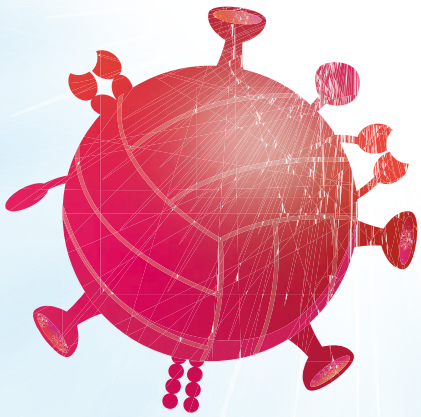
REJECTION AND TOLERANCE AFTER LIVER TRANSPLANTATION



Aafke Duizendstra

Rejection and Tolerance after Liver Transplantation

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It seems impossible, until it is done

- Nelson Mandela -

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CHAPTER 1.

General Introduction and Outline

General introduction

The liver

The liver is a vital organ with many different functions, which supports almost every other organ in the body. The functions of the liver include protein, carbohydrate and lipid metabolism, bile formation, alcohol and drugs metabolism, and hormone catabolism. Blood from the gastrointestinal tract is transported by the portal vein to the liver. The liver has a crucial function to filter out bacterial, fungal and viral antigens derived from the gastrointestinal tract. These antigens, and intact micro-organisms that have passed damaged intestinal epithelium, are phagocytosed and degraded by Kupffer cells, the macrophages present in the liver, and an (adaptive) immune response in the liver is often avoided. Hence, the liver is considered an immunologically privileged organ.[1]

Because of the specific functions in the body, the liver is prone to many hepatic diseases. Acute hepatitis can be caused by viral infections e.g. Epstein-Barr virus and cytomegalovirus, parasites e.g. Toxoplasma gondii, poisons e.g. aflatoxins, drugs e.g. paracetamol, or alcohol. Chronic hepatitis can be caused by cholestatic diseases e.g. primary sclerosing cholangitis and biliary cirrhosis, viral infections e.g. Hepatitis B or C virus, autoimmune disease, drugs, alcohol or metabolic disorders.[1]

The immune system

The immune system consists roughly of an innate and adaptive part. The innate immune system is the initial response to foreign invaders, and prevents, controls or reduces infection by huge numbers of pathogens. It recognizes several common molecular structures typically present on microbes. The main cells belonging to the innate immune system are neutrophils, macrophages, basophils and eosinophils. Natural killer cells (NK cells) are innate immune lymphocytes that recognize infected or unhealthy (e.g. pre-cancerous) cells in the body by sensing a disturbed balance of receptors present on the cell and subsequently kill their targets. One of these receptors are class I human leukocyte antigen (HLA) molecules, which are under healthy conditions present on almost all types of cells. Antigen presenting cells (APCs), such as dendritic cells and macrophages, link the innate with the adaptive immune system.[2]

T-cells

The adaptive immune system consists of T and B lymphocytes, of which each clone expresses antigen receptors (T-cell receptors or immunoglobulins respectively) with an unique specificity, unlike the cells from the innate immune system. Macrophages and dendritic cells present processed peptides, derived from pathogens found in their near vicinity, in their class I or class II HLA molecules, after which the cells migrate into peripheral lymphoid organs. In an adaptive immune response, naive CD4+ and CD8+ T-cells migrate into peripheral lymphoid

organs, where they are activated by antigenic peptides presented by APCs that are exclusively specific for their T-cell receptor (Figure 1; Signal 1). Presentation of antigenic peptides in class I HLA molecules (HLA-A, -B and -C) can only activate CD8+ T-cells, whereas antigenic peptides presented in class II HLA molecules (HLA-DR, -DQ and -DP) can only activate CD4+ T-cells. Class I HLA molecules are expressed on nucleated cells in the body, whereas class II HLA molecules are only constitutively expressed on APCs, but may be induced by e.g. inflammatory signals on other cell types.

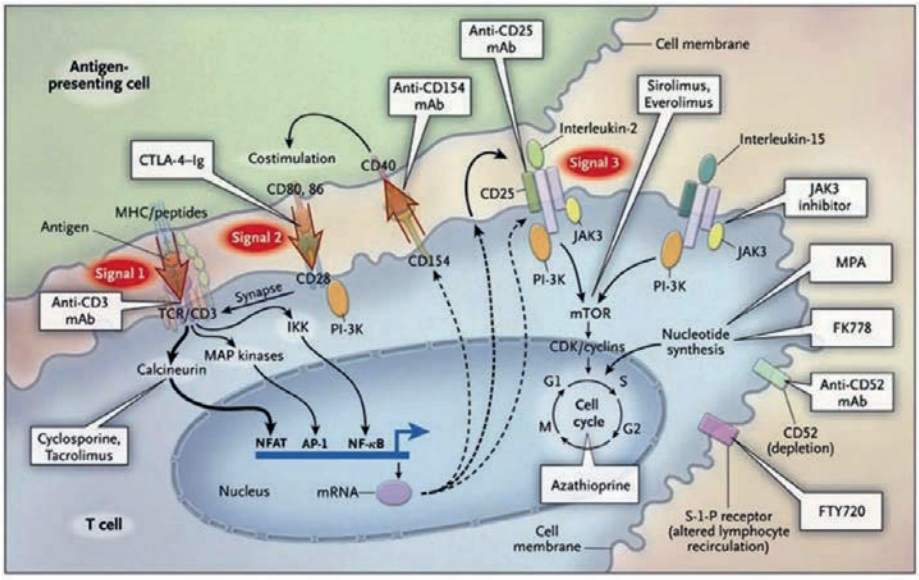

Figure 1 T-cell activation with three signal model and site of actions by immunosuppressive drugs. In red the three signals are indicated needed for appropriate T-cell activation. These include activation of the T-cell receptor (TCR) by HLA-antigen-complexes presented on antigen presenting cells, interaction of CD28 on T-cells with CD80/86 on antigen presenting cells, and production of auto-stimulant interleukin 2 (IL2) by T-cells. White boxes contain immunosuppressive drug therapies and indicate where (alloreactive) T-cell responses are inhibited. Reproduced with permission from Halloran, New England Journal of Medicine, 2004; 351:2715-2729, Copyright Massachusetts Medical Society.[3]

Cells of the immune system communicate by cell-to-cell contact via receptor-ligand interactions or through production of soluble factors, e.g. cytokines and chemokines. In addition to T-cell receptor activation, naive T-cells need co-stimulation via specific receptors, such as CD28, for appropriate activation (Figure 1; Signal 2). After activation, T-cells produce auto-stimulant interleukin 2 (IL2) (Figure 1; Signal 3), and proliferate and differentiate into short-lived effector T-cells that eliminate the antigens, and long-lived memory T-cells that whenever an antigen is encountered again, mediate a rapid and enhanced immune response less in need for co-stimulation. CD137 (or 4-1BB) is a co-receptor that is upregulated on T-

cells when activated, and when the receptor is engaged by its ligand present on APCs, T-cell proliferation and cytokine production are enhanced.[4] CD4+ effector helper T-cells can produce many different kinds of cytokines, such as IFNγ, IL4, IL13 and IL17, which all activate other immune cells, such as macrophages, eosinophils and neutrophils. CD8+ effector cytotoxic T-cells recognize and kill specific target cells with class I HLA molecules that present antigenic peptides, by releasing granules containing perforin and granzymes. Memory T-cells are comprised of central memory (CM), effector memory (EM) and terminally differentiated effector memory (EMRA) cells, which are each found in different sites of the body and can be discriminated by differential expression of CCR7 and CD45RA on the cell surface. During ageing memory T-cells accumulate in the body and replace naive T-cells.[2]

Another type of T-cells that comprises less than 5% of the T-cell population are γδT-cells. These T-cells are characterized by γ and δ T-cell receptor chains, whereas conventional T-cells harbor α and β T-cell receptor chains. These γδT-cells do not recognize peptides presented in HLA molecules, but recognize lipids, alkyl amines, small phosphorylated molecules and some proteins, and are commonly found in tissues. Specific subsets of γδT-cells produce cytokines and chemokines, whereas other subsets kill target cells using perforin and granzymes.[2] Some pathogens, for instance cytomegalovirus, influence the composition of circulating immune cells with sustained expansion of pathogen specific CD8+ T-cells, γδT-cells and NK cell subsets.[5,6]

B-cells and antibodies

Naive B-cells migrate into the follicles of peripheral lymphoid organs. When they encounter antigens presented by APCs that are exclusively specific for their B-cell receptors IgM or IgD, they become activated. After activation B-cells proliferate and differentiate into antibody-secreting plasma cells and memory B-cells. At the start of activation the antibody-secreting plasma cells secrete low affinity isotype IgM antibodies against the antigens encountered. B-cells require help from CD4+ follicular helper T-cells for an optimal antibody response against protein antigens. After T-cell help, the expanded antibody-secreting plasma cells go into heavy chain isotype class switching and start producing a huge amount of isotype IgG antibodies with increased affinity towards the antigens, and under certain situations also IgA and IgE antibodies. The receptor CD40 ligand (CD40L) present on helper T-cells stimulates the affinity maturation of the IgG antibodies in B-cells. With every subsequent antigen encounter the memory B-cells secrete larger amounts of antibodies, and heavy chain isotype class switching and affinity maturation are also increased. The IgG antibodies enter the circulation in order to neutralize and eliminate antigens, microbes or toxins. Upon binding of an IgG antibody to its target (opsonization), a back Fc part is exposed, which can be recognized by cells expressing receptors for the Fc part. Neutrophils or macrophages with Fc receptors can phagocytose and kill opsonized targets, whereas NK cells can destroy cells opsonized with antibodies (antibody-dependent cell-mediated cytotoxicity). Furthermore, opsonization of cells with antibodies can activate the complement system. The complement system is a tightly regulated cascade of events that leads to attraction of immune cells and inflammation, disruption of the opsonized cellular membrane and subsequent death of cells. Four IgG

antibody subtypes are known (IgG1, IgG2, IgG3 and IgG4), which all have slightly different functions with respect to opsonization, complement activation and antibody-dependent cell-mediated cytotoxicity.[2]

Immunological tolerance and regulatory T-cells

Whenever lymphocytes interact with an APC presenting antigenic peptides, the cells either become activated and an immune response is induced, or the lymphocytes are inactivated or eliminated and immunological tolerance is induced. This is determined by the antigenic properties, the maturation state of the antigen-specific immune cells and the type of stimulation the immune cells receive during antigen encounter. Memory T-cells that encounter their specific antigen target numerous times eventually become exhausted and are unable to mount an effective immune response. In general, prolonged systemic exposure to high doses of antigens without co-stimulation tends to induce tolerance. In this way the T-cells are stimulated via their T-cell receptor by the antigens, but do not receive co-stimulatory signals from APCs, and the T-cells become anergic (functional unresponsive), go into apoptosis, or differentiate into CD4+ regulatory T-cells.

Regulatory T-cells (Tregs) inhibit immune responses initiated by effector T-cells. Tregs derived from the thymus mostly inhibit self-reactive CD4+ T-cells and are termed natural Tregs, whereas peripherally induced Tregs in response to specific antigens are termed induced Tregs. TGF-β and IL2 promote development of CD4+ Tregs, and they constitutionally express CD25, the high affinity IL2 receptor alpha subunit, and the transcription factor FoxP3, critical for the development and function of most Tregs.[2] Since in humans activated effector T-cells also transiently express CD25 and FoxP3, a proposed combination of FoxP3 and CD45RA receptor expression is used to discriminate CD4+ activated effector helper T-cells, and two types of CD4+ Tregs (activated and resting Tregs).[7] Another type of Tregs are Tr1 cells, which express surface markers LAG3 and CD49b, but do not constitutionally express FoxP3.[8] T-cell immune responses are suppressed by Tregs in several ways, such as production of cytokines or (indirect) cell-to-cell contact via receptors. Tregs produce TGF-β, which inhibits the activation of macrophages and neutrophils, and inhibits the proliferation and effector function of T-cells. They also produce IL10, which inhibits activated macrophages and dendritic cells, and expression of their co-stimulatory ligands for T-cells. Co-inhibitory receptor CTLA4 expressed on Tregs can inhibit APC activation and suppresses co-stimulation of CD4+ or CD8+ T-cells via different mechanisms.[2]

Liver transplantation

Acute or chronic liver disease may lead to liver failure, for which a liver transplantation is the sole treatment option. In the Netherlands a total of around 3000 liver transplant recipients have received a donor liver since 1979. The current overall graft and patient survival rates are 90%, 80% and 70% after 1, 5 and 10 years of transplantation respectively.[9] Unfortunately, the majority of chronic liver diseases can recur in the donor liver graft and seriously hamper the

overall survival late after transplantation. These include primary sclerosing cholangitis and biliary cirrhosis, autoimmune hepatitis, and Hepatitis B infection.[10,11] After liver disease recurrence, a re-transplantation is the only treatment option.

Immunosuppressive drugs and its side effects

The transplanted donor liver graft is recognized as foreign by the immune system of the recipient. In order to prevent severe rejection in the first few weeks after transplantation, prophylactic induction treatment is administered around the time of transplantation. Induction treatments include lymphocyte depleting agent rabbit antithymocyte globulin (rATG) and non-depleting agents basiliximab and methylprednisolone.[1] Calcineurin inhibitors tacrolimus (Tac) and cyclosporine A (CsA), inosine monophosphate dehydrogenase (IMPDH) inhibitor mycophenolate mofeteil (MMF, pro-drug of MPA) and corticosteroid prednisone are the most potent and most commonly life-long used maintenance immunosuppressive drugs after liver transplantation (Figure 1). Other less commonly used maintenance immunosuppressive drugs are mTOR inhibitors everolimus and sirolimus, and IMPDH inhibitor azathioprine (Figure 1).[12,13]

Unfortunately, use of immunosuppressive drugs is associated with severe side effects in a significant proportion of liver transplant recipients. The side effects for short term immunosuppressive drugs use are persistent infections, and after long term use metabolic disorders (e.g. diabetes, dyslipidemia), renal dysfunction, cardiovascular disease, and malignancies arise in a significant proportion of recipients.[14-18] Short-term survival rates after liver transplantation improved significantly over the last two decades because of improved surgical techniques and optimized immunosuppressive drug regimens.[19] However, morbidity and mortality rates more than one year after liver transplantation showed no improvement and are still noticeably higher than the general population.[20] Thus, most liver transplantation centers attempt to gradually reduce immunosuppressive drugs over time in order to decrease its side effects. However, immunosuppressive drug reduction is associated with an increased risk of graft rejection.

Graft rejection after transplantation

Diagnosis of rejection

Despite use of immunosuppressive drugs, rejection of the donor graft could occur. After liver transplantation liver function serum parameters bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (AP) and γ-glutamyltransferase (γGT) are regularly measured and monitored in order to detect an upcoming rejection episode. After an elevation of these parameters is observed, a liver biopsy is often performed to diagnose the cause, and in case of rejection to specify which form(s) are present in the donor graft. Acute rejection with T-cell mediated rejection and antibody mediated rejection, or chronic rejection can all arise in the donor liver graft, after which an appropriate rescue treatment of immunosuppressive drugs is administered to the recipient.

Direct and indirect HLA alloantigen recognition

HLA molecules are the most polymorphic proteins encoded by the human genome. Furthermore, extremely high proportions (1-2%) of recipient T-cells are capable of recognizing a foreign HLA molecule, of which the majority are cross-reactive memory T-cells that elicited a response against other antigens. Therefore, recognition of donor HLA molecules or fragments thereof by immune cells of the recipient is considered the cause of almost all severe rejections. Other donor polymorphic proteins can elicit weaker or slower immune responses compared to HLA molecules and they are called minor histocompatibility antigens. Donor antigens recognized by the immune system of the recipient are called alloantigens. Donor antigens can be recognized in three ways: direct, indirect and semi-direct alloantigen recognition. Direct recognition is the stimulation of T-cells of the recipient with alloantigens presented by HLA molecules of donor cells (Figure 2A). This direct stimulation could be by (allo)antigens presented in HLA molecules of the donor that are similar to the recipient's, by a combination of antigenic peptides presented in foreign HLA molecules of the donor, or by the foreign HLA molecules of the donor alone. The latter two both comprise the recipient's T-cell response against intact conformational donor HLA molecules. The indirect recognition is the stimulation of T-cells of the recipient with alloantigens presented by recipient APCs, which are fragments of donor HLA molecules or other minor donor-specific antigens (Figure 2B). Until donor APCs are replaced by recipient APCs in the donor liver, de novo induction of allogenic recipient T-cells by direct alloantigen recognition occurs (<6 months after transplantation). De novo induction of allogenic recipient T-cells by indirect alloantigen recognition occurs mostly later (>6 months) after transplantation. The third way in which donor-antigens could be recognized is the semi-direct alloantigen recognition (or cross-dressing) (Figure 2C). In this recently acknowledged recognition in animal and human studies, donor leukocytes and other cells release small extracellular vesicles that contain intact donor HLA molecules that are taken up and presented by recipient APCs, after which alloreactive recipient T-cells are activated.[21-26] This semi-direct alloantigen recognition after liver transplantation is transient and short-lived (1-4 days) when measured in peripheral blood, but could be a continuous local phenomenon in the liver graft.[23,25] All three alloantigen responses against donor HLA are believed to be important for the development of (severe) rejection after liver transplantation.[2,27,28]

T-cell mediated rejection

T-cell mediated rejection occurs in about a third of all liver transplant recipients in the first few weeks, in about 20% of all recipients in the first months and in about 10% of all recipients late after transplantation.[29] In T-cell mediated rejection, (memory) T-cells respond to HLA alloantigens and receive co-stimulation by APCs. Alloreactive CD4+ T-cells start producing cytokines and damage the liver graft by inducing inflammation as described earlier. After direct alloantigen recognition CD8+ T-cells kill allograft cells expressing donor class I HLA molecules by releasing granules containing perforin and granzymes, and after direct and indirect recognition CD8+ T-cells secrete inflammatory cytokines contributing to graft damage.[2] T-cell mediated rejection is quantitatively estimated by histological analysis of liver

biopsies according to Banff criteria using the Rejection Activity Index (RAI) score that includes portal, bile duct and venous endothelial inflammation and damage.[30] In general, T-cell mediated rejection can be adequately treated by intravenous administration of methylprednisolone or enhanced dosing of conventional immunosuppressive drugs. However, memory T-cells and alloreactive CD4+ T-cells involved in the indirect alloantigen response are more resistant to immunosuppressive drugs compared to CD8+ T-cells involved in the direct alloantigen response.[2] Adequately treated T-cell mediated rejection early after liver transplantation does not adversely impact patient or liver allograft survival, but late T-cell mediated rejection could lead to graft failure and mortality.[29]

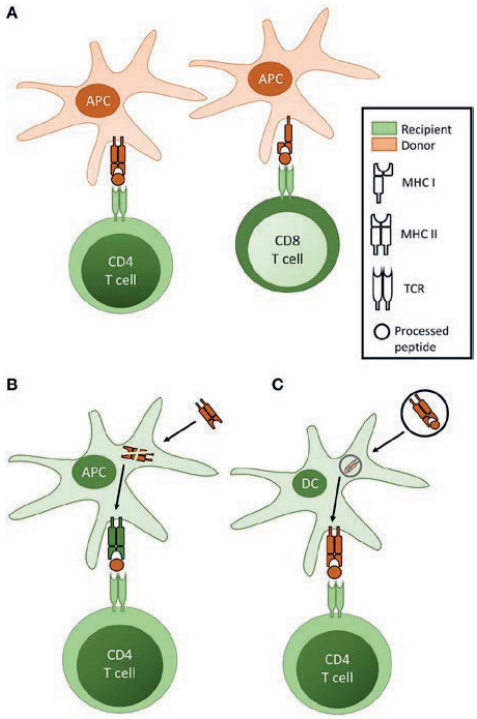

Figure 2 Direct, indirect and semi-direct alloantigen recognition by recipient T-cells. Direct (A), indirect (B) and semi-direct (C) alloantigen presentation towards recipient T-cells are indicated. In orange the donor antigen presenting cells (APCs), donor HLA molecules and donor processed antigens (spheres) are depicted. In green the recipient antigen presenting cells, recipient T-cells and recipient HLA molecules are presented. Reproduced with permission from Siu et al, Frontiers in Immunology, 2018; 9:2548, Copyright Frontiers Media SA.[31]

Antibody mediated rejection

In most antibody mediated rejections B-cells recognize foreign donor HLA molecules, internalize and process these proteins, and present them towards helper T-cells that were previously activated by dendritic cells in an indirect antigen response. Hence, B-cells secrete

many IgG antibodies with increased affinity towards the donor HLA antigens. These antibodies are called de novo donor-specific antibodies (DSAs). Preformed DSAs are already present before liver transplantation because of previous exposure to foreign HLA molecules, e.g. during pregnancy or upon blood transfusion. Alloantibodies cause acute graft rejection by binding donor (HLA) molecules expressed on hepatocytes, endothelial cells and cholangiocytes in the liver graft, after which opsonization, complement activation and antibody-dependent cell-mediated cytotoxicity occur as described earlier.[2] Unlike in other types of solid organ transplantation, many DSA positive liver transplant recipients do not experience clear symptoms of rejection.[32] Nevertheless, concurrent presence of DSAs and subclinical T-cell mediated rejection, a mild form of rejection without elevating liver function parameters, is associated with more severe graft inflammation and fibrosis.[33] Antibody mediated rejection can be diagnosed by scoring C4d deposition, a component of the complement cascade, in the portal microvasculature. However, antibody mediated rejection can also occur without activation of the complement system. In about 3-5% of liver grafts experiencing dysfunction late (> 6 months) after transplantation antibody mediated rejection is diagnosed. Its incidence in liver allografts is very low compared to for instance kidney allografts, since the liver is relatively resistant to antibody mediated rejection.[30]

Chronic rejection

Chronic rejection develops in about 2-5% of the liver grafts several months or years after transplantation. It is a severe deleterious complication after liver transplantation, characterized by foamy arteriopathy and progressive bile duct loss (ductopenia). In general, chronic rejection is not associated with development of fibrosis or cirrhosis.[34] Chronic rejection does traditionally not respond to treatment with immunosuppressive drugs, hence often a retransplantation is the only treatment option.[30] Nonetheless, in some cases involving multiple forms of rejection including chronic rejection, a standard retransplantation is not sufficient and rejection recurs. Until now little is known on how to approach such complex situations. To date, the mechanisms causing chronic rejection are under-investigated. The likely mechanisms involved in chronic rejection are persistent secretion of cytokines by alloreactive T-cells and subsequent inflammation and attraction of leukocytes, persistent antibody mediated rejection or chronic viral infections.[2]

Markers predicting acute rejection

Adequately treated acute rejection early after liver transplantation does not adversely impact patient or liver allograft survival, but late acute rejection is associated with a higher risk of graft failure and mortality.[29] Thus, early diagnosis and prevention of rejection is paramount for graft and patient survival. A regularly taken liver biopsy in order to predict development of acute rejection is not feasible, since it is an invasive procedure that could lead to severe clinical complications. Therefore, non-invasive soluble blood markers predictive for acute rejection early after liver transplantation, such cytokines, chemokines, receptors and cell adhesion molecules, have been investigated. These markers include tumor necrosis factor

(TNF), IL10, CXCL10, CXCL11, CD44 and IL6.[35-40] However, the investigated biomarkers were commonly also elevated prior to a severe infection, hence until now none of the biomarkers have been implemented in the clinic due to its lack of specificity.[39,41-43] Infections are much less prevalent late after transplantation, but soluble non-invasive biomarkers predictive for acute rejection late after liver transplantation were never investigated. Graft rejection is a complicated process that involves different parts of the immune system. Therefore, it is highly unlikely that one immunological serum biomarker could predict an acute rejection episode late after liver transplantation. A first exploration of an extensive panel of soluble immunological proteins in serum would be useful to identify combinations of immunological factors that might be promising to validate in subsequent studies.

The immune-privileged liver graft

In contrast to lung, kidney and heart transplantation, liver transplantation in general does not require matching of HLA between donor and recipient to prevent or dampen severe forms of rejection.[44] Furthermore, less rigorous immunosuppressive drug regimens are often necessary to prevent allograft rejection after liver transplantation compared to other solid organ transplantations. Acute and chronic rejection, including antibody mediated rejection, also occur less often after liver transplantation compared to other types of solid organ transplantation.[45] Moreover, it has been shown that liver grafts prevent rejection or dampen an ongoing rejection in lung, kidney and heart allografts from the same donor.[46-48] This is probably all due to the immunologically privileged state of the liver, hence the liver is so called 'tolerogenic'.

Operational immunological tolerance

Most liver transplantation centers attempt to gradually reduce immunosuppressive drugs over time in order to decrease its side effects. Occasionally, however, liver transplant recipients need to be weaned from immunosuppressive drugs altogether because of life-threatening medical complications. Additionally, the burden of chronic adherence to immunosuppressive drugs leads in some cases to non-compliance and subsequent complete weaning.[45] In the majority of recipients, complete immunosuppressive drug weaning leads to graft rejection and requires re-installment. However, a minority of liver transplant recipients develop spontaneous operational tolerance towards their graft, a long-term state defined by absence of (acute) rejection episodes while completely weaned from immunosuppressive drugs. Subsequently, a few clinical trials confirmed the possibility of achieving immunological tolerance to donor liver grafts in about 40% of adult and 60% of pediatric careful electively weaned liver transplant recipients.[49-54] The possibility of achieving immunological tolerance after liver transplantation is probably due to the immunologically privileged state of the liver, since it is rarely achieved after all other solid organ transplantations. Whether complete weaning of immunosuppressive drugs could reduce its related side effects after liver transplantation is still unclear. Limited number of studies have assessed the long-term impact of weaning on co-morbidities late after liver transplantation, and contradictory findings have

been reported for tolerant adult and pediatric liver transplant recipients.[49,55-60] This could be due to the lack of matched clinical parameters between the study groups in these studies. A study investigating the influence of complete immunosuppressive drug weaning on its related side effects in tolerant liver transplant recipients compared to a completely matched control group of liver transplant recipients on regular immunosuppressive drug regimen has not been performed yet.

Tolerant liver transplant recipients and liver fibrosis

One study suggested that pediatric tolerant liver transplant recipients weaned from immunosuppressive drugs are more prone to develop liver fibrosis compared to control recipients on regular immunosuppressive drug regimen.[61] Despite absence of further evidence in pediatric and adult tolerant liver transplant recipients,[55-57,62-64] clinicians fear development of severe fibrosis after complete immunosuppressive drug weaning, due to e.g. subclinical rejection, a mild form of rejection without elevating liver function parameters.

Liver fibrosis is a reversible wound-healing response to persisting injury and is characterized by the alteration and accumulation of the extracellular matrix (ECM). ECM structural components in the liver are collagens, proteoglycans, laminins and fibronectins. The ECM provides a support network for the surrounding cells that include epithelial cells (hepatocytes and cholangiocytes), endothelial cells, Kupffer cells, immune cells and hepatic and stellate cells (HSCs).[65,66] In the ECM TIMPs are present, which inhibit MMPs that degrade the ECM structural proteins. In a fibrotic liver the balance between MMPs and TIMPs is disturbed.[67] A variety of liver cells produce main growth factors PDGF and TGF-β1, which are implicated in HSC activation and collagen synthesis. When HSCs become activated upon chronic liver damage, they differentiate into fibroblast-like cells and upregulate production of TIMPs and hyaluronic acid.[65,66,68] Other proteins, such as BAFF, are implicated in lung and skin fibrosis in humans, and could therefore also be implicated in liver fibrosis.[69,70] All above mentioned proteins involved in (liver) fibrosis can be measured in the peripheral blood, and some are already used as a non-invasive biomarker for the assessment of liver fibrosis.[71,72] Fibrosis could eventually lead to cirrhosis, that in contrast to fibrosis, has a high morbidity and mortality.

A liver biopsy is the golden standard for detecting liver fibrosis. However, as mentioned earlier, this is an invasive procedure that could lead to severe clinical complications. Therefore, transient elastography, a type of ultrasound that measures the liver's stiffness, has recently been validated for the assessment of liver fibrosis in immunocompetent individuals with various liver diseases.[73-75] A few studies have concluded that transient elastography can also be used to discriminate between development of no or mild fibrosis and significant fibrosis after liver transplantation.[71,76,77] A study to assess liver fibrosis in adult tolerant liver transplant recipients by transient elastography and measuring different fibrosis related peripheral blood markers has not been performed yet.

Identifying tolerant liver transplant recipients

Many studies have tried to elucidate the mechanisms by which operational immunological tolerance is achieved and which biomarkers could identify tolerant liver transplant recipients. The clinical factors favoring immunosuppressive drug weaning and tolerance are time after transplantation, higher age at time of transplantation and normal histology of the liver graft prior to complete weaning.[78] Nevertheless, these factors provide insufficient sensitivity and specificity to identify tolerant liver transplant recipients accurately. In order to safely wean liver transplant recipients from immunosuppressive drugs, biomarkers accurately identifying tolerant recipients that have developed immunological tolerance towards their graft are needed. Gene expression in the liver graft or peripheral blood mononuclear cells (PBMCs) and circulating immune cells in relation to operational tolerance have been investigated, but soluble immune-system related markers present in peripheral blood of tolerant liver transplant recipients were never examined.

Liver graft gene expression

A few studies have investigated presence of specific immune cells or gene expression profiles in the liver graft of tolerant liver transplant recipients. In the graft of tolerant liver transplant recipients CD4+FoxP3+ T-cells were elevated[64,79] and expression of proinflammatory genes was downregulated in the years after complete weaning compared to baseline level.[64] Tolerant and non-tolerant liver transplant recipients differed in the intragraft expression of genes involved in the regulation of iron homeostasis.[80] In another study the intragraft Vδ1/Vδ2 γδT-cell gene expression ratio was elevated in tolerant recipients versus control recipients with regular immunosuppressive drugs.[81] For identification of tolerant liver transplant recipients according to their liver graft gene expression profiles or presence of immune cells, a liver biopsy is necessary. Therefore, it would be more safe and convenient to investigate whether peripheral blood markers could identify tolerant liver transplant recipients.

Peripheral blood gene expression

Several gene expression studies on circulating PBMCs were performed in relation to operational tolerance. Expression of FOXP3[82] and several immune system and NK cell related genes, such as KLRB1, KLRC4, SLAMF7, NKG7, KLRF1, OSBPL5, IL2RB and IL8,[83,84] in PBMCs were significantly different in tolerant recipients versus non-tolerant or control recipients using regular immunosuppressive drugs. A drawback of these studies is that they lack matching of parameters between study groups that are known to influence the composition of immune cells, such as cytomegalovirus infection and recipient age. Furthermore, of most expressed genes different isoforms exist and the isoform specificity of the detected gene expressions in these studies is not reported. Hence, these gene expression markers have a need to be validated in another study that take matching of important parameters and isoforms of gene expression into account.

Circulating cells of the immune system

Circulating immune cells were heavily investigated in tolerant liver transplant recipients. Elevated percentages of circulating B-cells expressing CD19 were present in tolerant liver transplant recipients versus control recipients with regular immunosuppressive drugs.[85] One study reported an elevated ratio of two distinct types of circulating dendritic cells in successfully weaned tolerant liver transplant recipients compared to a control group of recipients using regular immunosuppressive drugs,[86] but this difference was not reported in another study.[87] The type of dendritic cells that were elevated, plasmacytoid dendritic cells, were suggested to have a low capacity to stimulate T-cells and therefore induce immunological tolerance.[88,89] Another study indicated that HLA-G, a non-classical HLA class I molecule expressed on dendritic cells that represses T-cell stimulation, was elevated on dendritic cells of tolerant liver transplant recipients compared to a control group of recipients using regular immunosuppressive drugs.[90] This could indicate that operational tolerance towards the liver graft could arise due to inhibition of T-cells by dendritic cells.

Several studies have investigated circulating T-cells to identify tolerant liver transplant recipients. Higher relative numbers of circulating CD4+CD25^{high} T-cells,[82,85,87] CD4+FoxP3+ T-cells[87], CD4+CD25++CD45RA+ T-cells[91], CD4+CD25++CD127^{dim} T-cells[92], and a higher Vδ1/Vδ2 γδT-cell ratio[82,85,87] in blood of adult or pediatric recipients were implied to discriminate between tolerant and control groups of liver transplant recipients. These data suggest that Tregs and γδT-cells might play a role in the development or maintenance of operational tolerance. However, many of these studies lack matching of parameters that are known to influence the composition of circulating immune cells, such as cytomegalovirus serostatus.[5,6] Furthermore, phenotypic and/or functional characteristics of donor-specific T-cells and their association with operational tolerance after liver transplantation is under-investigated. Two studies reported lack of proliferation of total CD4+ T-cells upon stimulation with alloantigens when compared to third party antigens (donor-specific hyporesponsiveness), but donor-specific CD4+ or CD8+ T-cells have never been studied in more detail at the single-cell level.[92,93] Upon activation of CD4+ and CD8+ T-cells CD137 is expressed, and it has been proven that this marker can accurately identify alloreactive T-cells in kidney and liver transplant recipients, independent of effector function differences.[94-96] Therefore, a study on (CD137 positive) donor-reactive T-cells in tolerant liver transplant recipients that are carefully matched with other study groups needs to be performed.

Aim and outline

The first aim of this thesis is to investigate biomarkers by which operational immunological tolerance can be recognized and consequences of complete immunosuppressive drug weaning, by comparing tolerant liver transplant recipients to completely matched groups of other liver transplant recipients and/or healthy controls. The second aim is to assess whether rejection episodes after liver transplantation can be prevented by changing a standard retransplantation protocol or by proteomic screening of soluble markers that could predict an upcoming late acute rejection.

In **Chapter 2** a case report is presented where we applied HLA matching between donor and recipient in combination with a more rigorous immunosuppressive drug induction regimen as a treatment option to improve graft and patient survival, instead of a standard retransplantation. In **Chapter 3** we investigated whether an extensive proteomic screening of soluble serum markers could predict an upcoming late acute rejection or development of tolerance in liver transplant recipients. In **Chapter 4** the clinical benefits of complete immunosuppressive drug weaning on its related side effects in tolerant liver transplant recipients are described. In **Chapter 5** it was examined whether tolerant liver transplant recipients develop fibrosis in the donor liver graft after complete weaning of immunosuppressive drugs, by performing transient elastography and measurements of serum markers related to liver fibrosis. In **Chapter 6** presence of circulating immune cells, the donor specific T-cell immune response by expression of activation induced CD137 and presence of DSAs were assessed in tolerant liver transplant recipients and other matched study groups. In **Chapter 7** the goal was to investigate expression of tolerance gene profiles in PBMCs suggested by previous studies, in our cohort of tolerant liver transplant recipients and other matched study groups.

CHAPTER 2.

HLA matching and rabbit antithymocyte globulin as induction therapy to avoid multiple forms of rejection after a third liver transplantation

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Abstract

Background:
Despite immunosuppressive drug regimens, T cell-mediated rejection, antibody-mediated rejection with donor-specific antibodies, and chronic rejection occur after liver transplantation (LTx). Rejection may significantly impact allograft survival and often a standard re-LTx is required. However, in some cases rejection recurs. Little is known on how to approach this and which aspects to consider.

Case:
Here we describe a case in which two successive liver grafts were lost due to T cell-mediated rejection, possible antibody-mediated rejection with de novo donor-specific antibody formation, and chronic rejection that occurred within a month. In an attempt to avoid recurrence with the third graft, we decided to administer a more rigorous immunosuppressive drug induction regimen with rabbit antithymocyte globulin, while applying HLA matching between recipient and donor. This resulted in rejection free survival for 337 days until a mild T cell-mediated rejection occurred, which could then be easily treated with high dose steroids. Graft survival is now at least 683 days without chronic rejection, antibody-mediated rejection or de novo donor-specific antibody formation.

Conclusion:
In conclusion, when a liver graft is lost due to multiple forms of rejection short after LTx, the combination applied in this case could be considered as a viable option to improve graft and patient survival instead of a standard re-LTx.

Introduction

Liver transplantation (LTx) is the only treatment for end stage liver disease. Prophylactic induction treatment is administered around the time of LTx in order to prevent severe rejection during the first few weeks after transplantation. Since there is no consensus on a specific induction treatment worldwide, multiple treatment approaches exist, such as lymphocyte depleting agents alemtuzumab and rabbit antithymocyte globulin (rATG) and non-depleting agents basiliximab and methylprednisolone.[97]

Despite induction and maintenance immunosuppressive drug (IS) regimens, T-cell-mediated rejection (TCMR), antibody-mediated rejection (AMR) and chronic rejection (CR) occur after transplantation. The formation of donor-specific antibodies (DSAs) against human leukocyte antigen (HLA) types of the donor graft is common across all solid organ transplantations.[98] Unlike in other types of solid organ transplantation, many DSA positive LTx recipients do not experience clear symptoms of acute or chronic rejection. Nevertheless, both preformed and de novo DSAs have been associated with lower graft survival[32] and an increased risk of acute and chronic rejection (CR)[99], including AMR.[100] Acute AMR diagnosis requires four criteria and according to the BANFF scoring system this indicates a probability of AMR.[30] TCMR is quantitatively estimated by the Rejection Activity Index (RAI) score that includes portal, bile duct and venous endothelial inflammation. CR is a severe deleterious complication after LTx, characterized by foamy arteriopathy and progressive bile duct loss (ductopenia). In contrast to TCMR, CR does traditionally not respond to IS treatment.[30]

In only a minority of LTx recipients severe acute or chronic rejection after transplantation due to alloreactivity against donor HLA, leads to graft failure. For these patients, a retransplantation (re-LTx) is the only treatment available.[101,102] Nonetheless, in some cases a standard re-LTx is not sufficient and recurrence of rejection occurs. Until now little is known on how to approach this complex situation. Here we present a case where a third transplantation with HLA matching between recipient and donor in combination with a change in induction regimen was necessary to circumvent the severe TCMR, AMR, de novo DSA formation, and CR that arose after the first two liver transplantations.

Case

A 35-year old Caucasian male with a history of several cholestatic complications due to primary sclerosing cholangitis (PSC), was transplanted with a ABO-compatible donation after brain death (DBD) split liver in September 2017 at our institution. The donor liver had five HLA mismatches with the recipient (Table 1). As our standard protocol indicates, methylprednisolone 500 mg was administered during implantation and basiliximab 20 mg i.v. was administered within 6 hours after reperfusion, and repeated at day 4 after LTx. The following maintenance IS regimen was administered after LTx: daily oral prednisolone 20 mg and mycophenolate mofetil 2x 1000 mg; at day 5 tacrolimus at a dose of 0.1 mg per kg (2x 4 mg) was added; at day 7 prednisolone was reduced to 10 mg, which was continued for 3 months. After two adequate tacrolimus trough levels (8-15 µg/L), mycophenolate mofetil was discontinued. After 25 days the patient experienced symptoms of rejection, including a fever and substantially increasing liver enzyme abnormalities. A liver biopsy was evaluated by a liver pathologist and a severe acute TCMR and CR (Figure 1A; Table 2) were diagnosed. As a rescue treatment 1000 mg of methylprednisolone was administered for three consecutive days. Unfortunately, liver enzyme abnormalities did not decrease and a follow-up liver biopsy indicated that the rejection episode was not resolved (Figure 1B; Table 2). As a consequence, a second liver transplantation was needed. In the explant (Figure 1C; Table 2) clear TCMR with central perivenulitis and CR with ductopenia were present. There was also a suspicion of acute AMR[30] because of a combined C4d-score + h-score of 3 in the explant (Supplementary Figure 1; Table 2) and presence of de novo DSAs against donor HLA (Table 1) in the blood of the recipient.

Fifty-five days after the first LTx, a second LTx was performed with a ABO-compatible DBD donor liver. It is common practice for LTx not to consider the donor HLA type in combination with the recipient's. The second donor liver had four HLA mismatches with the recipient (Table 1). Of these four, three HLA mismatches were similar to those of the first donor liver. Similar induction treatment and maintenance IS regimen were given as described above. Again, the IS trough levels were adequate (6-15 µg/L). Two weeks after the second LTx, the patient again experienced increasing liver enzyme abnormalities. A liver biopsy (Figure 1D; Table 2) indicated again TCMR and CR. An AMR was also considered, because of DSA positivity (Table 1). As a rescue treatment methylprednisolone was administered as described above and an additional 65 g of i.v. immunoglobulins for two consecutive days thereafter. The patient responded to some degree to the rescue treatment according to the RAI score in the follow-up biopsy, but the CR episode and central perivenulitis did not resolve (Figure 1E; Table 2). Eventually, the graft could not be saved. In the explant (Figure 1F; Table 2) clear TCMR and CR with ductopenia and foamy arteriopathy were present. Despite a C4d score of 3 in the explant (Supplementary Figure 1; Table 2) and DSA positivity, acute AMR could not be confirmed due to a zero h-score.[30]

Table 1 The HLA types of the recipient and the three liver donors, their HLA mismatches and the DSAs present.

Recipient	A1 A2 B7 B8 DR2 (DR15) DR6 (DR13) DQ1 DQ6	HLA mismatch with recipient				
		A	B	DR	DQ	Total
Donor 1	A1 A19 (A32) B7 B8 DR3 (DR17) DR4 DQ2[ab] DQ3[ab] (DQ8)	1	0	2	2	5
Donor 2	A2 A19 (A32) B7 B15 (B62) DR2 (DR15) DR4 DQ1 (DQ6) DQ3[ac] (DQ8)	1	1	1	1	4
Donor 3	A2 B7 B16 (B38) DR2 (DR15) DR6 (DR13) DQ1 DQ6	0	1	0	0	1

In bold the HLA mismatches of donor with recipient are indicated. [a]de novo DSAs against the HLA type of the donor present in the recipient after LTx. [b]Mean fluorescence intensity measured: 6400 for both. [c]Mean fluorescence intensity measured: 1000. DSAs were measured in pre- and post-LTx serum samples by a standardized Luminex single antigen test.

Table 2 Histology of biopsies and explants.

Indicative of	TCMR	Transition of AR to CR	CR	AMR	
Biopsy (B) or explant (E)	RAI score	Central Perivenulitis	Ductopenia (% of portal field)	C4d score[c]	h-score
B1a	5[a]	+++	Yes (90%)	ND	1
B1b	3[a]	+++	Yes (90%)	0-1	1
E1	7	+++	Yes (80-90%)	2	1
B2a	5[a]	++(+)	Yes (90%)	0	1
B2b	1[a]	++(+)	Yes (90%)	2-3	0
E2	5[a]	+++	Yes (>95%)[b]	3	0
B3a	7	+(+)	No	0	1
B3b	2	-	No	0	0

Biopsies after 1st (B1), 2nd (B2) and 3rd (B3) LTx, before (a) and after (b) rescue treatment, and explants after 1st (E1) and 2nd (E2) LTx are depicted. [a]Bile duct inflammation could not be determined due to ductopenia. Therefore, RAI scores are without bile duct inflammation score. [b]Foamy arteriopathy present towards hilus. [c]C4d was determined in formalin-fixed paraffin-embedded biopsies by immunohistochemistry with a monoclonal antibody. Due to a shortage of biopsy tissue before rescue treatment after first LTx, C4d staining could not be performed. – none, + mild, ++ moderate, +++ severe. AMR, antibody-mediated rejection; AR, acute rejection; CR, chronic rejection; h-score, histopathology-score; ND, not determined; RAI, rejection activity index; TCMR, T-cell-mediated rejection.

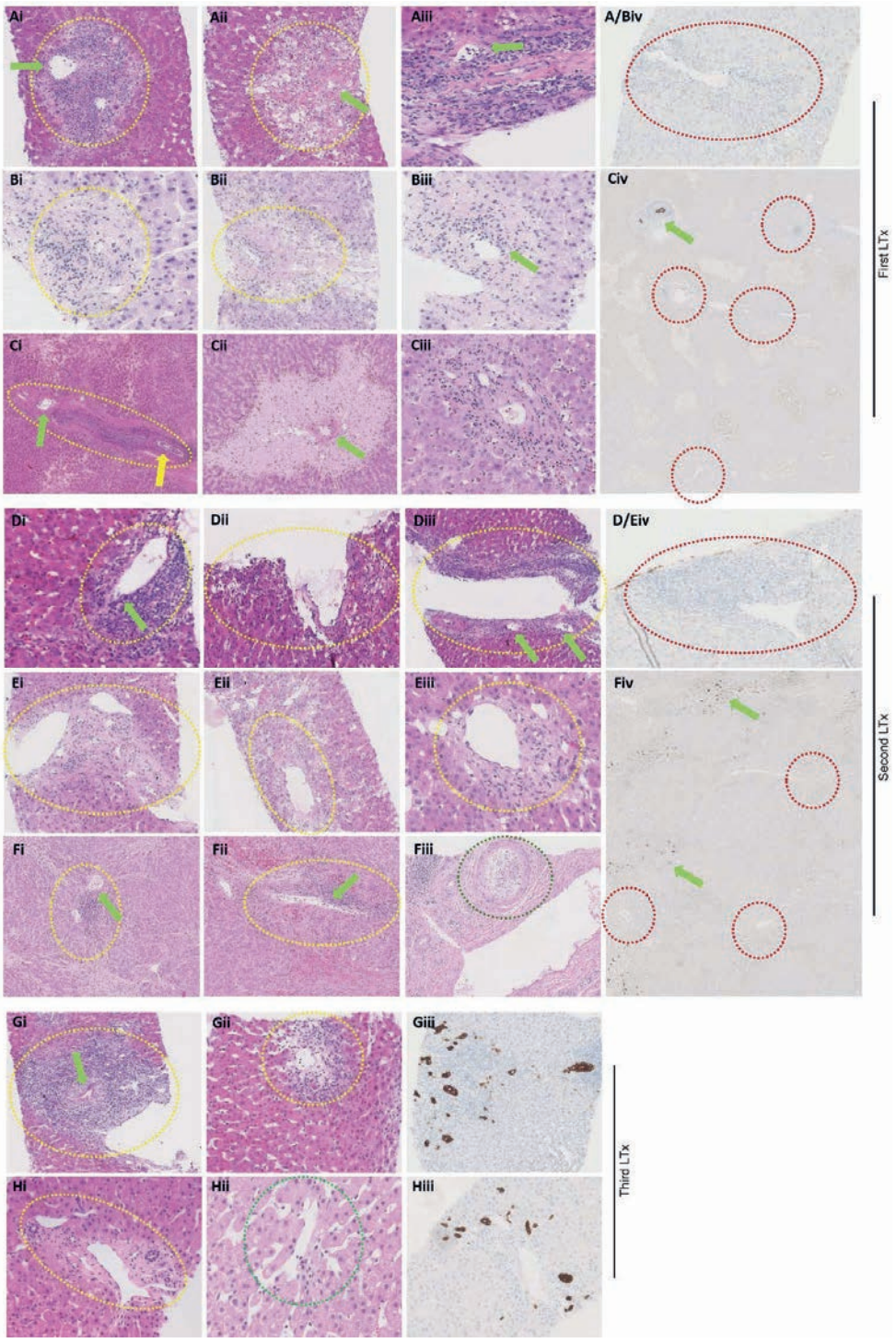

Ai
Aii
Aiii
A/Biv
Bi
Bii
Biii
Civ
Ci
Cii
Ciii
First LTx
Di
Dii
Diii
D/Eiv
Ei
Eii
Eiii
Fiv
Fi
Fii
Fiii
Second LTx
Gi
Gii
Giii
Hi
Hii
Hiii
Third LTx

Figure 1 Biopsies and explants after first and second LTx indicate multiple forms of rejection, whereas biopsies after third LTx indicate TCMR only. Biopsies before (A) and after (B) rescue treatment and explant (C) after first LTx. A, B, C. i: TCMR with prominent portal inflammatory cell infiltrates. Yellow dotted circle: portal triad, Green arrow: endotheliitis, Yellow arrow: bile duct inflammation and damage. A, B, C. ii: TCMR with central perivenulitis, obvious hepatocyte dropout and mild/moderate inflammatory infiltrates. Yellow dotted circle: perivenular area, Green arrow: central vein. A, B. iii: Focal microvasculitis (green arrow), suggestive of AMR. C. iii: Portal area without recognizable bile duct, indicative of CR. A/B, C. iv: Keratin 7 (#790-4462, Clone: SP52, Ventana Medical Systems) immunostaining indicates obvious loss of bile ducts and the lack of ductular reaction in the portal areas (red dotted circles), indicative of CR. Green arrow: a single portal field in the explant liver with native bile duct present. Biopsies before (D) and after (E) rescue treatment and explant (F) after second LTx. D, E, F. i: TCMR with prominent (D) and (F) and mild (E) portal inflammatory cell infiltrates. Yellow dotted circle: portal triad, Green arrow: endotheliitis. D, E, F. ii: TCMR with central perivenulitis, hepatocyte dropout and moderate inflammatory infiltrates. Yellow dotted circle: perivenular area, Green arrow: central vein endotheliitis. D, E. iii: Focal microvasculitis (D, green arrow), suggestive of AMR. In E no recognizable microvasculitis. Yellow dotted circle: perivenular area. F. iii: Portal area with foamy arteriopathy (green dotted circle), indicative of CR. D/E, F. iv: Keratin 7 immunostaining indicates the obvious loss of bile ducts and the lack of ductular reaction in the portal areas (red dotted circles), indicative of CR. Green arrow: areas with ductular metaplasia. Biopsies before (G) and after (H) rescue treatment after third LTx. G. i: TCMR with prominent portal inflammatory cell infiltrates. Yellow dotted circle: portal triad, Green arrow: bile duct inflammation and damage. G. ii: TCMR with central perivenulitis and mild/moderate inflammatory infiltrates. Yellow dotted circle: perivenular area. H. i, ii: No signs of rejection in the portal (dotted yellow circle) and pericentral area (green dotted circle). G, H. iii: Keratin 7 immunostaining highlights the presence of the original bile ducts. Abbreviations: AMR, antibody-mediated rejection; CR, chronic rejection; LTx, liver transplantation; TCMR, T-cell-mediated rejection.

To save the patient's life, a different approach seemed to be needed. After a literature search, and multidisciplinary meetings with experts in the area of transplantation and rejection, an alternative protocol was developed. In order to minimize the possibility of developing TCMR, CR and possible AMR due to pre-existing DSAs or de novo DSAs, it was decided to accept only a donor liver with minimal HLA mismatches with the recipient. Fortunately, a suitable ABO-compatible DBD donor liver, with only one HLA mismatch with the recipient (Table 1), was available 189 days after the second transplantation. The second change was the type of induction therapy given around the time of LTx. A case report by Yamada et al.[103] indicated that rATG induction therapy resulted in minimal TCMR and no AMR after the second (non-HLA-matched) transplantation, while the first liver transplant was lost due to severe AMR. A study performed by Kubal et al.[104] indicated that with the use of rATG lower rejection rates were observed compared to other induction therapies. Therefore, instead of administering methylprednisolone and basiliximab, 1.5 mg/kg i.v. rATG (as used in the case report of Yamada et al.) was administered on day 1 and 3 after LTx. Maintenance IS regimen was administered as described above. With this adjusted protocol the patient did not develop CR, nor de novo DSAs and AMR after the third LTx (Figure 1G; Table 2). Unfortunately, a mild acute TCMR developed after 337 days, which quickly and fully resolved after administering standard 1000 mg methylprednisolone for three consecutive days, as was confirmed by a follow-up liver biopsy (Figure 1H; Table 2). Maintenance IS regimen was set to tacrolimus 5 mg and prednisolone 7.5 mg. For at least 346 days thereafter (683 days in total), the patient is stable with the third donor liver graft.

Discussion

Here we describe a young patient with PSC in which the two first liver transplants led to TCMR, CR and possible AMR after 25 and 13 days respectively, including de novo DSA formation. The allograft response against donor HLA was probably the cause of severe rejection and graft loss after both LTx. There is no clear protocol and/or study in the literature that describes how to approach and resolve such difficult situations. Based on available information and experience in our transplant unit, we chose to match the HLA type of the donor as closely as possible to the HLA of the recipient, and to apply another induction regimen post-operatively. In this way, the allograft response against donor HLA was limited after the third LTx. The adjusted protocol led to rejection-free survival for up to 337 days, absence of DSA formation and graft survival for at least 683 days. At day 337 a TCMR was diagnosed, but the patient responded well to the standard rescue treatment, in contrast to the earlier episodes of rejection observed after the first and second LTx. We speculate that the extent of the rejection after the third LTx was limited due to HLA matching, as is supported by the absence of DSA formation and CR, and that this contributed to a rapid clinical response after rescue treatment with methylprednisolone.

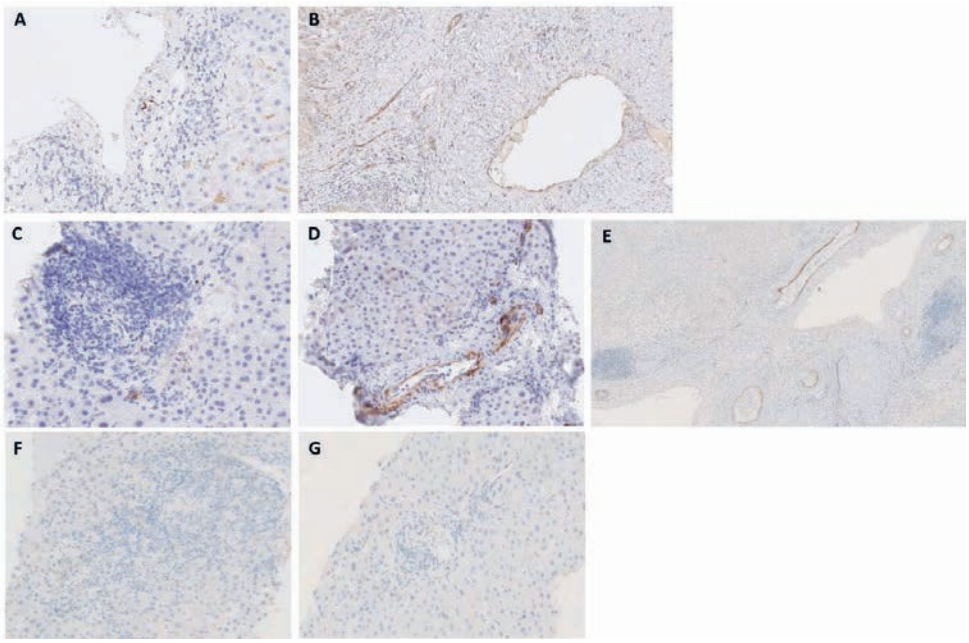
Instead of the standard induction treatment of methylprednisolone and basiliximab, rATG was used around the third LTx. Although rare in liver transplantation, rATG is a lymphocyte-depleting polyclonal antibody commonly used and indicated for the (induction) treatment of acute renal allograft rejection. It causes a persistent increase of regulatory T-cells and a prolonged reduction in effector CD4+ T-cells and B-cells.[105] On the other hand, methylprednisolone works by preventing overall inflammation, whereas basiliximab prevents activation of T-cells. Yamada et al.[103], described that with the use of rATG as induction regimen, an AMR and subsequent second liver graft loss was prevented. In addition, several other studies in liver[104] and kidney[106] transplantation have indicated that with rATG lower rejection rates were observed compared to other induction regimens. However, for some sensitized kidney transplant recipients rATG (with rituximab) induction treatment alone was not sufficient to avoid an AMR.[107] Therefore, matching of HLA between recipient and donor was added to our adjusted protocol. We believe that this rigorous method of induction therapy, aside from HLA matching, was needed to avoid de novo DSA formation with AMR and CR and to succeed with a re-LTx.

Although definite criteria have been described for the diagnosis of acute AMR after LTx[30], its lower incidence compared to other solid organ transplants, the (technical) difficulties in unmasking a C4d staining and the relatively often patchy expression of C4d (C4d score 2 and 3 show only minimal of focal staining), make a confident histopathological diagnosis on a liver biopsy challenging.[108] On the other hand, CR with absolute ductopenia within a month after both LTx, was very striking in this case. Other etiologies/diseases leading to vanishing bile ducts were considered in the differential diagnosis, but both the lack of ductular reaction and an appropriate clinical history made them incompatible with this case. Moreover, retrospectively the identification of foamy arteriopathy in the big (peri)hilar arterial branches in the second explant specimen, also pointed towards CR. Having a multidisciplinary team

with experts in early recognition and application of a suitable treatment is paramount for solving such complicated cases with different types and severity of rejection.

From this case we can conclude that whenever a liver graft is (repeatedly) lost due to severe acute TCMR, possible AMR, DSA formation and chronic rejection short after LTx, HLA matching between donor and recipient in combination with a more rigorous IS regimen should be considered as a primary treatment option instead of a standard re-LTx, in order to improve graft and patient survival.

Supplementary Material



Supplementary Figure 1 C4d positivity in biopsies and explants after the first two LTx, but not after the third LTx. C4d staining in biopsy after rescue treatment (A) and explant (B) after first LTx, biopsies before (C) and after (D) rescue treatment and explant (E) after second LTx, and biopsies before (F) and after (G) rescue treatment after third LTx. Endothelial C4d expression is observed in A, B, D, and E. Due to shortage of biopsy tissue before rescue treatment after first LTx, C4d staining could not be performed. C4d staining was determined in formalin-fixed paraffin-embedded biopsies by immunohistochemistry with a monoclonal antibody (#760-4803, Clone: SP91, Cell Marque). LTx, liver transplantation.

CHAPTER 3.

An altered serum proteome in liver transplant recipients long before acute rejection; a pilot study

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Submitted in Transplant International

Abstract

Background:
Late after transplantation liver transplant (LTx) recipients can develop late acute rejection (AR), but other recipients can be weaned off immunosuppressive drugs (IS). There are currently no serum markers predicting future progression to late AR or graft tolerance.

Methods:
Using a multiplex proximity extension assay we quantified 92 immunological proteins in serum of LTx recipients. A stable non-tolerant group (n=8) that progressed to biopsy-confirmed AR in 4 months-1.3 years following serum collection, a stable control group (n=23) under regular IS, a stable group under minimal IS (n=10), a group with operational tolerance not requiring IS (n=15), and healthy controls (n=14) were included.

Results:
A combination of fifteen serum proteins discriminated LTx recipients that were stable (long before elevated liver function parameters) at sample collection but progressed to late AR, from all other groups. Serum levels of HO1, TIE2 and ICOSLG were sufficient to predict eventual AR in all but one of the non-tolerant LTx recipients. A proteomic profile associated with development of graft tolerance was not found.

Conclusion:
This explorative pilot study suggests that an altered serum proteomic profile is associated with late AR in LTx recipients long before clinical symptoms appear, and potentially useful for guiding clinical decision making.

Introduction

Liver transplantation (LTx) is the only treatment option for end-stage liver failure. The use of immunosuppressive drugs (IS) after LTx is essential to avoid graft rejection. Acute rejection (AR) is common in the first few weeks after LTx, whereas late AR is less common and occurs in about 20% of all LTx recipients, especially when IS is reduced to ameliorate its adverse effects.[29] Early AR does not adversely impact patient or liver allograft survival if adequately treated. However, late acute T-cell mediated rejection (aTCMR) could lead to graft failure and mortality.[29] Thus, early diagnosis and prevention of late AR is paramount for graft and patient survival.

A regularly taken liver biopsy in order to predict development of AR is not feasible, since it is an invasive procedure and it could lead to severe clinical complications. Therefore, non-invasive soluble markers for AR, such as soluble cytokines, chemokines, receptors and cell adhesion molecules present in the blood have been investigated.[43,109,110] Soluble tumor necrosis factor (TNF)[35], interleukin (IL) -10[36,37,39], (C-X-C motif) ligand (CXCL) 10[36], CXCL11[37], Cluster of Differentiation (CD) 44[40] and IL-6[38] were reported to be elevated in serum or plasma of LTx recipients 2-4 days prior to diagnosis of rejection. However, all published studies have focused on occurrence of AR early after LTx. Soluble non-invasive biomarkers for indication of AR late after LTx were not investigated. Furthermore, some of the above mentioned biomarkers were elevated prior to a severe infection.[39,41-43] Hence, until now none of the biomarkers have been implemented in the clinic due to lack of specificity for AR prediction. However, since bacterial and fungal infections are much less prevalent late after LTx, soluble immunological factors might be useful for early detection of late AR.

Chronic IS exposure leads to severe side effects, hence most LTx centers attempt to gradually reduce IS over time. The majority of LTx recipients experience graft rejection during IS minimization or complete weaning and are considered non-tolerant to their liver graft. This is also the case for recipients that suffer from late AR episodes while treated with adequate maintenance IS. However, a minority of stable LTx recipients can be safely weaned from IS, and have developed spontaneous immunological operational tolerance to their graft.[49] Several studies have investigated whether tolerant differ from non-tolerant LTx recipients, or could be identified within a larger cohort of LTx recipients on regular IS regimen by measuring immune system related markers. Immune cell subsets and gene expression within peripheral blood were broadly investigated,[111] but not soluble peripheral blood markers.

Graft rejection is a complicated process that involves different parts of the immune system. Therefore, it is highly unlikely that one immunological biomarker in serum could predict an upcoming late AR or identify tolerant LTx recipients. A first exploration of an extensive panel of immunological serum proteins would be useful to identify combinations of immunological factors that might be promising to validate in subsequent studies. The multiplex Proximity Extension Assay (PEA) technology from Olink[112] has been successfully used to explore characteristics of several different diseases[113-115] and biomarkers for AR in kidney transplant recipients.[116] The purpose of this explorative study was to perform an extensive proteomic

screening of soluble immune system related serum markers in non-tolerant and tolerant LTx recipients, stable LTx recipients with regular or minimal IS regimen and healthy individuals, using this PEA technology.

Patients and Methods

Study participants and materials

Serum samples from four different groups of LTx recipients late after LTx and a healthy control group were collected. Serum samples from adult operational tolerant LTx recipients (TOL; n=15) that were followed at the outpatient clinic at the Erasmus University Medical Centre between 2014 and 2020 were collected (MEC 2014-232). Samples were obtained at a median of 15 years with IQR 13 - 17.5 after LTx and at a median of 3 years with IQR 1.5 - 5.5 after complete IS weaning (Table 1). TOL were completely weaned off IS for medical reasons or non-compliance between 2008 and 2019 and did not experience AR. Acute rejection was defined as at least a two-fold increase in serum bilirubin, aspartate aminotransferase or alanine transaminase, alkaline phosphatase or γ-glutamyltransferase, that normalized upon adequate IS regimen, since protocol biopsies after complete IS weaning were not taken because of possible complications related to the procedure. A liver biopsy was performed in five tolerant LTx recipients because of possible rejection as indicated by increasing liver enzymes, at on average 3.1 ± 2.2 years after complete weaning. In all cases rejection was excluded according to BANFF criteria. Furthermore, a group of non-tolerant LTx recipients (nonTOL; n=8) was included that developed AR after (partial) IS weaning or while being on a regular maintenance IS regimen between 2009 and 2019 and for which a serum sample was available. Seven out of eight nonTOL LTx recipients were selected because of a liver biopsy confirmed rejection assessed using BANFF criteria. One nonTOL LTx recipient was prospectively weaned from IS and a subsequent AR was defined as described for TOL, and liver enzymes normalized after IS re-installment. Serum samples collected at a time point with stable liver enzymes prior to (4 months - 1.3 years) the AR episode and during the AR episode were used (MEC 2020-0572). Serum samples at the stable time point were collected at a median of 3.5 years with IQR of 2 - 17 years after LTx. A control group of stable LTx recipients (CTRL; n=23) with regular dual or mono IS regimen (Table 1) and a group of stable LTx recipients (MIN; n=10) with minimal mono IS regimen (Table 1) were also included (MEC 2014-232). Serum samples of CTRL were collected at a median of 14 years with IQR 12 - 19 years after LTx and those of MIN were collected at a median of 15 years with IQR 13 - 19 years after LTx. Minimal immunosuppressive drug trough levels were considered to be Tacrolimus <2.5 µg/L, Mycophenolate mofeteil <1.0 mg/L and Cyclosporine <60 µg/L (based on personal communication with Dr. R.J. de Knegt, liver physician). Both CTRL and MIN did not experience rejection episodes for at least 5 years before and 4 years after blood collection. A healthy control group (HC; n=14) was also included in the study (MEC 2012-022). Blood samples were centrifuged (2500 rpm; 10 minutes) and obtained sera were stored at -80°C until further use. Clinical and laboratory information was retrieved from electronic patient records. From all participants informed consent was received. This study was conducted in accordance with the Declaration of Helsinki and approved by the medical ethics committee of Erasmus MC.

Proximity Extension Assay technology

Serum samples were analyzed using the Proximity Extension Assay (PEA) technology designed by Olink (Uppsala, Sweden).[112] In this assay 92 proteins of the Immuno-oncology panel, consisting of a combination of pro- and anti-inflammatory proteins, were simultaneously measured in 1µl of serum. This PEA technology used pairs of oligonucleotide-labeled antibodies specific for each target protein that bound to each of the target proteins present in the sample. The oligonucleotides in close proximity hybridized, were extended by a DNA polymerase, and amplified using a realtime polymerase chain reaction (PCR). These amplifications were quantified by microfluidic qPCR (Fluidigm Biomark HD system, San Francisco, USA). Internal controls were added to each sample and included two incubation controls, one extension control and one detection control. The incubation controls consisted of two non-human proteins to monitor all three steps in the PEA technology. An extension control was used for normalization of the Ct values. This control consisted of an antibody independent of protein binding, linked to two matched oligonucleotides for immediate proximity-dependent hybridization and extension. A detection control that consisted of a synthetic double-stranded template specifically monitored the detection step. When one or more internal controls deviated from a pre-determined range by Olink, samples were removed before statistical analysis. The external inter-plate control, a pool of 92 matching oligonucleotide pairs, was included on both plates and was used for a second normalization step of the Ct values. In the final normalization step the values were set relative to a correction factor that is determined by Olink for each batch of conjugated PEA antibodies. A Normalized Protein eXpression (NPX) Log2 scale was calculated to minimize both intra- and inter-assay variations. In the NPX scale higher protein concentrations correlate to higher NPX values. Values lower than 3 standard deviations above the detection limit calculated with a calibration curve were excluded from analysis.

Statistical analyses

Characteristics of the study groups were analyzed with Pearson Chi-Square or Kruskal-Wallis rank test by using IBM SPSS software version 25 (SPSS Inc., Chicago, USA). Proteomic statistical analyses were performed with R version 3.6.3 (Foundation for Statistical Computing, Vienna, Austria). Comparisons between nonTOL Stable and Rejection groups were analyzed with a paired t-test and p-values were corrected with Benjamini-Hochberg's procedure. Comparisons between multiple groups were performed using an ANOVA and p-values were corrected for multiple testing using Benjamini-Hochberg's procedure. After correction P-values less than 0.05 were considered statistically significant (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$). Volcano plots were created with R. Graphs were created with GraphPad Prism 8 version 8.4.3 (GraphPad Software Inc., San Diego, USA). The hierarchical cluster analysis with heatmap for discriminating nonTOL and CTRL groups was created with the public Galaxy server Version 3.0.1 R gplots package.[117] Principal component analysis (PCA) using direct oblimin factor rotation of the 15 proteins that were significantly different between nonTOL and the other groups was performed using IBM SPSS software version 25.

Results

Characteristics of the study groups

Characteristics of the control (CTRL) group with regular IS regimen, minimal IS (MIN) group, stable non-tolerant (nonTOL) group at a time point (4 months - 1.3 years) before rejection, operational tolerant (TOL) group and healthy controls (HC) are presented in Table 1. Age, sex, years after LTx, primary disease and (last) IS used did not significantly differ among groups. All LTx recipients were at least 12 years after transplantation, except for a few recipients in the nonTOL group (Table 1 and 2). The IS regimen in nonTOL was more similar to CTRL than to MIN (Table 1). Some nonTOL LTx recipients were intentionally weaned off IS for medical reasons and developed an AR episode, whereas others had lower trough levels most likely because of non-compliance, but all developed an AR episode (Table 2). Values of liver function parameters during blood collection were generally below the upper limit of normal for CTRL, MIN and TOL LTx recipients, and occasionally enhanced values were attributed to other causes than AR (Figure 1). Liver function values of the nonTOL group were considerably elevated during the rejection episode. The values were generally stable and below the upper limit of normal prior to rejection, and if slightly elevated, causes other than AR were attributed. Viral and bacterial infections present a month before and after blood collection were limited for all study groups (Supplementary Table 1), and fungal infections were completely absent.

Table 1 Characteristics of the study groups.

	CTRL	MIN	nonTOL	TOL	HC	P-value
Demographics	n=23	n=10	n=8	n=15	n=14	
Male (%)	69.6	50.0	75.0	73.0	71.4	0.74
Age in years	55 (32.5-63.5)	57 (43.8-61.8)	39 (29.8-50)	56 (43-67.5)	52 (45-63.8)	0.56
Years after LTx	14 (12-19)	15 (13-19)	3.5 (2-17)	15 (13-17.5)	NA	0.13
Years LTx - complete weaning	NA	NA	NA	11 (8-15.5)	NA	
Years complete weaning - end follow-up	NA	NA	NA	3 (1.5-5.5)	NA	
Primary disease (%)					NA	0.26
Cholestatic disease	30.4	20.0	25.0	33.3		
Virus-related	30.4	70.0	25.0	26.7		
Hepatocellular carcinoma	13.0	0.0	0.0	26.7		
Cryptogenic cirrhosis	13.0	0.0	25.0	13.3		
Toxicity-induced	8.6	0.0	12.5	0.0		
Metabolic-related	4.3	0.0	12.5	0.0		
Rupture	0.0	10.0	0.0	0.0		
IS last used (%)					NA	0.51
Tac	65.2	70.0	50.0	46.7		
CsA	0.0	20.0	12.5	6.7		
MMF	8.7	10.0	0.0	6.7		
Aza	0.0	0.0	0.0	13.3		
Tac and MMF	13.0	0.0	25.0	13.3		
CsA and MMF	4.3	0.0	0.0	0.0		
Pred and Tac	8.7	0.0	0.0	0.0		
Tac and Evero	0.0	0.0	12.5	0.0		
Aza and CsA	0.0	0.0	0.0	6.7		
Unknown	0.0	0.0	0.0	6.7		
IS mono therapy trough levels				NA	NA	
Tac µg/L	3.2 - 7.8	1.2 - 2.5	1.9 - 7.3			
MMF mg/L	>2.9	0.9	NA			
CsA µg/L	NA	52 - 58	122			

Percentages, concentrations or median years with 25th and 75th IQR are presented. Statistical analyses were performed with Chi-Square or Kruskal-Wallis rank test. Aza, azathioprine; CsA, cyclosporine A; CTRL, control group; Evero, everolimus; HC, healthy controls; IS, immunosuppressive drugs; LTx, liver transplantation; MIN, minimal IS group; MMF, mycophenolate mofetil; NA, not applicable; nonTOL, non-tolerant group; Pred, prednisolone; Tac, tacrolimus; TOL, tolerant group.

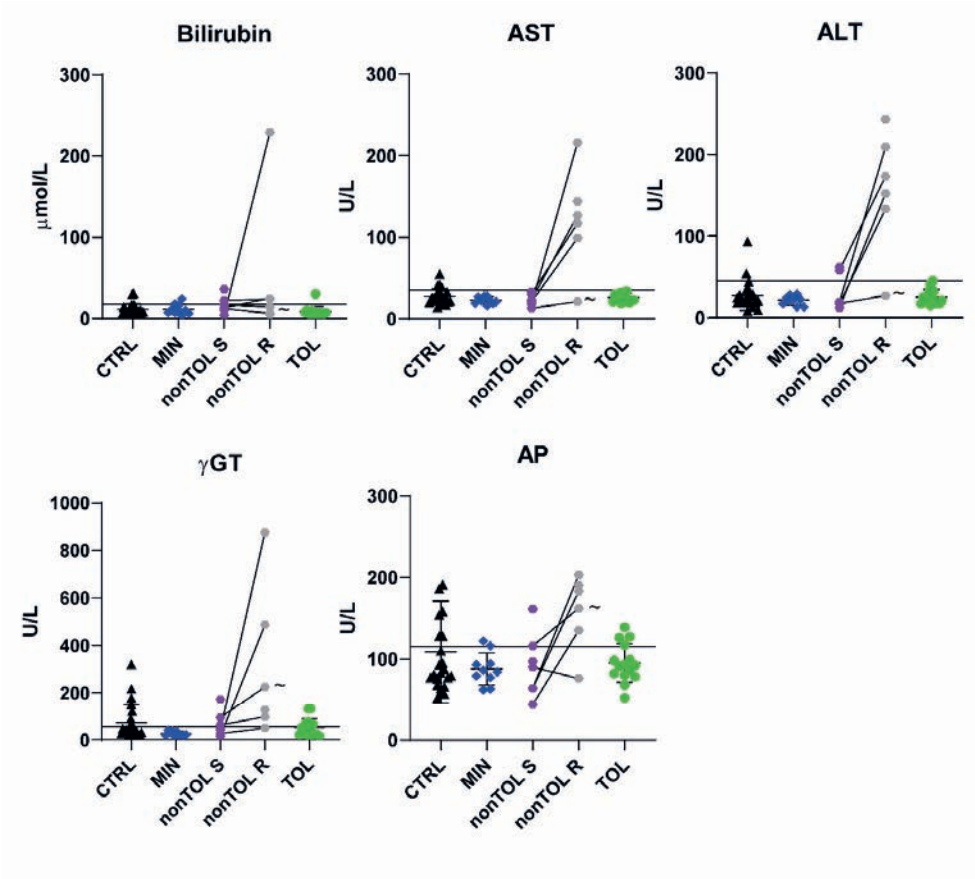


Figure 1 Liver function values of the different study groups. Values of liver function parameters bilirubin, AST, ALT, γGT and AP for CTRL, MIN, nonTOL at a stable (S) and at a rejection (R) time point, and TOL at time of blood collection are presented. Solid black line represents the upper limit of normal for each parameter. CTRL, MIN, stable nonTOL and TOL LTx recipients with values above the upper limit of normal were attributed to: Parkinson's disease, cholangitis, drug addiction, stenosis or very slightly chronically elevated levels without a two-fold increase. ~For this nonTOL LTx recipient γGT and AP levels increased considerably, but not AST and ALT. Since γGT and AP levels normalized upon reinstalling IS, AR was diagnosed as the cause of elevated γGT and AP. ALT, alanine transaminase; AP, alkaline phosphatase; AST, aspartate aminotransferase; CTRL, control group; γGT, γ-glutamyltransferase; LTx, liver transplantation; MIN, minimal IS group; nonTOL, non-tolerant group; TOL, tolerant group.

Significantly different soluble protein levels could predict an upcoming acute rejection in non-tolerant LTx recipients

Ninety-two immune-system related proteins were simultaneously measured using the PEA technology in serum samples of the study groups. From the 92 proteins, serum levels of 15 proteins were significantly different in the stable nonTOL group a long time prior to rejection, compared to various other groups (Figure 2; Supplementary Table 2 for proteins that not significantly differed). Soluble CD244, Caspase 8 (Casp8), Inducible T Cell Costimulator Ligand (ICOSLG) and Angiopoietin-1 receptor (TIE2) were significantly higher, whereas TNF, TNF-related weak inducer of apoptosis (TWEAK), CD40L, Chemokine (C-C motif) ligand (CCL) 19, Angiopoietin-1 receptor (TIE2), Galectin 1 (Gal1) and Matrix Metalloproteinase-12 (MMP12) were significantly lower in stable nonTOL compared to CTRL, MIN, TOL and HC groups (Figure 2A). Furthermore, IL8, Programmed Cell Death 1 (PDCD1) and Latency-associated peptide transforming growth factor β1 (LAP TGFβ1) were significantly higher, whereas Heme Oxygenase-1 (HO1) and CXCL11 were significantly lower in stable nonTOL compared to CTRL LTx recipients, but not to all other groups (Figure 2B). HO1 and CXCL11 were significantly lower in stable nonTOL compared to MIN LTx recipients, whereas the other three soluble proteins were not. PCA of the 15 proteins revealed three components that completely separated nonTOL from all other groups that clustered together. Despite considerable variability within the nonTOL group the three components accounted for 65.9% of the variance (Figure 2C) between the groups. A volcano plot of the proteomic analysis indicated that all serum protein levels were similar in nonTOL long before and during AR, except for Gal1 levels (Figure 3A). Gal1 serum levels were reduced in nonTOL long before AR, but decreased significantly further during rejection (Figure 3B). Unfortunately, a proteomic biomarker profile that distinguished TOL LTx recipients was not observed (Figure 2 and data not shown). The nonTOL group was most similar to the CTRL group regarding dual or mono IS regimen and IS through levels (Table 1). Therefore, the 15 serum proteins that significantly differed between nonTOL and CTRL were used to create a heatmap with hierarchical clustering analysis (Figure 4A), in which nearly all stable nonTOL LTx recipients clustered together. Implementing measurements of 15 different proteins in the clinic is costly and time consuming, therefore we reduced the number of proteins needed to obtain a similar clustering profile between nonTOL and CTRL. Serum levels of HO1, TIE2 and ICOSLG (Figure 4B) resulted in similar clustering and together were able to predict an upcoming late AR in all of the stable nonTOL LTx recipients except for one.

Table 2 Characteristics of the nonTOL LTx recipients during stable liver function values and the later rejection episode.

	Stable and/or Rejection group[a]	Years after LTx	Age	Sex	IS medically weaned	Reason weaning	Trough levels Stable	Trough levels Rejection	Biopsy
nonTOL1	Both	17	71	Male	Yes	Skincancer	Tac 1.9 µg/L	Tac <1.0 µg/L	Indication on elevated liver enzymes and normalization after IS re-installment
nonTOL2	Rejection	1	30	Female	Yes	EBV primary infection	Tac 3.1 µg/L; Evero 2.8 µg/L[b]	Tac <1.0 µg/L	aTCMR (RAI 5); possibly start of chronic rejection
nonTOL3	Both	2	29	Female	No	NA	Tac 5.1 µg/L	Tac 12.3 µg/L	aTCMR (RAI 5-6)
nonTOL4	Stable	17	18	Male	Yes	HEV infection	Tac 3.0 µg/L	Tac 2.0 µg/L[c]	aTCMR (RAI 7)
nonTOL5	Both	4	45	Male	No	NA	Tac 4.3 µg/L; MMF 1.38 mg/L	Tac 3.5 µg/L	aTCMR (RAI 5-6)
nonTOL6	Both	17	53	Male	No	NA	CsA 122 µg/L	CsA 66 µg/L	aTCMR (RAI 6)
nonTOL7	Stable	2	59	Male	No	NA	Tac 5.0 µg/L; MMF 0.64 mg/L	Tac 3.1 µg/L[c]	aTCMR (RAI 5-6)
nonTOL8	Both	3	33	Male	No	NA	Tac 7.3 µg/L	Tac 4.0 µg/L; Pred	aTCMR (RAI 5)

[a]Blood was collected at a time point with stable liver function values and/or at a rejection time point for proteomic analyses. [b]At this time point not enough blood was collected for proteomic analysis. [c]At this time point blood was not collected, no proteomic analysis could be performed, and IS trough levels measured a few weeks before the rejection episode are indicated. aTCMR, acute T cell mediated rejection; Aza, azathioprine; CsA, cyclosporine A; EBV, Epstein-Barr virus; Evero, everolimus; HEV, Hepatitis E virus; IS, immunosuppressive drugs; LTx, liver transplantation; MMF, mycophenolate mofetil; NA, not applicable; nonTOL, non-tolerant group; Pred, prednisolone; RAI, rejection activity index; Tac, tacrolimus; TOL, tolerant group.

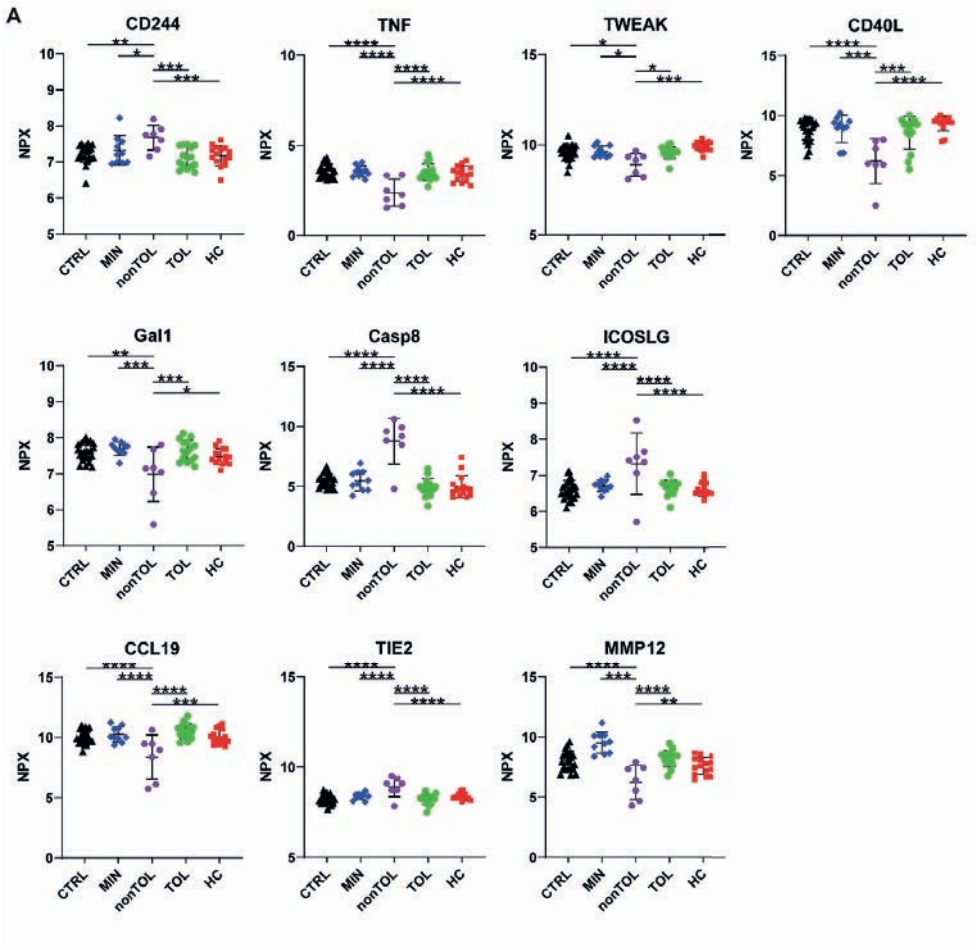
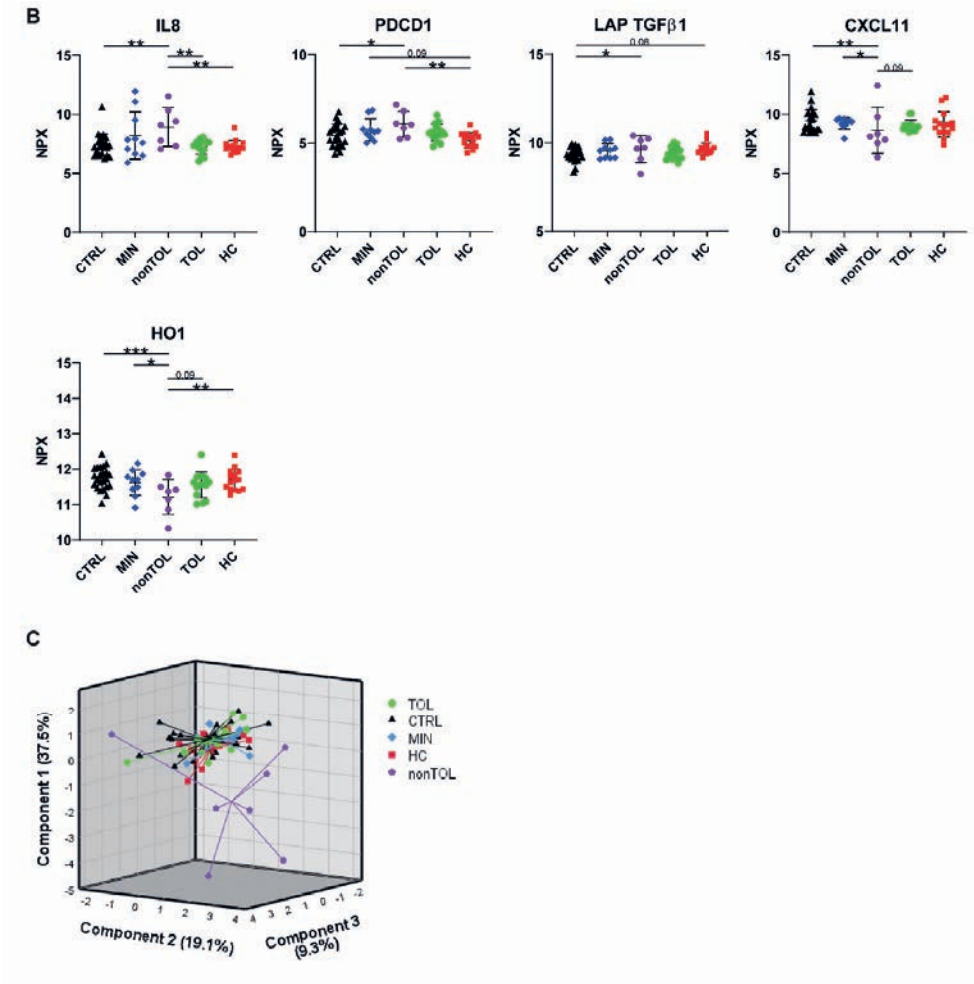


Figure 2 Fifteen soluble proteins were significantly different in stable non-tolerant LTx recipients compared to control LTx recipients. In A the NPX values of ten soluble serum proteins that were significantly different between stable nonTOL and all other groups are presented. In B the NPX values of five significantly different soluble serum proteins between stable nonTOL and CTRL, but not all other groups, are presented. NPX values with Log2 scale were calculated with several normalization steps for Ct values derived with realtime polymerase chain reaction. Higher protein concentrations correlate to higher NPX values. In C a principal component analysis is presented of the fifteen proteins measured in CTRL, MIN, stable nonTOL, HC and TOL. Rotated component matrix analysis was performed using direct oblimin factor rotation. On the axes the contributed percentage of the variance between groups by that component is indicated. CTRL, control group; LTx, liver transplantation; MIN, minimal IS group; nonTOL, non-tolerant group; NPX, Normalized Protein eXpression; TOL, tolerant group.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PDCD1
LAP TGFβ1
CXCL11
HO1
NPX
CTRL
MIN
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Component 3 (9.3%)
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HC
nonTOL

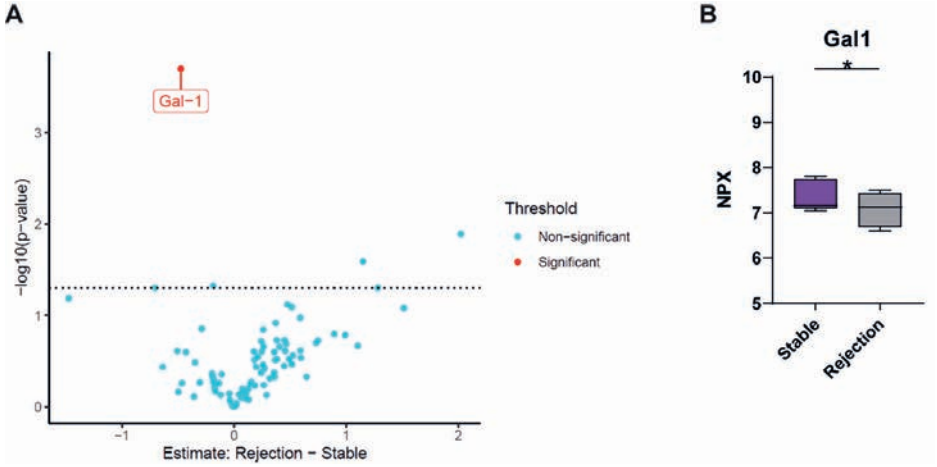
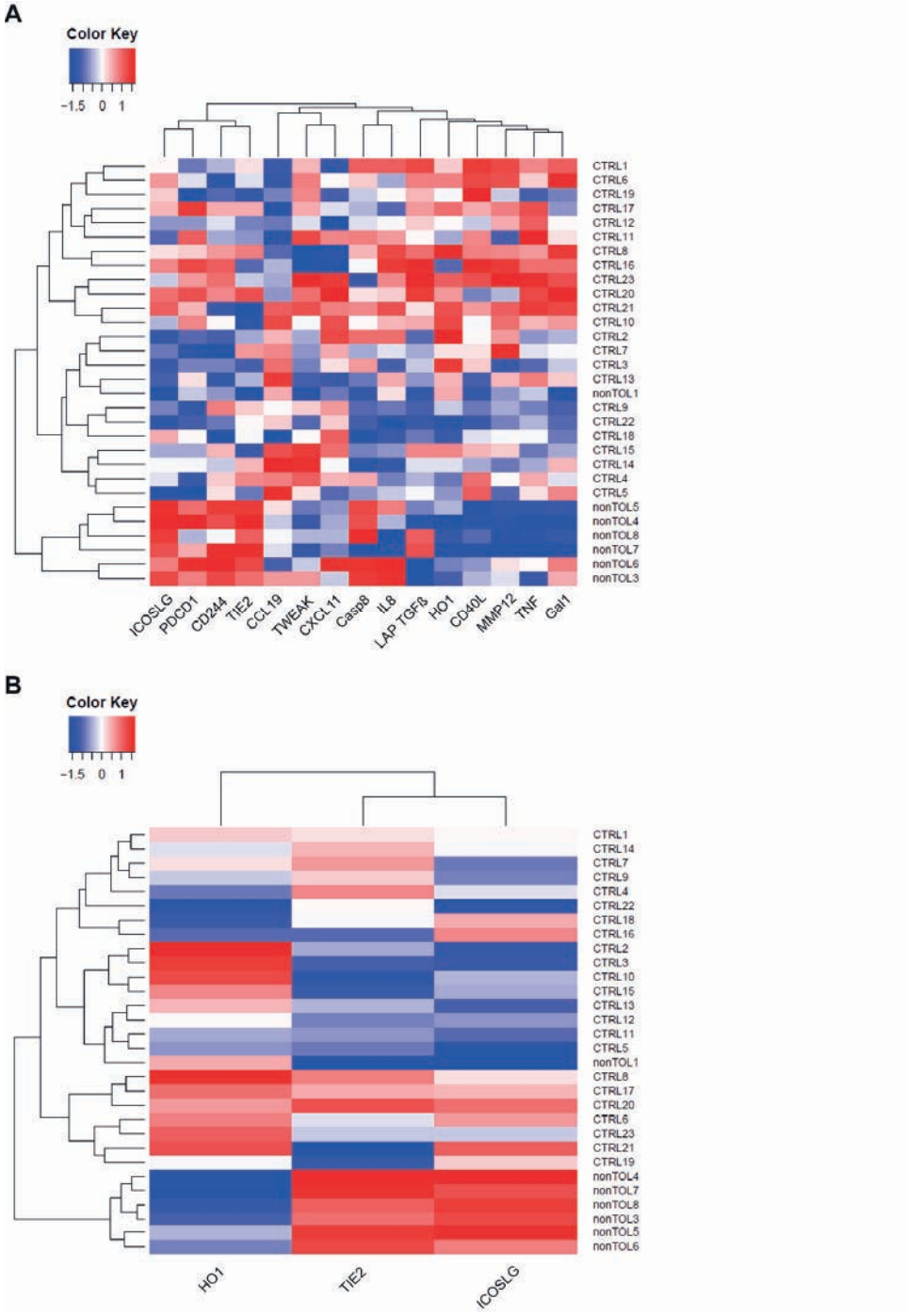


Figure 3 In non-tolerant LTx recipients only Galectin 1 serum levels were significantly different between the rejection episode and a stable time point prior to rejection. In A a volcano plot is presented that identified only Galectin 1 serum protein as significantly different between the stable time points 4 months - 1.3 years prior to rejection and rejection time points in nonTOL. The volcano plot, with statistical significance on y-axis and magnitude of change in NPX values on x-axis, presents non-corrected different proteins above the dotted line. In red the significantly different proteins corrected for multiple testing with Benjamini-Hochberg's procedure are indicated. In B the measured NPX values of the significantly different serum protein between stable and rejection nonTOL groups, Galactin1, is presented in boxplots (Min-Max whiskers). NPX values with Log2 scale were calculated with several normalization steps for Ct values derived with realtime polymerase chain reaction. Higher protein concentrations correlate to higher NPX values. LTx, liver transplantation; nonTOL, non-tolerant group; NPX, Normalized Protein eXpression.

Figure 4 Proteomic analysis of non-tolerant LTx recipients could predict an upcoming late acute rejection. In A hierarchical clustering analysis of 15 significantly different soluble serum proteins between stable nonTOL and CTRL is depicted. In B hierarchical clustering analysis of 3 significantly different soluble serum proteins between stable nonTOL and CTRL resulted in similar clustering as in A. Heatmaps with hierarchical clustering analysis were created with the public Galaxy server R gplots package with Euclidean distance method and Complete hierarchical clustering method. CTRL, control group; LTx, liver transplantation; nonTOL, non-tolerant group.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nonTOL4
nonTOL7
nonTOL8
nonTOL3
nonTOL5
nonTOL6
HO1
TIE2
ICOSLG

Discussion

In this pilot study we performed a first proteomic screening of soluble immune system related peripheral blood markers in non-tolerant and tolerant LTx recipients late after LTx and three other study groups, using the PEA technology from Olink. Unfortunately, an immune profile identifying tolerant LTx recipients could not be established, since no significant differences were observed between TOL LTx recipients and all other groups. In total we found 15 soluble proteins that were already significantly different in the stable nonTOL group a long time prior to rejection, compared to a stable control group of LTx recipients with regular IS regimen. PCA indicated that a combination of the 15 serum proteins completely separated nonTOL from all other groups, despite variability within the nonTOL group that may reflect the involvement of a complicated immune system in an upcoming rejection episode. Clustering analysis of these 15 proteins revealed that all stable nonTOL LTx recipients, except for one, clustered together when compared to a stable control group of LTx recipients with regular IS regimen. Of these 15 proteins HO1, TIE2 and ICOSLG were sufficient to predict an upcoming late AR in all but one of the nonTOL LTx recipients with stable liver function values a long time prior to rejection.

We were the first to investigate soluble immune system related peripheral blood proteins in tolerant LTx recipients. Until now only immune cell subsets and gene expression within peripheral blood were broadly investigated.[111] Unfortunately, we could not establish a tolerance profile that identifies tolerant liver transplant recipients using this explorative proteomic approach. Possibly, the state of operational tolerance is less prominently represented by soluble immune system related proteins, as for example for a rejection episode, and therefore no significant differences were observed in soluble proteins for TOL. Presence or absence of specific types of alloreactive peripheral blood immune cells and their function, rather than soluble proteins solely, are likely to be involved in operational tolerance. Therefore, additional studies have to be performed to investigate these (as of yet unknown) immune cells and their interaction with the liver graft in order to identify tolerant LTx recipients within a larger cohort of LTx recipients.

Non-invasive soluble markers for diagnosis of AR have been investigated,[35-40,43,109,110] but the investigated proteins were occasionally also elevated prior to a severe infection short after LTx, and therefore none of the biomarkers have been implemented in the clinic.[39,41-43] In our study active viral and bacterial infections were minimally present and fungal infections were completely absent for all study groups, hence our proteomic analyses late after LTx were minimally influenced by infections.

In our study we found that serum levels of 15 soluble proteins were significantly different between the stable nonTOL and CTRL group. In addition, in PCA the combination of the 15 serum proteins separated the stable nonTOL group from all other groups, long before liver function parameters were elevated and an AR episode was diagnosed with a liver biopsy. This suggests that either long before an AR episode becomes apparent rejection processes are already ongoing in these LTx recipients, or that these soluble serum proteins are indicative of

recipients with a higher inherent sensitivity to graft rejection. By including the proteomic profile after full clearance of the AR episode this could possibly be investigated in the future. Of these 15 proteins HO1, TIE2 and ICOSLG were sufficient to predict an upcoming late AR in most of the stable nonTOL LTx recipients. These three proteins were not only significantly different between the nonTOL and CTRL group, but also to the MIN group. This could indicate that nonTOL LTx recipients could be identified long before the AR episode by measuring these three soluble serum proteins in a larger cohort of LTx recipients, regardless of dual and/or mono IS regimen or the height of their IS trough levels.

HO1 exerts cytoprotective effects with anti-oxidative and anti-inflammatory functions.[118] Nevertheless, high pre-transplant expression levels in the liver have been correlated with more graft injury [119] and a higher risk for AR [120] short after LTx. In contrast, low post-reperfusion HO1 expression levels in the liver graft have been correlated with an increased hepatocellular death, deteriorated liver function and decreased recipients' survival.[121,122] In our study the soluble HO1 protein was significantly lower in stable nonTOL LTx recipients that experienced rejection compared to all other LTx recipients that did not experience rejection in the near future. This could indicate that adequate soluble HO1 levels indeed exert cytoprotective effects and could contribute to prevention of an acute rejection episode late after LTx. The TIE2 tyrosine kinase receptor binds the angiopoietin family of growth factors, is largely specific to endothelial cells and higher expression of TIE2 in the tumor tissue is correlated to stabilization of tumor blood vessels and therefore tumor growth.[123] Unfortunately, nothing is known about the involvement of TIE2 in liver transplantation and acute rejection. In our study the soluble TIE2 protein was significantly higher in stable nonTOL LTx recipients that experienced rejection in the near future compared to all other LTx recipients. Since higher soluble TIE2 levels in blood could indicate increased shedding of receptors from the liver tissue or a higher expression in liver tissue it is unclear what biological function this tyrosine kinase receptor could have in nonTOL LTx recipients experiencing rejection. ICOSLG is mostly expressed on dendritic cells, macrophages and B-cells.[124] ICOSLG is a ligand for the inducible co-stimulatory molecule ICOS that is expressed on activated T-cells.[125] In one study, monotherapy with a blocking anti-ICOS antibody significantly prolonged liver graft survival in a rat model by inhibiting the activation and proliferation of graft infiltrating CD4 and CD8 T cells.[126] This could imply that the significantly higher soluble serum ICOSLG levels found in stable nonTOL LTx recipients long before AR, may have activated graft infiltrating T-cells and gradually led to development of an AR episode, despite the (supposable adequate) IS regimen in these LTx recipients. It is clear that the biological mechanisms of these serum proteins and their involvement in the development of AR late after LTx are largely unknown.

The strength of our explorative study is that we are the first to investigate an extensive panel of immunological non-invasive soluble serum proteins in tolerant and non-tolerant LTx recipients late after LTx. We performed these proteomic analyses using the PEA technology from Olink, that has been validated for serum samples, has a higher specificity and sensitivity than other multiplex assays, and the inter variability between assays is negligible.[112] A limitation of the study is the small and heterogeneous group of nonTOL LTx recipients. Furthermore, it is preferable that the group of nonTOL LTx recipients would be compared to

a group of stable CTRL LTx recipients at a shorter time point late after transplantation. Despite these limitations, the combination of 15 serum proteins completely separated the stable nonTOL LTx recipients prior to an AR episode from all other groups. Another study limitation is that we analyzed a pre-selection of 92 proteins instead of performing a discovery study by analyzing a broader array of proteins. Nevertheless, the Immuno/Oncology panel consists of a broad range of pro- and anti-inflammatory proteins, likely to be involved in immunological processes studied.

We performed an explorative pilot study that suggests that a distinctive profile of soluble immune system related serum markers could predict an upcoming late AR in most LTx recipients long before clinical symptoms appear. The results should be validated in a much larger homogeneous cohort of non-tolerant LTx recipients. After validation, these serum biomarkers may be used in the clinic to regularly monitor LTx recipients and identify LTx recipients at risk for an upcoming AR episode long before clinical symptoms appear, and should not be exposed to IS reduction.

Acknowledgements

We would like to express our gratitude to the liver transplant nurses of the Department of Gastroenterology and Hepatology at the Erasmus University Medical Centre for their efforts to successfully collect all blood samples. We also would like to thank the Department of Viroscience at the Erasmus University Medical Centre for making archived serum samples of LTx recipients available.

Supplementary Material

Supplementary Table 1 Active infections present a month before and after blood collection in each group.

	Infections			
CTRL	Pneumonia	Cholangitis	Bladder infection	Stomach flu
MIN	Active HBV infection			
nonTOL	EBV primary infection			
TOL	Common cold	Active HHV-8 infection		

CTRL, control group; EBV, Epstein-Barr virus; HBV, Hepatitis B virus; HHV-8, Human Herpesvirus type 8; MIN, minimal IS group; nonTOL, non-tolerant group; TOL, tolerant group.

Supplementary Table 2 Non-significant proteins nonTOL versus other groups.

non-significant proteins			
ADA	CRTAM	IL2	MCP-4
ADGRG1	CSF-1	IL4	MMP7
ANGPT1	CXCL1	IL5	MUC16
ANGPT2	CXCL5	IL7	NCR1
ARG1	CXCL9	IL10	NOS3
CAIX	CXCL10	IL12	PDGF subunit β
CCL3	CXCL13	IL12Rβ1	PDL1
CCL4	CX3CL1	IL13	PDL2
CCL17	DCN	IL15	PGF
CCL20	EGF	IL18	PTN
CCL23	FasL	IL33	TNFRSF4
CD4	FGF2	KIR3DL1	TNFRSF9
CD5	Gal9	KLRD1	TNFRSF12A
CD8a	GZMA	LAG3	TNFRSF14
CD27	GZMB	LAMP3	TNFRSF21
CD28	GZMH	MIC-A/B	TRAIL
CD40	HGF	MCP-1	VEGF-A
CD70	IFNγ	MCP-2	VEGFR-2
CD83	IL1α	MCP-3	

CHAPTER 4.

Immunosuppressive drug withdrawal late after liver transplantation improves the lipid profile and reduces infections

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Abstract

Background:
Lifelong treatment with immunosuppressive drugs (IS) after transplantation is accompanied by severe side effects. A limited number of studies have investigated the effect of IS withdrawal on IS-related comorbidities after liver transplantation (LTx) and the results are contradictory.

Methods:
We determined the clinical effects of complete IS withdrawal in operationally tolerant LTx recipients who discontinued IS 10.8 ± 5.1 years after LTx (n=13), compared to a perfectly matched control group still on IS (n=22). Tolerant recipients have been IS- and rejection-free for 4.0 ± 2.8 years.

Results:
We found no deterioration of liver function after complete IS withdrawal. Moreover, IS withdrawal in tolerant recipients resulted in lower LDL levels and reduced *de novo* infection rates, whereas this was not observed in the control group. Furthermore, persistent infections in individual recipients were successfully resolved by IS withdrawal. Tolerant recipients also had significantly less *de novo* infections after IS withdrawal compared to recipients kept on IS in the same follow-up period. Unfortunately, no improvement of kidney function, lower rates of *de novo* occurrences of diabetes, hypertension, cardiovascular diseases and malignancies were observed in the tolerant group after IS withdrawal compared to the control group in the same follow-up time period.

Conclusion:
IS withdrawal late after LTx reduces infection rates and LDL levels, but other IS-related side effects persist late after LTx. An accurate tolerance immune profile enabling identification of tolerant LTx recipients eligible for safe IS withdrawal earlier after transplantation is needed to prevent development of irreversible IS-related side effects.

Introduction

Liver transplantation (LTx) is the only treatment for end stage liver disease. In order to prevent allograft rejection after transplantation, use of immunosuppressive drugs (IS) is indispensable. However, in a significant proportion of LTx recipients, long-term use of IS leads to severe side effects such as (persistent) infections, metabolic disorders (e.g. diabetes, dyslipidemia), renal dysfunction, cardiovascular disease and malignancies.[14-18,127] Short-term post-transplant survival rates after LTx significantly improved over the last two decades due to improved surgical techniques and optimized immunosuppressive drug regimens.[19] However, morbidity and mortality more than one year after LTx have showed little improvement and is still substantially higher compared to the general population.[20] Since most causes of morbidity and mortality are related to IS therapy, most centers attempt to gradually reduce IS over time after LTx.

Occasionally, LTx recipients spontaneously develop operational tolerance to their graft, a state in which IS can be completely withdrawn without the occurrence of an acute rejection episode. This was first observed when individuals were withdrawn from IS for medical reasons or due to non-compliance.[53] Subsequently, a few clinical trials confirmed the possibility of achieving immunological tolerance to allogenic liver grafts in about 40% of selected adult[49,51,53] and 60% of selected pediatric LTx recipients electively withdrawn from IS.[53]

Whether complete withdrawal of IS in LTx recipients could reduce IS-related side effects after LTx is still controversial. The number of studies that have assessed the long-term impact of IS withdrawal late after LTx on IS-related comorbidities is limited, and contradictory findings are reported for both adult[49,56,57,59,60] as well as pediatric[53,55,58] recipients. Moreover, a study investigating the influence of IS withdrawal on IS-related side effects in adult LTx recipients compared to a completely matched control group on regular IS therapy has not been performed yet. For this reason, the purpose of this study was to determine the effect of complete IS withdrawal in tolerant recipients late after LTx on liver function, kidney function, lipid metabolism, and occurrence of diabetes, hypertension, cardiovascular disease, malignancies and infections compared with a completely matched control group maintained on IS.

Patients and Methods

Study design

The study cohort included in this retrospective single center study consisted of all operationally tolerant (TOL) adult LTx recipients that visited the outpatient LTx clinic of Erasmus MC between 2014 and 2017 (n=13). Operational tolerance was defined as complete withdrawal of IS or unmeasurable IS trough levels associated with non-compliance for at least six months without the occurrence of an acute rejection episode. Tolerant recipients had been completely withdrawn of IS between 2008 and 2017. In order to avoid risks associated with liver biopsies after transplantation, protocol biopsies were not taken during or after complete IS withdrawal in this study. However, in four TOL recipients a biopsy was taken on indication of elevated liver enzymes in the time period after complete IS withdrawal (on average 3.1y ± 2.2). All biopsies were evaluated by a pathologist and in all cases rejection was excluded and an alternative diagnosis was given. A control (CTRL) group of LTx recipients with regular IS regimen (n=22) was matched to the TOL group for gender, age, time after LTx, cytomegalovirus (CMV) serostatus and primary disease (Table 1). For each tolerant LTx recipient, one or two (when available) control LTx recipient(s) was matched. No other inclusion or exclusion criteria were used. All patients, both TOL and CTRL, were seen with regular intervals at the outpatient LTx clinic of Erasmus MC. All clinical and laboratory information was retrieved from electronic patient records. Follow-up of patients ended in December 2017. All patients gave written informed consent to participate in the study. This study was approved by the medical ethics committee of Erasmus MC (MEC 2014-232) and conducted in accordance with the 1975 Declaration of Helsinki.

Laboratory assessments

The following parameters were analyzed for both groups: total bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (AP) and gamma-glutamyl transferase (GGT) for liver function, creatinine and estimated glomerular filtration rate (eGFR; derived from MDRD formula with four variables[128]) for kidney function, glycated hemoglobin (HbA1c) for glycemic index, and low-density lipoproteins (LDL), high-density lipoproteins (HDL), cholesterol and triglycerides for lipid metabolism. Blood levels of above mentioned parameters (except for HbA1c levels) were evaluated one year before (-1), shortly before (0) and two and four years after (2, 4) complete IS withdrawal for the TOL group and at matching time points after LTx for the CTRL group. HbA1c levels were included two years before (-2), shortly before (0) and two years after (2) complete withdrawal for the TOL group and at matching time points after LTx for the CTRL group, since information was not available for other time points.

Side effects

Occurrence of events possibly related to IS was analyzed during the complete post LTx period and before and after complete IS withdrawal in TOL recipients or matching time points after LTx in CTRL recipients. *De novo* development of malignancies and cardiovascular diseases was noted when diagnosed. *De novo* development of diabetes and hypertension was noted when disease-specific medication had been given. Infections were noted when a PCR confirmed viral infection or a positive culture confirmed a bacterial, parasitic or fungal infection, and when appropriate treatment had been given. Total number of infections was divided by total number of years in the time period analyzed for each individual. The recipients who received a liver transplant because of HBV have been HBV-DNA negative after LTx. Recipients remained HBV-DNA negative either without antiviral therapy or after immediate antiviral therapy with anti-HBV hyper-immunoglobulins combined with either lamivudine, entecavir or tenofovir. For the recipients transplanted for HCV induced cirrhosis recipients either cleared the virus spontaneously after LTx, or were cured after direct antiviral therapy in 2015.

Statistical analysis

Statistical analyses were performed using IMB SPSS statistics version 24. Wilcoxon signed-rank test was used for comparing values of liver function, kidney function and metabolic parameters and infections for either the TOL or CTRL group in time. Mann-Whitney rank-sum test was used for comparing time points between the two groups for these parameters. For gender, CMV status, primary disease, diabetes, hypertension, cardiovascular disease and malignancies the 2-sided Fisher's exact test was used to compare both groups, whereas the McNemar's test was used to compare occurrence within the TOL or CTRL group before and after withdrawal. Data are presented as mean ± SD or percentage.

Results

Patient characteristics

The study group of tolerant LTx recipients consisted of all recipients who visited the Erasmus MC LTx outpatient clinic between 2014 and 2017 and who had not received any IS therapy for at least six months without indication of graft rejection (n=13) (Supplementary figure 1). The control group (n=22) consisted of LTx recipients on regular IS regimen matched with the tolerant group for gender, age, follow-up time after LTx, CMV serostatus, and primary liver disease (all $p>0.05$). Demographic characteristics of the tolerant (TOL) and control (CTRL) group of LTx recipients are depicted in Table 1. Two-third of both the TOL and CTRL group were male, which is representative of the total Erasmus MC LTx cohort. Age at LTx was 38.8 ± 17.5 years for TOL and 36.0 ± 17.9 years for CTRL and time from LTx to end follow-up was 14.9 ± 3.7 years for TOL and 14.5 ± 5.6 years for CTRL. Most individuals were transplanted because of cholestatic or virus related liver disease. TOL recipients were completely withdrawn from IS 10.8 ± 5.1 years after LTx due to non-compliance, persistent infection or renal dysfunction and have been IS- and rejection-free for 4.0 ± 2.8 years.

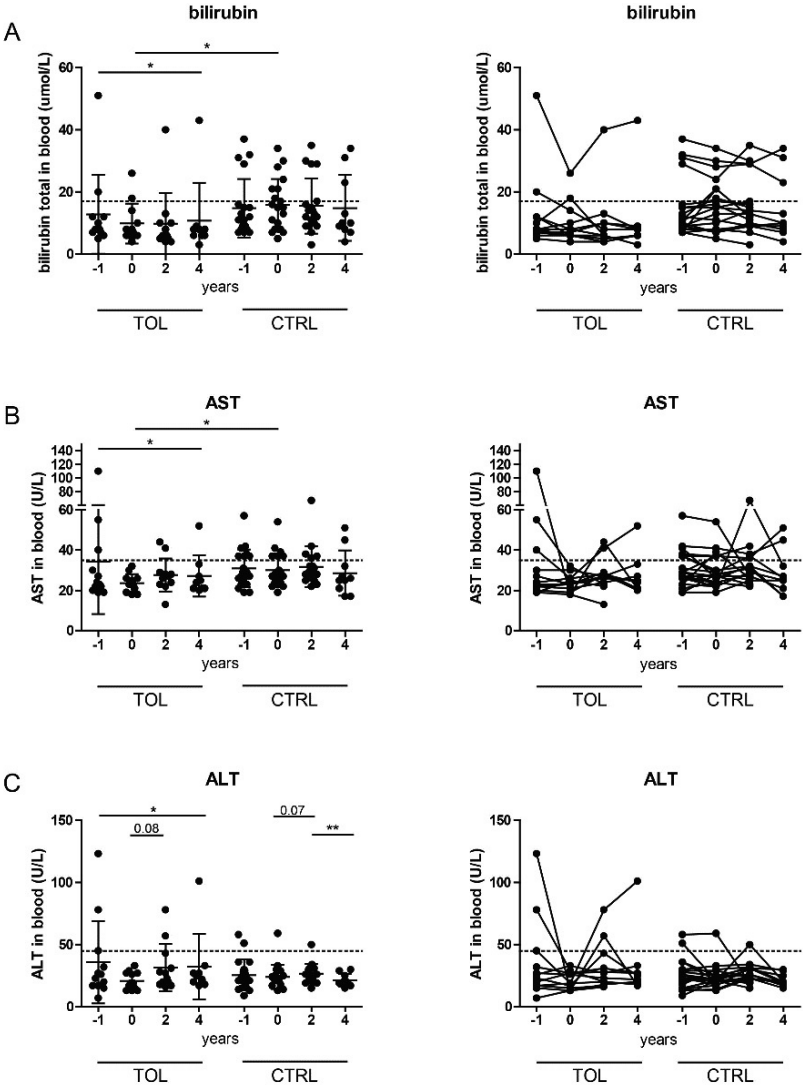
Liver function

In the first two years after complete IS withdrawal slight to moderate, but transient, elevations of hepatocellular enzymes AST and ALT were observed in two tolerant LTx recipients (Supplementary figure 2), but these were due to other medical complications and not to rejection activity. Since shortly before complete IS withdrawal the IS trough levels were low in the tolerant group (Supplementary figure 1), one year before complete IS withdrawal was included in the analysis of all clinical parameters. Overall, liver function parameters bilirubin, AST and ALT improved in TOL recipients four years after complete IS withdrawal compared to one year before complete withdrawal (Figure 1A-C), whereas this was not observed in the CTRL group. AP levels increased in the TOL group four years after complete IS withdrawal compared to one year before, but in the CTRL group also an increase in AP levels was observed when comparing time point 4 versus time point 0 (Figure 1D). Moreover, AP levels did not differ between both groups four years after IS withdrawal. GGT levels were significantly increased in the CTRL group, whereas only an increasing trend in the TOL group was observed four years after complete withdrawal compared to before withdrawal (Figure 1E). In conclusion, based on bilirubin, AST and ALT parameters no deterioration of liver graft function was observed in TOL after complete IS withdrawal.

Table 1 Characteristics of the study group (TOL) and control (CTRL) group.

Parameters	TOL	CTRL	*P-value*
Demographics			
n	13	22	
Male (%)	69.2	68.2	1.00
Age at LTx (mean ± SD)	38.8 ± 17.5	36.0 ± 17.9	0.82
Years LTx - complete withdrawal (mean ± SD)	10.8 ± 5.1	n/a	
Age complete withdrawal (mean ± SD)	49.6 ± 16.9	n/a	
Complete withdrawal - end of follow-up (mean ± SD)	4.0 ± 2.8	n/a	
Years LTx - end of follow-up (mean ± SD)	14.9 ± 3.7	14.5 ± 5.6	0.42
CMV+ serostatus (%)			
Recipient pre LTx	58.3	54.5	1.00
Recipient post LTx - end of follow-up	66.7	77.3	0.69
Donor	53.8	54.5	1.00
Primary disease (%)			
Cholestatic disease[a]	38.5	22.7	0.69
Virus-related	30.8	36.4	1.00
Hepatocellular carcinoma	15.4	18.2	0.68
Cryptogenic cirrhosis	15.4	13.6	1.00
Drug-induced	0.0	4.5	1.00
Metabolic-related	0.0	4.5	1.00
Reason complete withdrawal (%)			
Non-compliance	46.2	n/a	
Persistent infection[b]	38.5	n/a	
Renal dysfunction	15.4	n/a	
IS last used (%)			
Tac	53.8	72.7	
CsA	7.7	4.5	
MMF	0.0	9.1	
Aza	7.7	0.0	
Tac and MMF	15.4	4.5	
Pred and MMF	0.0	4.5	
Pred and Tac	0.0	4.5	
Aza and CsA	7.7	0.0	
Unknown	7.7	0.0	

Statistical analyses were performed with Mann-Whitney rank-sum or 2-sided Fisher's exact test. Abbreviations: TOL, tolerant group; CTRL, control group; Tac, tacrolimus; CsA, cyclosporine A; MMF, mycophenolate mofetil; Aza, azathioprine; Pred, prednisolone. [a] Includes primary sclerosing cholangitis, secondary biliary cirrhosis, biliary atresia, biliary anastomosis. [b] Includes viral(n=4) and parasitic(n=1) infection.


A
bilirubin
bilirubin total in blood (umol/L)
years
TOL
CTRL
B
AST
AST in blood (U/L)
C
ALT
ALT in blood (U/L)
0.08
0.07
**

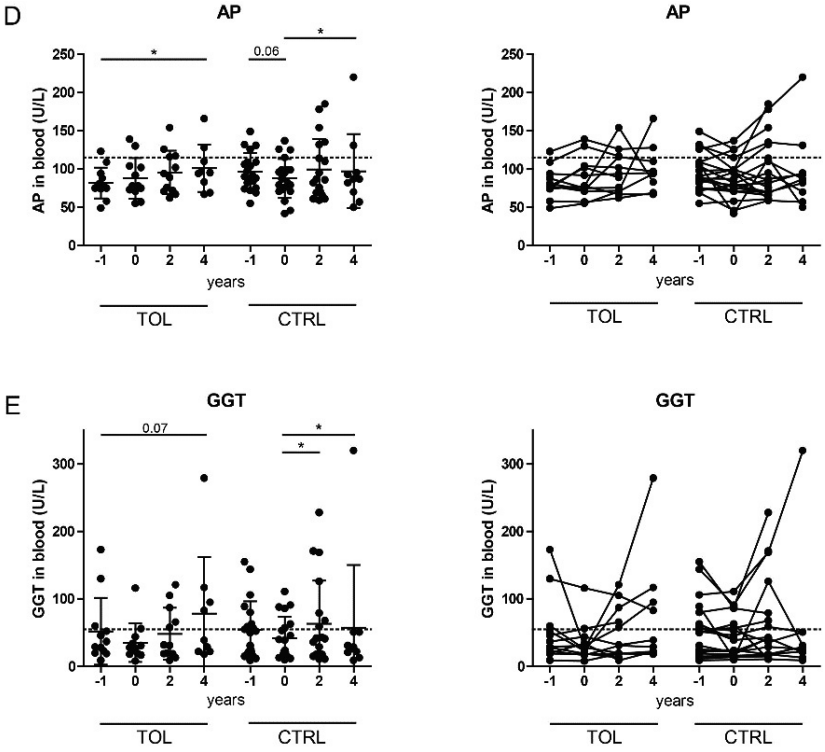


Figure 1 Liver function parameters bilirubin, AST and ALT improve after complete IS withdrawal. Bilirubin (A), AST (B), ALT (C), AP (D) and GGT (E) levels were analyzed one year before (-1), right before (0) and two and four (2, 4) years after complete IS withdrawal in the tolerant group (TOL) or at matching time points in the control group (CTRL). Left graphs indicate values of individual recipients and mean ± SD for each time point. Right graphs indicate longitudinal course of each individual. Dotted lines indicate the normal healthy range of the indicated parameter. Statistical analyses were performed using Mann-Whitney rank-sum or Wilcoxon signed-rank test. * $p < 0.05$ ** $P < 0.01$

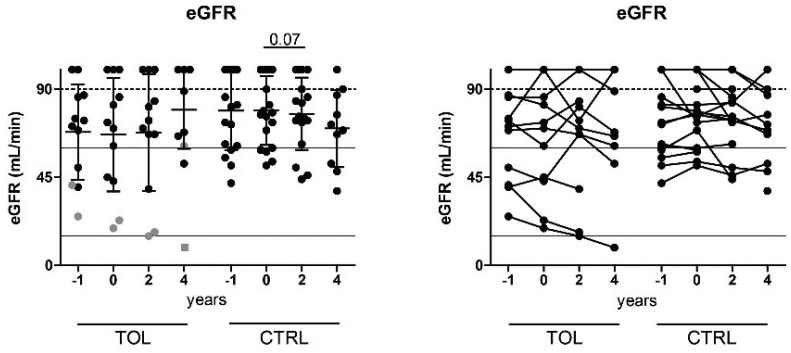


Figure 2 The eGFR does not improve in the tolerant group after complete IS withdrawal. Levels of eGFR were analyzed one year before (-1), right before (0) and two and four (2, 4) years after complete IS withdrawal in the tolerant group (TOL) or matching time points in the control group (CTRL). Left graphs indicate values of individual recipients and mean ± SD for each time point. Right graphs indicate longitudinal course of each individual. Dotted lines indicate the normal healthy range of the indicated parameter and solid lines represent a eGFR of 15 and 60. Grey dots indicate renal dysfunction when values were measured. Grey square indicates that hemodialysis was given, and this value is not included in mean ± SD and statistics. Statistical analyses were performed using Mann-Whitney rank-sum or Wilcoxon signed-rank test.

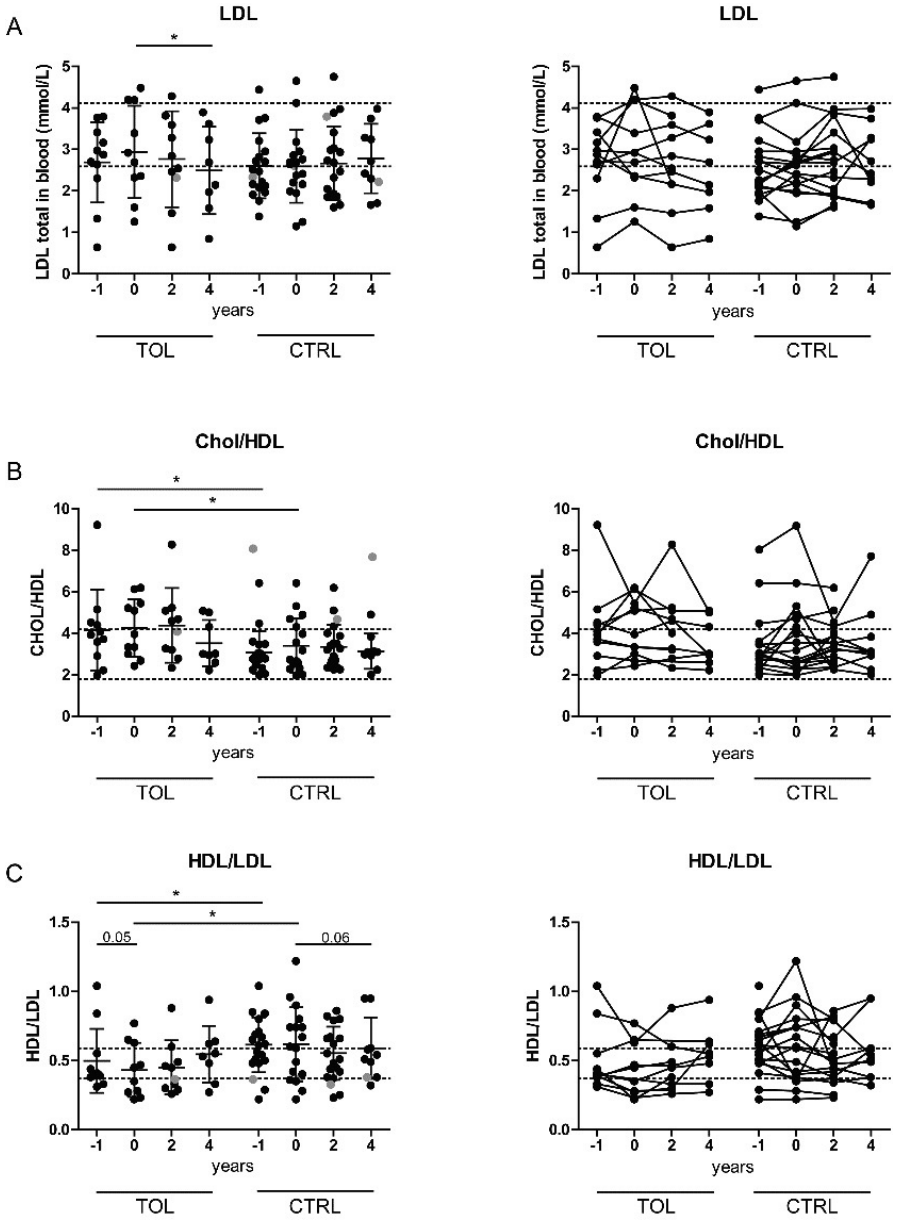
Table 2 In all tolerant LTx recipients withdrawn for medical reasons complete IS withdrawal had a positive effect on IS-related morbidity.

Recipient	Primary reason withdrawal	Secondary reason withdrawal	Result after withdrawal
TOL 1	Norovirus infection	Renal dysfunction	Infection resolved
TOL 2	EBV related lymphoma	n/a	Resolved
TOL 3	HHV-8 related Kaposi sarcomas	n/a	Mitigated
TOL 4	Active EBV infection	n/a	Resolved
TOL 5	Microsporidia infection	n/a	Resolved
TOL 6	Renal dysfunction	n/a	Stable
TOL 7	Renal dysfunction	n/a	Mitigated

IS-related side effects

Kidney function, measured as eGFR, as well as creatinine levels, did not improve in TOL after complete IS withdrawal (Figure 2, Supplementary Figure 3). However, deterioration of renal function was mitigated in one individual, and a stable renal function was induced in another individual after complete IS withdrawal (Table 2). In TOL recipients a significant decrease in LDL levels was observed four years after complete withdrawal, whereas this was not observed within the CTRL group (Figure 3A). Before IS withdrawal cholesterol/HDL ratios were significantly higher in TOL recipients, whereas HDL/LDL ratios were significantly lower in TOL individuals compared to CTRL recipients (Figure 3B and 3C). However, these differences between groups disappeared after complete IS withdrawal. For both groups no significant changes in HDL, cholesterol or triglyceride levels were observed (Supplementary figure 4A-C). HbA1c levels did not improve over time after complete IS withdrawal (Figure 3D). Unfortunately, no reduction was observed in *de novo* cardiovascular disease or malignancies, nor was a significant reduction in *de novo* occurrence of diabetes and hypertension found in the TOL group after IS withdrawal compared to CTRL recipients during the same post-LTx period (Table 3). Interestingly, all TOL recipients that acquired diabetes, developed this within two years after LTx, whereas none developed diabetes after complete IS withdrawal.

In the TOL group, the total number of infections per year was significantly reduced after complete IS withdrawal compared to before (Figure 4A), whereas such a decrease was not observed in the CTRL group. Furthermore, total numbers of infections after complete IS withdrawal in the TOL group were significantly lower than the CTRL group in the same follow-up time period. Moreover, in every TOL LTx recipient withdrawn from IS because of a persistent infection, the infection was fully resolved after IS withdrawal (Table 2). When the total numbers of infections per year were split into bacterial and viral infections, a decreasing trend in infection rate was observed for both types of infections in the TOL group, whereas only the viral infections rate decreased in the CTRL group (Figure 4C).


A
LDL
LDL total in blood (mmol/L)
years
TOL
CTRL
B
Chol/HDL
CHOL/HDL
C
HDL/LDL
0.05
0.06

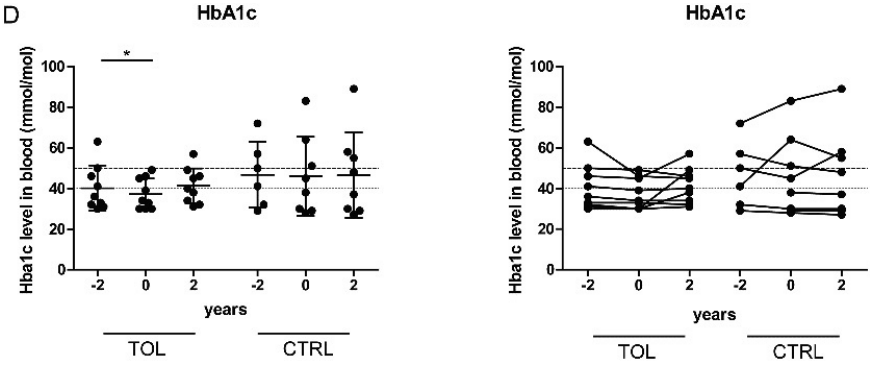
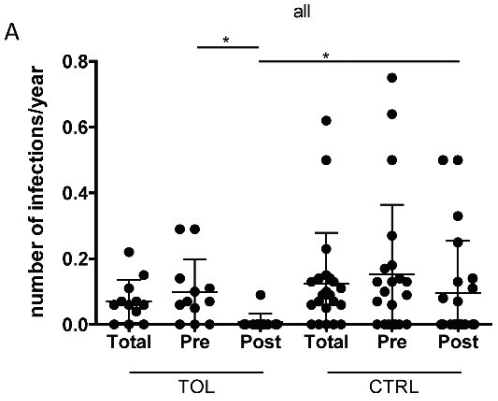
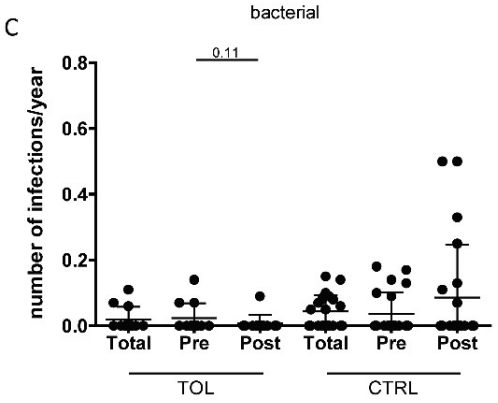
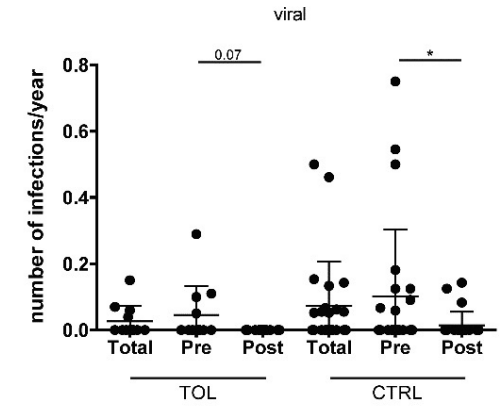


Figure 3 LDL levels significantly decrease in the tolerant group after complete IS withdrawal. LDL (A), Cholesterol/HDL (B), HDL/LDL (C) and HbA1c (D) levels were analyzed one year before (-1), right before (0) and two and four (2, 4) years after complete IS withdrawal in the tolerant group (TOL) or matching time points in the control group (CTRL). Left graphs indicate values of individual recipients and mean ± SD for each time point. Right graphs indicate longitudinal course of each individual. Dotted lines indicate the normal healthy range of the indicated parameter. Values in grey are influenced by statin-use and are not included in mean ± SD and statistics. Statistical analyses were performed using Mann-Whitney rank-sum or Wilcoxon signed-rank test. * $p < 0.05$


A
all
number of infections/year
0.8
0.6
0.4
0.2
0.0
*
*
Total
Pre
Post
Total
Pre
Post
TOL
CTRL


C
bacterial
number of infections/year
0.8
0.6
0.4
0.2
0.0
0.11
Total
Pre
Post
Total
Pre
Post
TOL
CTRL


viral
number of infections/year
0.8
0.6
0.4
0.2
0.0
0.07
*
Total
Pre
Post
Total
Pre
Post
TOL
CTRL

B

TOL		CTRL	
Pre	Post	Pre	Post
viral	**bacterial**	**viral**	**viral**
HSV	pneumonia	HEV	CMV (2)
EBV (2)	campylobacter	HSV	EBV
VZV		CMV (4)	**bacterial**
norovirus		EBV (3)	colitis
bacterial		VZV (2)	bladder infection (3)
peritonitis		asterovirus (2)	osteomyelitis
bladder infection		enterovirus	cholangitis (4)
pneumonia		norovirus	
Q fever		**bacterial**	
fungal		colitis	
dermatomycosis (2)		bladder infection (4)	
parasitic		pneumonia (2)	
microsporidium		cholangitis (2)	

Figure 4 Total number of infections significantly decrease in the tolerant group after complete IS withdrawal compared to before withdrawal and to the control group. A) Total number of infections per year in the collected time period for each individual recipient with mean ± SD. Total follow-up time after LTx and before or after IS withdrawal were included. B) All types of infections with occurrence (n) in both groups presented in A. C) Total number of infections presented in A split into bacterial and viral infections. Statistical analyses were performed with Mann-Whitney rank-sum or Wilcoxon signed-rank test. * $p < 0.05$

Table 3 No reduction in *de novo* occurrence of diabetes, hypertension, cardiovascular complication or malignancy rate is observed in the TOL group after IS withdrawal.

Complication		TOL	CTRL	*P value TOL vs CTRL*
Diabetes	Total %	23.1	13.6	0.65
	Pre withdrawal %	23.1	9.1	0.34
	Post withdrawal %	0.0	4.5	1.00
	P value Pre vs Post	0.50	1.00	
Hypertension	Total %	46.2	27.3	0.29
	Pre withdrawal %	38.5	13.6	0.12
	Post withdrawal %	7.7	13.6	1.00
	P value Pre vs Post	0.06	1.00	
Cardiovascular[a]	Total %	23.1	13.6	0.65
	Pre withdrawal %	0	4.5	1.00
	Post withdrawal %	23.1	9.1	0.34
	P value Pre vs Post	0.50	1.00	
Malignancy[b]	Total %	30.8	18.2	0.43
	Pre withdrawal %	15.4	13.6	1.00
	Post withdrawal %	15.4	4.5	0.54
	P value Pre vs Post	1.00	0.63	

De novo development of diabetes and hypertension was enlisted when disease-specific medication had been given. *De novo* development of malignancies and cardiovascular diseases were enlisted when diagnosed. Percentages of patients with events during total follow-up time after LTx and pre or post IS withdrawal time points are depicted. Statistical analyses were performed with the 2-sided Fisher's exact test or McNemar's test. [a]Includes myocardial infarction, aneurysm, atrioventricular block. [b]Includes lung carcinoma, colon carcinoma with liver metastasis, renal cell carcinoma, yolk sac tumor, testicular seminoma, Kaposi sarcoma, lymphoma.

Discussion

In this study we compared a group of TOL LTx recipients completely withdrawn from IS for on average four years to a completely matched CTRL group on regular IS regimen. A significant decrease in total number of infections and LDL levels was observed in the TOL group after complete IS withdrawal. Furthermore, total numbers of infections after complete IS withdrawal in the TOL group were significantly lower than in the CTRL group in the same time period. Moreover, complete IS withdrawal successfully resolved all persistent infections in individual recipients. Thus even late withdrawal (on average 11 years after LTx) of IS may offer benefits for LTx recipients.

Here we observed a significant lower *de novo* infection rate and persistent infections were all resolved after complete IS withdrawal in the TOL group, which resulted in significantly fewer infections compared to the control group in the same follow-up time. This finding is supported by two follow-up studies of the Tor Vergata clinical trial,[56,57] in which eight adult stable HCV-positive LTx recipients were successfully withdrawn from IS, whereas 26 required IS re-installment and were considered non-tolerant. After 6.5 years and 10 years of IS-free follow-up, significantly fewer recurrent infections were found in the tolerant group compared to the nontolerant group. However, only recurrent infections were analyzed in their study and not *de novo* infections. In contrast, Benitez et al. from 2013[49] reported that there was no significant difference in numbers of infections requiring in-hospital admission and treatment between the tolerant and nontolerant recipients three years after initiation of their IS withdrawal study. One explanation for the contradictory results could be the difference between the definitions of infections used in these and our studies; infections requiring in-hospital admission vs recurrent infections vs *de novo* infections. Another reason could be the difference in matching between the two groups in the study of Benitez et al., in which the control group consisted of nontolerant LTx recipients in which IS was re-installed. This control group differed in age at withdrawal, time after LTx and gender compared to the tolerant group, whereas these variables are perfectly matched in our study.

The LDL levels of the TOL group significantly decreased after complete IS withdrawal but no significant changes were found in total cholesterol, triglyceride levels and HDL levels. In accordance with our data, Benitez et al. observed no differences between the tolerant and nontolerant group in hypercholesterolemia and hypertriglyceridemia. Furthermore, our data are confirmed by the Tor Vergata withdrawal study, in which cholesterol and triglyceride levels also did not improve in the tolerant group after complete IS withdrawal. However, none of the hitherto published IS withdrawal studies investigated LDL levels. Thus, we are the first to report that LDL levels do improve after IS withdrawal leading to a more favorable lipid profile after IS withdrawal.

Nevertheless, *de novo* occurrence of cardiovascular disease was not reduced after IS withdrawal in the TOL group compared to the CTRL group. In addition, we found no reduction in *de novo* occurrence of diabetes, hypertension and malignancies after IS withdrawal in the TOL group compared to the CTRL group in the same follow-up time period. Similarly, Benitez

et al. did not observe significant differences between tolerant and nontolerant LTx recipients in the occurrence of all four mentioned parameters after IS withdrawal. In contrast, the 6.5- and 10-year follow-up of the Tor Vergata IS withdrawal study showed a significantly lower incidence of new onset cardiovascular diseases and diabetes in tolerant compared to non-tolerant recipients. These discrepancies may be due to earlier IS withdrawal after LTx in the Tor Vergata study (5.3 years) and therefore shorter IS toxicity versus the later IS withdrawal after LTx in our study and Benitez et al. (about 11 years). Another reason could be the type of recipients included in the studies. In the Tor Vergata study, only LTx recipients with HCV as primary disease and HCV RNA serum positivity after LTx were included. Recipients with deteriorated liver function, cirrhosis or other hepatic or non-hepatic diseases after LTx were excluded. In our study and in the study performed by Benitez et al. recipients were included that had co-morbidities due to IS and Benitez et al. also included recipients that had a higher risk of developing a neoplasm. Tryphonopoulos et al. in 2010[60] did not observe significant differences in *de novo* neoplasms between the tolerant and rejector group after IS withdrawal, which is similar to our results and Benitez et al. These data may suggest that pre malignant cell changes were already formed in the immunosuppressed state before IS withdrawal, which evolved into malignancies after withdrawal.

The follow-up time of our study is probably too short to observe a significant decrease in malignancies and cardiovascular disease after IS withdrawal. Also, the higher incidence of cardiovascular disease after withdrawal compared to before in the TOL group in our study is probably related to the higher incidence of diabetes and hypertension prior to withdrawal within these recipients. Overall, this possibly indicates that recipients with a pre-existing disease are more prone to developing co-morbidities and consequently the positive effects of IS withdrawal can be less evident than it actually is in our study. A combination of a long time period between LTx and IS withdrawal and a short follow-up time in the study by Benitez et al., Tryphonopoulos et al. and our own study could obscure positive effects of IS withdrawal on *de novo* occurrence of cardiovascular diseases and malignancies.

Similarly to Benitez et al., we did not observe an improvement of GFR after IS withdrawal in TOL LTx recipients. In contrast, Pons et al. in 2009[59] found that the GFR increased significantly after IS withdrawal, whereas in non-tolerant recipients the GFR decreased significantly. One explanation for these discrepancies could be the time between LTx and IS withdrawal and induced toxicity. IS withdrawal was performed on average of 3.4 years after LTx in the study by Pons et al., whereas in our study and Benitez et al. recipients were withdrawn on average 11 years after LTx. Another explanation could be that in the study performed by Pons et al. none were withdrawn from IS because of renal dysfunction, whereas in our study and by Benitez et al. some recipients were withdrawn because of these co-morbidities. Both of the tolerant groups are thus more biased, and it is therefore possible we do not observe an improvement in GFR after IS withdrawal. IS minimization and complete withdrawal should occur as soon as possible after LTx to limit the IS-related nephrotoxicity in LTx recipients, since it may then be still largely reversible.

The strength of our study is that we compared a TOL group with a completely matched CTRL group, and can therefore eliminate potential confounders. Multiple significant differences

between important factors, such as age, gender, primary disease and time after LTx, were present in all other studies that investigated clinical effects of IS withdrawal by comparing tolerant adult LTx recipients with a nontolerant or rejecter group. However, our study also has some limitations. We performed a retrospective study with a small cohort of TOL LTx recipients. There is probably a population bias in our TOL group, since about half of the TOL LTx recipients were withdrawn from IS for medical reasons, and recipients with a pre-existing disease could be more prone to co-morbidities. Furthermore, the time period between LTx and IS withdrawal in the tolerant group is extensive and may result in persistent IS-related morbidities. However, this is a limitation of most other follow-up studies, except for two.[49,59] Despite these limitations we do find positive effects of IS withdrawal in our TOL group. When compared to other studies it does suggest that benefits of IS withdrawal could be more extensive when IS withdrawal is performed earlier.

Unfortunately, time after LTx is a strong predictor of tolerance after LTx, i.e. the longer the time after LTx, the higher the chance of being tolerant towards the liver graft.[49] For example, no more than 13% of selected stable LTx recipients were tolerant when transplanted shorter than six years ago. Therefore, in order to withdraw LTx recipients safely from IS earlier after LTx, TOL recipients need to be identified carefully from a larger group of LTx recipients with regular IS regimen. Therefore, an accurate tolerance identification profile enabling identification of LTx recipients eligible for safe IS withdrawal early after transplantation is needed. Different studies already examined possible markers to identify these TOL LTx recipients.[80,82,84,87,92,129,130] However, an accurate immune profile that could be validated in independent studies has not been determined yet. When these TOL LTx recipients can be recognized earlier and withdrawn from IS, more IS-related side effects could be reversed or avoided.

CHAPTER 5.

Minimal development of liver fibrosis in adult tolerant liver transplant recipients late after immunosuppressive drug weaning and transplantation

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*Shared last authors

Submitted in Clinical Transplantation

Abstract

Background:
Operationally tolerant liver transplant (LTx) recipients can be weaned off immunosuppressive drugs (IS) without development of graft rejection. However, it is feared that liver graft fibrosis might develop after complete IS weaning. The purpose of this small single-center study was to assess liver fibrosis in adult tolerant LTx recipients long after LTx and IS weaning.

Methods:
Liver fibrosis was assessed in adult tolerant LTx recipients (n=9) using non-invasive transient elastography and measurements of multiple pro- and anti-fibrotic serum markers associated with liver fibrosis. Data was collected of two subsequent years on average 8 and 9 years after IS weaning and on average 19 and 20 years after transplantation. Healthy individuals (n=9) matched for age and sex were included as a reference for fibrosis related serum markers.

Results:
Transient elastography indicated that 7 out of 9 tolerant LTx recipients had no or minimal liver fibrosis (F0-F1), whereas two recipients had moderate or severe liver fibrosis (F2-F3). Most fibrosis related serum markers in tolerant LTx recipients were within or close to the range obtained for healthy individuals and were relatively stable across time.

Conclusion:
This small single-center study indicates that most adult tolerant LTx recipients have no or minimal liver graft fibrosis long after transplantation and IS weaning, and their fibrosis related serum marker profile indicates an absence of a pro-fibrotic status.

Introduction

Liver transplantation (LTx) is the only treatment option for end stage liver disease. Use of immunosuppressive drugs (IS) is necessary to prevent allograft rejection after LTx. However, in a substantial number of LTx recipients chronic IS exposure is associated with adverse effects, and morbidity and mortality rates more than one year after LTx are still noticeably higher in LTx recipients compared to the general population.[20] Therefore, most LTx centers attempt to gradually reduce IS over time. Sporadically, LTx recipients spontaneously develop operational tolerance to their graft, a long-term state defined by absence of (acute) rejection episodes while completely free of IS.[49]

One study suggested that pediatric tolerant LTx recipients weaned off IS are more prone to develop fibrosis in the liver graft compared to pediatric LTx recipients on regular IS regimen.[61] Despite absence of any further evidence that pediatric or adult tolerant LTx recipients develop fibrosis after weaning of IS,[49,55-57,62-64,131] clinicians fear development of severe fibrosis and a potential subsequent loss of the liver graft after complete IS weaning.

The golden standard for detecting liver fibrosis is a biopsy. However, a liver biopsy is an invasive procedure that could lead to severe clinical complications. The non-invasive transient elastography (TE), that measures liver stiffness as a measure for fibrosis, has been validated in immunocompetent individuals with various liver diseases.[73-75,132-134] A few studies have investigated the possibility of applying TE in LTx recipients to measure fibrosis, and concluded that TE can discriminate between LTx recipients that develop no or mild fibrosis (F0-F1) and recipients that develop significant fibrosis (F2 or higher).[71,76,77,135]

Liver fibrosis results from an uncontrolled healing response to persisting injury and is characterized by excessive accumulation and altered composition of extracellular matrix (ECM).[65] The key ECM components in the liver are collagens, laminins, proteoglycans, fibronectins and matricellular proteins. The ECM is dynamic and provides a structural and biochemical support network for the surrounding cells.[65,66] The surrounding cells include epithelial cells (hepatocytes and cholangiocytes), endothelial cells, Kupffer cells, immune cells and hepatic and stellate cells (HSCs).[66] Development of donor-specific antibodies (DSAs) against donor human leukocyte antigen (HLA) types early after LTx is associated with liver fibrosis.[136] Fibrosis could eventually lead to cirrhosis, that in contrast to fibrosis, is associated with increased morbidity and mortality.[66]

In case of chronic liver damage, platelet-derived growth factor (PDGF) and transforming growth factor β1 (TGF-β1) are produced by a variety of liver cells and stimulate HSC to differentiate into myofibroblasts and to produce ECM components (a.o. collagen and hyaluronan (HA)).[65,66] In addition, activated HSCs produce tissue inhibitors of metalloproteinases (TIMPs), particularly TIMP1.[65,66,68] TIMPs inhibit matrix metalloproteinases (MMPs), a large family of proteases that are collectively capable of degrading all ECM structural proteins.[137,138] A disturbed balance between MMPs and TIMPs is associated with liver fibrosis.[67] Two studies indicated that hepatic gene expression or serum levels of TIMP1 were higher in pediatric LTx recipients with severe liver fibrosis.[139,140] HA could

be used as a non-invasive biomarker for the assessment of liver fibrosis in immunocompetent individuals and LTx recipients.[72,141] B-cell activating factor (BAFF), implicated in lung and skin fibrosis,[69,70,142] could possibly also be implicated in the development of liver fibrosis. Whether adult tolerant liver transplant recipients develop a pro-fibrotic state characterized by abnormal levels of pro- and anti-fibrotic serum markers after complete IS weaning has never been investigated.

The purpose of this small single-center study was to assess liver fibrosis in tolerant LTx recipients long after LTx and IS weaning, with non-invasive TE and multiple soluble serum markers that are all implicated in the biological processes of liver fibrosis.

Patients and Methods

Patient cohort

Adult operational tolerant LTx recipients (TOL; n=9) were followed at the outpatient clinic at the Erasmus University Medical Centre between 2014 and 2021. TOL were weaned off IS for medical reasons or non-compliance between 2008 and 2016. Recipients were defined as operationally tolerant when IS was ceased for at least one year without occurrence of a rejection episode. Acute rejection was defined as at least a two-fold increase in serum bilirubin, aspartate aminotransferase or alanine transaminase, alkaline phosphatase or γ-glutamyltransferase, as protocol biopsies after complete IS weaning were not taken because of possible complications related to the procedure. Patient blood samples were collected at two subsequent years, of which the first collection occurred on average 8.2 ± 3.0 years after complete IS weaning. Blood was also collected from a group of healthy individuals (n=9) that were matched at a 1:1 ratio to the TOL group for age and sex (age mean ± SD: 55.8 ± 13.0; 77.8% male). Blood samples were centrifuged (2500 rpm; 10 minutes) and obtained sera were stored at -80°C until further use. At the days of blood collection, liver fibrosis was measured using transient elastography. Clinical and laboratory information was retrieved from electronic patient records. From all participants informed consent was obtained. This study was conducted in accordance with the Declaration of Helsinki and approved by the medical ethics committee of Erasmus MC (MEC 2014–232; MEC-2012-022).

Soluble serum protein measurements

Soluble proteins TGFβ-1, HA, PDGF-BB, BAFF, MMP1, MMP2, MMP9 and TIMP1 were measured in two subsequent yearly collected serum samples (referred to as year 1 and year 2) of TOL LTx recipients and serum samples of healthy individuals. Thawing of serum samples was kept to a maximum of two times for all measurements and paired samples were analyzed in the same assay. Concentrations of TGF-β1 were determined using the Human TGF-β 1 DuoSet ELISA (R&D systems, Minneapolis, USA) according to manufacturer's protocol, except for Streptavidin HRP and TMB Peroxidase. In this protocol latent TGF-β1 is activated, hence total TGF-β1 is measured. Serum samples were diluted 4x and after measuring corrected for the dilution factor. Concentrations of HA were determined using Hyaluronan DuoSet ELISA (R&D systems) according to manufacturer's protocol, except for Streptavidin HRP and TMB Peroxidase. Serum samples were diluted 2x and after measuring corrected for the dilution factor. Streptavidin HRP (BD Biosciences, San Jose, USA) and KPL TMB Peroxidase Substrate (SeraCare Life Sciences, Milford, USA) were used for each ELISA according to manufacturer's protocol. ELISA assays were measured using a BioTek Reader Elx800 (BioTek, Winooski, USA). Concentrations of PDGF-BB, BAFF, MMP1, MMP2, MMP9 and TIMP1 were determined using Human Magnetic Luminex Assay (R&D systems) according to manufacturer's protocol. Serum samples were diluted 50x and after measuring corrected for the dilution factor. Luminex assays were measured using a Bio-Plex MAGPIX Multiplex Reader (Bio-Rad, Veenendaal, the Netherlands).

Transient Elastography measurements

Transient elastography (TE) measurements were performed on the same day of blood withdrawal for measurements of fibrosis related markers in serum. TE was performed using Fibroscan Touch 502 (Echosens, Paris, France) according to current standards.[133,134] TE was considered reliable when 10 measurements were obtained with IQR below 30% of the median value. Results were expressed in kilopascal (kPa) and converted to Metavir scores for development of fibrosis after transplantation. Values between 2-7 kPa are referred to as F0-F1, 7-10.5 kPa as F2, 10.5-17 kPa as F3, and values >17 kPa are referred to as F4.[76,135] Two tolerant LTx recipients were excluded from TE analysis: one due to primary sclerosing cholangitis recurrence and one due to the inability to obtain reliable TE results (this recipient had received a left liver lobe only, where due to the anatomic position TE measurement is influenced by the cardiac contractions).

Donor-specific antibodies

Donor-specific antibodies (DSAs) were measured in retrospectively derived serum samples initially collected for diagnostic purposes (MEC 2018-1597) pre LTx and 1-2 years before the collection of the two subsequent yearly serum samples used for analysis of fibrosis related factors. Screening of HLA Class I (HLA-A or HLA-B) or HLA Class II (HLA-DQ or HLA-DR) DSAs was performed using the Lifecodes Lifescreen Deluxe (LMX) kit according to the manufacturer's protocol (Immucor Transplant Diagnostics Inc.). Positive samples were measured with Luminex single antigen assay using HLA class I and class II antigen beads (LABscreen One Lambda, Canoga Park, CA, USA) according to manufacturer's protocol. Antibodies measured with a MFI of >1000 were considered positive.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 8 version 8.4.3 (GraphPad Software Inc., San Diego, USA). The normality of the distribution of the data was determined by the Shapiro-Wilk normality test. Differences between yearly measurements were analyzed by either the paired t-test or Wilcoxon signed-rank test.

Results

Characteristics of the study group

Transient elastography (TE) and fibrosis related serum marker measurements in tolerant (TOL) LTx recipients were performed at two subsequent years and are referred to as year 1 and year 2. Values of liver function parameters were normal for most TOL LTx recipients (Figure 1). Values above the upper limit of normal were attributed to causes other than rejection. Characteristics of the TOL group are presented in Table 1. At year 1 the TOL LTx recipients were on average 19.0 ± 3.8 years after LTx, and on average 8.2 ± 3.0 years after complete IS weaning and without development of a rejection episode. DSAs developed in 4 out of 9 (44.4%) TOL LTx recipients, and were all de novo.

Table 1 Characteristics of the tolerant (TOL) LTx recipients.

	TOL
Demographics	n=9
Male n (%)	7 (77.8)
Age in years[a]	56.7 ± 15.1
Years after LTx[a]	19.0 ± 3.8
Years complete IS weaning - year 1[a]	8.2 ± 3.0
Primary disease n (%)	
Cholestatic disease	3 (33.3)
Virus-related	3 (33.3)
Hepatocellular carcinoma	2 (22.2)
Cryptogenic cirrhosis	1 (11.1)
IS last used n (%)	
Tacrolimus	5 (55.6)
Azathioprine	1 (11.1)
Tacrolimus and mycophenolate mofetil	1 (11.1)
Azathioprine and cyclosporine A	1 (11.1)
Unknown	1 (11.1)
HLA mismatches recipient/donor[a]	
A + B	3.3 ± 0.9
DR + DQ	2.4 ± 1.0
DSA positive n (%)	4 (44.4)

Characteristics of the study group at the first year of blood collection and transient elastography measurements are presented as n with percentages or [a]mean ± SD. DSAs; donor-specific antibodies; HLA, Human Leukocyte Antigen; IS, immunosuppressive drugs; LTx, liver transplantation; TOL, tolerant group.

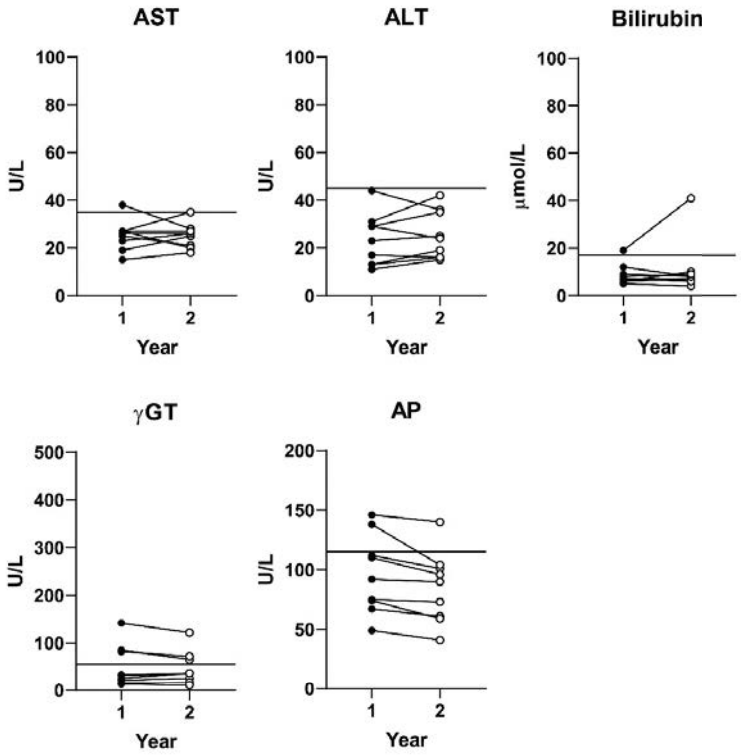

Figure 1 Liver function values of the tolerant LTx recipients. Values of liver function parameters AST, ALT, γGT, AP and bilirubin of tolerant LTx recipients at the two subsequent years of blood collection are presented. Solid black line represents the upper limit of normal for each parameter. Values above the upper limit of normal were attributed to very slightly chronically elevated levels without a two-fold increase. ALT, alanine transaminase; AP, alkaline phosphatase; AST, aspartate aminotransferase; γGT, γ-glutamyltransferase; LTx, liver transplantation.

Most tolerant liver transplant recipients developed no or minimal liver fibrosis

TE measurements did not significantly differ between year 1 and year 2, and thus values were relatively stable. TE measurements indicated that 5 out of 9 (55.6%) tolerant LTx recipients developed no or mild fibrosis (F0-F1) at year 1 (Figure 2A). At year 2 this increased to 7 out of 9 (77.8%) TOL LTx recipients, and this is mainly due to the two LTx recipients that were previously diagnosed with moderate fibrosis (F2) at year 1, but were at the borderline of no to mild fibrosis (Figure 2A). One TOL LTx recipient had TE outcomes corresponding to moderate fibrosis (F2) at both time points and one TOL LTx recipient progressed from moderate (F2) to severe fibrosis (F3). Altogether, TE measurements indicated that most (77.8%) TOL LTx recipients developed no or mild fibrosis at year 2 after on average 9 years of complete IS weaning and 20 years after transplantation. However, the TOL LTx recipient with the highest fibrosis score at year 1 did progress a little further at year 2. Age, years after LTx, years after complete IS weaning, number of HLA mismatches between recipient and donor, and percentage and type of donor-specific antibodies and height of MFI did not notably differ between the TOL LTx recipients with F0-F1 and F2/F3 fibrosis stages at year 2 (data not shown).

Most fibrosis related serum markers in tolerant liver transplant recipients were within or close to the range observed for healthy individuals

Serum concentrations of pro-fibrotic growth factors TGF-β1 and PDGF-BB measured in TOL LTx recipients were mostly in or below the range measured for healthy individuals matched for age and sex (Figure 2B). In contrast, concentrations of pro-fibrotic BAFF were higher in almost all TOL LTx recipients compared to healthy individuals, but were quite stable between year 1 and year 2. Pro-fibrotic HA concentrations in TOL LTx recipients were mostly non-detectable or within the range observed for healthy individuals, except for one TOL LTx recipient with F0-F1 at year 1. Pro-fibrotic TIMP1 concentrations in TOL LTx recipients were mostly within or near the range measured in healthy individuals and were relatively stable across time. Concentrations of anti-fibrotic MMP1 and MMP9 in TOL LTx recipients were mostly within the range observed for healthy individuals, whereas for anti-fibrotic MMP2 concentrations were mostly below the narrow range. For all three MMPs most values were relatively stable across time. Values of serum fibrosis related markers measured for TOL LTx recipients with F2 and F3 TE fibrosis stage at year 2 did not deviate from the values obtained from the TOL LTx recipients with no or minimal fibrosis (F0-F1). Altogether, this data indicated that for TOL LTx recipients most fibrosis related serum markers were within or close to values obtained for healthy individuals and relatively stable across time.

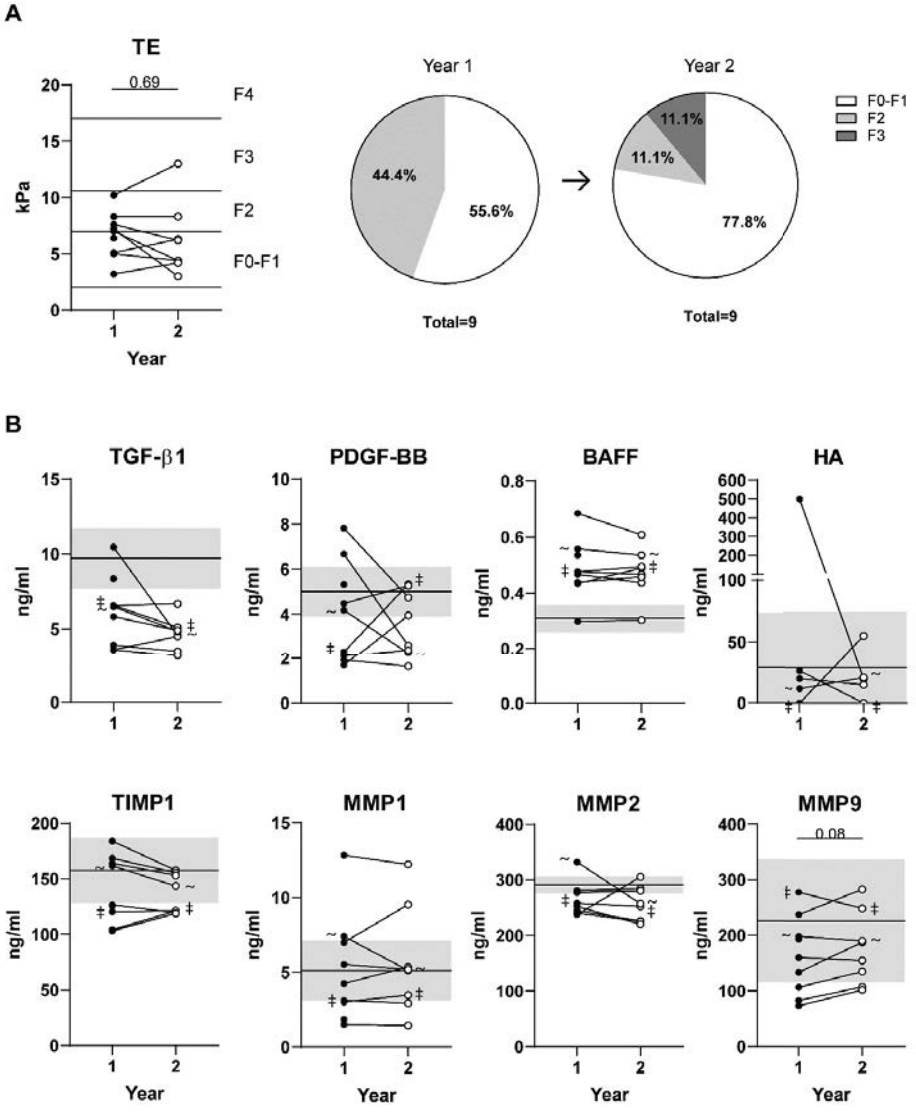
A
TE
0.69
F4
F3
F2
F0-F1
kPa
Year
Year 1
Year 2
44.4%
55.6%
11.1%
11.1%
77.8%
F0-F1
F2
F3
Total=9
Total=9
B
TGF-β1
PDGF-BB
BAFF
HA
TIMP1
MMP1
MMP2
MMP9
0.08
ng/ml
Year

Figure 2 Transient elastography and fibrosis related serum markers indicate that most tolerant LTx recipients did not develop liver fibrosis late after transplantation. Transient elastography and serum markers related to fibrosis were measured at two subsequent years in tolerant LTx recipients on average 19.0 (year 1) and 20.0 (year 2) years after transplantation and 8.2 (year 1) and 9.2 (year 2) years after complete IS weaning. In A transient elastography measurements in kPa and their associated Metavir score (F0-F4) in tolerant LTx recipients are presented. In B levels of serum markers related to (liver) fibrosis in tolerant LTx recipients are presented with the mean (solid line) and 95% CI (grey area) of fibrosis related markers measured in matched healthy individuals. Serum levels of LTx recipients with F2 (‡) and F3 (~) fibrosis stage assessed with transient elastography at year 2 are indicated. For one tolerant LTx recipient (F2 → F0-F1 fibrosis stage) the markers could not be measured at year 2. IS, immunosuppressive drugs; LTx, liver transplantation; TE, transient elastography.

Discussion

In this small single-center study transient elastography indicated minimal development of fibrosis in the liver graft of 7 out of 9 adult tolerant LTx recipients on average 9 years after complete IS weaning and 20 years after LTx. This is supported by several studies on pediatric and adult tolerant LTx recipients, in which protocol liver biopsies indicated that the incidence of fibrosis in the liver graft of tolerant LTx recipients after complete IS weaning late after LTx did not differ from baseline level or non-tolerant LTx recipients.[49,55,57,62-64,131] In fact, one study even reported a slower liver graft fibrosis progression rate in tolerant LTx recipients compared to non-tolerant LTx recipients 10 years after transplantation upon liver biopsy assessment.[56] Only one study published by Yoshitomi et al,[61] suggested a higher incidence of liver graft fibrosis in pediatric tolerant LTx recipients compared to a control group of pediatric LTx recipients on maintenance IS late after transplantation. However, this study should be interpreted with caution. Firstly, biopsies were taken from the tolerant group on average 10 years after transplantation and from the control group on average 4.3 years after transplantation. Secondly, the pediatric tolerant LTx recipients were transplanted at a significantly younger age compared to the control group. Multiple studies have shown that liver fibrosis progresses in a significant proportion of pediatric LTx recipients on regular maintenance IS regimen and that there is a strong positive association with time after transplantation or younger age at transplantation.[143-148] This would explain the discrepancy in data on development of liver fibrosis in (pediatric) LTx recipients observed between the study published by Yoshitomi et al. and all other studies that compared more similar study groups. To date, a positive association between liver fibrosis progression and time after transplantation or age of the recipient at transplantation is not reported for adult LTx recipients on regular IS regimen or adult tolerant LTx recipients.

Pro-fibrotic TGF-β1 and PDGF-BB are implicated in activation of HSCs in the liver, that in turn upregulate synthesis of TIMP1, HA and collagens.[65,66,68] A previous study indicated that TGF-β1 plasma levels were significantly higher in pediatric patients with significant (Metavir >F2) liver fibrosis compared to patients without or minimal liver fibrosis (Metavir F0-F1) awaiting liver transplantation.[149] We are the first to measure serum TGF-β1 levels in adult tolerant LTx recipients late after transplantation. We observed lower levels of TGF-β1 in TOL LTx recipients compared to matched healthy individuals. Together with the normal or reduced PDGF-BB serum levels, these data suggest absence of a pro-fibrotic status in these TOL LTx recipients. In contrast to TGF-β1 and PDGF-BB levels, serum levels of BAFF were higher in tolerant LTx recipients compared to the range observed for healthy individuals, but unlike in skin and lung fibrosis [69,70,142], it is unclear whether BAFF is involved in liver fibrosis. In pediatric LTx recipients hepatic gene expression of MMP2, MMP9 and TIMP1 was higher in recipients with liver fibrosis assessed with liver biopsies compared to recipients without liver fibrosis 15 years after transplantation.[140] This difference was not reflected in serum levels where MMP9 and TIMP1 concentrations were not significantly different. Higher levels of MMP expression in severe fibrotic liver grafts seems counterintuitive. Next to that, serum levels of MMPs and TIMPs were much higher in pediatric LTx recipients compared to a control group of day-

surgery patients without evidence of metabolic, gastrointestinal or hepatobiliary diseases. We are the first to measure MMP and TIMP levels in adult tolerant LTx recipients late after transplantation. In our study, serum levels of MMP1, MMP2, MMP9 and TIMP1 in adult tolerant LTx recipients were overlapping with serum levels of matched healthy individuals. Another study indicated that serum levels of TIMP1 were higher in pediatric LTx recipients with a higher liver allograft fibrosis score assessed with liver biopsies.[139] This was not observed in our own data, since most of the tolerant LTx recipients developed no or minimal liver fibrosis assessed with TE. HA is used as a non-invasive biomarker for the assessment of liver fibrosis in immunocompetent individuals.[72] HA levels in serum of LTx recipients were able to distinguish between presence of liver fibrosis (>F2) and no or mild liver fibrosis (F0-F1) independent of etiology assessed with liver biopsies.[141] In our study we observed no or mild liver fibrosis with TE in most adult TOL LTx recipients and serum levels of HA were within the range of matched healthy individuals. Hence, TE and fibrosis related serum markers indicated that liver fibrosis is minimally present in adult TOL LTx recipients on average 9 years after complete weaning and 20 years after transplantation.

To our knowledge this is the first study on the assessment of liver fibrosis in adult tolerant LTx recipients with TE in combination with serum fibrosis related markers long after LTx and IS weaning. A strength of the study is that the levels of serum fibrosis related markers of TOL LTx recipients were compared to healthy individuals matched on age and sex. Furthermore, in this study two subsequent years were included that demonstrated that the measured serum markers were relatively stable across time and levels correlated with absence of fibrosis since much overlap was observed with the healthy individuals. Another strength of this study is that presence of de novo DSAs and fibrosis could be studied. Weaknesses of the study are the small population of TOL LTx recipients included, and lack of protocol liver biopsies to confirm the operational tolerant state of the LTx recipients and absence of liver fibrosis in most recipients. A few studies indicated that in up to 85% of the biopsies late after liver transplantation histological abnormalities were observed without elevated liver function parameters, and that this could point to presence of subclinical rejection.[150] However, liver grafts experiencing subclinical rejection showed heterogeneous histology and gene expression, which indicates a complex and as of yet unknown process.[33] Furthermore, the clinical significance of subclinical rejection and a possible relation to graft damage is still unclear,[150,151] although one study suggests that subclinical rejection is benign in adult LTx recipients.[152] We cannot exclude presence of subclinical rejection in the liver graft of the TOL LTx recipients, but in most of the TOL LTx recipients liver graft fibrosis was absent. Another weakness of our study is that the measured serum fibrosis related markers are not specific for liver fibrosis, hence fibrosis in other organs could influence the results. Nevertheless, elevated concentrations of some serum markers such as HA and TIMP1 have been associated with (severe) fibrosis in the liver (graft) in other studies.[72,139-141,153] Furthermore, TE used in this study to assess liver fibrosis in adult tolerant LTx recipients has not been validated yet for the assessment of liver graft fibrosis in a large independent cohort of LTx recipients, but several studies have indicated that TE can be used to diagnose liver fibrosis in LTx recipients.[71,76,77,135]

From this study we can conclude that most adult tolerant LTx recipients have no or minimal development of liver graft fibrosis long after transplantation and long after IS weaning, according to both their TE and serum liver fibrosis marker measurements. These results are supported by similar observations from other centers. We suggest that adult and pediatric tolerant liver transplant recipients are distinct populations, since adult and pediatric liver transplant recipients differ in their clinical course (late) after transplantation.

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CHAPTER 6.

Activated CD4+ T-cells and highly differentiated alloreactive CD4+ T-cells distinguish operationally tolerant liver transplant recipients

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Abstract

Background:
Spontaneous operational tolerance to the allograft develops in a proportion of liver transplant (LTx) recipients weaned off immunosuppressive drugs (IS). Several studies have investigated whether peripheral blood circulating T-cells could play a role in the development or identify operational tolerance, but never characterized alloreactive T-cells in detail due to the lack of a marker for these T-cells.

Methods:
In this study we comprehensively investigated phenotypic and functional characteristics of alloreactive circulating T-cell subsets in tolerant LTx-recipients (n=15) using multiparameter flowcytometry and compared these to LTx-recipients on IS (n=23) and healthy individuals (n=16). Activation-induced CD137 was used as a marker for alloreactive T-cells upon allogenic stimulation.

Results:
We found that central and effector memory CD4+T-cells were hyporesponsive against donor and third party splenocyte stimulation in tolerant LTx-recipients, whereas an overall hyperreponsiveness was observed in alloreactive terminally differentiated effector memory CD4+T-cells. In addition, elevated percentages of circulating activated T-helper cells were observed in these recipients. Lastly, tolerant and control LTx-recipients did not differ in donor-specific antibody formation.

Conclusion:
A combination of circulating hyperresponsive highly differentiated alloreactive CD4+T-cells and circulating activated T-helper cells could discriminate tolerant recipients from a larger group of LTx-recipients.

Introduction

Liver transplantation (LTx) is the sole treatment option for end-stage liver disease. Over the last few decades immunosuppressive drugs (IS) substantially improved short-term graft and patient survival.[154] However, long-term use of IS leads to various serious side effects and adversely affects quality of life.[14,17,155] Therefore, most transplantation centers attempt to gradually reduce or even completely cease IS over time.[53] Several clinical trials have shown that part of LTx-recipients that are completely weaned off IS develop operational tolerance towards their graft, a long-term state defined by absence of (acute) rejection episodes while free of IS.[49,52,54]

Compared to other solid organ grafts, the transplanted liver facilitates operational tolerance.[156] Preformed or de novo donor-specific antibodies (DSAs) against donor human leukocyte antigen (HLA) types have been associated with an increased risk of acute and chronic rejection.[99] Nevertheless, many DSA positive LTx-recipients do not experience rejection, and DSAs have even been detected in tolerant LTx-recipients.[62,157]

Several studies have investigated whether immune system-related peripheral blood markers could identify the LTx-recipients that have developed immunological tolerance towards their graft. Higher relative numbers of circulating CD4+CD25^{high} T-cells,[82,85,87] CD4+FoxP3+ T-cells[87] and CD4+CD25++CD127^{dim} cells,[92] and a higher Vδ1/Vδ2 γδT-cell ratio[84,85,87] in blood of adult or pediatric recipients were implied to discriminate between tolerant LTx-recipients and (non-tolerant) LTx-recipients with IS. These data suggest that regulatory T-cells and γδT-cells might play a role in the development and/or maintenance of operational tolerance. However, many of these studies lack matching of parameters that are known to influence the composition of circulating immune cells, such as cytomegalovirus (CMV) infection,[158,159] when comparing tolerant with control groups of LTx-recipients.

Whilst donor-specific T-cells critically contribute to liver graft rejection, their association with operational tolerance after LTx is being under-investigated. Lack of proliferation of total CD4+T-cells upon stimulation with donor-antigens when compared to third party antigens (donor-specific hyporesponsiveness) was reported,[92,93] but donor-specific responses of CD4+ or CD8+ T-cells have never been studied in more detail in tolerant LTx-recipients. CD137 is expressed by activated CD4+ and CD8+ T-cells upon interaction with antigen-presenting cells, and it has been proven that this marker can identify all alloreactive T-cells in kidney and liver transplant recipients.[94-96]

The purpose of this study is to comprehensively investigate phenotypic and functional characteristics of circulating (anti-donor-antigen-specific CD137+) T-cell subsets and DSAs in operationally tolerant LTx-recipients, and compared these immunological markers to well-matched control groups.

Patients and Methods

Study design and participants

In this study cohort all adult operationally tolerant LTx-recipients (TOL; n=15) followed at the outpatient clinic at the Erasmus University Medical Centre between 2014 and 2020 were included. TOL LTx-recipients were weaned off IS for medical reasons or non-compliance between 2008 and 2019 (Table 1). Four LTx-recipients were prospectively completely weaned off IS (Supplementary Table 1). Recipients were defined as operationally tolerant when IS was ceased for at least one year without occurrence of a rejection episode. Protocol biopsies after complete IS weaning were not taken in this study, because of possible complications related to the procedure and patient reluctance. Therefore, acute rejection was defined as at least a two-fold increase in serum bilirubin, aspartate aminotransferase or alanine transaminase, alkaline phosphatase or γ-glutamyltransferase. A liver biopsy was performed in 5 tolerant LTx-recipients because of possible rejection as indicated by increasing liver enzymes, at on average 3.1 ± 2.2 years after complete weaning. In all cases rejection was excluded using BANFF criteria. A control group of stable LTx-recipients (CTRL) on regular IS regimen (n=23), and a healthy control group (HC; n=16), were included in the study and both matched to the TOL group based on their sex, age and CMV-seropositivity. For the CTRL group also time after LTx and primary disease were matched to the TOL group. No other inclusion or exclusion criteria were used. Heparinized blood samples were collected from all participants. From TOL blood samples were collected at a time point at least one year after complete IS weaning. From CTRL blood samples were collected at matched time points with TOL for time after LTx. The CTRL LTx-recipients did not experience rejection episodes for at least 5 years before and 4 years after blood collection. From the recipients prospectively weaned blood was collected before the start, during and six months after IS weaning. Clinical and laboratory information was retrieved from electronic patient records. From all participants written informed consent was received. This study was conducted in accordance with the Declaration of Helsinki and was approved by the medical ethics committee of Erasmus MC (MEC 2014–232; MEC-2012-022).

Donor and third party T-cell stimulation

For each donor-specific stimulation, donor splenocytes that had been collected and stored in liquid nitrogen at the time of LTx, were used. To account for non-specific HLA stimulation, third party splenocytes with the same number but different HLA mismatches with the recipient as between recipient and donor, were used. Recipient PBMCs and splenocytes were thawed according to our standard protocol. Splenocytes were depleted of CD3+ cells using MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Efficiency of T-cell depletion (>98% was accepted for further use) was determined by staining with CD3 FITC conjugated antibody (clone sk7; BD Biosciences, San Jose, USA) and measured at a BD FACS Canto II flow cytometer (BD Biosciences). After resting, PBMCs and CD3-depleted donor or third party splenocytes were co-cultured in a 1:1

ratio with 2 million cells each overnight (±14h) in RPMI 1640 Medium Glutamax (Thermo Fisher Scientific, Waltham, USA) with human serum (Sanquin, Amsterdam, the Netherlands). As a negative control, PBMCs only were included. As a positive control, PBMCs were stimulated with 50ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, Saint Louis, USA) and 1µg/ml Ionomycin (IONO) (Sigma Aldrich). All cell cultures received co-stimulation with 1µg/ml α-CD49d (Purified NA/LE mouse; BD Biosciences) and 1µg/ml α-CD28 (Low endotoxin; Bioconnect). In addition, 1:400 Golgi stop (BD Biosciences) was added to each culture. Alloreactive recipient T-cells were detected by measuring activation-induced CD137.

Antibody staining and flowcytometry

See supporting information.

Donor-specific HLA class I and class II antibodies

See supporting information.

Statistical analyses

Statistical analyses were carried out with GraphPad Prism 8 version 8.4.3 (GraphPad Software Inc., San Diego, USA) or IBM SPSS software version 25 (SPSS Inc., Chicago, USA). The normality of the distribution of the data was determined by the Shapiro-Wilk normality test. Differences between two groups were analyzed by either the t-test or Mann-Whitney U test. Statistical analyses of three independent groups or more were performed with one-way ANOVA or Kruskal-Wallis, with a Bonferroni or Dunn's posttest. Statistical analyses within groups were performed with one-way ANOVA or Friedman test, with a Bonferroni or Dunn's posttest. Differences in discrete nominal data between groups were analyzed by the two-sided Fisher's Exact Test or Pearson Chi-Square test. Figures were created with GraphPad Prism 8 version 8.4.3. Principal component analysis was performed using IBM SPSS software version 25.

Results

Patient characteristics

In this study operationally tolerant (TOL) LTx-recipients were compared with a control (CTRL) group of stable LTx-recipients on regular IS regimen and a healthy control (HC) group. All three groups were matched for age, sex, CMV-serostatus and CTRL and TOL also for time after LTx and primary disease. Therefore, no significant differences between these parameters were observed (Table 1). The TOL group completely ceased IS 12.3 ± 6.7 years after LTx, and has been IS- and rejection-free for 3.6 ± 2.9 years. The number of recipients with biopsy-proven acute rejection episodes early after LTx (< 2y post LTx) in TOL and CTRL did not significantly differ. The numbers of HLA mismatches between recipient and donor were similar between both groups for HLA Class I and II.

Tolerant LTx-recipients have higher relative numbers of circulating activated T-helper cells

Percentages of circulating CD3+ cells (data not shown), CD4+ and CD8+ T-cells did not differ between HC, CTRL and TOL (Figure 1A). Several studies reported a higher proportion of CD4+FoxP3+CD25+ T-cells in TOL compared to control groups.[82,85,87] Indeed, here in TOL the proportion of FoxP3+CD25+ T-cells within CD4+T-cells was significantly higher than CTRL (Figure 1B), but only a higher trend was observed versus HC. Since CD4+FoxP3+CD25+ T-cells could contain regulatory T-cells (Tregs) and activated T-helper cells (aTh), we elucidated this further by discriminating aTh, resting Tregs (rTreg) and activated Tregs (aTreg) within CD4+T-cells using FoxP3 and CD45RA expression[7] (Figure 1C). In TOL a significantly higher percentage of circulating aTh was present compared to CTRL and HC, whereas percentages of aTreg and rTreg were similar among these groups. Even though a higher percentage of aTh is observed in TOL, their capacity to produce IFNγ and IL17 was similar among all groups (Figure 1D). No significant differences in percentages of circulating CD4+LAG3+CD49b+ type 1 regulatory T-cells (Tr1), late differentiated CD4+ or CD8+ CD28- T-cells and CD4+ or CD8+ T-cells expressing co-stimulatory immune checkpoint ICOS, or co-inhibitory immune checkpoints PD1 or CTLA4 (Supplementary Figure 1A-F) were observed between groups, except for higher numbers of CD4+CXCR5+ICOS+ follicular T-helper cells in TOL compared to HC. Furthermore, no significant differences in differentiation status of circulating CD4+ or CD8+ T-cells, and perforin and/or granzyme B expressing T-cells were observed between groups (Supplementary Figure 2A,B).

Table 1 Characteristics of the study group (tolerant LTx-recipients; TOL), control group (recipients with IS regimen; CTRL) and healthy control group (HC).

	HC	CTRL	TOL	P-value
Demographics	n=16	n=23	n=15	
Male (%)	75.0	65.2	73.0	0.58[a]
Age in years end follow-up (mean ± SD)	53.3 ± 15.0	49.7 ± 17.7	52.9 ± 16.4	1.00[d]
Years LTx - end follow-up (mean ± SD)	NA	14.6 ± 5.4	16.1 ± 5.4	0.62[c]
Years LTx - complete IS weaning (mean ± SD)	NA	NA	12.3 ± 6.7	
Years complete IS weaning - end follow-up (mean ± SD)	NA	NA	3.6 ± 2.9	
Primary disease (%)				0.84[a]
Cholestatic disease	NA	21.7	33.3	
Virus-related	NA	34.8	26.7	
Hepatocellular carcinoma	NA	21.7	26.7	
Cryptogenic cirrhosis	NA	13.0	13.3	
Drug-induced	NA	4.3	0.0	
Metabolic-related	NA	4.3	0.0	
CMV+ serostatus (%)				
Recipient pre LTx	NA	47.8	46.7	1.00[b]
Recipient end follow-up or healthy control	62.5	73.9	66.7	0.74[a]
Donor	NA	43.5	46.7	1.00[b]
Acute rejection (%)				
< 2y post LTx	NA	39.1	6.7	0.06[b]
> 2y post LTx	NA	8.7	0.0	0.51[b]
HLA mismatches recipient/donor				
A + B (mean ± SD)	NA	3.0 ± 0.9	3.5 ± 0.7	0.09[c]
DR + DQ (mean ± SD)	NA	2.8 ± 1.0	2.7 ± 1.0	0.68[c]
IS last used (%)				0.35[a]
Tac	NA	65.2	46.7	
CsA	NA	4.4	6.7	
MMF	NA	8.7	6.7	
Aza	NA	0.0	13.3	
Tac and MMF	NA	8.7	13.3	
Pred and MFF	NA	4.4	0.0	
Pred and Tac	NA	8.7	0.0	
Aza and CsA	NA	0.0	6.7	
Unknown	NA	0.0	6.7	

Statistical analyses were performed wih the Chi-Square[a], Fisher's exact [b], Mann-Whitney[c] or ANOVA and Bonferroni[d] test. Aza, azathioprine; CMV, cytomegalovirus; CsA, cyclosporine A; CTRL, control group; HC, healthy controls; HLA, human leukocyte antigen; IS, immunosuppressive drugs; LTx, liver transplantation; MMF, mycophenolate mofetil; NA, not applicable; Pred, prednisolone; Tac, tacrolimus; TOL, tolerant group.

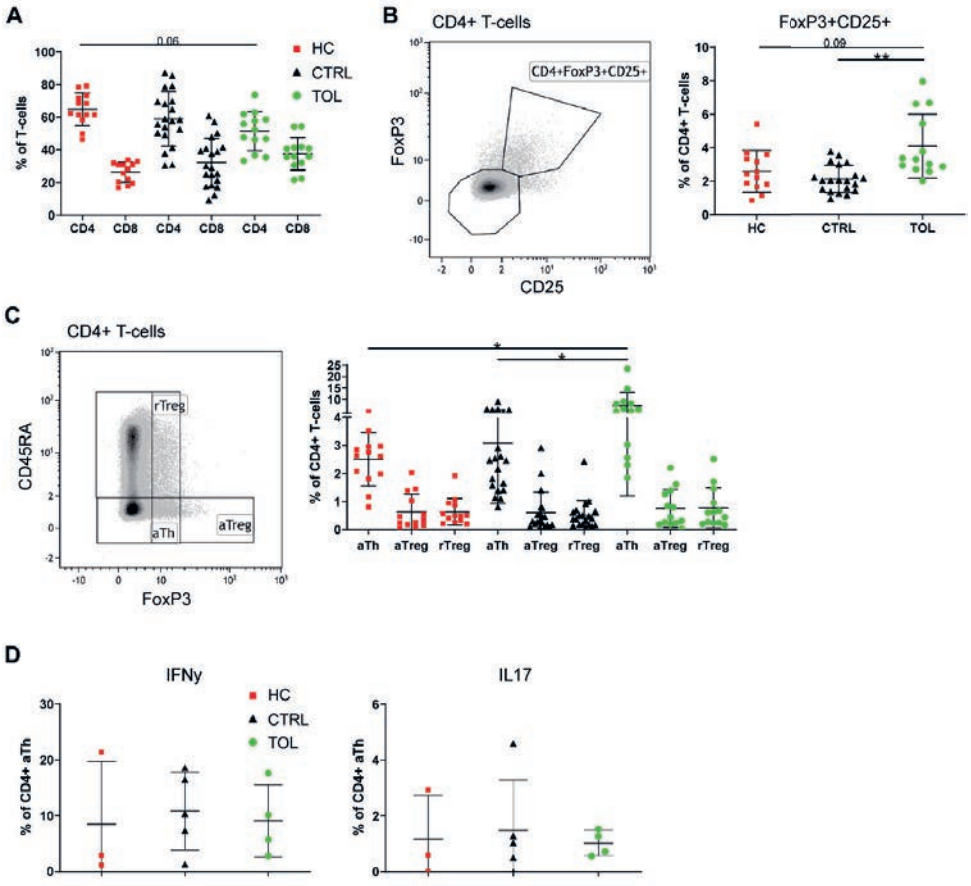


Figure 1 Higher relative numbers of circulating activated T-helper cells in tolerant LTx-recipients. Percentages of CD4+ and CD8+ T-cells (A), CD4+FoxP3+CD25+ T-cells and gating strategy (B), aTh, aTreg and rTreg defined by gating strategy FoxP3 and CD45RA (C) and IFNy or IL17 positive cells in PMA/IONO stimulated aTh (D) are presented. HC n=13, CTRL n=20, TOL n=13. Statistical analyses were performed with one-way ANOVA or Kruskal-Wallis and post-tests. *P < 0.05 **P < 0.01. Abbreviations: aTh, activated T-helper cells; aTreg; activated regulatory T-cells; CTRL, control LTx-recipients; HC, healthy control; IONO, ionomycin; LTx, liver transplantation; PMA, phorbol 12-myristate 13-acetate; rTreg, resting regulatory T-cells; TOL, tolerant LTx-recipients.

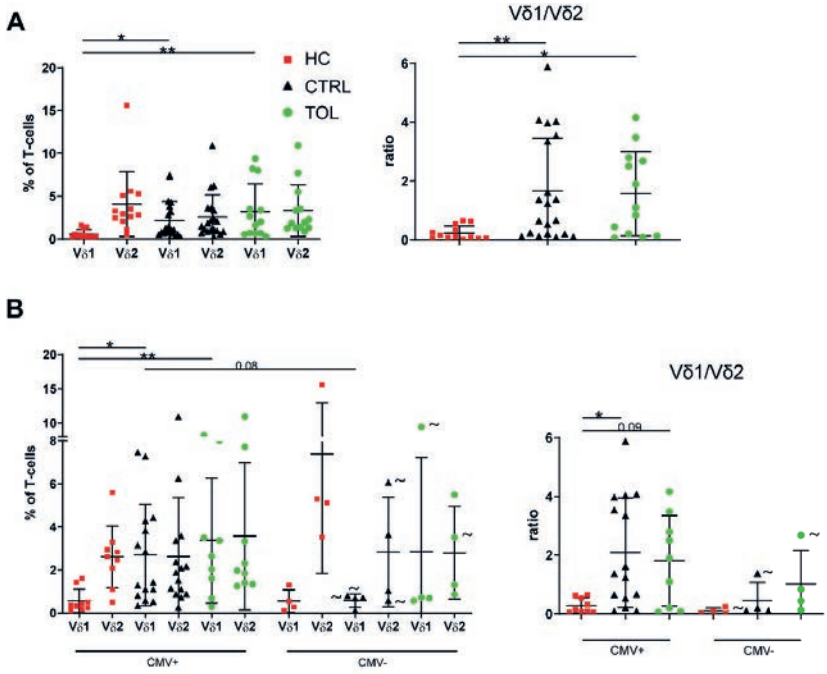


Figure 2 CMV-positive serostatus is associated with a higher Vδ1/Vδ2 γδT-cell ratio in all LTx-recipients. Percentages and ratios of Vδ1 and Vδ2 γδT-cells of entire groups (A) and in CMV+ and CMV- individuals sorted by serostatus at end of follow-up (B) are presented. ~These individuals are CMV- seronegative, but were transplanted with a CMV+ donor. HC n=13, CTRL n=20, TOL n=13 Statistical analyses were performed with one-way ANOVA, Kruskal-Wallis or Friedman and post-tests. *P < 0.05 **P < 0.01. Abbreviations: CMV, cytomegalovirus; CTRL, control LTx-recipients; HC; healthy control; LTx, liver transplantation; TOL, tolerant LTx-recipients.

CMV-seropositivity is associated with an increased Vδ1/Vδ2 γδT-cell ratio in LTx-recipients

Several studies reported that a higher Vδ1/Vδ2 γδT-cell ratio could discriminate tolerant from control or non-tolerant LTx-recipients.[84,85,87] We did not find a significant difference in the Vδ1/Vδ2 γδT-cell ratio between CTRL and TOL (Figure 2A). However, this ratio was significant lower in HC compared to both TOL and CTRL, and this was due to a significantly lower percentage of Vδ1 T-cells (Figure 2A) within CD3+ T-cells. In our study we chose to match the groups for, amongst others, the CMV-serostatus, since a CMV-infection profoundly influences the composition of circulating immune cell subsets.[159] When we sorted the individuals according to the CMV-serostatus at end of follow-up (Figure 2B), high Vδ1/Vδ2 γδT-cell ratios were indeed predominantly found in CMV-seropositive LTx-recipients of both CTRL and TOL. Within the CMV-seropositives, both TOL and CTRL have (significantly) higher percentages of Vδ1 γδT-cells and Vδ1/Vδ2 γδT-cell ratios compared to HC. This indicates that a CMV-seropositive serostatus in LTx-recipients is associated with an increased proportion of Vδ1 γδT-cells and thereby an enhanced Vδ1/Vδ2 γδT-cell ratio.

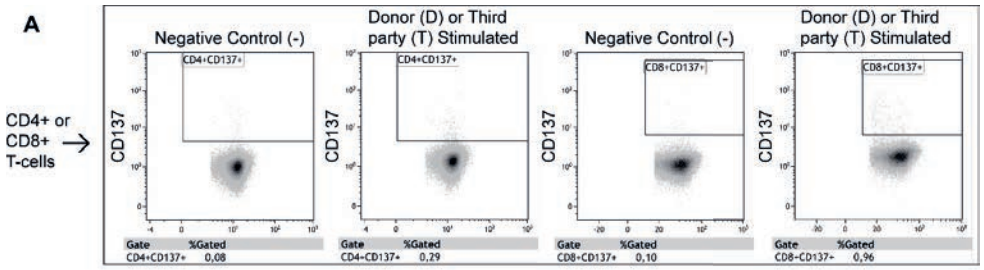
Alloreactive memory CD4+ T-cells are more terminally differentiated in tolerant LTx-recipients

Using activation induced CD137 expression as a surrogate marker for antigen-specific T-cells, we investigated T-cell responses against donor or HLA-mismatched third party splenocytes (Figure 3A; Supplementary Figure 3 for full gating strategy). Responses of CD4+ and CD8+ CD137+ T-cells against donor and third party splenocytes were detected in our assay, since the ratios of percentages of CD137-expressing T-cells upon allogenic stimulation over non-stimulated T-cells were higher than 1 for most individuals in all three groups. However, no significant differences were observed in ratios of donor (D) or third party (T) CD4+ or CD8+ CD137+ T-cell responses against non-stimulated (-) T-cells between groups (Figure 3B; Supplementary Figure 4). No significant differences were observed in donor against third party ratios (D/T) of CD4+ or CD8+ CD137+ T-cell responses, but donor-specific T-cell hyporesponsiveness was observed for some recipients in CTRL and TOL. Within CD4+CD137+ T-cells the proportions of Tregs and Th that responded to donor or third party splenocytes were similar (Figure 3C; Supplementary Figure 5). Functional alloreactive responses by measuring IFNγ-producing CD137-expressing T-cells were assessed. The ratios of CD137+ IFNγ-producing CD4+ and CD8+ T-cells after stimulation with donor or third party splenocytes were similar between CTRL and TOL (Figure 3D). Furthermore, the maximum production capacity of IFNγ within CD4+ or CD8+ CD137+ T-cells upon stimulation with PMA/IONO was similar among all groups (Figure 3E). Differentiation statuses of CD4+ or CD8+ CD137+ expressing T-cells were also assessed. Ratios of alloreactive CD4+ naive T-cells did not differ in TOL and CTRL (Figure 3F). In TOL CD4+ central memory T-cells (CM) and effector memory T-cells (EM) responded significantly less to both donor and third party splenocytes compared to CTRL. In contrast, CD4+ terminally differentiated effector T-cells (EMRA) responded significantly more to both donor and third party splenocytes in TOL compared to CTRL (Figure 3F). Within CD8+ T-cells no differences were observed in ratios of percentages activation induced CD137 expression of different differentiation statuses (Supplementary Figure 6).

Altogether, these results indicate that alloreactive memory CD4+T-cells of TOL are more differentiated compared to those of CTRL.

A tolerance profile discriminating tolerant LTx-recipients could be established

To investigate whether the phenotypic and functional differences in circulating (alloreactive CD137+) T-cells between TOL and CTRL could identify operational tolerance, a heatmap with hierarchical clustering analysis of these parameters was created (Figure 4A). Interestingly, TOL and CTRL completely separated based on these differences in circulating T-cells. Increased relative numbers of circulating aTh and elevated alloreactive responses of CD4 EMRA T-cells were the most discriminative characteristics of TOL compared to CTRL. TOL were clustered into two different groups, of which group I clearly displayed characteristics of overall hyporesponsiveness in CM and EM CD4+T-cells, whereas in group II more variability was present. This could indicate that LTx-recipients may develop spontaneous operational tolerance in more than one way. Group III and IV consisted of CTRL LTx-recipients, and differed in relative numbers of aTh, whereas the other markers were quite variable in both groups. Heatmap with hierarchical clustering analysis including alloreactive responses of healthy controls, but without the donor-reactive response, did not result in complete separate clustering of TOL and CTRL LTx-recipients, suggesting a contribution of donor-specific CD4+ T-cell subset responses (Supplementary Figure 7). Principal component analysis of all significantly different parameters between TOL and CTRL revealed two components that completely separated the two groups and in combination accounted for 70.6% of the variance (Figure 4B). In conclusion, the combination of alloreactive hyporesponsive and hyperresponsive subsets of CD4+T-cells and increased relative numbers of circulating aTh within CD4+T cells may be suited to discriminate TOL from a larger group of LTx-recipients.


A
CD4+ or
CD8+
T-cells
Negative Control (-)
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Negative Control (-)
Donor (D) or Third party (T) Stimulated
CD137
CD4+CD137+
CD8+CD137+
Gate %Gated
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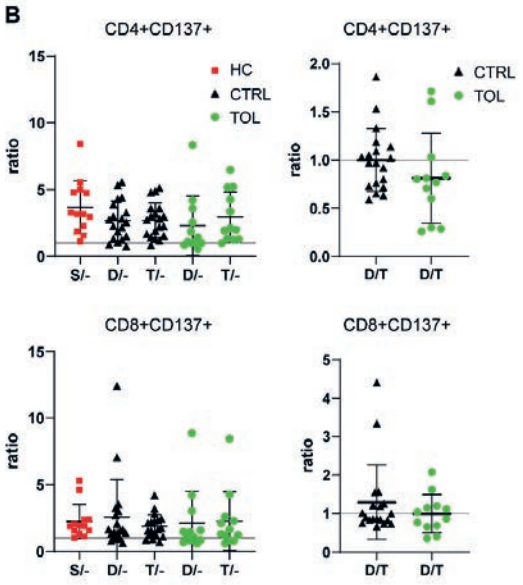
CD4+CD137+ 0,29
CD8+CD137+ 0,10
CD8+CD137+ 0,96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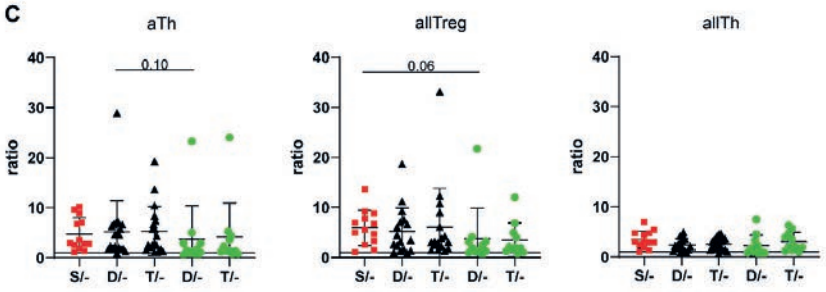
CD8+CD137+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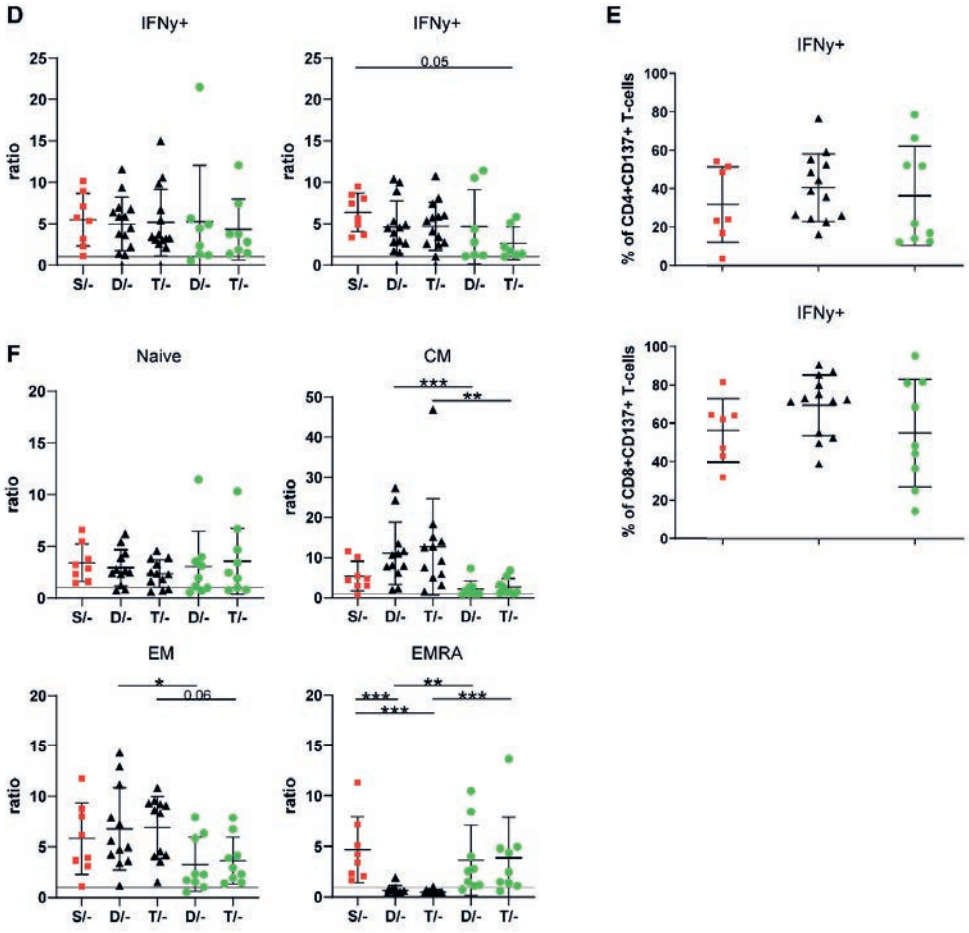
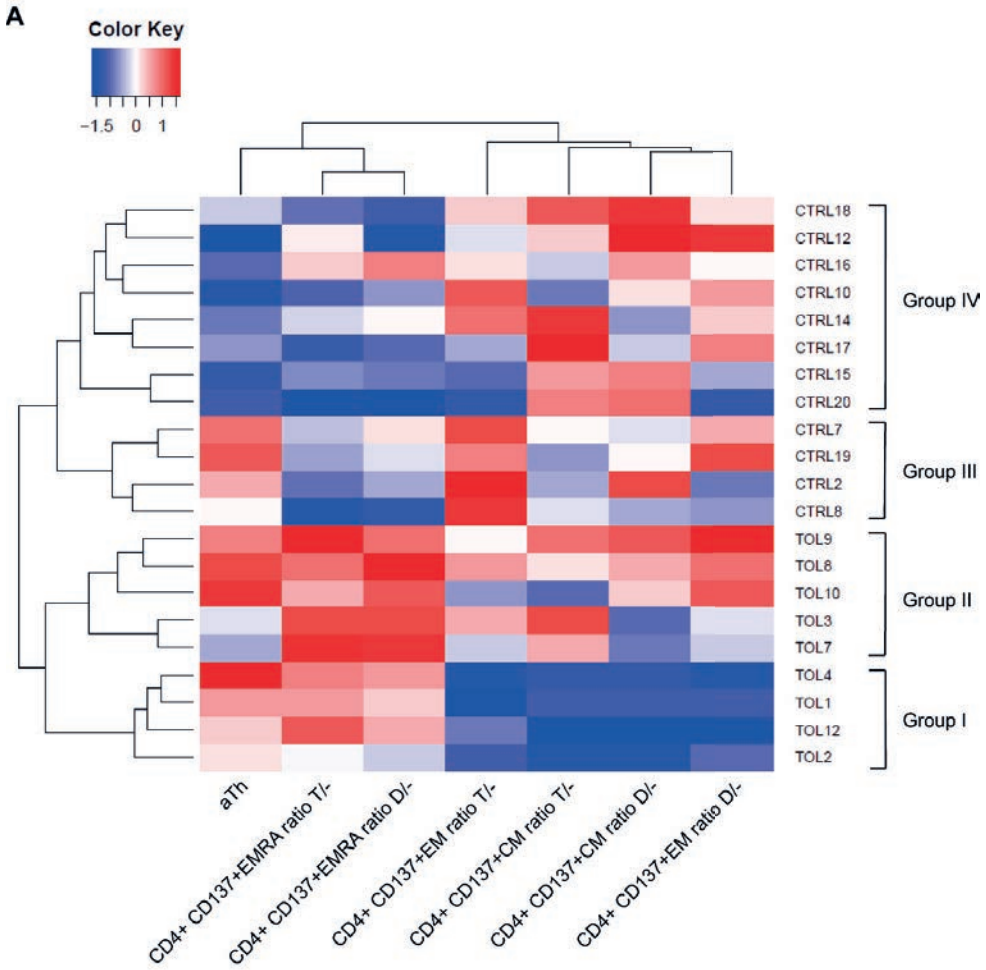


Figure 3 Alloreactive memory CD4+ T-cells are more differentiated in tolerant LTx-recipients. Representative dot plots indicating CD137 expression in CD4+ and CD8+ T-cells cultured in absence or presence of allogenic splenocytes presented in A. Ratios of CD137 expression in T-cells stimulated by allogeneic splenocytes S for HC, donor splenocytes D and third party splenocytes T against unstimulated T-cells - (S/-, D/- or T/-) and/or donor against third party (D/T) are presented in B-D and F. Ratios are presented for CD137 expressing CD4+ and CD8+ T-cells (B), CD137 expressing aTh, allTreg and allTh in CD4+ T-cells (C), CD137 expressing IFNy producing CD4+ (left) and CD8+ (right) T-cells (D) and CD137 expressing T-cell subsets naïve, CM, EM and EMRA in CD4+ T-cells (F). A solid line represents a ratio of 1. In E percentages of IFNy-positive cells in PMA/IONO stimulated CD4+ or CD8+ CD137+T-cells are presented. Panel B/C: HC n=13, CTRL n=19, TOL n=12; Panel D: HC n=8, CTRL n=13, TOL n=8; Panel F: HC n=8, CTRL n=12, TOL n=9. Statistical analyses were performed with one-way ANOVA, Kruskal-Wallis or Friedman and post-tests. *P < 0.05 **P < 0.01 ***P < 0.001. Abbreviations: aTh, activated T-helper cells; allTreg; all regulatory T-cells; CM, central memory T-cells; CTRL, control LTx-recipients; EM, effector memory T-cells; EMRA, terminally differentiated effector memory T-cells; HC; healthy control; IONO, ionomycin; LTx, liver transplantation; PMA, phorbol 12-myristate 13-acetate; TOL, tolerant LTx-recipients.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TOL3
TOL7
TOL4
TOL1
TOL12
TOL2
Group IV
Group III
Group II
Group I
aTh
CD4+ CD137+EMRA ratio T/-
CD4+ CD137+EMRA ratio D/-
CD4+ CD137+EM ratio T/-
CD4+ CD137+CM ratio T/-
CD4+ CD137+CM ratio D/-
CD4+ CD137+EM ratio D/-

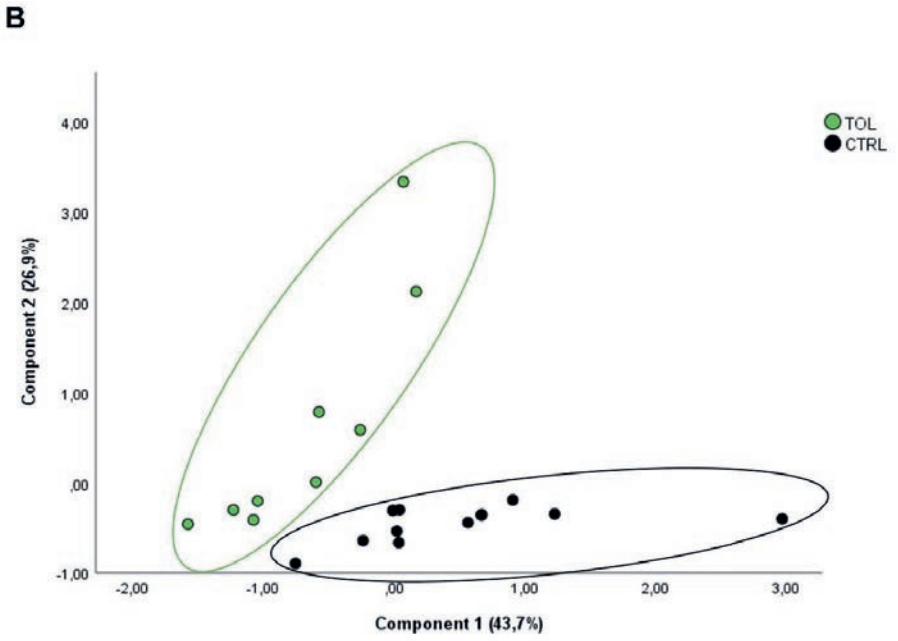


Figure 4 A tolerance profile discriminating tolerant LTx-recipients could be established. In A a heatmap with hierarchical clustering analysis is depicted of all LTx-recipients of whom all significantly different markers between TOL and CTRL were measured in this study. To avoid a selection bias, the LTx-recipients in which not all significantly different markers were measured were not included. Analysis was performed with the public Galaxy server Version 3.0.1 R gplots package with Euclidean distance method and Complete hierarchical clustering method. Data from each recipient was scaled with a Z-score according to total data of TOL and CTRL for that marker (Color key). In B principal component analysis of the significantly different markers between TOL and CTRL is depicted. Rotated component matrix analysis was performed using Varimax with Kaiser normalization. CTRL n=12, TOL n=9. Abbreviations: aTh, activated T-helper cells; CM, central memory T-cells; CTRL, control LTx-recipients; EM, effector memory T-cells; EMRA, terminally differentiated effector memory T-cells; TOL, tolerant LTx-recipients.

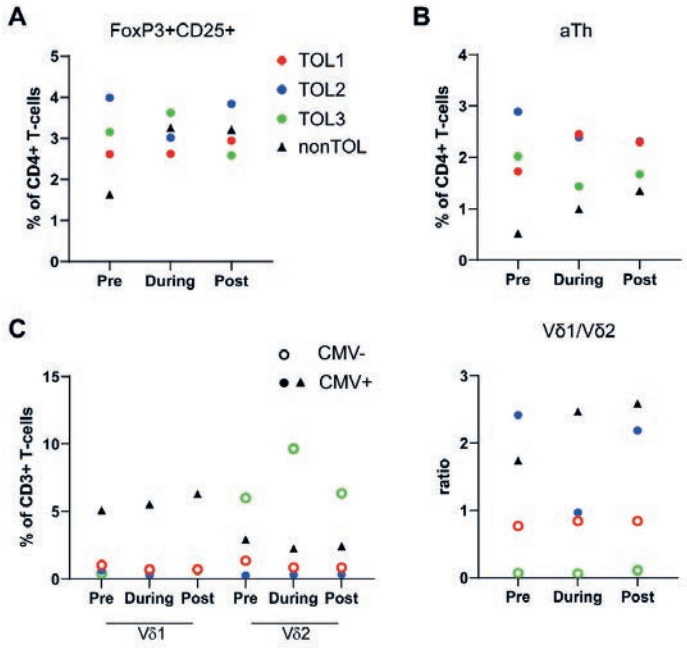
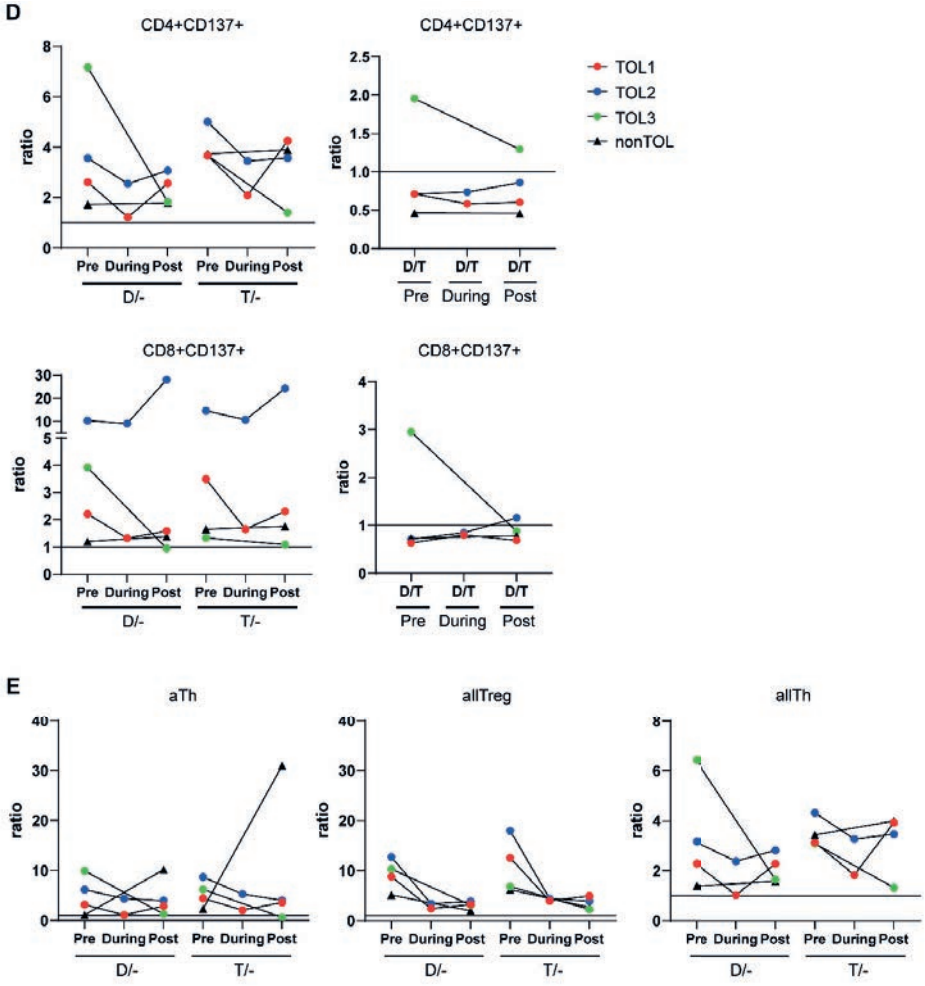


Figure 5 Findings in a small group of prospectively IS weaned group of LTx-recipients. Percentages of FoxP3+CD25+ in CD4+T-cells (A), aTh defined by FoxP3 and CD45RA expression (B) and percentages and ratios of Vδ1 and Vδ2 γδT-cells (C) pre, during and post IS weaning are presented. Ratios of T-cell responses against donor D and third party T splenocytes against unstimulated T-cells - (D/- or T/-) measured by flowcytometric determination of activation-induced CD137 are presented in D and E. Ratios are depicted for CD4+ and CD8+ CD137+T-cells (D), and CD137 expressing aTh, allTreg and allTh in CD4+T-cells (E). Abbreviations: aTh, activated T-helper cells; allTreg; all regulatory T-cells; CMV, cytomegalovirus; LTx; IS, immunosuppressive drugs; liver transplantation; TOL, tolerant LTx-recipients.


D
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CD4+CD137+
TOL1
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Pre During Post Pre During Post
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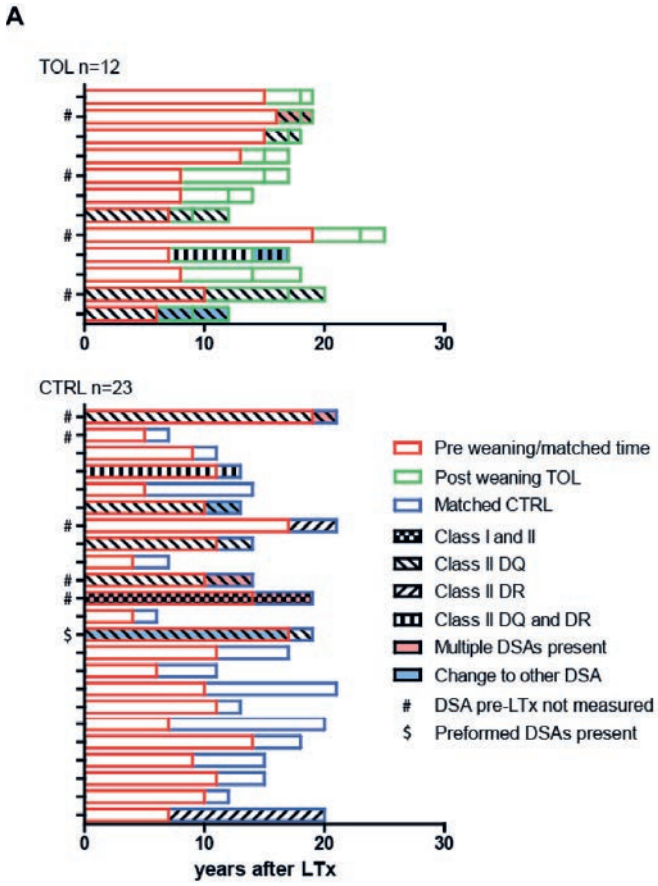
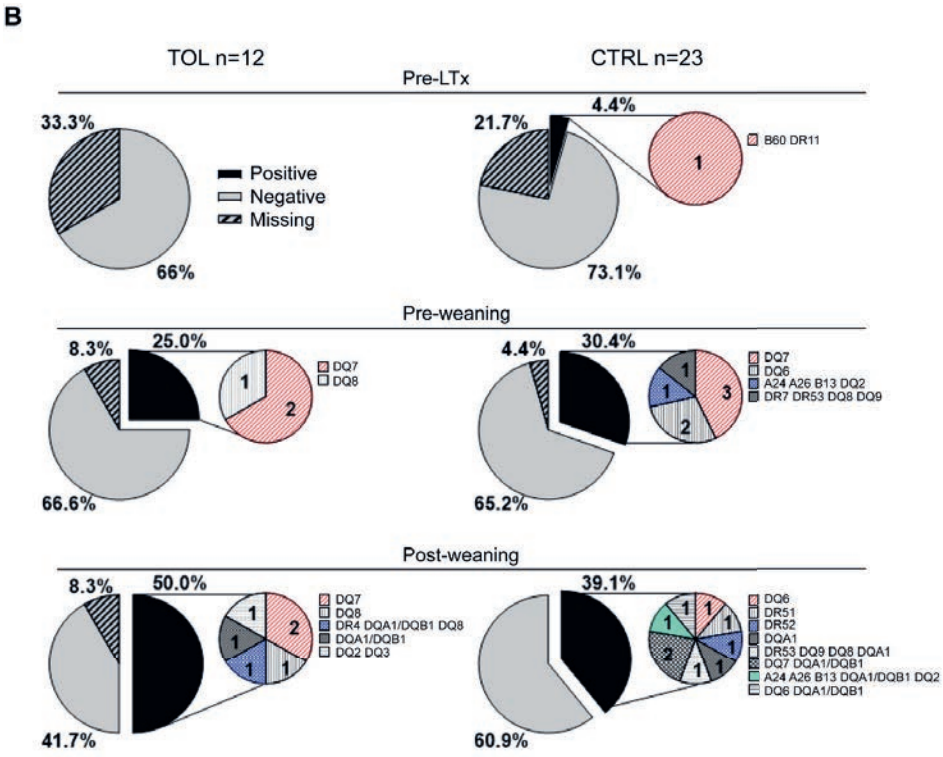


Figure 6 No significant differences are present in the development of DSAs in tolerant and control LTx-recipients. Development of DSA formation individually (A) and for the entire group with specific subtypes (B) in TOL pre, during and post IS weaning and in CTRL at matching time points are depicted. In C the cumulative MFI for each DSA+ LTx recipient is presented for TOL and CTRL. Statistical analyses were performed with two-sided Fisher's Exact Test or Pearson Chi-Square test. Pre vs post weaning: *a* = 0.06, *b,c* = 0.13. Abbreviations: CTRL, control LTx-recipients; DSAs, donor-specific antibodies; LTx; liver transplantation; MFI, mean fluorescence intensity; TOL, tolerant LTx-recipients.


B
TOL n=12
CTRL n=23
Pre-LTx
33.3%
66%
Positive
Negative
Missing
21.7%
4.4%
73.1%
B60 DR11
Pre-weaning
8.3%
25.0%
66.6%
DQ7
DQ8
4.4%
30.4%
65.2%
DQ7
DQ6
A24 A26 B13 DQ2
DR7 DR53 DQ8 DQ9
Post-weaning
8.3%
50.0%
41.7%
DQ7
DQ8
DR4 DQA1/DQB1 DQ8
DQA1/DQB1
DQ2 DQ3
39.1%
60.9%
DQ6
DR51
DR52
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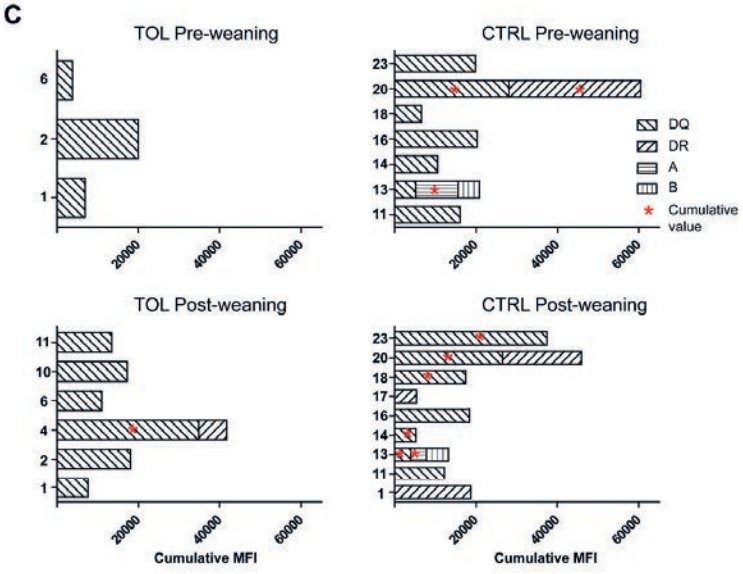
C
TOL Pre-weaning
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DQ
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CTRL Post-weaning
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40000
60000
Cumulative MFI

Findings in TOL and nonTOL before and during IS weaning

To investigate whether the immunological characteristics of TOL could be observed during IS weaning, we had the opportunity to collect blood from 4 LTx-recipients pre, during and post IS weaning. Three of these recipients appeared to be tolerant (Supplementary Table 1). The fourth recipient experienced highly elevated liver function values four months after IS cessation when blood was withdrawn, and was considered nonTOL. After regular IS regimen re-installment, liver graft function values normalized. Before IS weaning, circulating aTh were already enhanced in TOL compared to nonTOL, and this difference remained during the course of complete IS weaning (Figure 5A,B). For aTreg and rTreg no clear differences were observed between TOL and nonTOL in the course of IS weaning (Supplementary Figure 5D). These preliminary data suggest that elevations of aTh in TOL may already occur before IS weaning. We again observed that CMV-seropositive LTx-recipients, regardless of their TOL or nonTOL status, have a higher Vδ1/Vδ2 γδT-cell ratio compared to CMV-seronegative LTx-recipients (Figure 5C). In addition, similar to the data presented in Figure 3, no clear differences were observed in CD4+ or CD8+ CD137+ T-cell responses following donor and third party stimulation between TOL and nonTOL before or during weaning (Figure 5D, E). Unfortunately, due to shortage of samples CD4+ or CD8+ CD137+ alloreactive T-cell responses in different differentiation statuses could not be analyzed.

No significant differences in the development of DSAs between tolerant and control LTx-recipients

DSAs were measured in TOL pre-LTx, pre-weaning and post-weaning and for CTRL at matching time-points. Most of the DSAs that developed were de novo DSAs (Figure 6A). Only one CTRL had pre-formed DSAs (B60; DR11), but these completely disappeared after LTx. Just before complete IS weaning 25.0% of TOL had DSAs, and 30.4% of CTRL had one or more DSAs at matched time points (Figure 6B), indicating that TOL cannot be distinguished on basis of DSAs. Two out of five DSA+ CTRL developed more than one DSA, whereas within TOL none developed more than one DSA pre-weaning. Despite their operationally tolerant state, the number of DSA+ TOL doubled after IS weaning, whereas it only increased moderately in CTRL at matched time points, although this difference is not statistically significant. For both groups about half of the DSA+ individuals developed more than one DSA post weaning. Most of the de novo DSAs were against HLA Class II DR or DQ. Only one LTx recipient in the CTRL group developed DSAs against HLA Class I (Figure 6B). A shift to another DSA across time occurred within both groups (Figure 6A). No clear differences in mean cumulative fluorescence intensity (MFI) of DSAs were observed among groups pre and post weaning (Figure 6C). These data demonstrate that tolerance develops and is maintained despite development of DSAs.

Discussion

In this study we characterized circulating T-cells subsets that could play a role in the development or identify operationally tolerant LTx-recipients. We found that in TOL CM and EM CD4+T-cells displayed hyporesponsiveness, whereas EMRA CD4+T-cells displayed hyperresponsiveness against donor and third party stimulation, compared to CTRL. In addition, TOL exhibited an elevated proportion of circulating aTh compared to CTRL. Clustering analysis and principal component analysis revealed that the combination of these CD4+T-cell characteristics accurately discriminated TOL from CTRL. In contrast, no significant differences in alloreactive CD8+ T-cells or DSA formation were observed between TOL and CTRL.

As confirmed by other studies,[87,92] a significant higher proportion of circulating CD4+CD25+FoxP3+ T-cells was found in TOL compared to CTRL. However, upon further delineation of these cells it was found that circulating aTh were elevated in TOL compared to CTRL and HC. Furthermore, in a small cohort we found that these CD4+T-cells were already elevated pre weaning in TOL. The aTh subset of healthy and diseased individuals are cytokine-secreting nonsuppressive T-cells that transiently express FoxP3.[7] A robust FoxP3 expression requires DNA demethylation of the FOXP3 gene, as is found for conventional Tregs generated in the thymus. Induced Tregs are generated by specific antigen stimulation in combination with IL-2 and transforming growth factor β, and have an unstable FoxP3 expression.[160] Unfortunately, we cannot rule out that the elevated aTh subset in TOL are actually induced Tregs. The second novel finding of our study is that allogenic hyporesponsiveness was observed in CM and EM CD4+T-cells, whereas a allogenic hyperresponsiveness was found in the EMRA compartment of TOL. CD4+ CM T-cells exhibit a high proliferative capacity and poor effector function, whereas EM T-cells exhibit an immediate effector function and only a limited proliferative capacity.[161] Many studies hold Tregs responsible for induction and maintenance of immune tolerance,[162] and are investigating the therapeutic potential of Treg therapies in tolerance induction. Surprisingly, our data suggests that specific T-helper subsets might be associated with natural occurring tolerance. It could be that in TOL alloreactive CM and EM CD4+T-cells are either deleted, anergic, senescent or inhibited by Tregs.[163] Unfortunately this interaction between T-helper cells and Tregs could not be further investigated as this requires large numbers of cells. Until now data on CD4+ EMRA T-cells is sparse, nonetheless it has been suggested that these T-cells resemble CD8+ EMRA T-cells. They exhibit cytotoxic potential and can secrete multiple cytokines after activation, but have poor proliferative capacity, and are expanded during chronic viral infections.[164,165] Highly differentiated CD4+T-cells were also associated with low proliferative alloreactivity in kidney transplantation.[95] Previous studies have reported a proliferative donor-specific hyporesponsiveness within the total population of CD4+T-cells of tolerant LTx-recipients.[92,93] Our data suggests that this may be due to enrichment of alloreactive CD4+T-cells of TOL with EMRA, that exhibit poor proliferative responsiveness to alloantigens, explaining their involvement in operational tolerance. However, additional studies have to be performed to investigate the functionality of these alloreactive CD4+ EMRA T-cells.

Several studies reported that a higher Vδ1/Vδ2 γδT-cell ratio could discriminate tolerant from other LTx-recipients, without reporting the CMV-serostatus. CMV-infection influences the composition of immune cell subsets,[158,159] hence our groups were matched for the CMV-serostatus. Indeed, our results indicate that a positive CMV-serostatus is associated with an increased Vδ1/Vδ2 γδT-cell ratio due to an increase in circulating Vδ1 in LTx-recipients, regardless of a state of tolerance. This confirms previous studies that indicated that CMV-seropositivity is associated with an increased Vδ1/Vδ2 γδT-cell ratio in LTx-recipients.[166] These data also highlights the importance of matching groups on parameters that could influence the markers of interest.

In this study no significant differences in DSA formation over time, against HLA Class type I or II, or MFI of DSAs was observed between TOL and CTRL, which is confirmed by other studies.[49,62,157] DSAs against donor HLA have been associated with an increased risk of acute and chronic rejection[99] early after LTx. Our recipients were included on average 15 years after LTx, hence LTx-recipients with complications due to DSAs were possibly lost. An increase in DSA formation after complete IS weaning in TOL was observed, but this did not lead to clinical complications. This could be explained by formation of certain less harmful IgG subtypes, their potential weaker complement binding, and possibly a lower HLA antigen density in the liver[167] in TOL. These aspects have to be investigated in the future.

The strength of our study is that we are the first to investigate phenotypic as well as functional features of alloreactive T-cells in tolerant LTx-recipients in detail using CD137 as a surrogate marker for the total alloreactive T-cell compartment. Furthermore, we are the first to delineate further the elevated proportion of CD4+CD25+FoxP3+ T-cells present in TOL. We performed this study with completely matched groups for important clinical parameters, and thereby eliminated potential confounders. Our study has also limitations. Similar to other recent studies,[52,54] in our definition of operational tolerance, liver function tests were used instead of a protocol liver biopsy, due to concerns about possible complications. Subclinical rejection may have been undetected in this way, but in our study biopsies were taken from five TOL at some point after complete IS weaning and every type of rejection was excluded. We admit that CTRL LTx population could represent a mixed population of tolerant and nontolerant recipients, although, based on available literature[49,52,54], we expect that the majority of CTRL recipients is nontolerant. In addition, despite the possibility of a mixed CTRL population, we observed statistically significant differences in relative numbers of circulating aTh and in several alloreactive T-helper subsets between TOL and CTRL. We compared a TOL and CTRL group that differ in IS usage. Therefore, we included a group of healthy controls without an IS regimen to compare to TOL. The significant difference of circulating aTh in HC versus TOL, but not CTRL, indicates that the influence of IS is limited on the development on this subset of T-cells. Moreover, in a small group of LTx-recipients we found preliminary evidence that aTh were already increased in TOL before IS weaning. If IS usage would have inhibited alloreactivity of T-cells, we would have expected overall T-cell hyporesponsiveness in CTRL versus TOL. Instead we observed hyporesponsiveness of alloreactive CM and EM CD4+T-cells against both donor and third party splenocytes in TOL versus CTRL. T-cell responses against third party alloantigen in transplanted recipients can be compared to T-cell responses against HLA-mismatched alloantigen in HC. Notably, CM and EM CD4+ T-cell

responses against HLA-mismatched alloantigens in HC were not reduced compared to CTRL, suggesting that the observed hyporesponsiveness of CD4+T-cell subsets in TOL versus CTRL cannot be explained by absence of IS in TOL. In line with this one could argue that the actual difference in functional activity between CTRL and TOL would even have been larger than is observed now if CTRL would not have used IS regimen. We could not investigate unresponsive alloreactive T-cells or T-cell responses against indirectly presented alloantigens, since no reliable techniques are available. Finally, our prospective IS weaning cohort (n=4) was too small to draw a robust conclusion on whether enhanced numbers of aTh were discriminative of TOL before IS weaning.

In this study we identified enhanced frequencies of aTh and highly differentiated alloreactive CD4+T-cells in blood as new markers associated with of operational tolerance after LTx. Validating whether these T-cell markers can be used to discriminate TOL from nonTOL LTx-recipients on IS regimen requires a prospective study with a larger independent IS weaning cohort. Additional studies have to be performed to investigate the functionality of alloreactive CD4+ CM, EM and EMRA T-cells and their involvement in spontaneous operational tolerance.

Acknowledgements

We would like to express our gratitude to the liver transplant nurses of the Department of Gastroenterology and Hepatology at the Erasmus University Medical Centre for their efforts to successfully collect all heparinized blood samples. We also would like to thank the Department of Viroscience at the Erasmus University Medical Centre for making archived serum samples of LTx-recipients available and for determination of CMV IgG serostatus.

Supplementary Material

Patients and Methods

Antibody staining and flowcytometry

After thawing or overnight co-culturing, PBMCs were stained with fluorochrome-conjugated antibodies (Supplementary Table 2). Briefly, cells were washed and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) for 20 minutes at 4°C. When appropriate, cells were stained with fluorochrome-conjugated CCR7 antibody for 1h at RT. Subsequently, cell surface staining was performed for 20 minutes at 4°C. Thereafter, cells were permeabilized using the Foxp3 Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol, and subsequently stained for intracellular markers, including CD137, for 20 minutes at 4°C. All samples were measured using BD FACS Canto II (BD Biosciences) or Navios (Beckman Coulter, Pasadena, USA) flow cytometer and analysed with Kaluza Analysis Software version 2.1 (Beckman Coulter). Appropriate isotype and fluorescence minus one (FMO) controls were used for gating purposes.

Donor-specific HLA class I and class II antibodies

Serum samples from TOL pre-LTx, pre-weaning and post-weaning and samples from CTRL pre-LTx and at matching time points with TOL, were retrospectively derived from serum samples initially collected for diagnostic purposes (MEC 2018-1597). All samples were screened for HLA Class I (HLA-A or HLA-B) or HLA Class II (HLA-DQ or HLA-DR) DSAs using the Lifecodes Lifescreen Deluxe (LMX) kit according to the manufacturer's protocol (Immucor Transplant Diagnostics Inc.). Samples that tested positive were measured with a Luminex single antigen assay using HLA class I and class II antigen beads (LABscreen One Lambda, Canoga Park, CA, USA). A brief protocol is described elsewhere.[168] Antibodies measured with a MFI of >1000 were considered positive.

Tables and Figures

Supplementary Table 1 Characteristics of prospectively weaned LTx recipients.

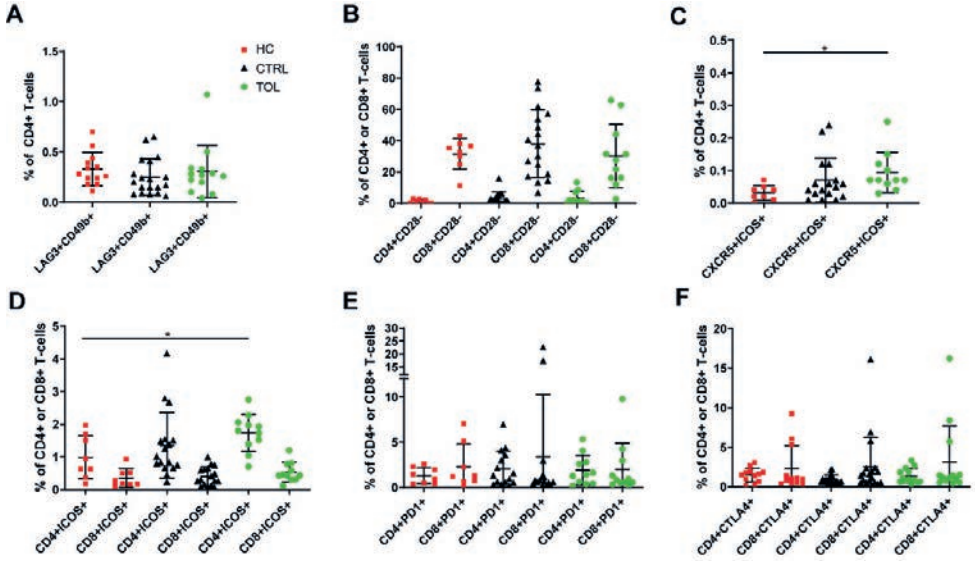
Demographics	TOL1	TOL2	TOL3	nonTOL
Male (%)	Male	Male	Female	Male
Age in years	28	67	33	71
Years after LTx	27	14	31	17
Years LTx - complete weaning	20	11	30	16
Years complete weaning - end follow-up	7	3	1	1
Primary disease	Cholestatic disease	Hepatocellular carcinoma	Cholestatic disease	Virus-related
CMV serostatus pre-LTx	Negative	Positive	Negative	Negative
CMV serostatus end follow-up	Negative	Positive	Negative	Positive
CMV serostatus Donor	Negative	Positive	Negative	Positive
IS last used	Aza	MMF	Aza	Tac → Tac
IS trough levels weaning	Unknown	Pre 1.9; During 1.1; Post <1.0 mg/L	Unknown	Pre 3.5; During 1.9; Post <1.0; Current 2.8 µg/L
Liver function values rejection	NA	NA	NA	Bili: 1.5x; γGt: 4x; AP: 1.5x elevated
HLA mismatches recipient/donor A+B	4	4	4	3
HLA mismatches recipient/donor DR+DQ	2	3	3	2

AP, alkaline phosphatase; Aza, azathioprine; Bili, bilirubin; CMV, cytomegalovirus; γGt, γ-glutamyltransferase; HLA, human leukocyte antigen; IS, immunosuppressive drugs; LTx, liver transplantation; MMF, mycophenolate mofetil; NA, not applicable; Tac, tacrolimus; TOL, tolerant group.

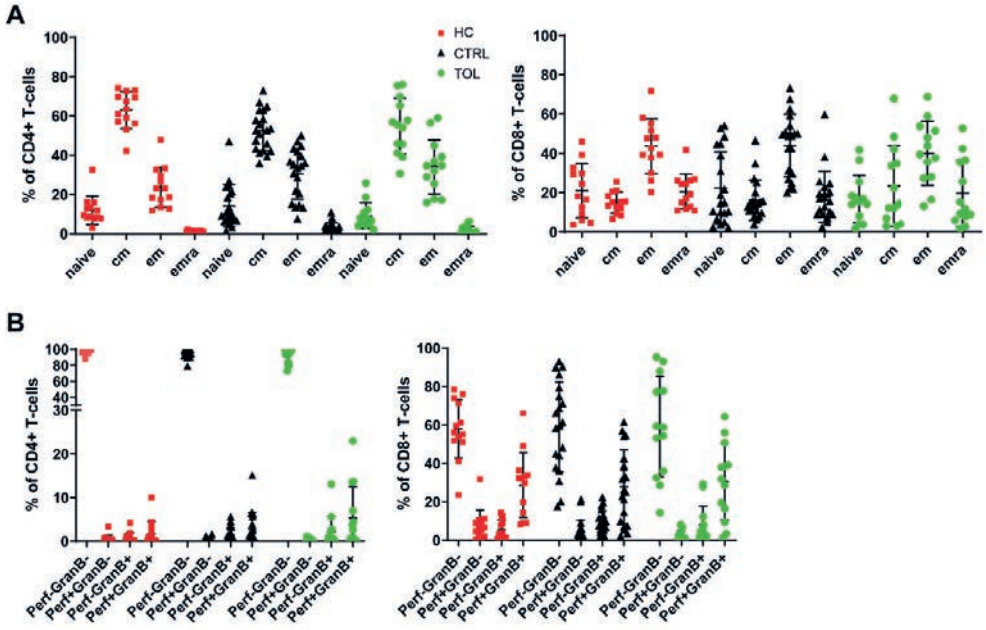
Supplementary Table 2 Fluorochrome-conjugated antibodies.

Antibody	Clone	Supplier
CD3 PE-CF594	UCHT1	BD
CD3 APC-eFluor780	SK7	eBioscience
CD4 AlexaFluor 700	13B8.2	Beckman Coulter
CD4 APC-eFluor780	OKT4	eBioscience
CD8 PerCP-Cy5.5	RPA-T8	eBioscience
CD8 FITC	SK1	eBioscience
CCR7 FITC	150503	R&D systems
CCR7 APC	150503	R&D systems
CD45RA APC-H7	5H9	BD
CD45RA FITC	HI100	eBioscience
CD25 PE-Cy7	BC96	eBioscience
CD49b APC	P1E6-C5	Biolegend
LAG3 PE	3DS223H	eBioscience
FoxP3 eFluor450	236A/E7	eBioscience
FoxP3 APC	236A/E7	eBioscience
CD137 BV-421	4B4-1	BD
mIgG1-BV421	X40	BD
Perforin APC-Cy7	dG9	Biolegend
Granzyme B Horizon V450	GB11	BD
Vδ1 APC	REA173	Miltenyi Biotec
Vδ2 PE	B6	BD
CXCR5 PerCP-Cy5.5	RF8B2	BD
ICOS PE-Cy7	ISA-3	eBioscience
PD1 APC	J105	eBioscience
CD28 PE	L293	BD
IFNγ PE	4S.B3	BD
mIgG1-PE	P3.6.2.8.1	eBioscience
IFNγ BV421	4S.B3	Biolegend
IL17A PECy7	BL168	Biolegend

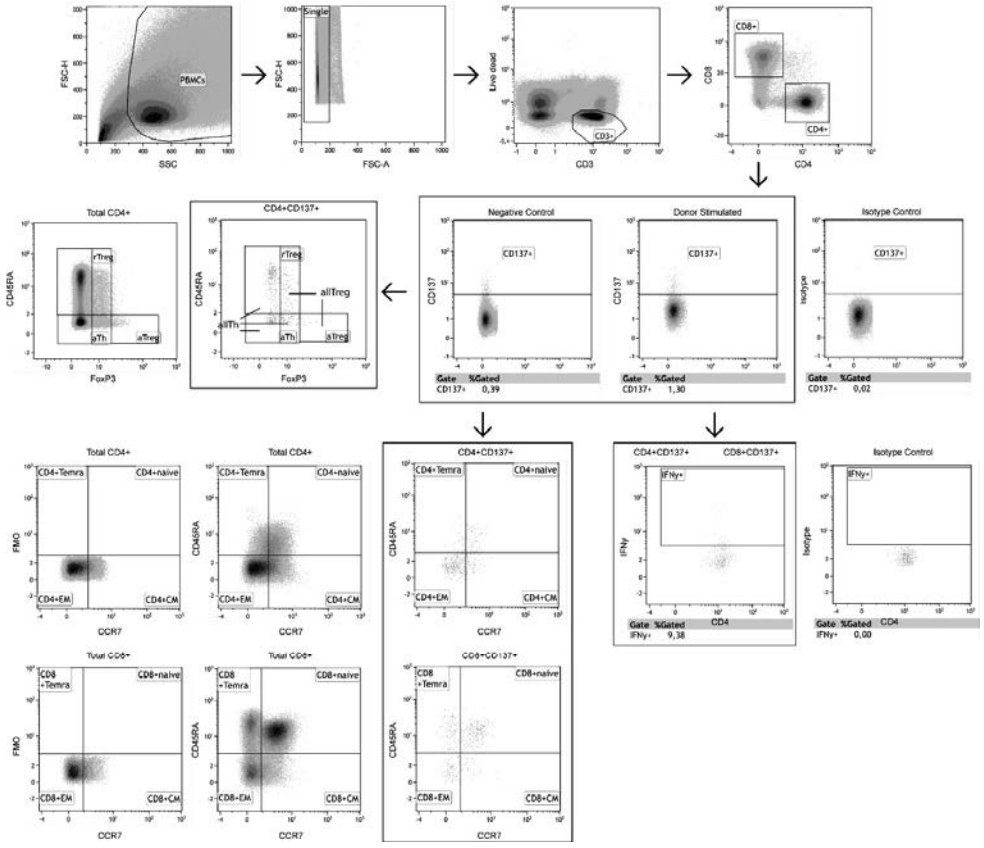
BD Biosciences, Erembodegem, Belgium; Beckman Coulter, Woerden, The Netherlands; eBioscience, Thermo Fisher Scientific, Waltham, USA; Miltenyi Biotec, Bergisch Gladbach, Germany; R&D systems, Minneapolis, USA.



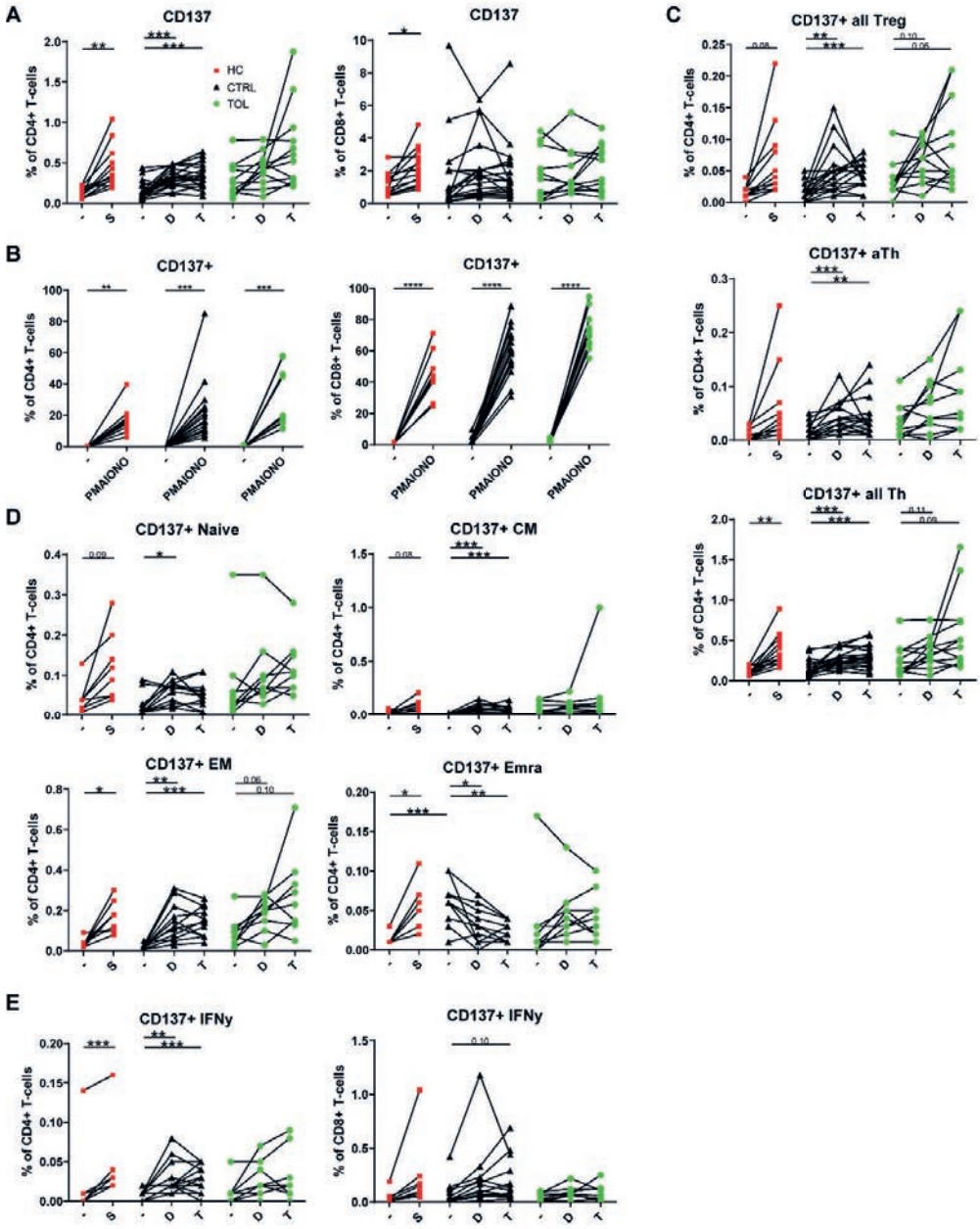
Supplementary Figure 1 Circulating Tr1, CD4+ or CD8+ CD28- T-cells, Tfh and CD4+ or CD8+ ICOS+, PD1+ or CTLA4+ T-cells. Percentages of Tr1 (A), CD4+ or CD8+ CD28- T-cells (B), Tfh (C) and CD4+ or CD8+ ICOS+ (D), PD1+ (E) or CTLA4+ (F) T-cells are presented. Statistical analyses were performed with one-way ANOVA or Kruskal-Wallis and post-tests. *P < 0.05 Abbreviations: CTRL, control LTx recipients; Tfh, follicular helper T-cells; Tr1, Type 1 regulatory T-cells; HC, healthy control; rTreg, resting regulatory T-cells; TOL, tolerant LTx recipients.



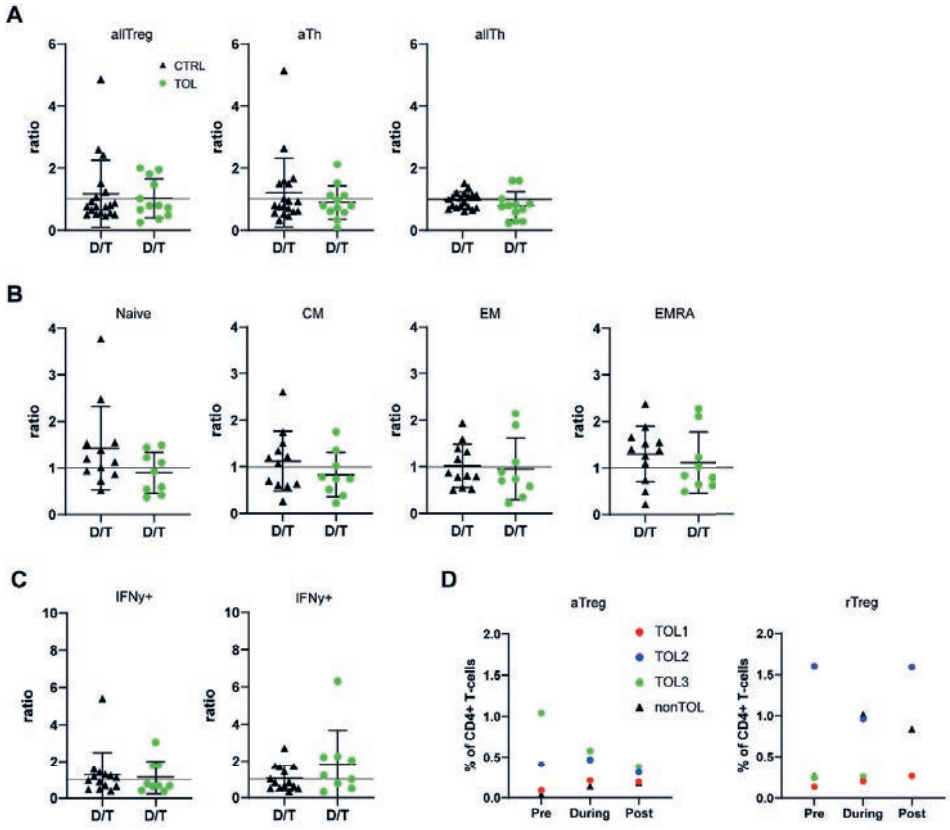
Supplementary Figure 2 Circulating CD4+ and CD8+ subsets, and perforin and granzyme B expressing T-cells. Percentages of subsets naïve, CM, EM and EMRA in CD4+ or CD8+ T-cells (A) and perforin and granzyme B expressing CD4+ or CD8+ T-cells (B) are presented. Statistical analyses were performed with one-way ANOVA or Kruskal-Wallis and post-tests. Abbreviations: aTh, activated T-helper cells; aTreg; activated regulatory T-cells; CM, central memory T-cells; CTRL, control LTx recipients; EM, effector memory T-cells; EMRA, terminally differentiated effector memory T-cells; HC, healthy control; rTreg, resting regulatory T-cells; TOL, tolerant LTx recipients.



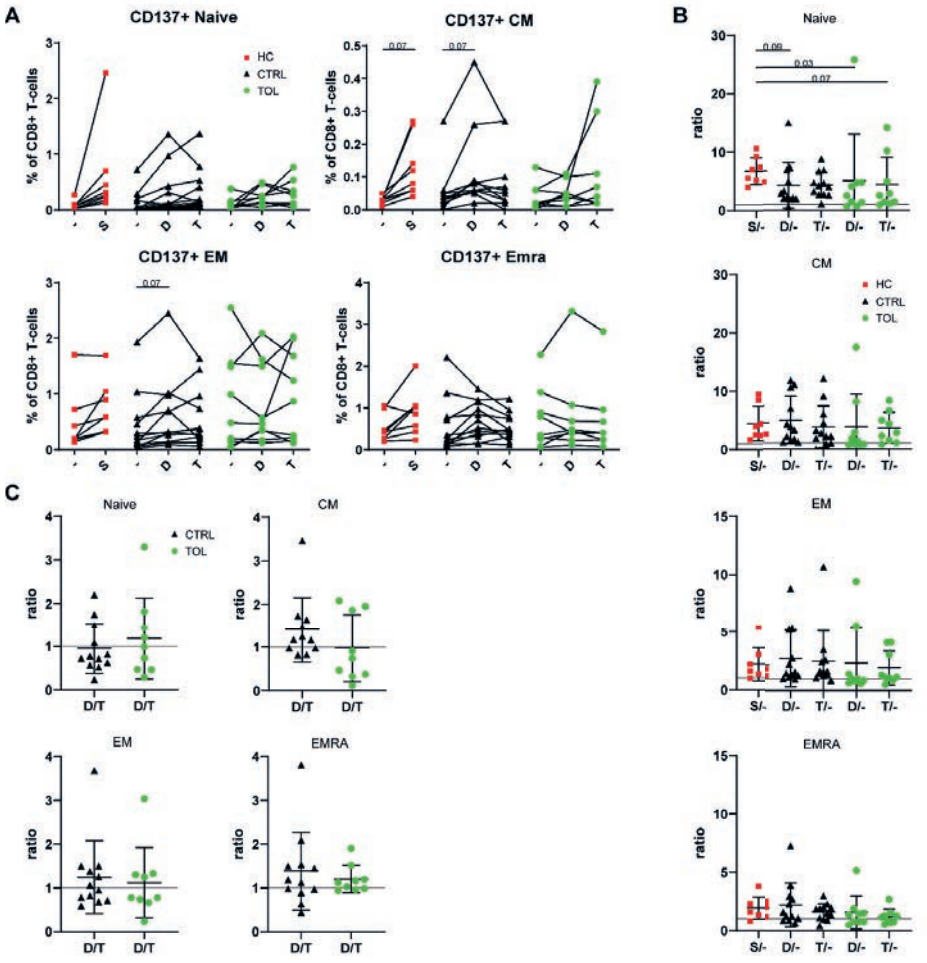
Supplementary Figure 3 Representative dot plots indicating the gating strategy of CD137 expressing alloreactive T-cells. Abbreviations: aTh, activated T-helper cells; allTreg; all regulatory T-cells; aTreg, activated regulatory T-cells; CM, central memory T-cells; EM, effector memory T-cells; EMRA, terminally differentiated effector memory T-cells.


A
CD137
% of CD4+ T-cells
HC
CTRL
TOL
CD137
% of CD8+ T-cells
B
CD137+
% of CD4+ T-cells
PMA/IONO
CD137+
% of CD8+ T-cells
C
CD137+ all Treg
% of CD4+ T-cells
CD137+ aTh
% of CD4+ T-cells
CD137+ all Th
% of CD4+ T-cells
D
CD137+ Naive
% of CD4+ T-cells
CD137+ CM
% of CD4+ T-cells
CD137+ EM
% of CD4+ T-cells
CD137+ Emra
% of CD4+ T-cells
E
CD137+ IFNy
% of CD4+ T-cells
CD137+ IFNy
% of CD8+ T-cells

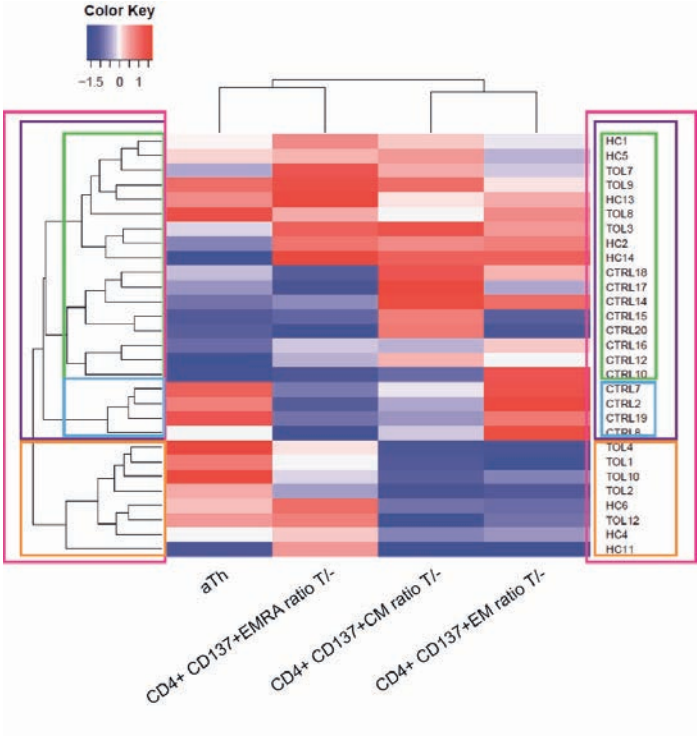
Supplementary Figure 4 Percentages of alloreactive CD4+ and CD8+ T-cells. Percentages of CD137 expression in T-cells stimulated by allogeneic splenocytes S for HC, donor splenocytes D and third party splenocytes T and unstimulated T-cells - are presented in A,C-E. Percentages are presented for CD137 expressing CD4+ and CD8+ T-cells (A), CD137 expressing aTh, allTreg and allTh in CD4+ T-cells (C), CD137 expressing T-cell subsets naïve, CM, EM and EMRA in CD4+ T-cells (D), and CD137 expressing IFNγ producing CD4+ (left) and CD8+ (right) T-cells (E). In B percentages are presented for CD137 expressing CD4+ and CD8+ T-cells stimulated with PMA/IONO. Statistical analyses were performed with one-way ANOVA, Kruskal-Wallis or Friedman and post-tests. *P < 0.05 **P < 0.01 ***P < 0.001. Abbreviations: aTh, activated T-helper cells; allTreg; all regulatory T-cells; CM, central memory T-cells; CTRL, control LTx recipients; EM, effector memory T-cells; EMRA, terminally differentiated effector memory T-cells; HC; healthy control; IONO, ionomycin; PMA, phorbol 12-myristate 13-acetate; TOL, tolerant LTx recipients.



Supplementary Figure 5 Ratios of CD137 expressing CD4+ alloreactive T-cell subsets and percentages of Tregs. Ratios (D/T) of CD137 expressing aTh, allTreg and allTh (A), subsets naïve, CM, EM and EMRA (B) and IFNy producing (C) CD4+ T-cells stimulated by allogeneic splenocytes S for HC, donor splenocytes D and third party splenocytes T against unstimulated T-cells -. Statistical analyses were performed with t-test or Mann-Whitney U test. Percentages of aTreg and rTreg defined by FoxP3 and CD45RA expression (D) pre, during and post IS weaning are presented. Abbreviations: aTh, activated T-helper cells; aTreg; activated regulatory T-cells; CM, central memory T-cells; CTRL, control LTx recipients; EM, effector memory T-cells; EMRA, terminally differentiated effector memory T-cells; rTreg, resting regulatory T-cells; TOL, tolerant LTx recipients.



Supplementary Figure 6 Percentages and ratios of alloreactive subsets of CD8+ T-cells. Percentages (A) or ratios (B,C) of CD137 expression in CD8+ T-cell subsets naïve, CM, EM and EMRA stimulated by allogeneic splenocytes S for HC, donor splenocytes D and third party splenocytes T against unstimulated T-cells - (S/-, D/- or T/-; D/T) are presented. Statistical analyses were performed with one-way ANOVA, Kruskal-Wallis or Friedman and post-tests, or t-test or Mann-Whitney U test. Abbreviations: CM, central memory T-cells; CTRL, control LTx recipients; EM, effector memory T-cells; EMRA, terminally differentiated effector memory T-cells; HC; healthy control; TOL, tolerant LTx recipients.



Supplementary Figure 7 A heatmap with hierarchical clustering analysis is depicted of healthy controls, tolerant and control LTx recipients. Circulating aTh and third party (T/-) CD4+ T-cell subset responses are depicted. Colored boxes indicate the groups that clustered together or separately. To avoid a selection bias, the LTx-recipients in which not all significantly different markers were measured were not included. Analysis was performed with the public Galaxy server Version 3.0.1 R gplots package with Euclidean distance method and Complete hierarchical clustering method. Data from each subject was scaled with a Z-score according to total data of TOL, CTRL and HC for that marker (Color key). Abbreviations: aTh, activated T-helper cells; CM, central memory T-cells; CTRL, control LTx-recipients; EM, effector memory T-cells; EMRA, terminally differentiated effector memory T-cells; HC, healthy controls; TOL, tolerant LTx-recipients.

CHAPTER 7.

Current tolerance-associated peripheral blood gene expression profiles after liver transplantation are influenced by immunosuppressive drugs and prior cytomegalovirus infection

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Abstract

Background:
Spontaneous operational tolerance to the allograft develops in a proportion of liver transplant (LTx) recipients weaned off immunosuppressive drugs (IS). Several previous studies have investigated whether peripheral blood gene expression profiles could identify operational tolerance in LTx recipients. However, the reported gene expression profiles differed greatly amongst studies, which could be caused by inadequate matching of clinical parameters of study groups. Therefore, the purpose of this study was to validate differentially expressed immune system related genes described in previous studies that identified tolerant LTx recipients after IS weaning.

Methods:
Blood was collected of tolerant LTx recipients (TOL), a control group of LTx recipients with regular IS regimen (CTRL), a group of LTx recipients with minimal IS regimen (MIN) and healthy controls (HC), and groups were matched on age, sex, primary disease, time after LTx, and cytomegalovirus serostatus after LTx. Quantitative Polymerase Chain Reaction was used to determine expression of twenty selected genes and transcript variants in PBMCs.

Results:
Several genes were differentially expressed between TOL and CTRL groups, but none of the selected genes were differentially expressed between HC and TOL. Principal component analysis revealed an IS drug dosage effect on the expression profile of these genes. These data suggest that use of IS profoundly affects gene expression in peripheral blood, and that these genes are not associated with operational tolerance. In addition, expression levels of SLAMF7 and NKG7 were affected by prior cytomegalovirus infection in LTx recipients.

Conclusion:
We found confounding effects of IS regimen and prior CMV infection, on peripheral blood expression of several selected genes that were described as tolerance-associated genes by previous studies.

Introduction

For end-stage liver disease a liver transplantation (LTx) is the sole treatment option. Since long-term use of immunosuppressive drugs (IS) could lead to several serious side effects and adversely impacts quality of life after transplantation, most transplantation centers attempt to gradually reduce or even completely wean IS over time.[14,17,53,155] Several clinical trials have shown that some LTx recipients can develop operational tolerance towards their graft, a long-term state where (acute) rejection episodes are absent after IS are fully weaned.[49,52,54]

In the last fifteen years considerable efforts have been made to identify noninvasive biomarkers of operational tolerance in LTx. Several studies have investigated whether tolerant LTx recipients could be discriminated from a control group or a non-tolerant group of LTx recipients with regular IS regimen by examining gene expression in circulating peripheral blood mononuclear cells (PBMCs).[80,82,84,87,130,169] Herein it was suggested that certain gene profiles related to the general immune system, natural killer (NK) cells, γδT-cells and regulatory T-cells (Tregs) could identify tolerant LTx recipients. Strikingly however, these gene profiles differed greatly amongst these studies. Several reasons may account for these differences. Firstly, in all studies, except the study of Bohne et al.[80], gene expression profiles of tolerant LTx recipients without IS regimen were compared to control or non-tolerant LTx recipients with IS regimen.[82,84,87,130,169] Therefore, gene expression profiles in the control or non-tolerant LTx recipients may have been affected by IS. Furthermore, thorough matching of parameters known to influence immune cell composition and gene expression, such as age, sex, IS usage, (viral) primary disease and prior cytomegalovirus (CMV) infection, between study groups was not performed. CMV infection constitutionally inflates memory(like) peripheral T-cell and NK cell compartments and circulating γδT-cells.[158,159] In addition, in kidney and liver LTx recipients with regular IS regimen a durable change in the circulating immune cell composition was observed after CMV infection.[166,170-174] Moreover, afore mentioned studies have used microarray and/or polymerase chain reaction (PCR) to study gene expression, but it is unclear which splice variants of the studied genes have been detected.

Therefore, the purpose of this study was to validate previously reported transcriptional profiles of immune system related genes in peripheral blood of tolerant LTx recipients. Validation was performed by comparing peripheral blood gene expression profiles of tolerant LTx recipients without IS, a control group of LTx recipients with regular IS regimen, a group of LTx recipients with minimal IS regimen to reveal possible effects of IS, and healthy controls. These groups were matched for important parameters known to influence immune cell composition and their gene expression in peripheral blood.

Patients and Methods

Study design and participants

In this study blood samples were collected from three different groups of adult LTx recipients late after LTx and an adult healthy control group. A group of operational tolerant LTx recipients (TOL; n=13) that were followed at the outpatient clinic at the Erasmus University Medical Center between 2014 and 2020 was included. TOL were completely weaned off IS for medical reasons or non-compliance between 2008 and 2019 and did not experience acute rejection. Acute rejection was defined as at least a two-fold increase in serum bilirubin, aspartate aminotransferase or alanine transaminase, alkaline phosphatase or γ-glutamyltransferase, that normalized upon adequate IS regimen. Protocol biopsies after complete IS weaning were not taken because of possible complications related to the procedure. In five tolerant LTx recipients a liver biopsy was performed because of possible rejection as indicated by increasing liver enzymes, at on average 3.1 ± 2.2 years after complete weaning. Rejection was excluded according to BANFF criteria. A control group of stable LTx recipients (CTRL; n=24) with regular dual or mono IS regimen and a group of stable LTx recipients (MIN; n=8) with minimal mono IS regimen were also included. These groups were matched to the TOL group for important parameters known to influence circulating immune cells (Table 1). IS mono therapy trough levels for CTRL were Tacrolimus 3.2-7.8 µg/L, Mycophenolate Mofetil >2.9 mg/L, and for MIN were Tacrolimus 1.2-2.5 µg/L, Cyclosporin A 58 µg/L, Mycophenolate Mofetil 0.9 mg/L. Both CTRL and MIN did not experience rejection episodes for at least 5 years before and 4 years after blood collection. Immunoglobulin G (IgG) antibodies to CMV in serum were measured with an enzyme immune assay (Biomerieux, VIDAS, Lyon, France). An outcome of ≥6 AU/mL was considered positive. A matched healthy control group (HC; n=7) was also included in the study. Clinical and laboratory information was retrieved from electronic patient records. Informed consent was received from all participants. This study was conducted in accordance with the Declaration of Helsinki and approved by the medical ethics committee of Erasmus MC (MEC 2014-232; MEC 2012-022).

Primers

Twenty-two immune system-related candidate genes (KLRB1, CD160, KLRC4, KLRF1, NKG7, IL2RB, IRF5, EGR2, CXCL8, ZBTB21, CX3CR1, OSBPL5, SLAMF7, ERBB2, UBD, FOXP3, SMAD2, SMAD3, TET1, TET2, HELIOS, NRP1) that have shown differential expression in TOL LTx recipients versus a control or non-tolerant group of LTx recipients in previous studies were selected.[80,82,84,87,130,169] Forward and reverse primers were designed with NCBI PrimerBLAST according to MIQE guidelines.[175] To prevent co-amplification of genomic DNA, intron-flanking primers or exon-exon junction primers were designed with target amplicon sequences of 80 to 150 bp with a maximum GC content of 65%. Forward and reverse primers were not modified and were purified with a desalt step (Merck, Darmstadt, Germany). Primer pairs were tested in duplo for their optimal annealing temperature and amplification efficiency using healthy control PBMC derived cDNA. Four temperatures were tested to determine the

optimal annealing temperature (56, 58, 60, 62°C) for each primer pair. Amplification efficiency was tested with a serial dilution series and efficiencies within the range of 90–110% were considered acceptable. Gel electrophoresis was performed to detect presence of unintended target amplicon sequences using a 2% agarose gel (Merck) with 10% TBE buffer (Thermo Scientific, Waltham, USA) and 1:100.000 DNA Stain G (SERVA, Heidelberg, Germany). Size of the PCR product was determined with a six times dilution using Blue/Orange Loading Dye (Promega, Madison, USA) and a 25bp DNA Step ladder (Promega), and compared with intended target amplicon sequence size to confirm specificity of the primer pair. Primer pairs for ERBB2 and UBD did not pass all tests, even after multiple attempts of re-designing primer pairs. Primer pairs for the remaining twenty selected genes and three housekeeping genes passed all tests and are presented in Supplementary Table 1. Most primer pairs targeted all splicing and transcript variants of the selected gene. Some selected genes required design of multiple primer pairs to target multiple splice variants (NKG7, ZBTB21, CX3CR1 and SLAMF7). For CX3CR1 and KLRF1 not all splice variants could be detected with selected primer pairs.

RNA isolation and generation of cDNA

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation (GE Healthcare, Little Chalfont, England), and were stored in RA1 lysis buffer (MACHEREY-NAGEL, Dueren, Germany) and β-Mercaptoethanol (Merck) at a concentration of $3x10^6$ cells at -80°C until further use. RNA was isolated with NucleoSpin RNA Mini (MACHEREY-NAGEL) according to standard protocol that includes a DNase step. Purity and quantity of isolated RNA was measured with NanoDrop (Thermo Scientific). A 260nm/280nm ratio of ~2.0 was considered pure RNA. cDNA was generated in 50µl (36ng/µl) using 5x PrimeScript™ RT Master Mix Perfect Real Time (Takara, Shiga, Japan) and SimpliAmp Thermal Cycler (Thermo Scientific). Concentration of cDNA was set to 2.5 ng/µl and stored at 4°C until short-term further use.

Quantitative Polymerase Chain Reaction

Gene expression was determined in triplo with 12.5ng cDNA/reaction using SYBR Select Master Mix for CFX (Thermo Scientific) measured by StepOnePlus Real-Time PCR System (Applied Biosystems Thermo Scientific) and analyzed with StepOne software version 2.3 (Applied Biosystems Thermo Scientific). Thermocycling parameters include a UDG activation step at 50°C for 2 min and AmpliTaq DNA Polymerase and UP Activation step at 95°C for 2 min. Thereafter, 40 PCR cycles with a denaturing step at 95°C for 15 sec, the determined optimal annealing temperature for 15 sec and extension at 72°C for 1 min continued. Thermocycling ended with a meltcurve stage with 95°C for 15 sec, 60°C for 1 min, after which an increase of 0.3°C/2sec to 95°C occurred. Gene expression was considered positive when <35 cycles were needed to detect a signal. After each measurement the meltcurve was examined to confirm exclusive amplification of the intended target gene. Expression of twenty selected genes was normalized with mean expression of three housekeeping genes (GAPDH, GUSB, and HPRT1; Supplementary Table 1) using the comparative Ct method.

Statistical analyses

Statistical analyses were performed with IBM SPSS software version 25 (SPSS Inc., Chicago, USA) or GraphPad Prism 8 version 8.4.3 (GraphPad Software Inc., San Diego, USA). The normality of the distribution of the data was determined by the Shapiro-Wilk normality test. Statistical analyses were performed with one-way ANOVA or Kruskal-Wallis, with a Bonferroni or Dunn's posttest. Differences in discrete nominal data between groups were analyzed by the Pearson Chi-Square test. Figures and heatmap were created with GraphPad Prism 8 version 8.4.3. Principal component analysis using direct oblimin factor rotation was performed using IBM SPSS software version 25.

Results

Patient characteristics

In this study operationally tolerant (TOL) LTx recipients were compared to a group of control (CTRL) LTx recipients with regular IS regimen, a group of LTx recipients with minimal IS monotherapy regimen (MIN) and a group of healthy controls (HC) (Table 1). These groups were all carefully matched for important parameters known to influence expression of immune system related genes. Therefore, the study groups did not differ in age, sex, time after transplantation, primary liver disease, and CMV serostatus of the donor and recipient before and after (at the time of blood collection) transplantation. Although the groups did not significantly differ in primary liver disease, the MIN LTx recipients harbor the highest prevalence of virus-related liver disease before LTx.

Use of immunosuppressive drugs affects gene expression of PBMCs

Relative gene expression of PBMCs was assessed for twenty genes and their transcript variants (annotation -1, -2 or -3) that were selected from previous studies in which their expression level was reported to be associated with operational tolerance after LTx (Supplementary Table 1; Figure 1A). Expression of TET1, TET2, NRP1, HELIOS, NKG7-1, NKG7-2, IRF5, EGR2, OSBPL5 and CX3CR1-1 genes did not differ among groups (Figure 1A). Expression of KLRC4 and SLAMF7-3 was significantly higher in HC compared to CTRL, but did not differ between other groups (Figure 1A, Supplementary Figure 1). Gene expression of KLRF1, SMAD2, CXCL8 and CD160 was significantly higher in MIN compared to TOL, CTRL and/or HC but did not differ between TOL and CTRL groups (Figure 1A, Supplementary Figure 1). This data might imply that expression of KLRF1, SMAD2, CXCL8, and CD160 could have been influenced by viral infections, since the majority of MIN LTx recipients were transplanted for virus-related primary liver disease. Gene expression of SMAD3, FOXP3, IL2RB, KLRB1, SLAMF7-1, SLAMF7-2, ZBTB21-1, ZBTB21-2 and CX3CR-2 was significantly different in TOL compared to CTRL (Figure 1A, B). Of these genes, expression of FOXP3, KLRB1, SLAMF7-1, SLAMF7-2 and CX3CR-2 also significantly differed between HC and CTRL. However, none of the twenty selected genes significantly differed between TOL and HC. This indicates that the differential expression of the genes between TOL and CTRL does not represent a tolerance-associated gene profile, but rather reflects a difference in IS usage. Principal component analysis of the nine significantly different expressed genes between TOL and CTRL revealed three components that separated CTRL from clustered HC and TOL, with MIN clustered in-between the groups (Figure 1C). This suggests that gene expression of SMAD3, FOXP3, IL2RB, KLRB1, SLAMF7-1, SLAMF7-2, ZBTB21-1, ZBTB21-2 and CX3CR-2 in CTRL and MIN LTx recipients is affected by the height of the IS through levels. This is most clearly observed in the stepwise increase of FOXP3, KLRB1, and SLAMF7-1 expression from CTRL to MIN to TOL and HC (Figure 1B).

Prior CMV infection affects gene expression of PBMCs

Our study groups were all carefully matched for important parameters known to influence expression of immune system related genes, such as prior CMV infection. To study the influence of prior CMV infection on gene expression, TOL and CTRL LTx recipients were divided according to their CMV serostatus at the time of blood collection late after LTx (Figure 2). Expression of SMAD3, FOXP3, KLRB1, KLRF1, CD160, CX3CR1-2 and ZBTB21-2 in CMV seropositive TOL differed (significantly) from CMV seropositive CTRL LTx recipients (Figure 2A). Expression of SMAD3 did significantly differ between TOL and CTRL CMV seronegative LTx recipients. Expression of other genes did not significantly differ between TOL and CTRL CMV seronegative LTx recipients, which is probably due to the low number of CMV seronegative individuals. These results could indicate that these genes are influenced by the use of IS, but not prior CMV infection. In contrast, gene expression of SLAMF7-1, SLAMF7-2 and SLAMF7-3 splice variants were significantly higher in CMV seropositive TOL compared to CMV seropositive CTRL and CMV seronegative TOL (Figure 2B). Expression of NKG7-1 tended to be higher in CMV seropositive TOL and CTRL versus their CMV seronegative counterpart (Figure 2C). Expression of NKG7-2 splice variant tended to be higher in CMV seropositive CTRL compared to CMV seronegative CTRL and was significantly higher in CMV seropositive CTRL compared to CMV seropositive TOL. These data show that prior CMV infection is associated with a higher relative gene expression of SLAMF7 and NKG7 in PBMCs of LTx recipients, but the increase in SLAMF7 expression in CMV seropositive CTRL was hampered by the use of IS. Principal component analysis of the eleven significantly different expressed genes and transcript variants between CMV seropositive TOL and CTRL revealed three components that separated CTRL from TOL, with most MIN in-between (Figure 2D). Strikingly, the CMV seronegative individuals positioned generally below the seropositive individuals for all three groups. Principal component analysis of the five SLAMF7 and NKG7 splice variants, which expression was influenced by prior CMV infection, revealed two components (Figure 2E). In this analysis the CMV seronegative LTx recipients clustered completely together, whereas CMV seropositive TOL and CTRL clustered partly separately with overlap of CMV seropositive MIN, indicative of an IS and a prior CMV infection effect on gene expression in PBMCs of these groups.

Full disclosure of analyzed splice variants of genes is necessary

In our study we carefully presented the splice variants of the selected and analyzed genes (Supplementary Table 1). It appeared that expression of CX3CR1-1 and CX3CR1-2, SLAMF7-1 – 3, and NKG7-1 and NKG7-2 (Figure 1B, 2A, 2C and Figure 3) splice variants were not similar to each other. Expression of SLAMF7-1 and SLAMF7-2, but not SLAMF7-3 (Figure 3), splice variants were significantly higher in TOL versus CTRL. Expression of CX3CR1-2 was significantly higher in TOL compared to CTRL, whereas CX3CR1-1 expression was not. Similarly, expression of CX3CR1-2 was significantly higher in CMV seropositive TOL compared to CMV seropositive CTRL, but not for CX3CR1-1 expression. These results indicate that it is important to always check and provide the analyzed data with splice variants for maximum transparency.

Table 1 Characteristics of the study groups.

	CTRL	MIN	TOL	HC	*P-value*
Demographics	n=24	n=8	n=13	n=7	
Male (%)	62.5	50.0	76.9	71.4	0.43
Age in years[a]	55.0 (30.5-58.5)	57.5 (37.8-64.8)	56.0 (43.0-68.5)	45.0 (25.0-54.0)	0.56
Years post-LTx[a]	14.5 (12.0-20.5)	15.5 (12.3-18.8)	15.0 (13.0-17.5)	NA	0.83
Years complete weaning - end follow-up[a]	NA	NA	4.0 (2.0-6.0)	NA	NA
Primary disease (%)				NA	0.61
Cholestatic disease	25.0	12.5	30.8		
Virus-related[b]	33.3	75.0	30.8		
Hepatocellular carcinoma	20.8	0.0	23.1		
Cryptogenic cirrhosis	12.5	0.0	15.4		
Toxicity-induced	4.2	0.0	0.0		
Metabolic-related	4.2	0.0	0.0		
Rupture	0.0	12.5	0.0		
IS (last) used (%)				NA	0.45
Tac	66.7	75.0	53.8		
CsA	4.2	12.5	7.7		
MMF	8.3	12.5	0.0		
Aza	0.0	0.0	7.7		
Tac and MMF	8.3	0.0	15.4		
Pred and Tac	8.3	0.0	0.0		
Pred and MMF	4.2	0.0	0.0		
Aza and CsA	0.0	0.0	7.7		
Unknown	0.0	0.0	7.7		
CMV seropositive (%)				ND	
Recipient pre-LTx	50.0	50.0	46.2		0.92
Recipient post-LTx	75.0	87.5	61.5		0.78
Donor	45.8	50.0	46.2		0.85

Percentages or [a]median years with 25th and 75th IQR are presented. Statistical analyses were performed with Chi-Square or Kruskal-Wallis rank test. [b] Viral infections include Hepatitis A, B or C virus, and Epstein Barr virus. Aza, azathioprine; CMV, cytomegalovirus; CsA, cyclosporine A; CTRL, control group; HC, healthy controls; IS, immunosuppressive drugs; LTx, liver transplantation; MIN, minimal IS group; MMF, mycophenolate mofetil; NA, not applicable; ND, not determined; Pred, prednisolone; Tac, tacrolimus; TOL, tolerant group.

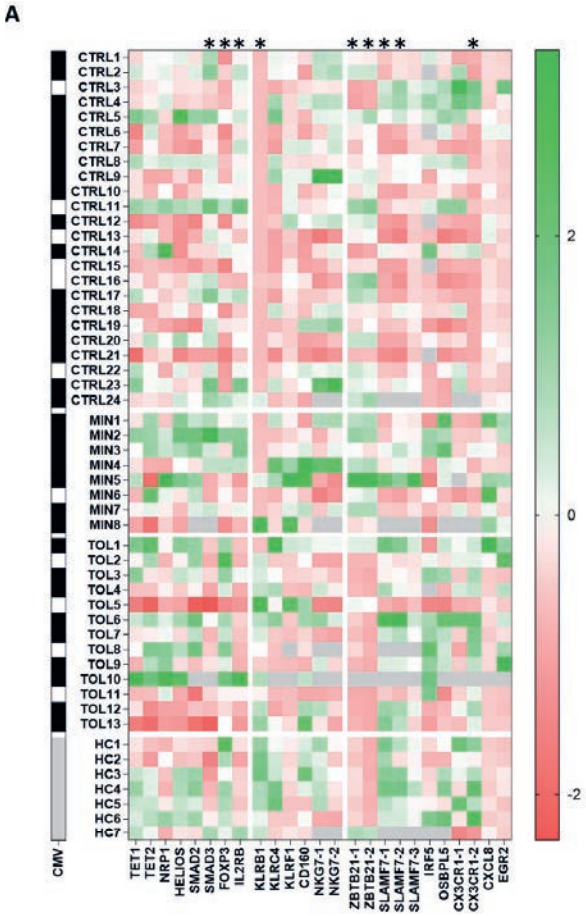
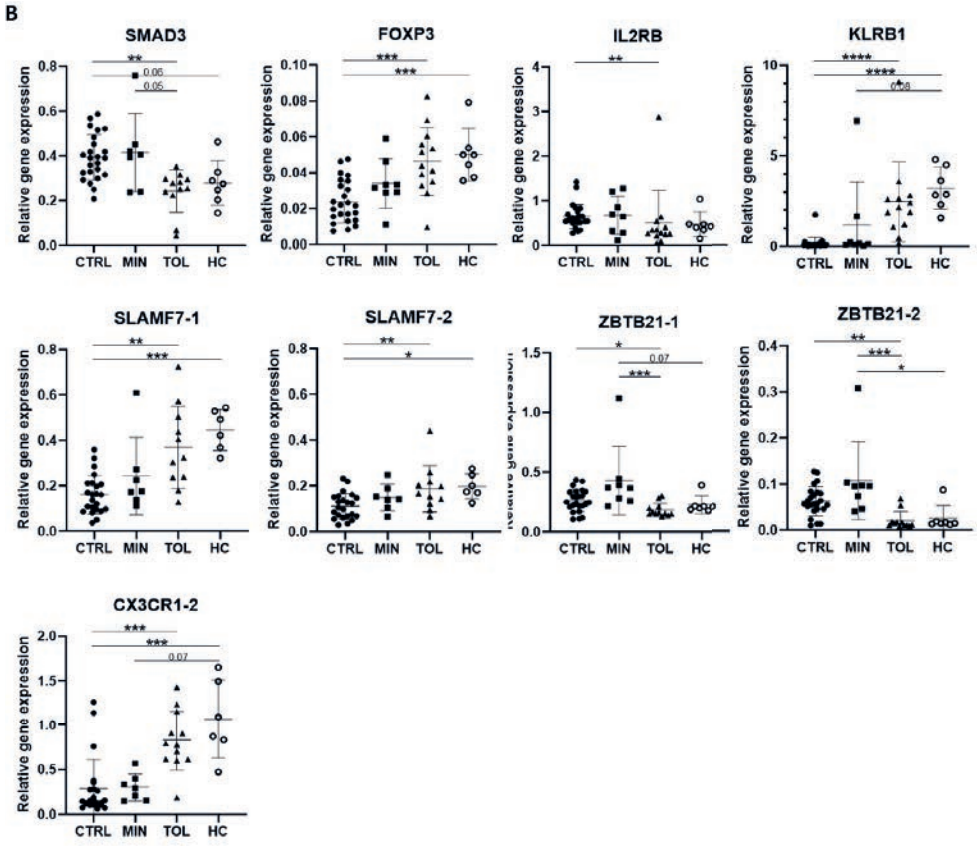
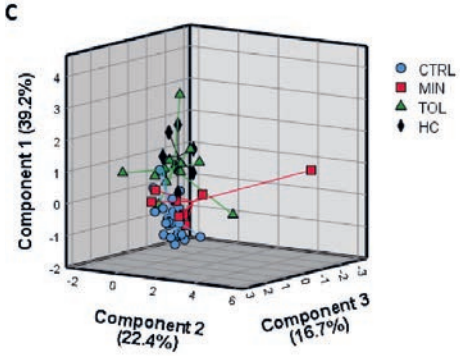
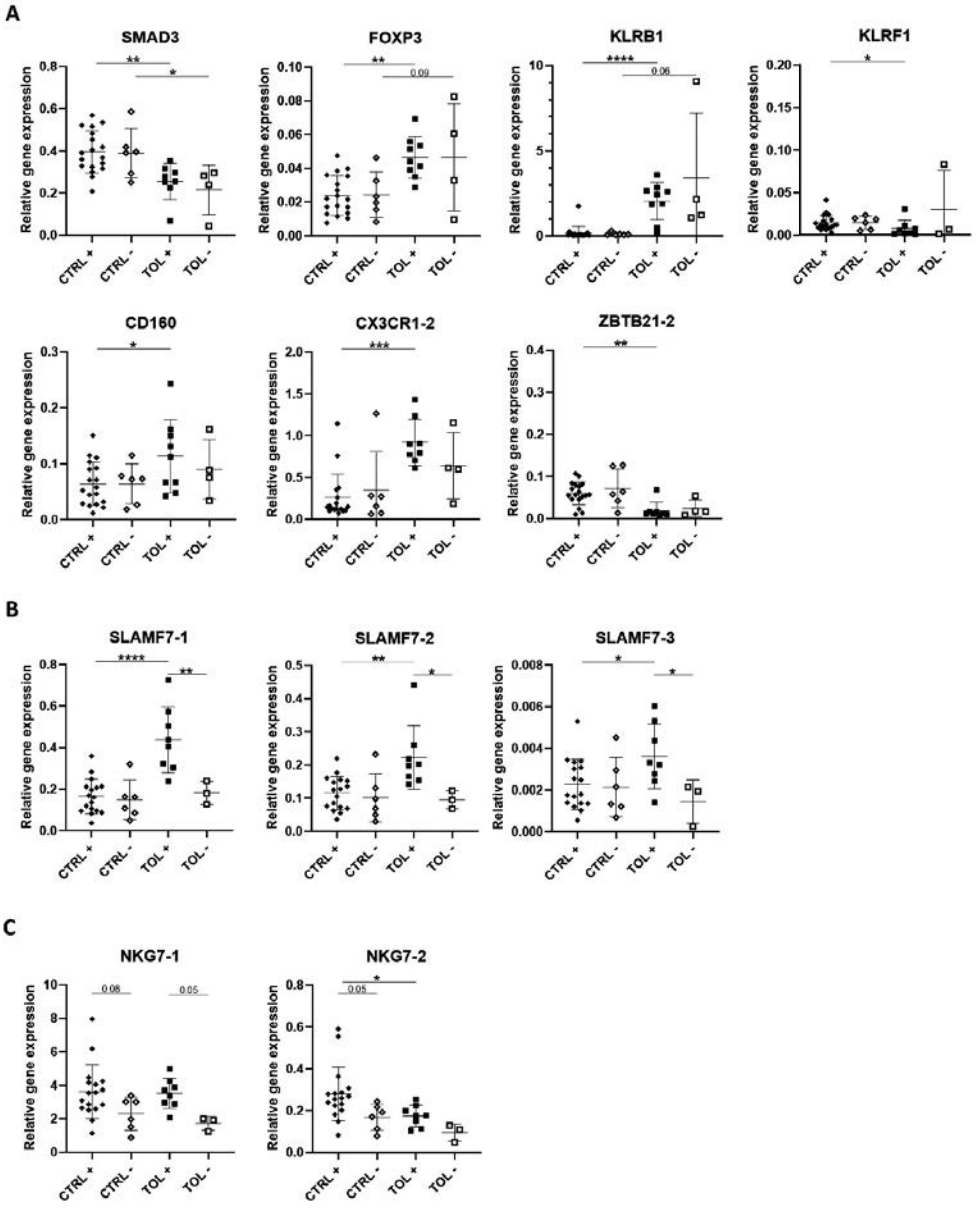


Figure 1 Use of immunosuppressive drugs affects gene expression. (A) A heatmap is presented with Z-scores derived from relative expression of each gene compared to its mean expression in all subjects. Green squares indicate an upregulation and red squares indicate a downregulation compared to the mean of the indicated gene. On the left black squares indicate CMV seropositivity, whereas white squares indicate CMV seronegativity for each study subject. Grey squares indicate that gene expression or CMV seropositivity was not determined. The annotation with -1 or -2 indicate that different splice variants of that gene are included. * Relative gene expression of indicated gene was significantly different between TOL and CTRL LTx recipients. (B) The nine significant differentially relatively expressed genes between TOL and CTRL are presented. The annotations with -1 or -2 indicate that different splice variants of that gene are included. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 (C) Principal component analysis of all study groups with the nine significant differentially expressed genes between TOL and CTRL LTx recipients is depicted. Rotated component matrix analysis was performed using direct oblimin factor rotation. On the axes the contributed percentage of the variance between groups by that component is indicated. CMV, cytomegalovirus; CTRL, control LTx recipients; HC, healthy control; LTx, liver transplantation; MIN, minimal IS regimen LTx recipients; TOL, tolerant LTx-recipients.


B
SMAD3
FOXP3
IL2RB
KLRB1
SLAMF7-1
SLAMF7-2
ZBTB21-1
ZBTB21-2
CX3CR1-2
Relative gene expression
CTRL
MIN
TOL
HC


C
CTRL
MIN
TOL
HC
Component 1 (39.2%)
Component 2 (22.4%)
Component 3 (16.7%)


A
SMAD3
FOXP3
KLRB1
KLRF1
CD160
CX3CR1-2
ZBTB21-2
B
SLAMF7-1
SLAMF7-2
SLAMF7-3
C
NKG7-1
NKG7-2
Relative gene expression
CTRL +
CTRL -
TOL +
TOL -

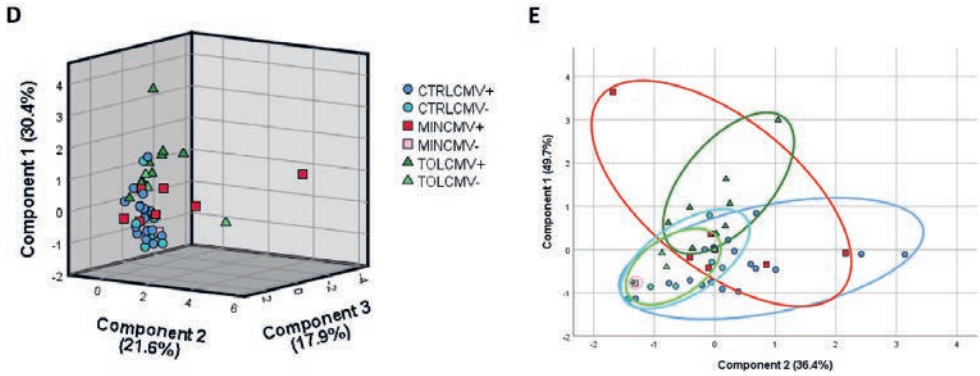


Figure 2 Prior cytomegalovirus infection affects gene expression of PBMCs. (A) Seven significant differentially expressed genes between CMV seropositive TOL and CTRL LTx recipients are presented. (B) Differentially expressed SLAMF7 with its splice variants in CMV seropositive TOL is depicted. (C) Differentially expressed NKG7 with its splice variants in CMV seropositive TOL and CTRL is depicted. (D) Principal component analysis of expression of the eleven gene variants which were significantly differentially expressed between CMV seropositive TOL and CTRL LTx recipients is depicted. (E) Principal component analysis of splice variants of SLAMF7 and NKG7 presented in B and C is depicted. (D,E) Rotated component matrix analysis was performed using direct oblimin factor rotation. On the axes the contributed percentage of the variance between groups by that component is indicated. The annotation with -1 or -2 indicates that different splice variants of that gene are included. + indicates CMV seropositivity, - indicates CMV seronegativity. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. CMV, cytomegalovirus; CTRL, control LTx recipients; HC, healthy control; LTx, liver transplantation; MIN, minimal IS regimen LTx recipients; TOL, tolerant LTx-recipients.

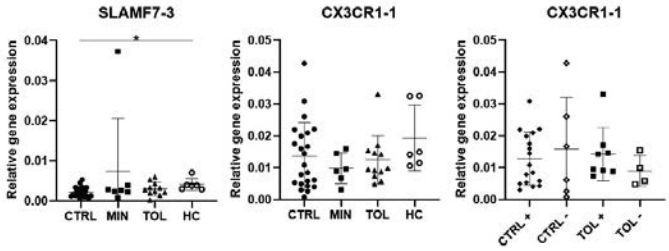


Figure 3 Differential splice variant expression of selected genes. Splice variants SLAMF-3 and CX3CR1-1 differ in their gene expression from splice variants SLAMF-1, SLAMF7-2 and CX3CR1-2. The annotation with -1 or -2 indicate that different splice variants of that gene are included. + indicates CMV seropositivity, - indicates CMV seronegativity. * P<0.05 CMV, cytomegalovirus; CTRL, control LTx recipients; HC, healthy control; LTx, liver transplantation; MIN, minimal IS regimen LTx recipients; TOL, tolerant LTx-recipients.

Discussion

Here we studied peripheral blood expression of twenty different immune system related genes described in previous studies suitable for identification of tolerant LTx recipients. These genes include KLRB1, KLRC4, KLRF1, CD160, NKG7, FOXP3, IL2RB, SMAD2, SMAD3, TET1, TET2, HELIOS and NRP1, IRF5, EGR2, CXCL8, ZBTB21, CX3CR1, OSBPL5 and SLAMF7.[80,82,84,87,130,169] Our study indicates that previously reported differential expressions of these genes between tolerant and non-tolerant LTx recipients may have been profoundly influenced by differences in IS regimen, prior CMV infection, and potentially other differences between the study groups.

In our study expression of NKG7, IRF5, EGR2, OSBPL5, CX3CR1-1, TET1, TET2, NRP1 and HELIOS did not differ among groups. We performed our study using carefully matched study groups for important clinical demographics and characteristics known to influence expression of immune system related genes. The afore-mentioned studies lack thorough matching of such parameters. In the studies of Martínez-Llordella et al 2007, Martínez-Llordella et al 2008, Pons et al 2008, Lozano et al 2011, Bohne et al 2012 and Revilla-Nuin et al 2017 [80,82,84,87,130,169] age, sex, primary liver disease, time after LTx, and prior CMV infection, were either not described or greatly differed between study groups. These parameters could all have had a confounding effect on the reported differential gene expression between the study groups, and could explain the discrepancies observed with our study. This is also supported by the notion that in all of the above mentioned studies different gene expression profiles, with little common genes, were found that supposedly identified tolerant LTx recipients. That matching of clinical parameters is important is also illustrated by our data, where significantly higher expression levels of KLRF1, SMAD2, CXCL8, CD160 and ZBTB21 in the MIN group were observed, the LTx recipients that harbored the highest prevalence of viral liver disease before LTx. That viral liver diseases are capable of influencing peripheral blood gene expression is illustrated by Martínez-Llordella et al 2007 and Martínez-Llordella et al 2008, where Hepatitis C infection affected expression of many analyzed genes in tolerant, control and non-tolerant LTx recipients.[84,87] Another possible explanation for the discrepancies observed is that, in contrast to our study, three of the above mentioned studies[80,84,87] did not apply a correction factor for multiple statistical testing for data analysis and possibly have found statistical differences by chance. For a few genes, the discrepancies observed between different studies could also be due to assessment of different splice variants of the genes assessed. Unfortunately, it has not been reported which splice variants were analyzed in previously mentioned studies. That there are differences between expression of splice variants of several genes was illustrated by our own data, as well as those by others.[176] Therefore, it is important to always provide data concerning the analyzed splice variants for full disclosure and maximum transparency when publishing research.

In our study gene expression of SMAD3, FOXP3, IL2RB, KLRB1, SLAMF7-1, SLAMF7-2, ZBTB21-1, ZBTB21-2 and CX3CR1-2 did significantly differ in TOL compared to CTRL LTx recipients. However, none of these genes significantly differed in expression between TOL and HC, suggesting an influence of IS. Principal component analysis revealed that the transcriptional

profiles of these genes of MIN LTx recipients clustered in between CTRL and both the TOL and HC groups. This suggests that even the height of the IS trough levels affects expression of these genes, which was clearly observed for FOXP3, KLRB1 and SLAMF7-1. This could explain the differential expression between tolerant LTx recipients off IS and other LTx recipients on IS found in other studies. That IS influences expression levels of the studied genes is also supported, but definitely not clearly stated, by the study of Bohne et al 2012,[80] where microarray analysis of tolerant and non-tolerant LTx recipients before prospective IS weaning resulted in a different tolerance associated gene profile in PBMCs compared to their own previous data on tolerant LTx recipients after IS weaning.[84,87,169] KLRB1, SLAMF7, and CX3CR1 genes, of which the expression levels we found to be suppressed by IS, were reported among tolerance-associated genes in peripheral blood in previous studies, but not in the prospective weaning study.[80] Moreover, one report admitted that peripheral blood gene expression patterns of TOL recipients without IS regimen appeared to be closer to those of healthy individuals than to those of non-TOL recipients with IS regimen.[84] Therefore, we suggest that future studies on tolerance-associated genes in peripheral blood should be performed before IS weaning in LTx recipients.

Several studies have suggested that circulating NK cells and γδT-cells are implicated in operational tolerance.[84,87,177] One study by Martínez-Llordella et al 2008[84] even suggested that three different gene profiles, including the mainly NK cell and γδT-cell related KLRF1, KLRB1, IL2RB, SLAMF7, NKG7 and CX3CR1 genes, in different combinations, could discriminate tolerant from non-tolerant LTx recipients after IS weaning.[84] In our study we found that expression of KLRB1, IL2RB, SLAMF7 and CX3CR1 is probably affected by use of IS. Moreover, we found that prior CMV infection was associated with a higher relative gene expression of SLAMF7 and NKG7. It is known that CMV infection in healthy subjects changes the composition of circulating immune cells with expansion of pathogen-specific CD8+ T-cells, γδT-cells and NK cell subsets.[6] CMV infection after kidney and liver transplantation induces similar long-lasting changes in these immune subsets.[5,6,170-174] As mentioned before, we carefully matched for demographical and clinical parameters, such as prior CMV infection, between our study groups. In the studies by Martínez-Llordella et al 2007 and Martínez-Llordella et al 2008 in which peripheral blood gene expression was studied, as well as in another study[85], the circulating Vδ1/Vδ2 γδT-cell ratio was higher in tolerant LTx recipients compared to control or non-tolerant LTx recipients, and was suggested to be a marker for tolerance. However, we[174] and others[166,171] have shown that a higher Vδ1/Vδ2 γδT-cell ratio is associated with prior CMV infection in LTx recipients. Recently, we also demonstrated that the Vδ1/Vδ2 γδT-cell ratio in peripheral blood does not differ between TOL and CTRL LTx recipients matched for CMV serostatus.[178] Therefore it is likely that in these previous studies the tolerant group of LTx recipients harbored more CMV seropositive LTx recipients than the control or non-tolerant groups of LTx recipients. Hence, the different gene expression of SLAMF7 and NKG7 found by Martínez-Llordella et al 2008[84] could be rather suitable for identification of CMV positive LTx recipients than tolerant LTx recipients.

In our study relative gene expression of Treg related markers TET1, TET2, NRP1 and HELIOS did not differ in TOL LTx recipients compared to all other study groups, whereas FOXP3 expression was significantly influenced by the use of IS. Although previously higher expression

of FOXP3 in blood of tolerant LTx recipients without IS compared to non-tolerant LTx recipients after reintroduction of IS regimen was reported, this difference was not observed when FOXP3 was measured before IS weaning,[82,130] supporting our observation that FOXP3 expression is suppressed by IS. In these previous studies, peripheral blood expression of TET2 and NRP1 were similar in tolerant and non-tolerant LTx recipients, comparable to our own data. HELIOS expression was reported to be enhanced in tolerant LTx recipients, but this difference was not observed before IS weaning.[82,130] Together with the other gene expression data this shows that the assessed markers related to Tregs, NK cells or γδT-cells are rather influenced by prior CMV infection and/or use of IS, than indicative of operational tolerance.

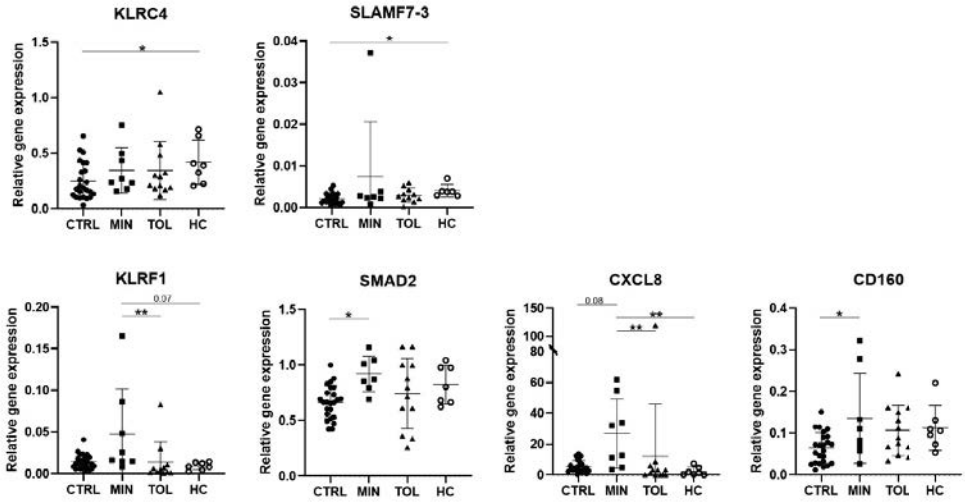
The strength of our study is that we carefully matched TOL, CTRL, MIN and HC study groups for important clinical parameters known to influence circulating immune cells and their gene expression, and thereby eliminated potential confounders. Furthermore, the selected forward and reverse primer pairs were thoroughly designed and tested for their optimal annealing temperature and amplification efficiency and specificity. This resulted in exclusion of ERBB2 and UBD genes. Another strength is that we used three references genes to study the relative gene expression of selected genes, in contrast to other studies that only used one reference gene[82,84,87], making our data more robust. Moreover, in our study we clearly state which splice variants of selected genes were measured for full disclosure and maximum transparency. Lastly, in contrast to other studies in this field, we used appropriate statistical analyses with correction for multiple testing to analyze our data. Our study also has some limitations. A weaker part of our study is that we included CTRL LTx recipients with regular IS regimen with unknown tolerance status to compare to tolerant LTx recipients without any IS regimen. However, it is expected that the majority of CTRL LTx recipients are non-tolerant towards their graft. Unfortunately, we did not have access to samples of TOL LTx recipients before IS weaning, which would have facilitated a better comparison of gene expression profiles between the different groups. To account for the difference in IS use, we included a minimal IS regimen group and a healthy control group. The small study group sizes in this study are another weakness, although this is not unusual when studying operational tolerance in LTx recipients. As in other recently published studies on operational tolerance,[52,54] liver function tests were used as an indicator of tolerance instead of protocol liver biopsies due to possible complications that could arise. The lack of protocol biopsies in our study may result in an absence of diagnoses of subclinical rejection. Subclinical rejection may also impact peripheral blood gene expression profiles. However, the clinical implications of subclinical rejection and a possible relation to graft damage are still unclear.[150-152]

Here we studied peripheral blood expression of Treg, NK cell and γδT-cells related genes described in previous studies that identified operational tolerance amongst LTx recipients. Unfortunately, we could not confirm their capacity to discriminate tolerant LTx recipients from control LTx recipients. Instead, we found a confounding effect of IS usage and prior CMV infection, on expression of many selected genes. In the future whole genome RNA sequencing should be performed on PBMCs of carefully matched tolerant and non-tolerant LTx recipients before IS weaning to identify a tolerance predicting gene expression profile suitable for selecting recipients eligible for prospective IS weaning.

Acknowledgements

We would like to express our gratitude to the liver transplant nurses of the Department of Gastroenterology and Hepatology at the Erasmus University Medical Centre for their efforts to successfully collect all heparinized blood samples. We also would like to thank the Department of Viroscience at the Erasmus University Medical Centre for determining the CMV IgG serostatus.

Supplementary Material



Supplementary Figure 1 Relative expression of genes differentially expressed between several study groups. Differentially expressed KLRC4, SLAMF7-3, KLRF1, SMAD2, CXCL8 and CD160 in CTRL, MIN, TOL and HC groups are depicted. * P<0.05, ** P<0.01 CTRL, control LTx recipients; HC, healthy control; LTx, liver transplantation; MIN, minimal IS regimen LTx recipients; TOL, tolerant LTx-recipients.

Supplementary Table 1 Analyzed genes with their transcript variants and forward and reverse primer sequences.

Gene	Other nomenclature	Number of splice variants	Name in graph	Forward primer	Reverse primer	Accession number	Splice variant
Housekeeping genes							
GADPH	G3PD; GAPD; HEL-S-162eP	8	NA	AAGGTCGGAGTCAACGGATTT	ACCAGAGTTAAAAGCAGCCCTG	NM_002046.7	Transcript variant 1
						NM_001289745.3	Transcript variant 3
						NM_001289746.2	Transcript variant 4
						NM_001357943.2	Transcript variant 7
GUSB	BG; MPS7	4	NA	CAGGTGATGGAAGAAGTGG	GTTGCTCACAAAGGTCACAG	NM_000181.4	Transcript variant 1
						NM_001284290.2	Transcript variant 2
						NM_001293104.2	Transcript variant 3
						NM_001293105.2	Transcript variant 4
HPRT1	HPRT; HGPRT	1	NA	GCTATAAATTCTTTGCTGACCTGCTG	AATTACTTTTATGTCCCCTGTTGACTGG	NM_000194.3	NA
NK-cell related function							
KLRB1	NKR; CD161; CLEC5B; NKR-P1; NKRP1A; NKR-P1A; hNKR-P1A	1	KLRB1	CTGGGTTGAGTGTTTCAGTGAC	TTTCTCTCGGAGTTGCTGCC	NM_002258.2	NA
CD160	NK1; BY55; NK28	1	CD160	AAGGTCTGGGTAATGCTGGTC	AGACTCATCTTGCTGTAGCTGT	NM_007053.3	NA
KLRC4	NKG2F; NKG2-F	1	KLRC4	CACTGCAAAGGTTTACTGCCAC	GTTCTGCTCCAGTACTCCAATACA	NM_013431.2	NA
KLRF1	NKp80; CLEC5C	4	KLRF1	GGAGTTCTGCCCAAACATCTC	TGGCATAGTACCAACAGGATCAA	NM_001291822.1	Transcript variant 1-s
NKG7	GIG1; GMP-17; p15-TIA-1	2	NKG7-1	TTCTGGTTTGAGGCTGTGGG	GTCACGTGGATGTAGCCTGATA	NM_005601.4	Transcript variant 1
			NKG7-2	GGACATCATATCAGGCCACG	CTGGGGACAAGGACAAGAGAG	NM_001363693.1	Transcript variant 2
Treg-related function							
TET1	LCX; CXXC6; bA119F7.1	1	TET1	ACTGCCAACCTTAGGGAGTAAC	ATGCCTCTTTCACTGGGTGAG	NM_030625.3	NA

TET2	MDS; IMD75; KIAA1546	2	TET2	TTACAACGCTTGGAAGCAGG	CTAGTTGAATTCAGCAGCTCAG	NM_001127208.2	Transcript variant 1
						NM_017628.4	Transcript variant 2
NRP1	NP1; NRP; BDCA4; CD304; VEGF165R	6	NRP1	AGCACCGAGAGAACAAGGTG	CCGCAGCTCAGGTGTATCAT	NM_003873.6	Transcript variant 1
						NM_001024628.2	Transcript variant 2
						NM_001024629.2	Transcript variant 3
						NM_001244972.1	Transcript variant 4
						NM_001244973.1	Transcript variant 5
						NM_001330068.1	Transcript variant 7
FOXP3	JM2; AIID; IPEX; PIDX; XPID; DIETER	2	FOXP3	CAAGTTCCACAACATGCGACC	GAAGGCAAACATGCGTGTGAA	NM_014009.3	Transcript variant 1
						NM_001114377.2	Transcript variant 2
IL2RB	CD122; IMD63; IL15RB; P70-75	1	IL2RB	TGGAGAGATGGCCACGGT	TTACATCCACAGGGTGGAGC	NM_000878.5	NA
HELIOS	ANF1A2; IKZF2; ZNF1A2; ZNFN1A2	7	HELIOS	GGAAACAGAGGCTATTGATGGCT	TGTCCATTGGGTGTGCTTGA	NM_016260.3	Transcript variant 1
						NM_001079526.2	Transcript variant 2
						NM_001371274.1	Transcript variant 3
						NM_001371275.1	Transcript variant 4
						NM_001371276.1	Transcript variant 5
						NM_001371277.1	Transcript variant 6
						NM_001387220.1	Transcript variant 7
SMAD2	JV18; MADH2; MADR2; JV18-1; hMAD-2; hSMAD2	3	SMAD2	CCGACACACCGAGATCCTAAC	GAGGTGGCGTTTCTGGAATATAA	NM_005901.6	Transcript variant 1
						NM_001003652.4	Transcript variant 2
						NM_001135937.2	Transcript variant 3
SMAD3	LDS3; LDS1C; MADH3; JV15-2; HSPC193; HsT17436	4	SMAD3	TGGACGCAGGTTCTCCAAAC	CCGGCTCGCAGTAGGTAAC	NM_005902.4	Transcript variant 1
						NM_001145102.1	Transcript variant 2
						NM_001145103.1	Transcript variant 3
						NM_001145104.1	Transcript variant 4
Other							

IRF5	SLEB10	7	IRF5	AGGGCTTCAATGGGTCAACG	ACGCCTTCGGTGTATTTCCC	NM_032643.4	Transcript variant 2
						NM_001098627.3	Transcript variant 3
						NM_001098629.3	Transcript variant 5
						NM_001098630.3	Transcript variant 6
						NM_001242452.3	Transcript variant 8
						NM_001347928.2	Transcript variant 9
						NM_001364314.2	Transcript variant 10
EGR2	CHN1; AT591; CMT1D; CMT4E; KROX20	5	EGR2	CCATCTTTCCCAATGCCGAAC	GGGAGATCCAACGACCTCTTC	NM_000399.5	Transcript variant 1
						NM_001136177.3	Transcript variant 2
						NM_001136178.1	Transcript variant 3
						NM_001136179.3	Transcript variant 4
						NM_001321037.2	Transcript variant 5
CXCL8	IL8; NAF; GCP1; LECT; LUCT; NAP1; GCP-1; LYNAP; MDNCF; MONAP; NAP-1; SCYB8	2	CXCL8	GAAACCACCGGAAGGAACCAT	AAACTGCACCTTCACACAGAGC	NM_000584.4	Transcript variant 1
						NM_001354840.2	Transcript variant 2
ZBTB21	ZNF295	5	ZBTB21-1	TGAAGACCGATAAACTCAAGCCA	CTGTCCTTTGAGACGCTCCT	NM_020727.5	Transcript variant 2
						NM_001320729.2	Transcript variant 4
			ZBTB21-2	TCGAGACTGAAGACCGAAGAC	CACTTTGATCCTCGCACACA	NM_001098402.2	Transcript variant 1
						NM_001098403.2	Transcript variant 3
						NM_001320731.2	Transcript variant 5
CX3CR1	V28; CCRL1; GPR13; CMKDR1; GPRV28; CMKBRL1	4	CX3CR1-1	TCTTCCACCATGAGCAGGC	GTGAAGGCCTCTAGTCGCTG	NM_001171171.1	Transcript variant 2
			CX3CR1-2	CAGAGGTTCCCTTGGCAGTC	TCCCAAAGACCACGATGTCC	NM_001337.3	Transcript variant 4
OSBPL5	ORP5; OBPH1	3	OSBPL5	ATCACCCTCATCGCTCTGTG	GGAAGACCCGTTCAGTGGG	NM_020896.4	Transcript variant 1
						NM_145638.3	Transcript variant 2
						NM_001144063.2	Transcript variant 3
SLAMF7		10	SLAMF7-1	ACAGAGTACGACACAATCCCTC	TTGGTGTGTCTGGCATCGTG	NM_021181.5	Transcript variant 1

	19A; CS1; CD319; CRACC					NM_001282589.1	Transcript variant 3
						NM_001282590.2	Transcript variant 4
						NM_001282591.1	Transcript variant 5
						NM_001282594.1	Transcript variant 8
						NM_001282595.1	Transcript variant 9
			SLAMF7-2	CAGTGGCTGACTTCCAGAGAG	GACCAGCTCTTTCACGGGTC	NM_021181.5	Transcript variant 1
						NM_001282588.1	Transcript variant 2
						NM_001282589.1	Transcript variant 3
						NM_001282592.2	Transcript variant 6
			SLAMF7-3	GTGGCTTCATTTCAGTGGCTG	CTTTGACAGGTGCTCTGTGAG	NM_001282590.2	Transcript variant 4
						NM_001282591.1	Transcript variant 5
						NM_001282593.1	Transcript variant 7
						NM_001282594.1	Transcript variant 8
						NM_001282596.2	Transcript variant 10

CHAPTER 8.

Summary, Discussion and Future Perspectives

Summary

In **Chapter 1** a General Introduction and Outline of this thesis is described. The first aim of this thesis was to investigate biomarkers by which operational immunological tolerance could be recognized and to investigate the consequences of complete immunosuppressive drug weaning, by comparing tolerant liver transplant recipients to completely matched groups of other liver transplant recipients and/or healthy controls. The second aim was to assess whether rejection episodes after liver transplantation could be prevented by changing a standard retransplantation protocol or by proteomic screening of soluble markers that could predict an upcoming late acute rejection.

In **Chapter 2** a case report is presented, in which a young liver transplant recipient lost two earlier liver transplants due to T-cell mediated rejection, chronic rejection and possible antibody mediated rejection with de novo DSA formation. The recipient allograft response against donor HLA, and/or other minor donor antigens, caused severe rejection that was unresponsive to rescue treatment. Unfortunately, there is no clear protocol and/or study in the literature that describes how to approach and resolve such difficult situations. During the third liver transplantation, we applied HLA matching between donor and recipient in combination with a more rigorous immunosuppressive drug induction regimen. In this way, the allograft response was limited with only a mild and treatable T-cell mediated rejection, and no development of chronic and antibody mediated rejection with de novo DSA formation. This led to long-term graft survival of at least 683 days.

In **Chapter 3** a pilot study is presented, in which we performed a novel proteomic screening of soluble immune system related serum markers in non-tolerant and tolerant liver transplant recipients late after transplantation and three other study groups, using the new highly sensitive multilex PEA technology from Olink. Unfortunately, we could not find an immune profile identifying tolerant liver transplant recipients. Nevertheless, fifteen serum proteins were significantly different between non-tolerant liver transplant recipients at a time point (4 months - 1.3 years) before elevated liver function parameters were observed, and stable control liver transplant recipients with regular immunosuppressive drug regimen. This suggests that either long before an acute rejection episode becomes apparent, rejection processes are already ongoing in these liver transplant recipients, or that these soluble serum proteins are indicative of recipients with a higher inherent sensitivity to graft rejection. Clustering analysis of these fifteen proteins revealed that HO1, TIE2 and ICOSLG proteins were sufficient to predict an upcoming late acute rejection in all but one of the non-tolerant liver transplant recipients with stable liver function values a long time prior to rejection.

In **Chapter 4** the clinical benefits of complete immunosuppressive drug weaning late after transplantation on its related side effects in tolerant liver transplant recipients were investigated. Unfortunately, no improvement in kidney function, or lower rates of de novo occurrences of diabetes, hypertension, cardiovascular diseases, and malignancies were observed in tolerant liver transplant recipients compared to matched control recipients on regular immunosuppressive drug regimen. However, we did observe a significant decrease in

the total number of infections and low-density lipoprotein levels in tolerant liver transplant recipients after complete immunosuppressive drug weaning. Furthermore, total number of infections were lower in the tolerant group after weaning compared to control recipients on regular immunosuppressive drug regimen in the same time period. Hence, weaning of immunosuppressive drugs late after liver transplantation could be beneficial for tolerant liver transplant recipients. Complete weaning of immunosuppressive drugs shorter after transplantation could possibly lead to more health benefits in tolerant liver transplant recipients. Hence, there is a need for a biomarker profile that accurately identifies tolerant liver transplant recipients earlier after transplantation.

In **Chapter 5** it was examined whether tolerant liver transplant recipients develop fibrosis in the donor liver graft after complete weaning of immunosuppressive drugs, by performing transient elastography and measurements of serum markers related to liver fibrosis. Transient elastography indicated that most adult tolerant liver transplant recipients developed no or at most mild fibrosis after on average 9 years of complete immunosuppressive drug weaning and 20 years after transplantation. Values of serum fibrosis related markers measured for tolerant liver transplant recipients were mostly within or close to values obtained for healthy individuals. Hence, we can conclude that most adult tolerant liver transplant recipients have minimal development of liver graft fibrosis long after transplantation and long after immunosuppressive drug weaning.

In **Chapter 6** numbers of circulating immune cell subsets, donor reactive T-cell subsets identified by expression of activation induced CD137, and presence of DSAs were assessed in tolerant liver transplant recipients and other matched study groups. Tolerant liver transplant recipients harbored lower numbers of alloreactive central memory (CM) and effector memory (EM) CD4+ T-cells and higher numbers of alloreactive terminally differentiated effector memory (EMRA) CD4+ T-cells compared to matched control liver transplant recipients on a regular immunosuppressive drug regimen. In addition, tolerant liver transplant recipients exhibited an elevated proportion of circulating activated T-helper cells (aTh) compared to control liver transplant recipients. Clustering analysis and principal component analysis revealed that the combination of these CD4+ T-cell characteristics allowed for an accurate discrimination of tolerant from control liver transplant recipients. In contrast, no significant differences in regulatory T-cells, alloreactive CD8+ T-cells or DSA formation were observed between the two groups.

In **Chapter 7** we investigated expression of tolerance-associated gene profiles in PBMCs suggested by previous studies, in our cohort of tolerant liver transplant recipients and other matched study groups. Unfortunately, we could not confirm their capacity to identify tolerant liver transplant recipients. Our results indicated that expression of the assessed genes were rather influenced by prior cytomegalovirus infection and/or use of immunosuppressive drugs, than indicative of operational tolerance.

Discussion and Future Perspectives

Rejection after liver transplantation

Antibody mediated rejection and C4d deposition

In 2016 the Banff working group introduced guidelines and consensus criteria for the diagnosis of liver allograft antibody mediated rejection.[30] Here, complement component 4d (C4d) tissue staining and interpretation are indicated. The criteria for establishing acute antibody mediated rejection in the donor liver are fairly strict. For diagnosis of definite antibody mediated rejection a histological pattern of injury with a diffuse microvascular C4d deposition should be visible in the liver graft biopsy, together with presence of DSAs in blood, and other diagnoses need to be excluded. However, it is technically difficult to unmask C4d deposition, and expression of C4d is often patchy in donor livers, which makes a confident histopathological diagnosis by pathologists on a liver biopsy challenging. Of note, in heart transplant recipients discrepancies were observed between C4d staining using immunofluorescence and immunohistochemistry,[179] hence the staining method used to detect C4d deposition could be of clinical significance. Moreover, antibody mediated rejection can also occur without activation of the complement system by antibody-dependent cell-mediated cytotoxicity of NK cells.[2] Therefore, presence of DSAs without a clear C4d tissue staining should be considered as a possible cause for severe (antibody mediated) rejection after liver transplantation in the clinic.

Avoiding graft loss in high risk recipients short after liver transplantation

Liver transplantation in general does not require matching of HLA between donor and recipient and less rigorous immunosuppressive drug regimens are often necessary to prevent or dampen severe forms of acute rejection, in contrast to lung, kidney and heart transplantation.[44,45] This is probably due to the immunologically privileged state of the liver that is needed to dampen immune responses towards antigens and micro-organisms transported from the intestine through the portal vein to the liver.[1] Nevertheless, the case report presented in this thesis indicated that combined presence of chronic rejection, possible antibody mediated rejection and T-cell mediated rejection after liver transplantation can be resistant to conventional treatment and could lead to detrimental outcomes. In the Netherlands there is a considerable shortage of donor organs, which results in an inevitable death among patients awaiting liver transplantation.[9] To prevent subsequent losses of liver grafts after re-transplantation, recipients at high risk for recurrent multiple forms of rejection should receive a HLA matched donor liver and a more rigorous induction immunosuppressive drug regimen as a primary treatment option at re-transplantation. Moreover, to avoid liver graft losses altogether, it should be investigated which patients are at high risk of developing multiple forms of rejection and subsequent loss of the primary donor liver graft short after transplantation. In the last few decades, human donor livers have been considered to be highly resistant against antibody mediated rejection caused by DSAs directed at donor HLA

molecules.[180] Recently however, studies not only indicated that preformed or de novo DSAs directed at donor HLA molecules in general are associated with detrimental outcomes in ABO-compatible liver transplantation,[168,181] but also higher mean fluorescent intensity (MFI) levels,[99] class II DSAs in general,[182] presence of DSA IgG3 subtypes,[183,184] and DSAs directed at non-HLA molecules in animal models.[185,186] In other solid organ transplantations, DSAs directed at non-HLA minor alloantigens are implicated in detrimental outcomes in recipients after transplantation.[187] The involvement of all DSAs in development of antibody mediated rejection or chronic rejection, especially in liver transplant recipients that develop (multiple forms of) rejection, should be investigated further.

Avoiding acute rejection in high risk recipients late after liver transplantation

In this thesis a pilot study is presented with a first proteomic screening of soluble immune system related serum markers in non-tolerant recipients late after liver transplantation. Graft rejection is a complicated process that involves different parts of the immune system. Therefore, it is highly unlikely that one immunological biomarker in serum could predict an upcoming late acute rejection. In our study, several serum proteins were already significantly different in non-tolerant liver transplant recipients long before a rejection episode became apparent through elevated liver function parameters. This suggests that rejection processes are already ongoing in these liver transplant recipients, or that these soluble serum proteins are indicative of recipients with a higher inherent sensitivity to graft rejection. Three of these serum proteins were already enough to predict an upcoming rejection episode in most non-tolerant liver transplant recipients. The results of the pilot study should be validated in a much larger homogeneous cohort of non-tolerant liver transplant recipients, and if possible in multiple transplantation centers. After validation, regular measurements of these proteins need to be implemented in the clinic, such that the liver transplant recipients at risk for development of rejection episodes can be identified and will be constrained from immunosuppressive drug reduction. The sensitive multiplex PEA technology used in this pilot study is rather expensive and time consuming. Therefore, alternative methods to measure these indicative proteins in the clinic should be investigated, such as ELISA or Luminex technology. The biological mechanisms and involvement of these serum proteins in the development of acute rejection late after liver transplantation are largely unknown. This pilot study provided us with a first glance of the early biological processes leading to acute rejection late after liver transplantation, and these mechanisms should be further investigated in the future.

Operational tolerance after liver transplantation

In this thesis clinical benefits of complete immunosuppressive drug weaning in tolerant liver transplant recipients late after transplantation were observed, but were rather limited unfortunately. With earlier immunosuppressive drug weaning, morbidity and mortality rates and costs for healthcare could be reduced further. To achieve this, one needs to know which liver transplant recipients are operationally tolerant towards their liver graft. The clinical

factors favoring immunosuppressive drug weaning and establishing tolerance are time after transplantation, higher age at time of transplantation and normal histology of the liver graft prior to complete weaning.[78] Nevertheless, these factors provide insufficient sensitivity and specificity to identify tolerant liver transplant recipients accurately.

Identification of circulating T-cells in operational tolerance

Many studies have tried to elucidate which biomarkers could identify tolerant liver transplant recipients. In these studies gene expression of the liver graft or peripheral blood mononuclear cells, or different subsets of circulating immune cells were investigated. In several of these studies an elevated proportion of circulating CD4+CD25+FoxP3+ T-cells was found in tolerant liver transplant recipients, which were regarded as regulatory T-cells (Tregs).[82,85,87] However, upon further delineation of these cells we found an elevated proportion of circulating activated T-helper cells (aTh) in tolerant liver transplant recipients compared to control liver transplant recipients on regular immunosuppressive drug regimen. In healthy and diseased individuals these CD4+ cells have been defined as cytokine-secreting nonsuppressive T-cells, characterized by low and transient expression of FoxP3 and absence of CD45RA.[7] In contrast, strongly suppressive activated Tregs are defined by high constitutive expression of FoxP3 and absent CD45RA, whereas resting Tregs are defined by constitutive expression of FoxP3 and CD45RA. In general, after antigen exposure T-cells either become activated and an immune response is induced, or T-cells are inactivated or eliminated and immunological tolerance against the antigen is induced. Immunological tolerance can be induced by prolonged systemic exposure to high doses of antigens and/or absence of appropriate co-stimulatory signals from APCs, after which the T-cells become anergic (functionally unresponsive), go into apoptosis, or differentiate into CD4+ Tregs.[2] The elevated proportion of aTh found in tolerant liver transplant recipients could therefore either be (donor-specific) anergic T-helper cells or an as of yet undefined special subset of Tregs induced in operational tolerance after liver transplantation. This specific subset of T-cells can be identified by studying the overall ability to respond towards donor antigens, the induction and type of cytokines produced, their possible suppressive function and demethylation of the *FOXP3* gene.[7,188] This needs to be investigated in the future.

Alloreactive T-cell subsets in operational tolerance

In this thesis it is observed that tolerant liver transplant recipients harbored less circulating alloreactive central memory (CM) and effector memory (EM) CD4+ T-cells, and more circulating alloreactive terminally differentiated effector memory (EMRA) CD4+ T-cells compared to control liver transplant recipients with regular immunosuppressive drug regimen. This might indicate that alloreactive CM and EM CD4+ T-cells are either deleted, anergic, senescent or inhibited by induced Tregs in tolerant liver transplant recipients. It could also indicate that there is a shift from CD4+ CM and EM alloreactive T-cells towards CD4+ EMRA alloreactive T-cells. Unfortunately, data on CD4+ EMRA T-cells is sparse, but it has been suggested that these T-cells resemble CD8+ EMRA T-cells. CD8+ EMRA T-cells have a poor

proliferative capacity, but display cytotoxic potential and can secrete multiple cytokines after activation.[164,165] Highly differentiated CD4+ T-cells were also associated with low proliferative alloreactivity in kidney transplantation.[95] Our data suggest that specific alloreactive T-helper subsets might be associated with natural occurring tolerance. Additional studies have to be performed to investigate the functionality of these alloreactive CD4+ EMRA T-cells in operational tolerance. In previous studies Tregs are held responsible for induction and maintenance of immune tolerance, and some studies are investigating the therapeutic potential of Treg therapies in tolerance induction after liver transplantation.[162] The interaction between alloreactive effector T-helper cells and Tregs in tolerant liver transplant recipients needs to be investigated further. The human immune system is a very complex network of biological processes. In addition, liver transplant recipients all over the globe are a very heterogeneous population with large differences in clinical and genetic parameters that influence the immune system. Therefore, it is highly likely that there is more than one way by which liver transplant recipients could develop spontaneous operational tolerance. This is supported by the notion that some recipients develop operational tolerance relatively short after transplantation (~3 years), whereas others develop operational tolerance later after transplantation.[49]

Development of operational tolerance

Speculation of one likely development of operational tolerance in liver transplant recipients is daring, but possible. One way operational tolerance might develop is through the semi-direct alloantigen recognition pathway short after transplantation. In this newly acknowledged recognition, donor leukocytes and other allograft cells release small extracellular vesicles that contain intact donor HLA molecules. These donor HLA molecules are then presented by recipient APCs, after which alloreactive recipient T-cells are usually activated.[31] This phenomenon is not only described in mice models,[21,22,24,26] but recently also for liver transplant recipients.[23,25] The liver is considered to be an immunologically privileged organ, since an (adaptive) immune response in the liver is often avoided to facilitate its function, e.g. to filter the portal vein.[1] In order to avoid an adaptive immune response in the liver, dendritic cells or Tregs need to avoid activation of effector helper T-cells, that occurs through production of inhibitory cytokines and/or cell-to-cell contact via inhibitory receptor-ligand interactions, such as PD1-PDL1 or CTLA-4-B7.[2] It is conceivable that an immunological privileged organ such as the liver, expresses more inhibitory ligands on their donor APCs or other cells. The acquired extracellular vesicles with HLA molecules from allograft cells by recipient APCs could therefore also contain ligands for inhibitory receptors, such as the recently investigated PDL1,[23] that could potentially inhibit activation of recipient T-cells directed at donor HLA molecules. This might lead to anergic or apoptotic alloreactive recipient T-cells, or induction of alloreactive recipient Tregs. A similar tolerance inducing semi-direct pathway has been described for maternal non-inherited antigens in a mice model, where extracellular vesicles released by maternal cells contain exosomal micro RNAs that mediate expression of inhibitory receptors on cells of the offspring.[189] The semi-direct alloantigen recognition after liver transplantation is transient and short-lived (1-4 days) when measured

in peripheral blood, but could be a continuous local phenomenon in the liver graft.[25] This semi-direct pathway with co-transfer of inhibitory molecules might lead to long-term operational tolerance towards the donor liver graft in at least some liver transplant recipients. This phenomenon needs to be extensively studied in the future.

Liver fibrosis in operational tolerance

Clinicians fear development of severe liver fibrosis and a potential subsequent loss of the liver graft after complete immunosuppressive drug weaning. In our small single-center study we concluded that most adult tolerant liver transplant recipients have no or at most minimal development of liver graft fibrosis long after transplantation and long after IS weaning. This is supported by other studies in pediatric or adult tolerant liver transplant recipients that did not show a higher liver fibrosis progression rate when compared to liver transplant recipients on regular immunosuppressive drug regimen.[49,55-57,62-64,131] Only one study suggested that pediatric tolerant liver transplant recipients weaned from immunosuppressive drugs are more prone to develop fibrosis in the liver graft compared to pediatric liver transplant recipients on regular immunosuppressive drug regimen.[61] In this study pediatric tolerant recipients were younger at the time of transplantation and biopsies were performed more than twice the time period after transplantation when compared to pediatric control recipients. It is known that there is a strong positive association between liver fibrosis progression and time after transplantation or younger age at transplantation in pediatric recipients, and could explain the discrepancy observed between this specific study and all other studies.[143-148] To date, a positive association between liver fibrosis progression and time after transplantation or age of the recipient at transplantation has not been reported for adult (tolerant) liver transplant recipients. Therefore, adult and pediatric liver transplant recipients should be regarded as distinct populations in studying operational tolerance, since adult and pediatric liver transplant recipients differ in their clinical course (late) after transplantation.

Subclinical rejection in operational tolerance

A few studies indicated that in up to 85% of the biopsies late after liver transplantation histological abnormalities were observed in stable liver transplant recipients.[150] This could indicate that subclinical rejection is present in most of the allografts, which is a mild form of rejection without elevating liver function parameters. Clinicians and researchers fear development of subclinical rejection after complete immunosuppressive drug weaning in tolerant liver transplant recipients. However, the clinical significance of subclinical rejection and whether it can result in graft damage is still unclear,[150,151] although one study suggests that subclinical rejection is benign in adult liver transplant recipients.[152] Subclinical rejection cannot be observed with regularly measured and monitored liver function parameters, hence many researchers and clinicians state that a liver biopsy is necessary to confirm the operational tolerant state in liver transplant recipients. Two studies indicated that subclinical rejection presented intermediate expression profiles of genes in the graft that were upregulated in T-cell mediated rejection.[33,190] This might indicate that subclinical rejection is

the preliminary stage of a full blown T-cell mediated rejection with elevated function parameters. Our proteomic pilot study in non-tolerant liver transplant recipients indicated that several serum proteins were already significantly different before elevated liver function parameters and T-cell mediated rejection were observed. This could indicate that these non-tolerant recipients experienced subclinical rejection at the time the serum proteins were measured. In this study our group of tolerant liver transplant recipients did not show any differences in serum proteins compared to stable groups of liver transplant recipients on regular or minimal immunosuppressive drug regimen, and healthy controls. When these three groups were examined using principal component analysis of the significantly different serum proteins found in non-tolerant recipients, a complete clustering overlap with the tolerant recipients was observed. This might indicate that in our group of tolerant liver transplant recipients subclinical rejection in the allograft was absent.

Validation of data in prospective immunosuppressive drug weaning trials

Clustering analysis and principal component analysis revealed that a combination of alloreactive CD4+ T-cell subset characteristics and the elevated circulating aTh compartment accurately discriminated tolerant from control liver transplant recipients. Nevertheless, these data have a need to be validated in a larger cohort of liver transplant recipients prospectively weaned from immunosuppressive drugs, preferably in multiple transplantation centers. In our study we compared a group of tolerant liver transplant recipients without immunosuppressive drugs and a group of control liver transplant recipients under regular immunosuppressive drugs. Our data on gene expression in peripheral blood of tolerant versus control liver transplant recipients indicated that immunosuppressive drugs have an effect on expression of the studied genes. This data is supported by the notion that in all previous published studies where gene expression was investigated in tolerant liver transplant recipients versus several control or non-tolerant groups either pre or post weaning, different gene profiles containing little common genes were found that identified tolerance.[80,82,84,87,130,169] This could suggest that the differences observed in (alloreactive) circulating T-cells of tolerant versus control recipients in our study have also possibly been influenced by the use of immunosuppressive drugs in control recipients. Therefore, our data needs to be validated primarily in a large prospectively weaned cohort of liver transplant recipients at the time point before (complete) immunosuppressive drug weaning. Our gene expression study indicated that weaning of immunosuppressive drugs to a minimal trough level slightly restores expression of the some of the studied genes in liver transplant recipients. Hence, some characteristics of operational tolerance (or acute rejection) might become visible with minimal immunosuppressive drug trough levels.

The pilot study with a first proteomic screening in non-tolerant recipients suggests that rejection processes are already ongoing before it is reflected in elevated liver function parameters, or that these soluble serum proteins are indicative of a higher inherent sensitivity to graft rejection. This might indicate that non-tolerant liver transplant recipients have an altered serum proteomic profile and immune system long before rejection when compared to a group of stable liver transplant recipients. In order to accurately identify tolerant liver

transplant recipients before immunosuppressive drug weaning in a large mixed cohort of liver transplant recipients, both control and non-tolerant liver transplant recipients need to be studied. Stable control liver transplant recipients should be considered as a separate, but equally important, group from non-tolerant liver transplant recipients. Tolerant liver transplant recipients might then be identified by the tolerance markers established in this thesis, or newly identified gene expression profiles or (alloreactive) circulating immune cells in peripheral blood. Even though matching of clinical parameters between study groups cannot be controlled during a prospective immunosuppressive drug weaning trial, this thesis has shown the importance of accurate matching of clinical parameters known to influence immune system related markers. For example, prior cytomegalovirus infection were associated with a higher Vδ1/Vδ2 γδT-cell ratio and a higher expression of certain genes in peripheral blood. Matching of age, sex, time after transplantation, primary disease and prior cytomegalovirus infection between study groups when determining tolerance-associated markers is recommended. A complete separate analysis of cytomegalovirus seropositive and seronegative recipients within each study group could be possible as well.

Requirements for prospective immunosuppressive drug weaning trials

First and foremost, to establish a successful immunosuppressive drug weaning program, a tight collaboration between eager and persevere clinicians and (basic) researchers is paramount. Several inclusion or exclusion selection criteria of adult liver transplant recipients need to established for a immunosuppressive drug weaning trial. Recipients with signs of primary sclerosing cholangitis and biliary cirrhosis, autoimmune hepatitis or other autoimmune disorders should be weaned with caution or excluded altogether. If the proteomic screening of serum from liver transplant recipients is validated and implemented in the clinic, recipients with a substantial risk for acute rejection can be largely excluded as well. Inclusion criteria could be recipients with stable liver function parameters, absence of rejection episodes for at least one year, and normal histology of the liver graft. Furthermore, time after transplantation favors immunological tolerance,[49] hence recipients >3 years after transplantation should be included exclusively.[54] Protocol biopsies should be performed pre weaning for determination of normal histology, and to exclude presence of (subclinical) rejection or liver fibrosis. Protocol biopsies should also be performed 1, 3, 5, and 10 years after complete immunosuppressive drug weaning or rejection, to monitor potential development of antibody mediated rejection, chronic rejection, subclinical rejection or liver fibrosis. During immunosuppressive drug weaning liver function parameters bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (AP) and γ-glutamyltransferase (γGT) should be regularly measured and monitored in order to detect an upcoming rejection episode. After significant elevation (>2.5 times baseline level) of one or more liver function parameters, a liver biopsy needs to be performed to diagnose the cause. In case of rejection, an appropriate rescue treatment or re-instatement of regular immunosuppressive drug regimen needs to administered. Significant elevation of γGT is the most specific and sensitive indication of acute rejection after immunosuppressive drug weaning.[150] During or after weaning, liver function parameters in tolerant liver transplant

recipients could be mildly elevated (<2.5 times baseline level), but this is often transient and returns to normal without therapeutic intervention.[150]

Improvements for research in general

Commonly, researchers try to explore new areas in their field of interest that were supposedly never investigated before. Therefore, negative results should also be published to make global research more efficient and accelerate scientific progress and development. Lower impact papers that contain negative results could contribute equally as positive results in higher impact papers to developing knowledge on a specific area of interest. Furthermore, new well-performed research that refutes previous published data should be regarded as valuable by editors and reviewers of scientific journals. In addition, for the significance of research in general, an unbiased approach towards new studies of highly motivated and talented researchers should be more important than other researchers' own interests, reputation or favoritism.

Appendices

Nederlandse Samenvatting

In dit proefschrift is mijn onderzoek naar afstoting en operationele tolerantie na levertransplantatie beschreven. **Hoofdstuk 1** is een algemene introductie over de lever, het immuunsysteem, levertransplantatie, immunosuppressieve medicatie, verschillende vormen van afstoting en hoe deze tot stand komen, operationele tolerantie en de ontwikkeling van leverfibrose, en de eerdere onderzoeken naar markers voor het identificeren van tolerante levertransplantatie patiënten.

In **Hoofdstuk 2** wordt een case report gepresenteerd, waarin we beschrijven hoe een jonge patiënt twee donor levers verloor door verschillende vormen van afstoting kort na twee opeenvolgende transplantaties. Beide keren traden er meerdere vormen van ernstige afstoting op en behandeling met verschillende immunosuppressieve medicaties liet geen verbetering van de situatie zien. In de literatuur stond niet beschreven hoe er gehandeld moet worden in dergelijke complexe situaties. Ter voorkoming van verlies van een derde donor lever, en mogelijk het leven van de patiënt, werd gebruikt gemaakt van een ander protocol voorafgaand aan de derde transplantatie. Er werd ingezet op een zo groot mogelijke overeenkomst van de HLA moleculen van de donor en ontvanger, iets waar normaal gesproken niet naar gekeken wordt bij levertransplantatie. Daarnaast werd er een meer rigoureuze immunosuppressieve inductie therapie gegeven dan bij de voorafgaande twee levertransplantaties. Na toepassing van deze maatregelen bij de derde transplantatie ontwikkelde de patiënt maar één type afstoting, die goed behandelbaar was. Dit resulteerde in een lange termijn overleving van de donorlever in de patiënt van tenminste de duur van onze studie (683 dagen).

In **Hoofdstuk 3** hebben we een onderzocht of er verschillen zijn in concentraties van eiwitten in het bloed van tolerante en niet-tolerante levertransplantatie patiënten in vergelijking met andere studiegroepen lang na transplantatie. Helaas is hierbij geen eiwitprofiel gevonden om tolerante patiënten te identificeren in een groot cohort van levertransplantatie patiënten. Wel hebben we vijftien significant verschillende concentraties van eiwitten gevonden in het bloed van niet-tolerante levertransplantatie patiënten, afgenomen op een tijdstip lang (4 maanden - 1.3 jaar) voordat verhoogde leverwaarden een indicatie voor afstoting gaven, in vergelijking met andere stabiele levertransplantatie groepen. Dit zou kunnen betekenen dat lang voordat een afstoting zichtbaar wordt, de eerste stappen van een afstoting zich al ontwikkelen, of dat patiënten die deze afwijkende concentraties van eiwitten in het bloed hebben gevoeliger zijn voor afstoting dan andere levertransplantatie patiënten.

In **Hoofdstuk 4** zijn de klinische voordelen van het volledig afbouwen van immunosuppressieve medicatie in tolerante levertransplantatie patiënten lang na transplantatie in kaart gebracht. Spijtig genoeg zagen we geen verbetering in nierfunctie of vermindering van diagnoses van diabetes, verhoogde bloeddruk, hart- en vaatziekten, of kanker in tolerante levertransplantatie patiënten in vergelijking met een controle groep van levertransplantatie patiënten. Wel zagen we een significante verlaging van het aantal (terugkerende) infecties en LDL waarden in het bloed na afbouw van immunosuppressieve

medicatie in tolerante levertransplantatie patiënten versus de controle groep van levertransplantatie patiënten met immunosuppressieve medicatie. Afbouw van immunosuppressieve medicatie korter na transplantatie zou kunnen leiden tot meer gezondheidsvoordelen in tolerante levertransplantatie patiënten. Om dit te bereiken moeten tolerante levertransplantatie patiënten accuraat geïdentificeerd kunnen worden in een grote groep van transplantatie patiënten.

In **Hoofdstuk 5** hebben we onderzocht of tolerante levertransplantatie patiënten leverfibrose ontwikkelen na volledige afbouw van immunosuppressieve medicatie. Fibroscan resultaten en bepaalde eiwitmetingen in het bloed wijzen op geen of weinig ontwikkeling van fibrose in de lever lang na volledige afbouw van de immunosuppressieve medicatie lang na transplantatie in tolerante patiënten. Dit betekent dat, in ieder geval met betrekking tot de ontwikkeling van leverfibrose, immunosuppressieve medicatie veilig kan worden afgebouwd in tolerante levertransplantatie patiënten.

In **Hoofdstuk 6** is onderzoek gedaan naar (donor-reactieve) circulerende T-cellen in het bloed van tolerante levertransplantatie patiënten en controle groepen. Bepaalde populaties van CD4+ T-cellen waren significant verhoogd (geactiveerde T-helper cellen en alloreactieve terminaal gedifferentieerde effector geheugen (EMRA) T-cellen) of verlaagd (alloreactieve centrale geheugen (CM) en effector geheugen (EM) T-cellen) in tolerante levertransplantatie patiënten in vergelijking met controle levertransplantatie patiënten met immunosuppressieve medicatie. Clustering analyse en principal component analyse toonden aan dat met behulp van deze CD4+ T-cel populaties tolerante levertransplantatie patiënten geïdentificeerd kunnen worden in een grote groep van levertransplantatie patiënten. Voor CD8+ T-cel populaties en ontwikkeling van donor-specifieke antilichamen zijn geen verschillen gevonden tussen de studie groepen. Deze resultaten kunnen wijzen op ontwikkeling van niet-alloreactieve anergische CD4+ helper T-cellen of een speciale subset van regulatoire T-cellen in tolerante levertransplantatie patiënten.

In **Hoofdstuk 7** hebben we geprobeerd om immuunsysteem gerelateerde gen profielen geassocieerd met tolerantie, zoals beschreven in eerdere studies, te valideren in ons cohort van tolerante levertransplantatie patiënten. Helaas bleek dat expressie van veel van deze genen beïnvloed werd door verschillende klinische en demografische parameters die bekend staan om het beïnvloeden van de samenstelling van de cellen het immuunsysteem, zoals het gebruik van immunosuppressieve medicatie en een eerdere infectie met het cytomegalovirus.

In **Hoofdstuk 8** wordt een uitgebreide discussie met toekomstperspectief van dit proefschrift beschreven. In de toekomst moet het eiwitprofiel gevonden in het bloed van niet-tolerante levertransplantatie patiënten, dat mogelijk voorspelt welke levertransplantatie patiënten een risico lopen op afstoting in de nabije toekomst, gevalideerd worden in een grotere groep levertransplantatie patiënten. Als dit het geval is kan het worden geïmplementeerd in de kliniek. Deze meting kan dan worden meegenomen tijdens de standaard poliklinische bloedafnamen. De markers ter identificatie van tolerante levertransplantatie patiënten die in dit proefschrift zijn gevonden moeten gevalideerd worden in een studie waarbij levertransplantatie patiënten prospectief worden afgebouwd van de immunosuppressieve

medicatie. Om de invloed van immunosuppressieve medicatie te minimaliseren en te onderzoeken of deze markers een voorspellende waarde hebben, moet er in tolerante levertransplantatie patiënten gekeken worden vóór volledige afbouw van de medicatie. Verder is het belangrijk om de tolerantie-markers te identificeren in studie groepen die in hun klinische en demografische parameters volledig op elkaar zijn afgestemd zodat deze een minimale invloed hebben.

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Publications

Duizendstra A.A., de Knegt R.J., Mancham S., Klepper M., Roelen D.L., Brand-Schaaf S.H., Boor P.P., Doukas M., de Man R.A., Sprengers D., Peppelenbosch M.P., Betjes M.G.H., Kwekkeboom J., Litjens N.H.R. (2021). Activated CD4+ T-cells and highly differentiated alloreactive CD4+ T-cells distinguish operationally tolerant liver transplant recipients. Liver Transplantation, Online ahead of print. DOI: 10.1002/lt.26188

Duizendstra A.A., de Knegt R.J., Betjes M.G.H., Coenen S., Darwish Murad S., de Man R.A., Metselaar H.J., Sprengers D., Litjens N.H.R., Kwekkeboom J. (2019). Immunosuppressive drug withdrawal late after liver transplantation improves the lipid profile and reduces infections. European Journal of Gastroenterology and Hepatology, 31(11), 1444-1451. DOI: 10.1097/MEG.0000000000001435

Duizendstra A.A., Doukas M., Betjes M.G.H., van den Bosch T.P.P, Darwish Murad S., Litjens N.H.R., Sprengers D., Kwekkeboom J. (2020). HLA matching and rabbit antithymocyte globulin as induction therapy to avoid multiple forms of rejection after a third liver transplantation. Clinics and Research in Hepatology and Gastroenterology, 2210-7401. DOI: 10.1016/j.clinre.2020.08.014

van der Heiden M., **Duizendstra A.**, Berbers G.A., Boots A.M., Buisman A.M. (2017). Tetanus toxoid carrier protein induced T-helper cell responses upon vaccination of middle-aged adults. Vaccine, 35(42), 5581-5588. DOI: 10.1016/j.vaccine.2017.08.056

PhD Portfolio

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Courses and Workshops	
Basic introduction on SPSS	2017
Annual Course on Molecular Medicine	2017
Workshop on Photoshop and Illustrator CS6	2017
Wetenschappelijke Integriteit voor PhD studenten	2017
Survival Analysis Course	2017
Biomedical English Writing Course for MSc and PhD-students	2018
Advanced course on Applications in flow cytometry	2018
Advanced Immunology	2018
Virology	2018
Workshop supervising students	2018
Workshop ‘People in Science’ for PhD candidates	2018
PhD day and Career Event	2018
RNA-seq workshop for beginners: from sequences to visualization using Galaxy	2019
Conferences	
Bootcongres Nederlandse Transplantatie vereniging	2017
Bootcongres Nederlandse Transplantatie vereniging	2018
BST18 Basic science in transplantation meeting	2018
ECI NVVI Fifth European Congress of Immunology	2018
Bootcongres Nederlandse Transplantatie vereniging	2019
Oral and Poster Presentations	
Oral presentation Bootcongres NTV	2018
Oral presentation 'Best Abstracts' Bootcongres NTV	2019
Oral presentation pitch/full presentation Bootcongres NTV	2019
Poster presentation European Congress of Immunology	2018
Poster presentation American Transplant Congress	2019
Scientific Grants	
Scholingsbeurs Nederlandse Transplantatie vereniging	2018
Gastrostart subsidie Nederlandse vereniging voor Gastro-enterologie	2019

Teaching activities

Supervision Internship MSc thesis Michelle van der Grift	2019

Seminars and Journal Club

Journal Club Department of Internal Medicine	2017-2021
Seminars Department of Gastroenterology and Hepatology	2016-2021
Seminars Department of Internal Medicine	2016-2021
Presentations Seminars and Journal Club	2017-2020
Weekly Research Group Meeting and Presentations	2016-2021

Curriculum Vitae

Aafke Duizendstra was born in Amsterdam on July 26th, 1989. During high school in Sittard, she completed VMBO in 2005, HAVO in 2007 and VWO in 2009, all with an economics background. During high school she developed a keen interest in biology, hence completed VWO with a nature and health background at ROC Leeuwenborgh Maastricht in 2010. Thereafter, she started a BSc Biology at the Wageningen University and Research Center. During this time she performed a BSc thesis under supervision of Dr. Geert Smant and Dr. José Lozano at the Department of Nematology at the Wageningen University. She finished her BSc degree with a specialization in Cell and Molecular Biology and a specialization in Human and Animal Health in 2014, after which she started a MSc Biology at Wageningen University. As a part of her MSc program she performed a thesis under supervision of Dr. Maria Forlenza at the Department of Cell Biology and Immunology at the Wageningen University. Here she investigated the interaction of Spring Viraemia Carp Virus and the Type I Interferon Response, with several different laboratory techniques e.g. cell culture, transfection, virus titration and rt-qPCR. She performed her final research internship under the supervision of Marieke van der Heiden and Dr. Annemarie Buisman at the National Institute for Public Health and the Environment (RIVM) in Bilthoven. Here she investigated the immune response after vaccination in middle-aged individuals with T-cell Elispot and flow cytometry, which resulted in her first publication. She finished her MSc degree, with a specialization in Health and Disease and an emphasis on Immunology and Virology, with honor in 2016. Thereafter, she started her PhD under supervision of Dr. Nicolle Litjens and Dr. Jaap Kwekkeboom as a joint project between Departments of Gastroenterology and Hepatology and Internal Medicine of the Erasmus University Medical Center in Rotterdam. The results of this PhD have been presented in this thesis. She attended a variety of courses through the Molecular Medicine Post Graduate School, supervised a MSc student, and visited and presented at several different conferences. During her PhD she also received a Gastrostart Grant from the Dutch Society of Gastroenterology (NVGE) to perform a pilot study on immune system related serum proteins in liver transplant recipients.

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- Toon Hermans -