from major injuries of the abdominal wall and loss of abdominal domain, we discovered that apart from the abdominal closure technique, the skin visibly rejected earlier than the intestine.

Therefore, an evolving, pioneering two-fold advantage of AWTX could be suggested: Firstly, a 'lead-time' advantage may be gained by treating rejection in the AWTX before it manifests in the intestinal graft and causes severe dysfunction, and secondly a distinction could be made between rejection and intestinal allograft infection, thus avoiding over immunosuppression. It is surprising to find such a hierarchy of immune reactivity in two equally immunogenic grafts from the same donor. Assumingly, the epidermal Langerhans cells have a highly immunostimulatory capacity by directly stimulating recipient T-cells, which may prioritize the induction of rejection in the skin. Possibly, the immediately initiated antirejection treatment for the skin may have protected the intestinal allograft. Other hypotheses are either an immunoprotective effect of the composite tissue graft on the intestine, or a deviation of rejection to the skin away from the intestinal graft.

A major advantage of the simultaneously transplanted skin is the potential distinction between intestinal allograft rejection and infection, avoiding misdiagnosis and immunosuppression peaks. Subsequently, an easier diagnosis and consecutive shorter hospital stays resulted in reduced morbidity and preserved kidney function in our cohort. Thus, a simultaneously transplanted vascularized skin graft may serve as remote access, patient-led, non-invasive tool to adjust immunosuppression without risking graft injury and may even improve long-term survival in eligible patients.

Despite this beneficial marker-effect of combined ITX and AWTX, this procedure should nevertheless stay reserved for patients in whom an additional AWTX is technically indicated. The abdominal closure with native abdominal wall and skin should remain the first approach.

However, the Oxford group has recently performed the additional transplantation of vascularized skin flaps, which are placed on the recipient's forearm and also serve as a sentinel rejection marker of the intestinal graft. Since the size of the VCA is however pivotal for the development of rejection, it remains to be investigated, whether the reduced size of a sentinel forearm flap is sufficient to achieve the same beneficial effects as a much larger abdominal wall graft. More studies are in progress to further determine the benefits and challenges of using a skin graft as a sentinel rejection marker for visceral allografts. Such projects give a promising perspective in that there is an increasing ascent in the research field of ITX, which is often triggered by newly identified similarities between ITX and other solid organ transplantations or ITX and gastroenterological diseases like IBD.

Beyond question, researchers in the field of ITX face considerable obstacles like small patient numbers, internationally different protocols for immunological monitoring and immunosuppression as well as the demanding task to correctly diagnose the different forms and stages of rejection. Nevertheless, many different ways of managing the immunological challenges of ITX have been presented so far. Larger prospective studies - ideally through international cooperation - are now required to further investigate promising hypotheses in order to pave the way for an improved long-term graft acceptance and patient survival after ITX.

IV. SUMMARY

Despite increasing numbers, ITX is still a rare form of solid organ transplantation. Due to the related immunological and surgical challenges it is only performed in specialized centres.

While short-term survival has considerably improved over the last decades, long-term survival is still hampered by the high immunogenicity of the intestine, chronic rejection and the consequences of an elevated immunosuppression over time.

The main focus of our studies was set on the investigation of factors that may influence long-term survival such as humoral immune responses, TNF-alpha triggered inflammatory graft injuries and eligible non-invasive rejection markers, in order to gain a better understanding on how to promote long-term graft acceptance in ITX.

In two presented studies on humoral immune responses, we could show that donor-specific HLAabs have an impact on allograft rejection and may – if not treated immediately – persist in the circulating blood, causing further graft injury and possibly chronic rejection. Interestingly, we further observed, that this vascular type of rejection, may be triggered or accelerated by non-HLAabs like AT_1R-Abs and ET_AR-Abs. Therefore, we established a regular screening system for HLA- and non-HLA antibodies pre-and post-transplant. In addition, we introduced a consequent treatment protocol including different agents to filter antibodies or deplete antibody-secreting cells like IVIG, PP, *rituximab* and *bortezomib* to be initiated upon the first antibody detection in order to prevent their persistence and subsequent long-term graft injury.

Based on an experimental rat model, we included the TNF-alpha inhibitor *infliximab* as an immunomodulator into our induction therapy. *Infliximab* was shown to attenuate IRI and to be an efficient additive to standard immunosuppression in the setting of acute rejection. Furthermore, in a clinical study *infliximab* was shown to sufficiently reduce the inflammatory processes observed in late allograft rejection and graft enteropathy. Whether graft enteropathy is already induced through the activated immune processes during IRI or whether it is based on similar mechanisms like NOD-2 mutations in Crohn's disease remains to be clarified in larger prospective studies.

Finally, we established a preclinical rat model for severe acute, moderate and severe chronic rejection and found that TCAIM, which has been identified as a potential marker for long-term graft acceptance in heart and kidney transplantation, may be similar beneficial in ITX. While TCAIM may potentially predict long-term graft acceptance at an early stage after transplantation, we additionally identified another non-invasive rejection marker: In the combined transplantation of a visceral graft like the intestine and a vascularized composite tissue graft like the abdominal wall we found that acute rejection manifested in the abdominal wall prior to the intestine and became visible before graft injury could spread. The underlying mechanisms of this delay have yet to be investigated in order to find out, whether remote sentinel skin grafts may become eligible also for ITX-candidates who do not require transplantation of an entire

abdominal wall. In any case, this sentinel effect proved not only useful to treat rejection at an early stage, but also to make the crucial differentiation between rejection and infection.

Despite several immunological challenges, ITX is no longer an experimental procedure. This is mirrored in the increasing number of transplantations and a constantly improving outcome. Nevertheless, the high immunogenicity of this organ clearly contains more immunological mechanisms and interactions than yet identified, so that translational approaches may often help to further clarify the underlying immune responses. Future studies will show whether there is still need for an overall extended immunosuppression, or whether pre-transplant risk stratifications may help to compose an individually tailored treatment regimen following the fine line between over- and under-immunosuppression.

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VII. ERKLÄRUNG GEMÄSS § 4 ABS. 3 (L) HABOMED

Hiermit erkläre ich,

- dass weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wird bzw. wurde,
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Dr. Undine A. Gerlach-Runge