Current Understanding of Alloimmunity of the Intestinal Graft.

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List of abbreviations.

AbMR	Antibody-mediated rejection.
ACr	Acute cellular response.
APC	Antigen-presenting cell.
ATG	Anti-thymocyte globulin.
CAE	Chronic allograft enteropathy.
CTV	Chronic transplant vasculopathy.
DAMP	Damage-associated molecular pattern.
DSA	Donor-specific antibodies.
GvHD	Graft-versus-host disease.
HLA	Human lymphocyte antigen.
IL	Interleukin.
ITx	Intestinal transplant(ation).
mAb	Monoclonal antibody.
MHC	Major histocompatibility complex.
MMF	Mycophenolate mofetil.
NK	Natural killer cell.
PAMP	Pathogen-associated molecular pattern.
sIgA	Secreted immunoglobulin A.
Th1	T-helper cell type 1.
TLR	Toll-like receptor.
TNF	Tumour necrosis factor.
Treg	Regulatory T-cell.

Abstract.

PURPOSE OF THIS REVIEW. This review focuses on the known mechanisms of alloimmunity that occur after transplantation and what is being done to improve graft and patient survival, particularly in the long term.

RECENT FINDINGS. The presence of mismatched antigens and epitopes might relate directly to the development of *de novo* donor-specific antibodies (DSA), and thus, rejection. In an abdominal wall transplant, the skin graft could be the first to show signs of rejection. The epithelial or endothelial cells are the main targets in acute and chronic rejection, respectively. Possible therapeutical targets are gut homing T cells and cells of the innate immune system. Chimerism development might mostly occur in isolated lymph nodes, but also in the epithelium, particularly after transplantation of bone marrow mesenchymal stromal cells.

SUMMARY. Ischemia-reperfusion, surgical injury, and bacterial translocation trigger the innate immune system, starting acute rejection. Interaction between donor and recipient immune cells generate injury and tolerance, which occur mostly in secondary lymphoid organs, lamina propria, and epithelium. Chronic rejection mostly affects the endothelial cells, generating graft dysfunction. DSA increase the risk of graft rejection both acutely and chronically, and the liver protects against their effects. Induction therapies deplete lymphocytes prior to implantation, and maintenance therapies inhibit T-cell expansion. Rejection rates are the lowest when depleting drugs and a combination of interleukin 2 receptor blockade, inhibition of T-cell expansion, and steroids are used as maintenance therapy. Chimerism and tolerogenic regiments that induce Tregs and prevent the development of DSA are important treatment goals for the future.

Introduction.

The small bowel can provoke a strong immune response. It is a transplantable organ with a delicate mucosal immune system harbouring large lymphoid-cell populations and lymphoid structures handling complex microbiota. Both factors are equally important for its high immunogenicity[8]. This article reviews the current knowledge of the mechanisms of rejection that are taking place in the graft after intestinal transplantation (ITx), how these problems are being approached, and what lies in the future of the field.

The normal mucosal immune response.

The immune response occurs in two forms: innate (antigen nonspecific) and adaptive (antigen specific) immunity, which constantly interact between each other. The main actors of the innate immunity of the gut are the microbiota, antigen presenting cells (APCs), T cells and B cells, Paneth cells, and the epithelium.

The microbiota act on invading organisms and the host's immunity itself[133]. APCs are comprised mainly of dendritic cells and macrophages. They present antigens and thus activate naive T cells in the gut's lymphoid organs. This is done through either a direct (APCs scavenging antigens) or an indirect pathway (M cells, goblet cells, and enterocytes gather antigens). Paneth cells are part of the intestinal crypts, regulate this environment and protect against microorganisms by releasing anti- microbial molecules[134]. The epithelium has a well-known barrier function that is also influenced by Toll-like receptors (TLR), present in both immune and epithelial cells[23].

The activated T cells expand and form two distinct populations: $CD4^+$ T-helper cells type 1 (Th1), which produce cytokines as tumour necrosis factor (TNF)-a, interferon-g, interleukin 1 (IL-1), IL-6, that generate an inflammatory environment, and $CD8^+$ cytotoxic T cells ($CD8^+$), which eliminate infected and dying cells through perforin, granzyme, and Fas ligand (Fas-L)[134]. Mucosal dendritic cell also activate B cells in lymphoid organs that produce and secrete immunoglobulin A (sIgA), contributing to the mucosal barrier against potential pathogens[38].

Regulatory T cells (Tregs), B cells, and $CD103^+$ dendritic cells are regulators of the immune response. Cells differentiate into these during the acute reaction and are part of the adaptive system. They have an important role in tolerance of own and environmental antigens, which is particularly important for gut and transplant immunity[2].

Memory T cells ($CD4^+$ and $CD8^+$), also part of the adaptive immune system, will be activated with exposure to the same antigens later in life. Subtypes of these are resident $CD4^+$ and $CD8^+$,

which exist in mucosal surfaces and lymphoid tissues, respectively, that make this reaction immediate and greater every time they are exposed to known antigens[135].

Immunology of the intestinal transplant.

Hyperacute, accelerated (both antibody-mediated and mainly affecting pre-sensitised patients), and humoral and/or cell-mediated acute rejection occur during the first months post-ITx. Although the entire graft is affected by this process, it can be patchy and occurs in the lamina propria[136,137].

Acute rejection.

Innate cellular alloimmunity is the earliest response after transplantation. The activation of this defence mechanism in ITx is the presence of donor antigens presented by APCs to recipient T cells. The uptake of these antigens can be from normal dying cells and aggravated by ischaemia-reperfusion injury, infection, or trauma[8,138]. The consequences of these injuries are the following (Figure 5.1)[134,139]:

(1) Damage-associated molecular patterns (DAMPs) in conjunction with microbial-induced signals (pathogen-associated molecular pattern, PAMPs) result in a proinflammatory and procoagulant environment after recognition by TLRs on dendritic cells (Figure 5.1 I)[113]. Recipients with mutations in intracellular PAMP's signalling molecules such as nucleotide-binding oligomerization domain 2 had an increased risk for immunologic graft loss in one study, although this could not be confirmed in a later one[134,140].

(2) Recipient monocytes and natural killer cells (NK) infiltrate the allograft within the first day posttransplant (Figure 5.1 II)[141]. The former differentiates into dendritic cells also without the need of DAMPs. Donor APCs (dAPCs) prime recipient naive T cells with allogeneic major histocompatibility complex (MHC) molecule-peptide complexes in the graft's own gut associated lymphoid tissue in mesenteric lymph nodes, Peyer's patches, or isolated lymph follicles in the lamina propria[142] with influence from its stromal cells[3]. This process is called direct pathway of antigen presentation (Figure 5.1 III, Figure 5.2 left). APCs range from monocytes to endothelial cells. The latter can provoke an acute immune reaction at any moment after transplant and have a role also in later stages (see Chronic rejection.)[143]. DAPCs migrate themselves to the recipient's lymphoid tissue as well ('passenger leukocytes') (Figure 5.1 P) as do NK cells, which interact with dendritic cells and could have a role in T-cell response[144].

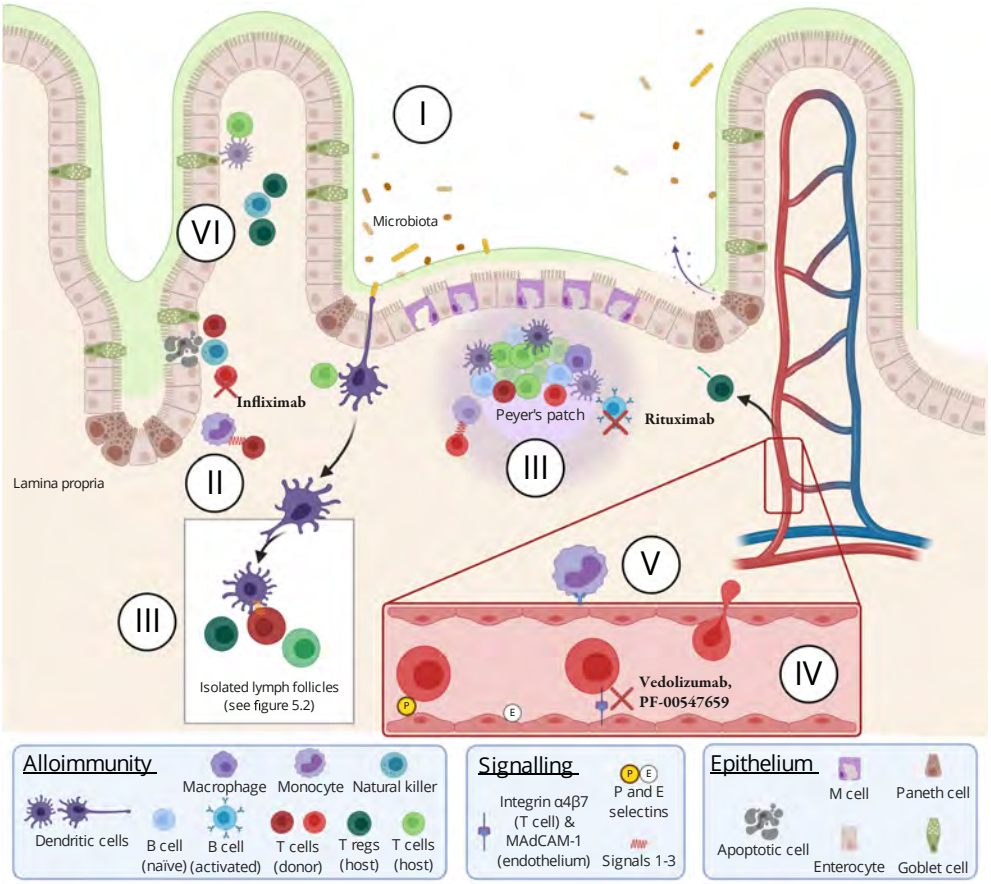

Figure 5.1. **The intestinal epithelium and lamina propria with normal and alloimmune reactions.** Further information can be found in the text and Table 5.1. (I–III) Dendritic cells present external antigens –DAMPs and PAMPs– and donor's antigens to host's and donor's naïve T-cells both from the lumen and the organ itself, also in lymphoid structures, such as the isolated lymph nodes and Peyer's patches. (III) Activated T- and B-cells will then expand. (IV) T-cells are captured by the endothelial cell through P- & E-selectins. Adhesion of the T-cell to the endothelial cell is mediated by its α4β7 integrin and the endothelial cells MAdCAM-1. T-cells ultimately migrate through the endothelium back to the graft ('gut-homing') and attack it generating crypt apoptosis. (V) In chronic rejection, monocytes and PMNs attack endothelial cells through TNF-α, recognizing MHC molecules (**Y**). (VI) Regulatory T-cells ("T regs") induce tolerance against own and foreign, benign antigens and have a role in graft tolerance as well. (**X**) Vedolizumab, an anti α4β7 monoclonal antibody, blocks the adhesion of the activated T-cell to the endothelium. Natalizumab works similarly by blocking the α4 subunit but is less gut-specific. PF-00547659 is an anti-MAdCAM-1 monoclonal antibody. Infliximab and rituximab are chimeric monoclonal antibodies against TNF-α and CD20, respectively. The former blocks the inflammatory activity by dendritic cells and T-cells and the latter, B-cell activity.

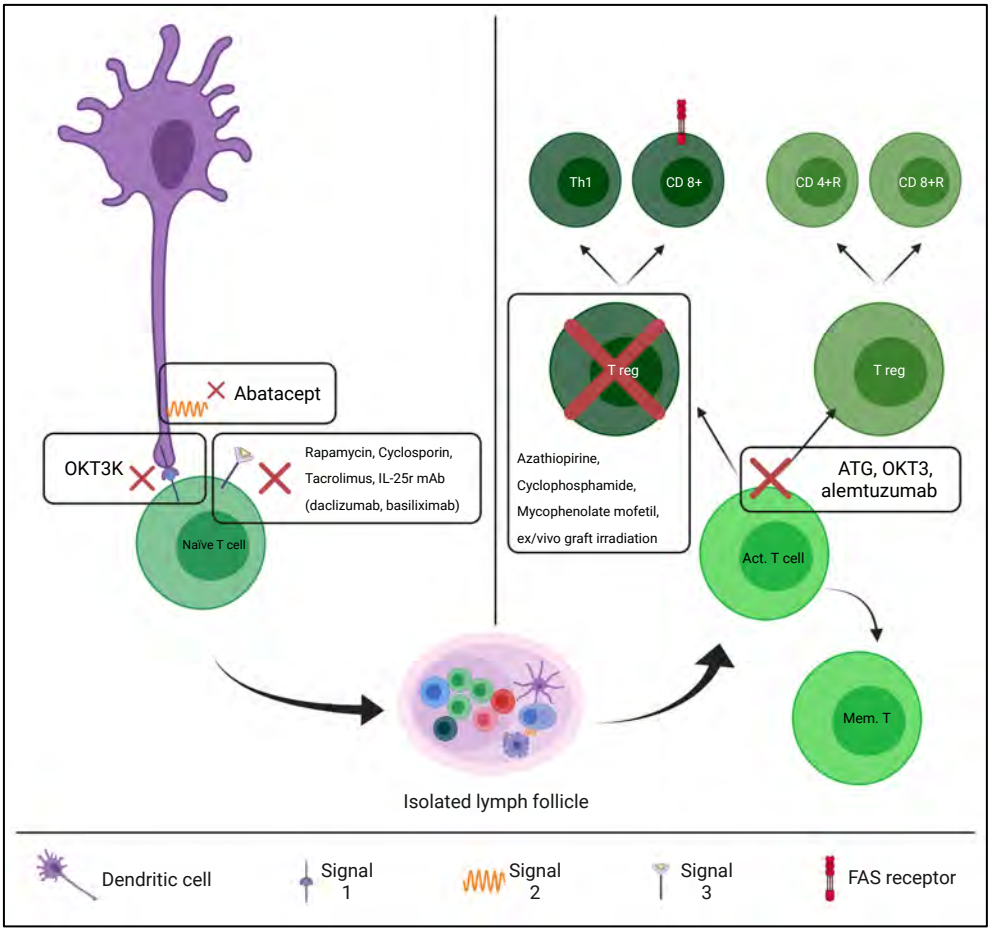

Figure 5.2. **Direct pathway of antigen presentation, detailed view**. Further information can be found in the text and on Table 5.1. (Left) APCs present antigens to naive T-cells through three signals, which can be blocked by the listed drugs next to the **X** symbol. After activation by APCs, T-cells migrate to secondary lymph organoids such as the isolated lymph follicle (pictured between panels). Signal 1: T-cell component is TCR, APC component is MHC þ antibody. Signal 2: T-cell component is CD28, APC component is CD80/CD8. Signal 3: T-cell component is CD25, ligand is IL-2. (Right) T-cells then replicate into either activated, regulatory ("T-regs") or memory T cells ("Mem T"). Tregs will then participate in tolerance induction for the graft. **X** shows were different lymphocyte-depleting therapies work. ATG therapy might induce a shift towards tolerant Tregs for the graft. IL, interleukin; mAb, monoclonal antibody; MHC, major histocompatibility complex; TCR, T-cell receptor.

(3) B cells, Th1, and $CD8^+$ are now activated. B cells differentiate into IgA-producing plasma cells[142], whereas T cells migrate to the graft ('gut-homing') (Figure 5.1 IV). NK and Th1 generate an inflammatory environment by secreting cytokines (Figure 5.2, right). $CD8^+$ induce crypt cell apoptosis (Figure 5.1 V) at large, a unique phenomenon of acute intestinal rejection[145,146].

(4) Donor and recipient macrophages get activated by uptake of antigens and contribute to the inflammatory environment (Figure 5.1 II)[147].

These steps comprise the acute cellular response (ACr), which is part of T-cell-mediated rejection[136]. This results in a mixed cellular infiltrate into the lamina propria, the most characteristic histologic finding in an acutely rejected graft[137]. The definition of ACr also includes crypt apoptosis and has been further classified into three categories by some authors, and clearly defined by [148] as mild (grade 1), moderate (grade 2), and severe (grade 3) depending on the grade of inflammation and whether the epithelium is affected or not.

The adaptive alloimmune response occurs when cells of the innate response system and those from this second line of adaptive immune response (primarily recipient-derived B cells and T cells) interact. Memory lymphocytes are present in high numbers in the lamina propria and get activated through both signals 1 and 2 by APCs. Other components of this type of alloimmunity are the endothelial cells, whose role is described later[23]. Of great importance is the high exposure to environmental and microbial antigens in the small bowel, which makes cross reactivity a phenomenon to take into account in the management of ITx[8].

Crypt apoptosis.

As previously mentioned, one of the unique characteristics of ITx is the presence of numerous (by definition, more than 6 apoptotic bodies per ten consecutive crypts) foci of apoptotic bodies in the crypts along the intestinal tract. For other transplants, crypt apoptosis is present as a sign of graft-versus-host disease (GvHD), albeit in fewer quantities[145].

These lesions are produced by $CD8^+$ attacking epithelial stem cells and results in ulceration of the mucosa. This attack is mediated by Fas and Fas-L injury and perforin-dependent granule-exocytosis (Figure 5.1 IV)[134]. It has been proposed that not only stem cells are attacked, but also T cells[137] and neuroendocrine cells[149], although the latter seem to be affected most importantly by the cold ischemia period[75].

Post-transplant monitoring.

Monitoring rejection is normally done by endoscopic assessment and biopsy, an invasive and discomforting exam for the patient. An endoscopy, in most cases performed through a temporary ileostomy, is still the golden standard for monitoring rejection. Measuring plasma citrulline and stool calprotectin levels are non-invasive monitoring tools. Both methods are not

specific regarding ITx, as they are markers for epithelial-cell loss or leucocytes in the stool caused by other gastrointestinal pathologies, respectively[150-152].

There has been increasing attention on the role of the skin from a vascularized composite allograft, when transplanting the abdominal wall[153]. There are two reasons for why this kind of transplant could be beneficial: it helps in the closing of the abdominal wall after the ITx surgery, and it could arguably serve as a tool for straightforward monitoring of the intestinal graft[153,154]. Although a controversial subject, there is evidence that points that a piece of donor skin is the most sensible for monitoring both acute rejection and GvHD. Applying this technique needs further exploration[155,156].

Chronic rejection.

Chronic allograft rejection (known in the case of ITx as chronic allograft enteropathy, CAE) is comprised of both immune and nonimmune processes that damage the organ mainly at a vascular level and occurs most frequently on isolated small bowel transplants. Endoscopically, there are ulcerative lesions and a flattening of the luminal surface (villous blunting). At the time of explant, a matted organ bloc with adhesions is to be expected[157].

The history of CAE starts with inflammation of the arteries, subsequently generating ischemic injuries due to obliterative arteriopathy, which can explain the macroscopic findings. Adhesions are caused by fibrosis of the parenchyma, a consequence of chronic inflammation that takes place after the first insults by cellular infiltrates. At a microscopic level, thus, there is evidence of interstitial fibrosis, leukocyte infiltration, and myointimal hyperplasia with intraluminal narrowing of the mesenteric, serosal, and submucosal vasculature[24,157,158].

The lesions found in blood vessels are also known as chronic transplant vasculopathy (CTV) and have also been characterized in other organs like heart, kidney, and lung. CTV is described to be an attack on endothelial cells by a mononuclear and polymorphonuclear infiltrate mediated by TNF-α. Endothelial cells express both types I and II of the MHC molecules (human lymphocyte antigens, or HLA), functioning as activators of memory and regulatory cells, but not of naive cells (Figure 5.1 V)[2,143]. The humoral immune system has an import- ant role in this, the recipient's immune system first encounters endothelial cells, which stimulate memory $CD8^{+}$[143]. This interaction induces apoptosis of the endothelial cells, which itself generates hyper-adhesiveness to and recruitment of leukocytes. This, in turn, induces a continuous fibroproliferative reaction[158].

Antibody-mediated rejection.

Antibody-mediated rejection (AbMR) is not well explored in the field of ITx and knowledge comes mainly from cardiac and renal transplantation. Research and treatment against graft loss has mostly focused on the cellular immune system, but there is evidence that its humoral counterpart has an important role on graft rejection, from immediate to chronic forms. AbMR is caused by donor-specific antibodies (DSA), mostly against HLA, but also against blood group and endothelial antigens. The presence of these has been associated with decreased allograft and patient survival and increased severity of the rejection episodes[159].

In the case of an accelerated and acute response, preformed as well as *de novo* antibodies generated by memory B cells are responsible for the damage[159]. Chronic ABMR has shown to appear after a period free of antibodies. An acute injury might awaken plasma cells that produce *de novo* DSA and start a long-lasting attack on the graft that will result in graft dysfunction[160]. This might also be the case for ITx although this has not been determined. Chronic ABMR might be an important factor in the low, long-term chronic intestinal graft survival rates[161]. It is important to note that patients with isolated small bowel transplants have worse outcomes than patients who also receive a liver transplant. This organ could be protective against ABMR by clearing out preformed DSA and by a lower rate of *de novo* DSA development[159].

The diagnosis of ABMR in ITx is based on the presence of plasma immunoglobulin G, ischaemic injury, mucosal congestion, and haemorrhage, and, especially, a strong crossmatch for HLA. Immunoglobulin deposits appear on injured micro vessels during rejection, according to [162] C4d, a product of the classic pathway of the complement system, also is deposited in their walls in other tissues during rejection. Therefore, it has been important in the diagnosis of this pathological entity in both kidney and heart transplants, but its importance for diagnosis in ITx has been argued. Even in renal transplantation, the utility of C4d for the diagnosis of ABMR is being put in doubt, and other markers, particularly the presence of HLA antibodies is being studied now[159,163].

Manipulation of alloimmunity.

To generate tolerance in transplantation, physiological immune mechanisms must be manipulated. The tolerance mechanisms are gestational, central, peripheral, and oral, of which there is experience at an experimental level in the ITx field for the latter three. Oral tolerance, natively to food antigens, seems to be the most promising way to promote allograft acceptance

in the case of the small bowel, as it is a mechanism own to the digestive tract. A list with molecules and cell interactions with respective treatment targets is available in Table 5.1[8,164].

Table 5.1. **The most relevant actors (cells, molecules, and cell interactions) in the alloimmunity of the intestinal transplant.** When available, treatment options are mentioned. For further information, see throughout text. APC, antigen presenting cell; DAMP, damage-associated molecular patterns; DC, dendritic cell; EC, endothelial cell; INF, interferon; mAb, monoclonal antibody; MAdCAM-1, mucosal vascular addressin cell adhesion molecule 1; MHC, major histocompatibility complex; NOD2, nucleotide-binding oligomerization domain 2; PAMP, pathogen-associated molecular pattern. [a]Mycophenolate mofetil reversibly inhibits inosine monophosphate dehydrogenase in B and T cells. [b]Rapamycin reduces the alloimmunogeneicity of ECs.

Cell 1	Molecule	Receptor	Cell 2	Drug	References
APC (signal 1)	MHC + Ab	TCR	Naïve T-cell	OKT3	1,2
APC (signal 2)	CD80/CD86	CD28	Naïve T-cell	CTLA-4-Ig (abatacept)	1-3
APC (signal 3)	Calcineurin, IL-2, mTOR	CD25	Naïve T-cell	Cyclosporin, tacrolimus, IL-25R mAb (daclizumab, basiliximab), rapamycin	1,2,4
Expanding T-cells	RAC-1, IMP		DNA	Azathiopirine, cyclophosphamide, MMF[a], *ex-vivo* graft irradiation	4
Depleting T-cells		CD3, CD52		Anti-thymocyte globulin, OKT3, alemtuzumab	8
Gut-homing lymphocytes	α4β7 integrin	MAdCAM-1	ECs	Vedolizumab, natalizumab, PF-00547659	18
DC	NOD2		Paneth cells		1
$CD4^+$, DC	TNF-α		EC[b], others	Rapamycin[b], infliximab	1,2,23,24
$CD4^+$	INF-γ		EC[b], others	Rapamycin[b]	1,2
$CD4^+$	IL-1				1
$CD4^+$, EC	IL-6		Tregs	Tocilizumab (IL-6R mAb)	1,2
$CD8^+$	Perforin				1
$CD8^+$	Granzyme				1
$CD8^+$	Fas-L	Fas	Epithelium		1
B-cell	sIgA, CD20			Rituximab (CD20)	38
Paneth cell	Human defensin 5				1
--	DAMPs, PAMPs	TLR	DC		1

Successful immunosuppressive therapies in ITx started in 1990 with the implementation of calcineurin inhibitors, such as ciclosporin and later tacrolimus, used in combination with steroids until 1995. This drug interferes with the signalling pathway that T cells use to express IL-2, thus, inhibiting the signal three of antigen presentation[4,164]. This strategy was further improved by using antibodies against the IL-2 receptor (daclizumab, basiliximab, see Figure 5.2, left).

As the intestine harbours huge amounts of lymphoid tissue, depleting strategies such as graft irradiation or cell-depleting agents, such as thymoglobulin (Anti-thymocyte globulin, ATG), OKT3, and anti-CD52 (alemtuzumab) was applied in both donors and recipients. As [8] explain this so-called tolerogenic immunosuppressive protocol began around 2001[4]. It consisted of a dose of alemtuzumab or ATG prior to implant and minimization of immunosuppression after ITx, excluding steroids and leading to an allegedly better balance toward tolerance by Tregs. Immunomodulation techniques by bone marrow augmentation (infusion of unmodified donor cells), and simultaneous liver or bone marrow transplantation were also tried to create this tolerogenic environment, generating 'macro chimerism' (>1% of donor cells in blood) or 'micro chimerism' (<1%).

Pirenne and Kawai [165] developed a multifactorial immunomodulatory approach that was first published in 2006. Donor-derived whole blood was transfused during transplant, followed by induction with IL-2 monoclonal antibody (mAb) and low doses of steroids and tacrolimus as maintenance. This method also generates a chimeric environment immediately at transplantation. It gave good results after following up to 8 years.

The current management of ITx is as follows: induction therapy with anti-CD25 (alemtuzumab) or lymphocyte-depleting agents (such as ATG), and maintenance therapy with tacrolimus and mycophenolate mofetil (MMF). According to the last Intestinal Transplant Registry Report, rapamycin as a maintenance drug improved ten-year graft survival by 20%, although its use combined to tacrolimus can cause severe proteinuria. Rapamycin and MMF help to modulate the expression levels of Th1 signature genes, as shown in a new study[166]. Pirenne and Kawai [99] mention the then Omaha group lead by A. Langnass and D. Sudan as the leaders in low rejection rates, using a combination of ATG in the donor, induction therapy with IL-2 receptor blockade in the recipient together with tacrolimus and low-dose steroids as maintenance. Although all these strategies improved short-term graft survival, long-term outcome is still poor, and the intestinal transplant rate dropped to 120 intestinal transplants per year worldwide since 2003[8,99,164,167].

To improve long-term survival, new maintenance strategies must be explored. The induction of (macro)chimerism and Tregs together with strategies to prevent the development of DSA are probably important treatment goals for the future.

New treatment strategies include cellular immune therapy, the most promising cell types that can be used, or donor-specific Tregs type 1, tolerogenic dendritic cells, suppressive macrophages, natural Tregs, and myeloid or mesenchymal stem cells[168]. These cells may participate in the NO-dependent maintenance phase of tolerance and suppress immune responses either directly by interacting with effector T cells or indirectly by promoting Tregs[169]. Mesenchymal stem cells seem to prevent GvHD and improve the state of the epithelial barrier[170,171].

The dynamics of the exchange of large amounts of donor and recipient's immune cells is unique in intestinal transplantation. Therefore, intervening T-cell trafficking of gut-homing T cells by using mAb against endothelial addressing cell adhesion molecules, such as mucosal vascular addressin cell adhesion molecule 1 or leucocyte homing molecules such as $\alpha4\beta7$[18] has high potential and needs to be explored as maintenance therapy in ITx.

Patients with DSA currently depend on mechanical antibody removal and strong immunosuppression, but a recent study shows that therapy with proteasome inhibitors (bortezomib) in kidney transplants can effectively reduce the amount of these antibodies[172,173] and could be useful in ITx scenario as well.

Conclusion.

Although significant progress has been made, a long-term successful transplantation of the small bowel remains a challenge due to the nature of the gut as an immunocompetent absorptive organ fully equipped to prevent enteral infection. The focus should be on the development of tolerogenic immunosuppressive strategies that imply the depletion of immunogenic cells in the intestinal graft, promoting the development of chimerism, blocking the re-entry of alloreactive gut-homing immune cells, and prevention of the development of DSA. Therefore, cell-depleting and cell-based immunotherapy together with treatments that prevent gut-homing of T-cells seems to be the most promising strategies for the future.

"Ik heb gewoon mijn leven terug."

De patiënt.