Molecular mechanisms behind the liver-induced acceptance of renal grafts in highly sensitized patients

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Cover picture: Dendritic cell (blue) interacting with T cell (yellow). Science Photo Library / IBL Bildbyrå.

ABSTRACT

Preformed antibodies directed at donor HLA are considered an absolute contraindication for kidney transplantation, because of the high risk of rejection. Thus, patients with high levels of HLA antibodies have little chance of receiving a kidney transplant. Recently it was demonstrated that an auxiliary liver graft from the same donor may protect a subsequently grafted kidney from these harmful antibodies. The aim of this thesis was to elucidate the mechanisms behind the kidney protection afforded by the auxiliary liver graft in highly sensitized patients. We focused on the activation of dendritic cells, because these cells, which reside in all peripheral tissues, play a key role in the initiation of an immune response.

This thesis demonstrates that gene expression in the liver graft correlates with clinical outcome: In patients without an acute rejection episode, 14 out of 45 investigated immunological genes were significantly higher expressed in the liver graft 4h after reperfusion, compared with patients that experienced an acute rejection episode within the first month. This indicates that high- and low-risk patients can be identified within hours after transplantation.

One gene of particular interest was indoleamine 2,3-dioxygenase (IDO), which is a tolerance-inducing enzyme previously found to play a key role in maintenance of semi-allogeneic pregnancy in mice. In our study, mRNA levels of IDO were strongly upregulated in patients after combined auxiliary liver-kidney transplantation and IDO expression in the liver graft correlated with clinical outcome. Furthermore, IDO activity was higher in patients after combined auxiliary liver-kidney transplantation and liver transplantation compared with patients undergoing kidney transplantation. Strongly increased serum levels of the anti-inflammatory cytokine interleukin (IL) 10 were also found after liver but not kidney transplantation. IL-10 has several immune inhibitory effects on dendritic cells. We found that IL-10 inhibited the production of chemokines MIG, IP-10 and I-TAC in monocyte-derived dendritic cells in vitro. When comparing different cytokine cocktails for dendritic cell maturation, we showed that MIG, IP-10 and I-TAC were essential for dendritic cell-mediated recruitment of natural killer (NK) cells. This is considered important for the initiation of a type 1 T helper cell response. We also showed that IL-10 treated dendritic cells, which expressed less of these chemokines, had reduced potential to activate NK cells.

Thus, the liver provides IDO and IL-10, both of which have the ability to reduce the immunostimulatory ability of dendritic cells, giving them a tolerance-promoting profile. We therefore suggest that the protective effect of an auxiliary liver in presensitized patients may, at least in part, be mediated by the liver-specific expression of IDO and IL-10.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Post-ischemic inflammatory response in an auxiliary liver graft protects against renal graft rejection in highly sensitized patients
Ingelsten M, Karlsson-Parra A, Björnson Granqvist A, Olausson M, Haraldsson B and Nyström J.
Submitted

II. Is indoleamine 2,3-dioxygenase important for graft acceptance in highly sensitized patients after combined auxiliary liver-kidney transplantation?
Ingelsten M, Gustafsson K, Oltean M, Karlsson-Parra A, Olausson M, Haraldsson B and Nyström J.
Transplantation, 2009. 88:911-919.

III. Recruitment and activation of natural killer cells in vitro by a human dendritic cell vaccine
Gustafsson K, Ingelsten M, Bergqvist L, Nyström J, Andersson A and Karlsson-Parra A.
Cancer Research, 2008. 68(14):5965-71.

IV. Rapid increase of IL-10 plasma levels after combined auxiliary liver-kidney transplantation
Ingelsten M, Gustafsson K, Olausson M, Haraldsson B, Nyström J and Karlsson-Parra A.
Manuscript

ABBREVIATIONS

APC	antigen presenting cell
CD40L	CD40 ligand
CTL	cytotoxic T lymphocyte
DC	dendritic cell
EGR2	early growth response 2
GM-CSF	granulocyte-macrophage colony stimulating factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HLA	human leukocyte antigen
HMGB1	high-mobility group box 1
HSP	heat shock protein
ICAM	intercellular adhesion molecule
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
IP-10	interferon-inducible protein 10
I-TAC	interferon-inducible T cell alpha chemoattractant
KIR	killer cell immunoglobulin-like receptor
MHC	major histocompatibility complex
MIG	monokine induced by interferon-γ
MLR	mixed leukocyte reaction
mRNA	messenger RNA
NFκB	nuclear factor of kappa light polypeptide gene enhancer in B cells
NFKBIA	NFκB inhibitor A
NK cell	natural killer cell
NKT cell	natural killer T cell
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PGE2	prostaglandin E2
Poly I:C	polyinosinic:polycytidylic acid
PRR	pattern recognition receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
TDO	tryptophan 2,3-dioxygenase
TGF	transforming growth factor
Th cell	T helper cell
Treg	regulatory T cell
TLR	toll-like receptor
TNF	tumor necrosis factor

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INTRODUCTION

RENAL TRANSPLANTATION

Every year around 1100 individuals in Sweden develop end-stage renal disease, often caused by diabetes mellitus or glomerulonephritis.[1] Approximately 8000 patients are under active uremic care today (45% in dialysis and 55% transplanted), and the numbers are growing.[1]

There are two forms of dialysis, peritoneal dialysis and hemodialysis. Both forms require continuous treatment either daily at home, or in the hospital three times a week, which greatly affects the individual's life and freedom. Hemodialysis is the most common form of dialysis. The normal continuous renal function is replaced by three treatments every week, where the patient's blood is filtered to remove waste products and excess fluid. The body is subjected to large variations in fluid balance with rapid loss of body weight after dialysis, orthostatic symptoms are also common. Dialysis is a life saving treatment; still it only corresponds to 5-15% of the normal renal function.[2] Consequently, some of the uremic symptoms often remains, e.g. fatigue, loss of appetite and itch. In young patients, dialysis can be a long term treatment, although it increases the risk for cardiovascular disease.[3] In elderly patients the expected survival is only a few years.[4]

Compared to dialysis a renal transplantation is advantageous in several aspects; from an economical viewpoint the cost for a renal transplantation and short term follow up are comparable to six months of dialysis. Thereafter the yearly cost for dialysis is about five-fold higher than the treatment given after transplantation.[5] Transplantation is also preferable in means of quality of life, and many transplanted patients feel their life has been given back to them.

A renal transplantation can be performed with a living or a deceased donor. The living donor is usually a close relative to the recipient, e.g. a parent or sibling. This is an advantage, as it increases the chance of genetic matching of the donor and recipient, thereby reducing the risk for rejection.[6, 7] Moreover, a graft from a living donor is in better condition than a graft from a dead donor, leading to improved graft function.[8]

The first successful renal transplantation was performed between identical twins in Boston in 1954. In Sweden, 12 000 kidney transplantations have been performed since the start in 1964.[9] Now, approximately 400 kidney patients are transplanted every year, one third with living donors.[9] The outcome has improved over the years, thanks to better immunosuppressive medication, improved diagnosis and treatment of rejections. Thus over 90% of the renal grafts are functional one year after transplantation today.[10] Still, chronic rejection, which may occur even after 10-20 years of stable graft function, remains a problem, with a slowly progressing loss of kidney function. After a graft loss the patient is referred to dialysis, and may be put back on the waiting list for a new transplantation. A patient can be

transplanted successfully several times. However, a transplant is recognized as an intruder by the recipient's immune system, due to the expression of donor tissue type antigens (human leukocyte antigens/HLA) on the graft surface, and there is a risk for developing antibodies against the donor HLA. Thus, with every graft loss the immune response of the recipient may be broadened to include even more different tissue types, making immunological matching increasingly difficult.

COMBINED AUXILIARY LIVER AND KIDNEY TRANSPLANTATION

A renal transplantation performed in the presence of antibodies directed against donor HLA may cause a hyperacute rejection when circulating antibodies in the patient immediately attack the foreign tissue.[11] HLA antibodies may have been induced by previous transplantations, blood transfusions and pregnancies.[12] For patients with high levels of HLA antibodies, the chance of finding a compatible kidney for transplantation is very small, and lifelong dialysis may be the only option. Attempts have been made to remove antibodies before transplantation.[13-17] However the efficacy of this treatment is variable, especially when it comes to highly sensitized patients (panel reactive antibodies > 70%) waiting for a non-living donation.

Liver grafts are more resistant to rejection than other solid organs and a liver graft can even be transplanted against a positive cross-match without risk of hyperacute rejection.[18-21] When transplanting both a liver and another organ simultaneously, it seems that the liver is able to protect the accompanying organs from the same donor from rejection in both clinical and experimental transplantation settings.[20, 22-29] Still, the exact mechanisms behind this phenomenon are unclear.

Encouraged by these findings, Olausson and colleagues demonstrated that a partial auxiliary liver graft can protect a subsequent kidney graft from the same donor against preformed lymphocytotoxic antibodies. [30] This suggested a new treatment option, with promising long term function, in highly sensitized patients waiting for a kidney transplant. The auxiliary liver-kidney transplantation procedure consists of transplantation of a partial liver graft, followed by kidney transplantation. The recipient undergoes a hepatectomy, removing the left lateral two segments, but leaving segments IV-VIII of the native liver untouched. The auxiliary liver graft, comprising the left lateral two segments, is then transplanted orthotopically, i.e. placed in the space created by the hepatectomy. After completion of the liver transplant procedure, a kidney graft from the same donor is transplanted in the groin. Kidney graft reperfusion takes place approximately 3 hours after auxiliary liver graft reperfusion.

So far, 23 highly sensitized patients with end-stage renal disease have received a kidney through combined auxiliary liver-kidney transplantation. 70% of the kidney grafts show excellent function today, with a follow up of 0.5-8 years – a truly remarkable outcome in such a high risk patient group.

THE IMMUNE SYSTEM

Innate and adaptive immunity

The immune system is a complicated network of biological processes that acts to defend the body against invading pathogens, cancer and for the maintenance of self-tolerance, thereby preventing autoimmune disorders.

It is divided into two phases; the fast acting innate immunity and the acquired adaptive immune response. The innate response is the first line defense system consisting of physical and chemical barriers (e.g. skin, mucus membranes and pH) and fast-reacting inflammatory mediators.[31] The inflammatory part of the innate immune system is triggered through pattern recognition receptors (PPRs)[32] by recognizing components that are conserved in wide ranges of invading microbes, so called pathogen-associated molecular patterns (PAMPs), or by exposure of cell fragments from injured or stressed cells.[33] This leads to activation of phagocytic cells and their subsequent production of pro-inflammatory cytokines. Leukocytes of the innate immune system include phagocytes (macrophages, neutrophils and dendritic cells), mast cells, eosinophils and natural killer cells (NK). Dendritic cells (DC) link the innate immune system to the adaptive system by migration to the secondary lymphoid organs, where they are able to activate T cells.[34] The activated T cells are thereafter able to attack the hostile invaders in an antigen specific manner, as discussed below.

Dendritic cells

DCs are the most important antigen-presenting cells (APC) in the immune system, compared to other APCs such as macrophages and B cells, since they are superior in activating naïve T cells that have not previously encountered any antigen. Thus, they play a key role in the induction of adaptive immune responses. DCs are a very heterogeneous cell population; they can be derived from bone marrow hematopoietic stems cell or circulating blood precursors. DCs are divided into several subsets based on their developmental origin and biological properties.[35] The division is not always clear, and the plasticity of DCs is large. In blood, there are plasmacytoid and myeloid DCs, which are characterized as immature and precursor DCs respectively. In addition, blood monocytes are able to differentiate into a myeloid like DC-type. DCs are found in most peripheral tissues, e.g. as Langerhans cells in the skin and interstitial DCs, and also in the lymphoid organs.

DC maturation

Immature DCs reside in the periphery where they efficiently internalize exogenous antigens, but have a low T cell stimulatory ability.[36, 37] During inflammation they undergo maturation, which increases their capacity to activate T cells (Figure 1).[38] Maturation of DCs is induced by environmental factors, such as proinflammatory cytokines e.g. granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL) 1β, tumor necrosis factor (TNF) α, but also bacterial or viral

components.[35] The latter are recognized by PRRs expressed on DCs. One important group of PRRs is the Toll-like receptors (TLR). These are transmembrane proteins that scan the extracellular environment for danger signals, such as PAMPs on microbes. TLRs can also be activated by endogenous ligands that are released from damaged cells.[39]

DC maturation induces several phenotypical changes in order to increase the T cell stimulatory ability, including a reduction in endocytic ability and upregulated expression of antigen-loaded MHC molecules and co-stimulatory molecules (e.g. CD80, CD86).[36, 37] It also increases the expression of chemokine receptor CCR7, which facilitates DC migration to the lymphoid organs in response to CCR7-ligands CCL19 and CCL21.[36, 37]

Immune regulation by dendritic cells

Mature DCs migrate to the lymphoid organs, where T cells are located. If a DC encounters a T cell with a T cell receptor specific for the antigen presented by the DC, the cells will form an immunological synapse, in which several activating signaling pathways are involved.[40-42] According to the two-signal model, postulated in 1970, activation of the T cell receptor by antigen presentation constitutes the first signal, but a second, co-stimulatory signal from the antigen presenting cells is needed for proper activation of the T cell.[43] Co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) on the DC, which bind to CD28 on the T cell, are the most important of these molecules. In this phase the immunogenic dendritic cells can direct the T cell response into the T helper 1 or T helper 2 pathway (discussed below), depending on the inflammatory stimuli in which the DC was maturated.[44]

It has become increasingly clear that DC ability to regulate an immune response depends on their origin, stage of maturation and tissue localization.[45] In the steady state, in the absence of inflammation, immature DCs continuously migrate from the periphery to the lymphoid organs, where they encounter T cells. The absence or low levels of co-stimulatory molecules expressed on immature DCs (lack of "signal two") make them unable to activate naïve T cells, leading to anergy or apoptosis of the antigen specific T cells.[46] Thus dendritic cells are not only the main instigators of an immune response, they also play a crucial role in the maintenance of peripheral T-cell tolerance.[47]

Major histocompatibility complex (MHC)

Histocompatibility antigens, in the human called human leukocyte antigens (HLA), are glycoproteins expressed at the surface of all nucleated cells. They function as antigen-presenting proteins and play a major role in the immune function. This is accomplished by displaying peptide fragments derived from proteins degraded within the cell to T-cells of the adaptive immune system.[48] The extremely high gene polymorphism that exists in the MHC alleles results in high diversity between individuals, making the chance of finding two unrelated individuals with the same HLA very low.[49] There are two forms of MHC-molecules, MHC I, which is

expressed on the surface of all nucleated cells, and MHC II, which is expressed on antigen presenting cells.

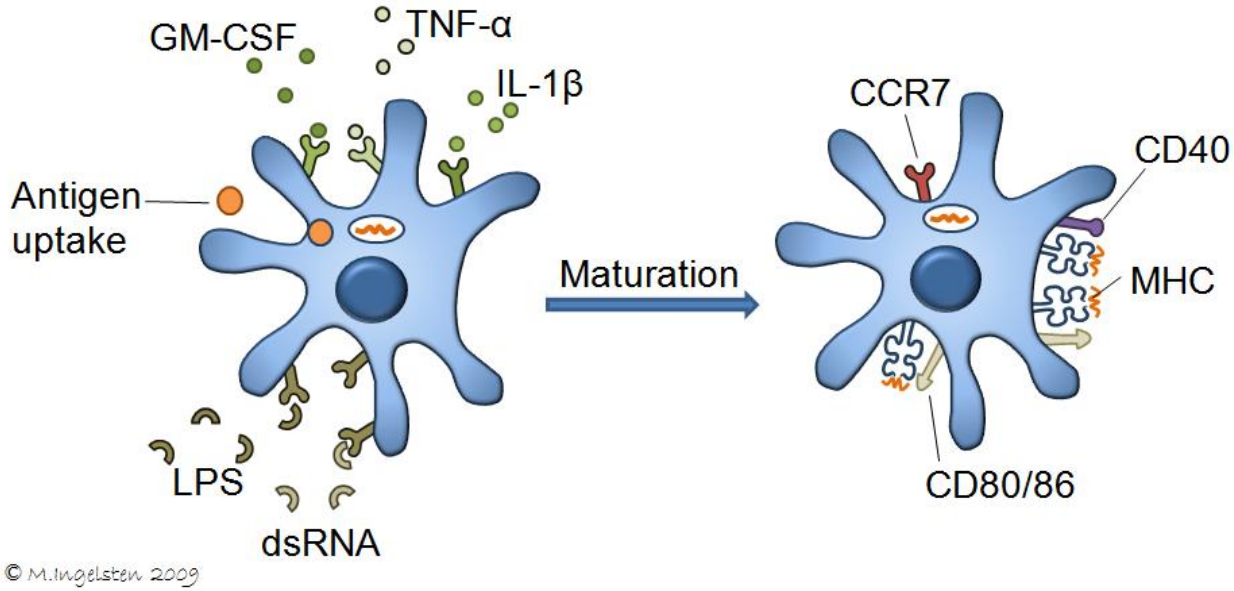

Figure 1. Dendritic cell maturation.

T cells

Approximately 75% of the blood lymphoid cells are T cells. T cells recognize foreign antigens expressed on MHC molecules via their T cell receptor. They originate from hematopoietic stem cells in the bone marrow that migrate to thymus to mature. During the maturation process the T cell receptor α- and β-chains are rearranged to a unique variant for each T cell, leading to a large variation in antigen specificity. Also, the cells start to express either the surface marker CD4 or CD8. In the periphery, CD4+ T cells recognize MHC class II molecules expressed on antigen presenting cells, whereas CD8+ T cells recognize antigens in the context of MHC class I molecules expressed on all nucleated cell types. When a naïve T cell, which has previously not encountered its specific antigen, is activated in the right context, it will start proliferate and become an effector cell (figure 2). CD8+ cells become cytotoxic T lymphocytes, whereas CD4+ cells become T helper cells.[50, 51] For a naïve T cell, mere antigen presentation is not enough for activation to occur. The process is tightly regulated, and only professional antigen presenting cells (e.g. DC) are able to provide the signals needed for efficient activation.

T helper cells

The main function for T helper (Th) cells is to activate and direct other immune cells by the release of cytokines. They contribute to the activation of cytotoxic T cells and macrophages and induce antibody class switch in B cells. Once the two-signal activation of a Th-cell is completed, it starts to express a functional IL-2 receptor and starts producing IL-2, a potent T cell growth factor. IL-2 acts in an autocrine and paracrine manner to induce proliferation of the T cells. After several

rounds of proliferation the Th cells will start to differentiate into Th1 or Th2 cells, depending on the cytokine environment. Th1 cells drive the cellular immunity and typically secrete interferon (IFN) γ, TNF-α and IL-2, whereas Th2 cells are important for humoral immunity and produce IL-4, IL-5, IL-6 and IL-10.[52]

Cytotoxic T lymphocytes

CD8+ T lymphocytes are activated by antigen presenting cells in a similar way as the T helper cells. But for a DC to efficiently activate a naïve CD8+ T cell, it must first interact with an antigen-specific Th cell.[53] After activation CD8+ T cells start to proliferate and become effector cells, also called cytotoxic T lymphocytes (CTL). These cells leave the lymphoid organ and start to circulate in the periphery, searching for infected cells to kill. Killing is executed by the release of granules filled with the cytotoxins perforin and granzyme B or by the expression of Fas-ligands, inducing apoptosis of the target cell.

Regulatory T cells

Regulatory T cells are a special subset of T cells that act to suppress the activation of the immune system. They downregulate the immune response after elimination of pathogens and they also repress potential self-reactive T cells to avoid autoimmunity. There are several subtypes of regulatory T cells, including natural Foxp3+ regulatory T cells (Foxp3+ Treg) and inducible types, such as Tr1 cells. Several publications indicate that Treg play a key role in the induction of transplantation tolerance.[54, 55]

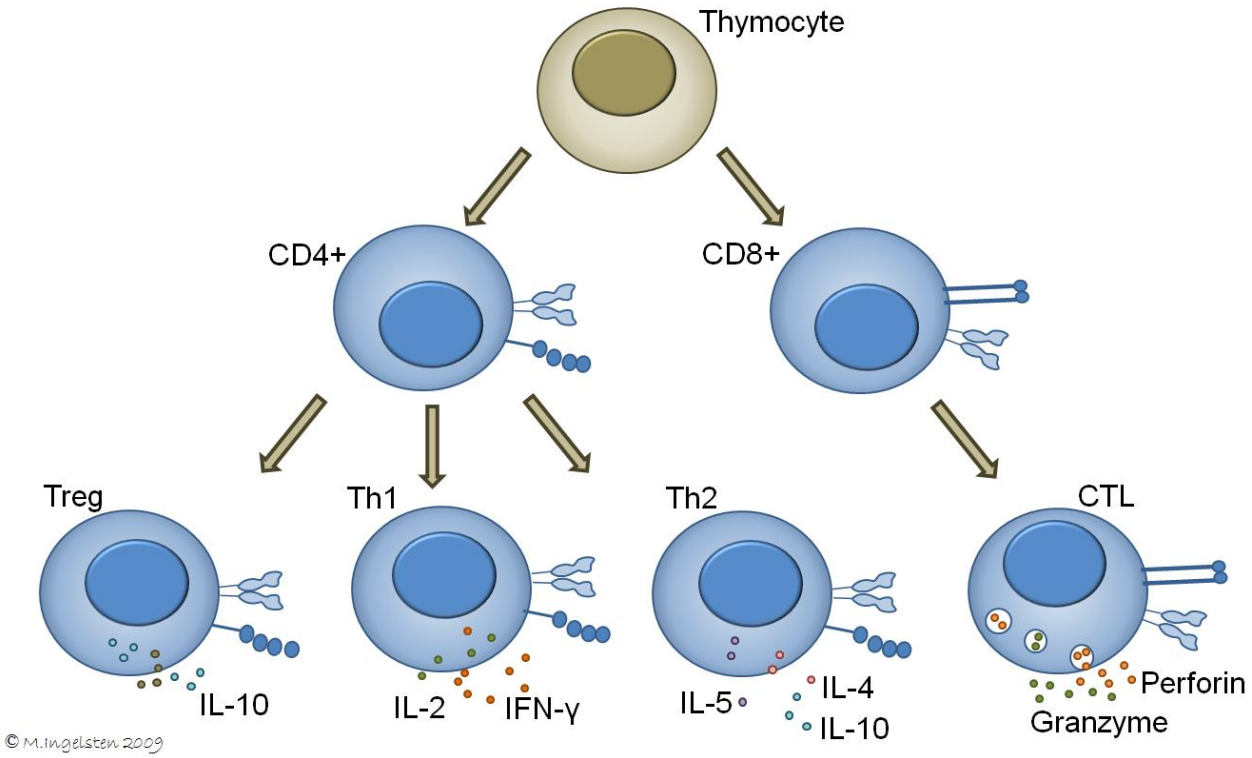

Figure 2. T cell development.

B cells

In blood, about 12% of the lymphocytes are B cells. Similar to T cells, B cells receive a unique antigen-specific B cell receptor during maturation through the process of rearrangement. When a B cell binds to its antigen, the B cell receptor is internalized and the antigen is degraded. These antigen-derived peptides bind to MHC II molecules and are presented on the cell surface. Thereby the B cell is able to interact with T cells with the same antigen specificity, which leads to full B cell activation.[56] Activated B cells proliferate and differentiate into antibody producing plasma cells.

Natural killer cells

NK cells constitute approximately 12% of lymphocytes in circulation. They are part of the innate immune system as they are not activated in an antigen specific manner. NK cells recognize target cells by their lack of MHC I molecules.[57] To avoid recognition by T cells, some tumors and virus infected cells downregulate the expression of MHC I. These cells may instead be recognized by NK cells. NK cells express several activating and inhibitory receptors, and the balance between inhibitory and activating signaling pathways determine the activation of NK cells.[58] Activating receptors are e.g. NKG2D and natural cytotoxicity receptors that recognize cellular stress-, infection- and transformation-induced molecules on the surface of target cells. Inhibitory receptors are e.g. killer cell Ig-like receptors (KIR) and C-lectin-type heterodimeric NKG2A receptor, which recognize MHC I molecules on the target cells.[59] When activated, NK cells are cytotoxic, and function in similar ways as cytotoxic T cells.

However, recent publications indicate that NK cells require additional activation for efficient priming.[60, 61] A stimulatory cross-talk between NK cells and mature DCs has been found in the lymphoid organs, where mature DCs stimulate NK cell cytotoxicity and NK cells aid DC maturation.[62-64] Moreover, NK cells may also aid DCs in the activation of T cells by providing IFN-γ that leads to Th1-deviation of naïve T cells.[65]

IMMUNE RESPONSE AFTER TRANSPLANTATION

Unspecific activation of the immune system

The surgical trauma and ischemia/reperfusion injury that is inevitable after solid organ transplantation leads to an activation of the innate immune system. This activation is independent of antigens and mediated by signaling via pattern recognition receptors, such as toll-like receptors.[66] TLRs are found on both parenchymal and non-parenchymal cells within the graft.[67] Several publications have demonstrated the importance of TLR4 in mediating reperfusion injury after solid organ transplantation.[68-70] Hypoxia has been reported to induce the release of high mobility group box-1 (HMGB1), an endogenous ligand for TLR4, suggesting a role for HMGB1 in the early events in ischemia/reperfusion injury.[70] Stimulation

of TLRs leads to immediate activation of a cascade of signaling events, involving the activation of transcription factors nuclear factor of kappa light polypeptide gene enhancer in B cells (NFκB) and activator protein-1, and the subsequent production of proinflammatory cytokines and chemokines as well as activation of passenger antigen-presenting cells.[71-73] The activation of macrophages and neutrophils leads to their engulfment of cell debris and release of large amounts of reactive oxygen species that further damage the graft.[74] The ischemia/reperfusion injury is initiated in a non-antigen specific way, and is similar in both allogeneic grafts (with a foreign tissue type) and syngeneic grafts (with the same tissue type)[75], but has major effect on allograft survival as it activates the adaptive immune response.

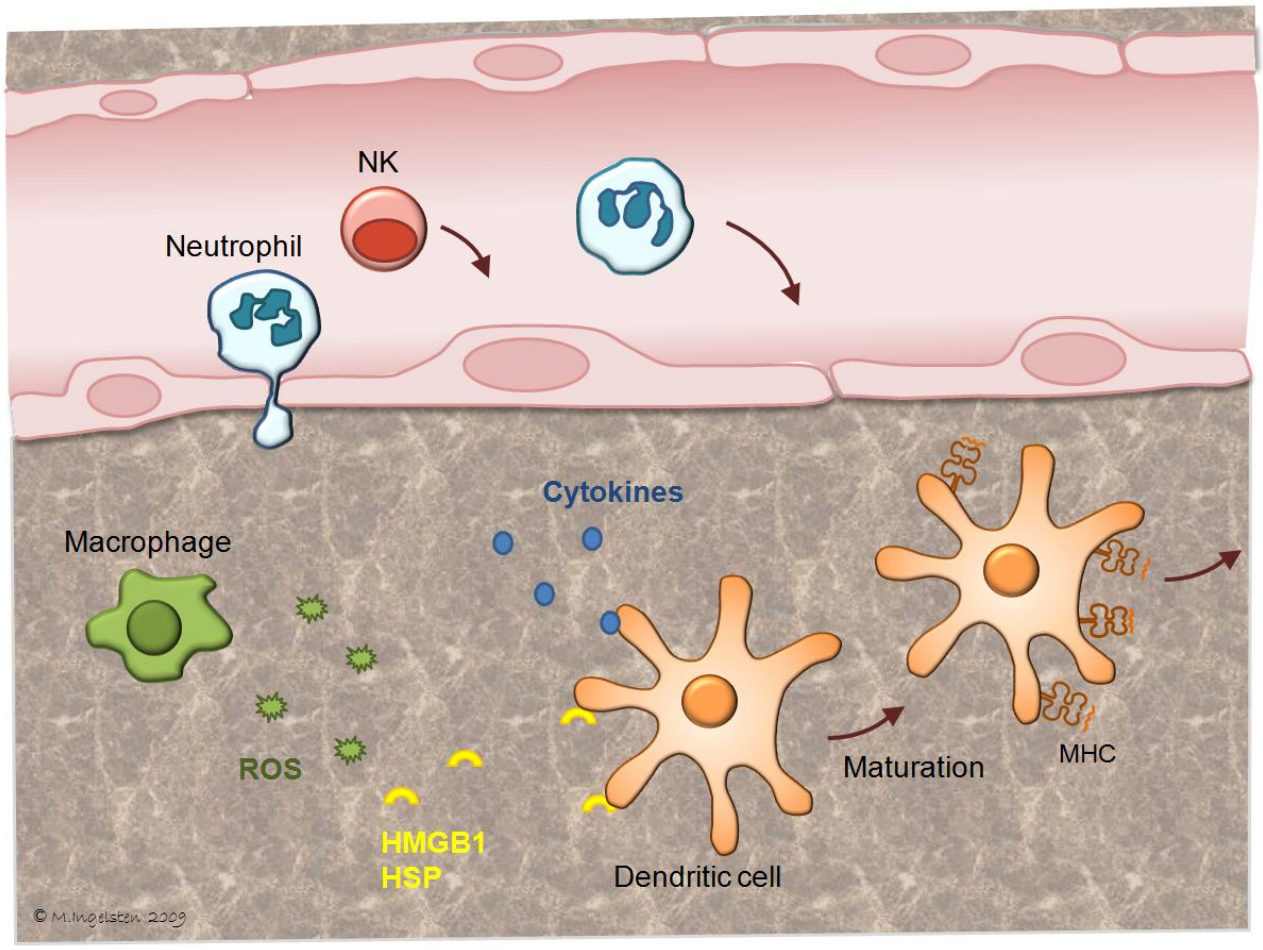

Figure 3. Initiation of an immune response after ischemia/reperfusion. Danger signals such as HMGB1 and heat shock proteins (HSP) are released due to surgical trauma and ischemia, leading to activation of leukocytes and endothelium. Activated endothelial cells, macrophages and neutrophils produce reactive oxygen species (ROS) and cytokines (e.g. TNF-α, IL-6, IFN, IL-1), which amplify the inflammatory environment and further maturate passenger dendritic cells. These latter migrate to the secondary lymphoid organs where they may initiate an allospecific immune response.

Antigen specific immunity in transplantation

The initial inflammatory process that takes place following transplant surgery is sufficient to initiate maturation and migration of graft-resident donor DCs (so

called passenger leukocytes) within the graft.[76, 77] These cells migrate to the secondary lymphoid organs in the recipient. Once in the T cell areas of the lymphoid organ they may activate naïve CD4+ and CD8+ T cells that recognize the foreign MHC molecules.[78] This mechanism, in which donor passenger cells expressing intact donor MHC is recognized by the recipient T cells, is known as the direct pathway of allorecognition (figure 4). It is responsible for a very strong proliferation of responder T cells due to the high proportion of T cells that recognize allogeneic MHC molecules. It is also an important initiator of acute rejection, as mentioned below.

A T cell response may also be initiated by the indirect pathway, where donor MHC molecules are degraded by the recipients own APCs and "non-self" peptides from the hypervariable MHC region are displayed in the context of self MHC (figure 4). Despite the name, this is the normal pathway for T cell stimulation by antigen presenting cells that occurs, e.g. after infection. The indirect pathway of allorecognition may occur after transplantation when the recipients own dendritic cells infiltrate the graft, acquire donor alloantigens from soluble MHC or fragments from apoptotic or necrotic cells, and migrate to the lymphoid organs where they activate T cells. While the donor antigen-presenting cells within a transplanted organ are depleted over time, reducing the impact of the directly activated immune response, the indirect pathway of allorecognition remains important, as the graft is continuously exposed to recipient APCs.[79] The indirect pathway has been considered mainly an initiator of late acute rejection and chronic graft rejection. For a proper B cell activation and antibody production to occur, indirectly activated Th cells are needed. Thus, the presence of HLA specific antibodies in sensitized patients indicates that the patients have been sensitized via the indirect pathway of allorecognition.[80]

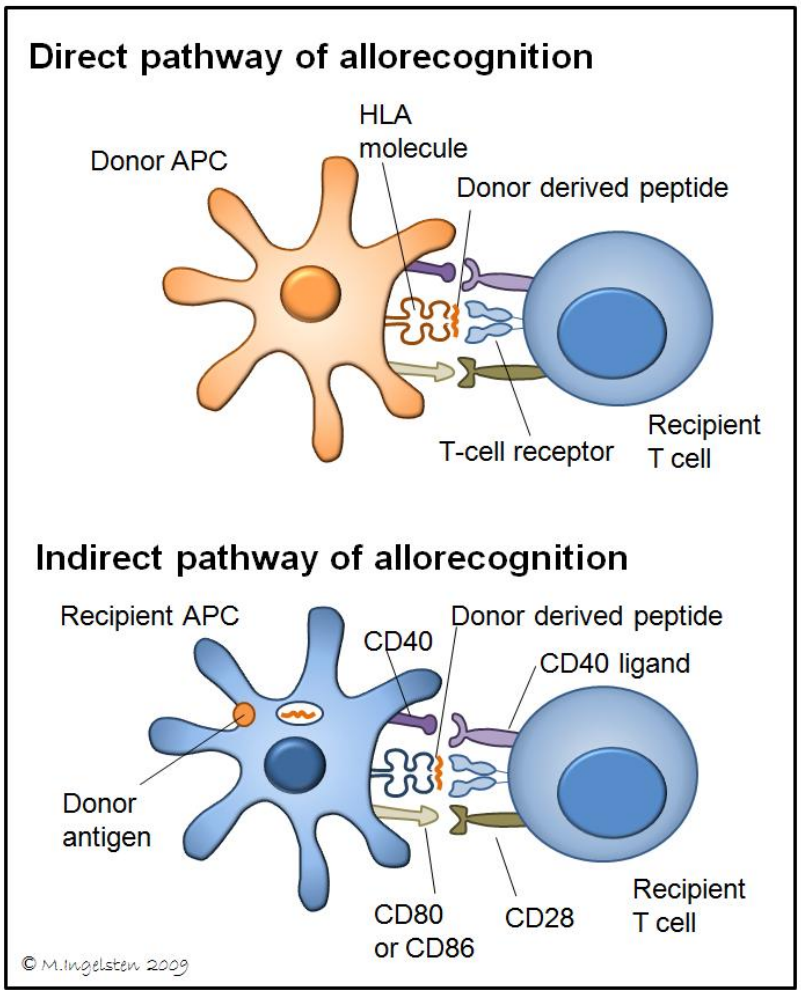

Figure 4.

Immunosuppression

Suppression of the immune system is essential for a successful transplantation. The need for immunosuppression is greatest the first months after transplantation, when passenger leukocytes that may stimulate an immune response are numerous and both direct and indirect presentation of alloantigens occur. Today's immunosuppressive medication acts in a non-specific manner, so that not only the rejection of the graft, but also the ability to generate an immune response against invading pathogens is impaired.

The immunosuppressive treatment may be divided into induction therapy, which is applied during the first weeks after transplantation, maintenance therapy that is used continuously throughout the graft life and rejection therapy that is started at the onset of rejections. Detailed discussions on immunosuppressive medications are beyond the scope of this thesis, but some of the major drugs are mentioned below.

Calcineurin inhibitors (Cyclosporin A, Takrolimus) revolutionized immuno-suppressive therapy in the seventies.[81] Calcineurin mediates the transcription signal for cytokine genes such as IL-2 and IFN-γ in T cells after stimulation.[82] Thus, calcineurine inhibitors suppress the activation and proliferation of lymphocytes.

Corticosteroids (Prednisolon, Methylprednisolon) have both anti-inflammatory and immunosuppressive effects.[83] The mechanisms are still unclear, but include inhibition of arachidonic acid metabolism and antigen-presentation of DCs. Steroids also reduce cytokine transcription and thereby inhibit lymphocyte activation.

Proliferation inhibitors (Mycophenolate mofetil) inhibit the enzyme inosine monophosphate dehydrogenase which is essential for synthesis of the nucleotide guanosine in lymphocytes.[84] In contrast, most other cell types can use alternative pathways for purine-synthesis. Thus, the anti-proliferative effect of mycophenolate mofetil is mainly directed to lymphocytes.

Antibodies (Basiliximab) directed against the IL-2 receptor α-chain (CD25). CD25 is expressed on T cells after activation, and crucial for T cell proliferation.[83] The immunosuppressive effect of Basiliximab is mediated by inhibition of IL-2 signaling in T cells.

Rejection

Hyperacute rejection

If the recipient has preformed antibodies directed towards donor endothelium a hyperacute rejection may occur.[11] The circulating antibodies bind within minutes to the graft endothelium, leading to the activation of coagulation cascades, causing destruction of the endothelial layers and occlusion of the blood vessels in the grafted organ. The process is instant, and the graft may be destroyed a few minutes to several hours postreperfusion. Antibodies causing hyperacute rejection are commonly directed against the ABO-antigens of the blood group system or HLA

class I antigens. ABO-antibodies are natural, preformed antibodies, whereas HLA antibodies have arisen from previous alloimmunizations, e.g. blood transfusions, previous transplantations and pregnancies. Hyperacute rejections are avoided by testing for any potential cross-match before transplantation. This can be done by FACS-analysis or by incubating serum from the recipient with HLA-expressing cells from the donor (B and T cells) together with complement and check for lysis of the donor cells.

Acute rejection

Acute rejection usually begins after the first week of transplantation, and the highest risk is during the first three months. The main players are T cells that are commonly activated by donor passenger DCs, as described above.[85] Graft infiltrating cells also include macrophages, NK cells, B cells and other leucocytes, such as eosinophilic granulocytes, which all cause destruction of the graft. Early acute rejection is correlated also to the presence of donor specific antibodies.[86] The main effector mechanisms in acute rejection are 1) cytotoxic CD8+ T cells, that bind allogeneic cells and causes cell death by perforin-granzyme B, 2) the presence of CD4+ T helper cells that produce pro-inflammatory cytokines and induce cell damage via activation of macrophages and 3) antibody-mediated destruction.[85]

Chronic rejection

Chronic rejection is a slowly progressing deterioration of graft function. Vascular inflammation and fibrosis are common constituents. The mechanisms are thought to be both immunological and non-immunological. Chronic rejection is associated with infiltration of mononuclear cells, mainly T cells and macrophages. Macrophages provide profibrotic factors and extra cellular matrix proteins, leading to the initiation of fibrosis.[87] Activation of T cells through the indirect pathway of allorecognition and development of antibodies directed against donor HLA might influence the process.[80, 85] Despite the improvements in immunosuppression in later years, the prevalence of chronic rejection has not been appreciably reduced.

The liver – a special case in transplantation

The liver is unique when it comes to transplantation, as it is resistant towards preformed graft-specific antibodies. A positive cross-match is therefore not considered a contraindication in liver transplantation.[6] A liver graft is even spontaneously tolerated across totally MHC incompatible barriers in a number of species.[18, 19, 88, 89] The liver is also able to prevent rejection of other organs transplanted from the same donor.[19, 20, 25] In some of these studies, subsequent transplants from the same donor were accepted, whereas third-party grafts were rejected, confirming induction of donor-specific tolerance.[24, 88-90] Also, similar to an immunosuppressive drug, a liver transplantation could reverse an ongoing rejection of a previously transplanted graft from the same donor strain.[23, 89, 91, 92] The quest for understanding the mechanisms behind the spontaneous induction of liver

tolerance has been a major goal of transplant immunology research ever since, as this knowledge could be exploited to greatly reduce the rate of rejection in clinical transplantation.

Several different mechanisms have been proposed for successful liver transplantation against a positive cross-match; including the unique architecture of the liver [29], the ability of the liver to absorb antibodies and shedding of soluble HLA which leads to removal of donor-specific antibodies by formation of immune complexes.[18, 93, 94] Induction of donor-specific suppressor cells (e.g. regulatory T cells) [95-97] and selective clonal deletion of donor-specific cytotoxic T lymphocytes have also been studied.[98-100] These mechanisms are not mutually exclusive; rather, a combination of these and other yet unrecognized factors are likely to be responsible for the spontaneous tolerance induction of a liver graft. However, these mediators have mainly been investigated in rodents, and it is unclear whether the observed mechanisms are responsible for the liver tolerance effect also in humans.

ORIGIN AND AIMS

The generation and testing of hypothesis that constitute this thesis originated from the following question, which is equally relevant and important whether posed in a clinical or a scientific context:

- How can a transplanted liver protect a kidney from acute rejection in a patient with preformed antibodies and a possible cross-match, and what are the specific molecular pathways involved?

Thus, the main focus of this thesis was to unravel mechanisms behind liver-induced acceptance of a renal graft in highly sensitized patients undergoing auxiliary liver-kidney transplantation, primarily by molecular studies on biopsies and sera, in combination with *in vitro* modeling.

Specific aims:

- To map transcriptional changes that relate to recruitment and activation of inflammatory cells in liver and kidney grafts in the early post-transplant period by global gene analysis and real-time PCR of biopsies collected from these patients before, during and after transplantation.
- To elucidate the meaning of key findings from the initial gene-study in auxiliary liver-kidney transplantation, and their potential contribution to the liver-mediated acceptance of a kidney.

METHODOLOGICAL CONSIDERATIONS

Detailed descriptions of materials and methods are given in each paper, and only those of particular importance for results are described below.

PATIENTS

Patients included in this thesis were patients undergoing combined auxiliary liver-kidney transplantation during the study period. All patients were previously transplanted and had developed high levels of multispecific HLA antibodies; hence they were unlikely to receive a cross-match negative kidney for transplantation. The liver was transplanted first, and the kidney was reperfused approximately 3 hours after the liver.

Biopsies were collected from both liver and kidney grafts and the patient's own liver at the following time points (Figure 5): Before reperfusion, 1 hour after reperfusion of the organs and 1 week later. Biopsies were also collected from the liver graft and the native liver 1 hour after kidney reperfusion (4 hours after liver reperfusion). All biopsies were immediately submerged in RNALater, to preserve mRNA integrity, and thereafter stored at -80°C. Sera and plasma were collected from the patients at multiple time points. Control sera were also collected from patients undergoing standard liver or kidney transplantation.

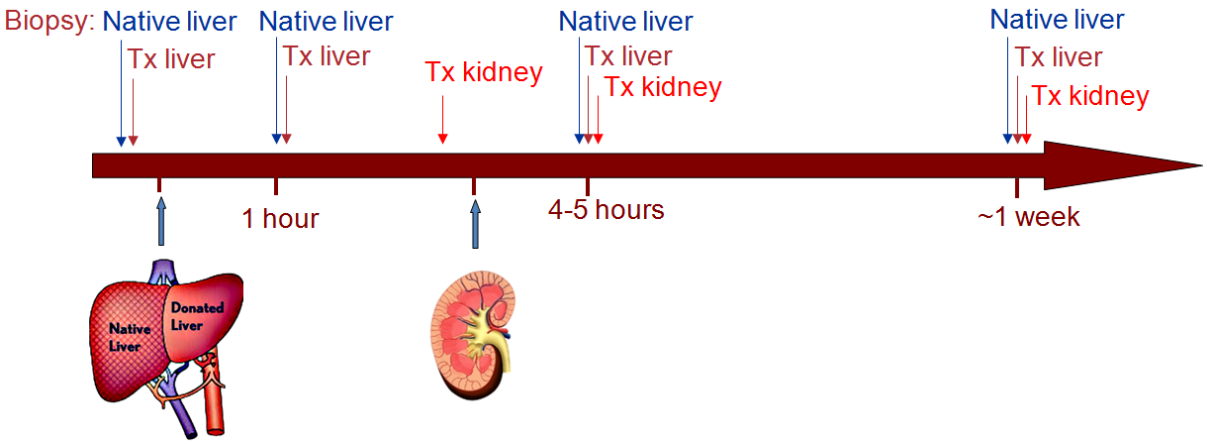

Figure 5. Biopsies collected during combined auxiliary liver-kidney transplantation.

REAL-TIME PCR

Quantitative real time PCR analysis was performed on patient material and cell cultures. The RNA quality of the samples was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies), figure 6. The patient material was investigated using Low Density Array cards, where 48 genes were analyzed in parallel in one sample including endogenous controls (18S ribosomal RNA, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). The sequence-specific primers and probes were pre-designed and tested by Applied

Biosystems. Low density arrays permits detection of a large number of genes in a very small amount of starting material, making it suitable for mRNA quantitation of small biopsy materials. Gene expression levels were normalized to 18S as it proved to be the most stable endogenous control in the human biopsy study. The comparative $\Delta\Delta C_T$ method of relative quantification was used to estimate the change of mRNA levels in the post-transplantation samples compared to the pre-transplantation levels (relative change or fold change). Thus, the patients function as their own controls.

Real-time PCR was also used to quantify mRNA expression in cell culture studies. In these experiments, gene expression levels were normalized to GAPDH, which was stable in this material. The standard curve method was used to estimate mRNA levels.

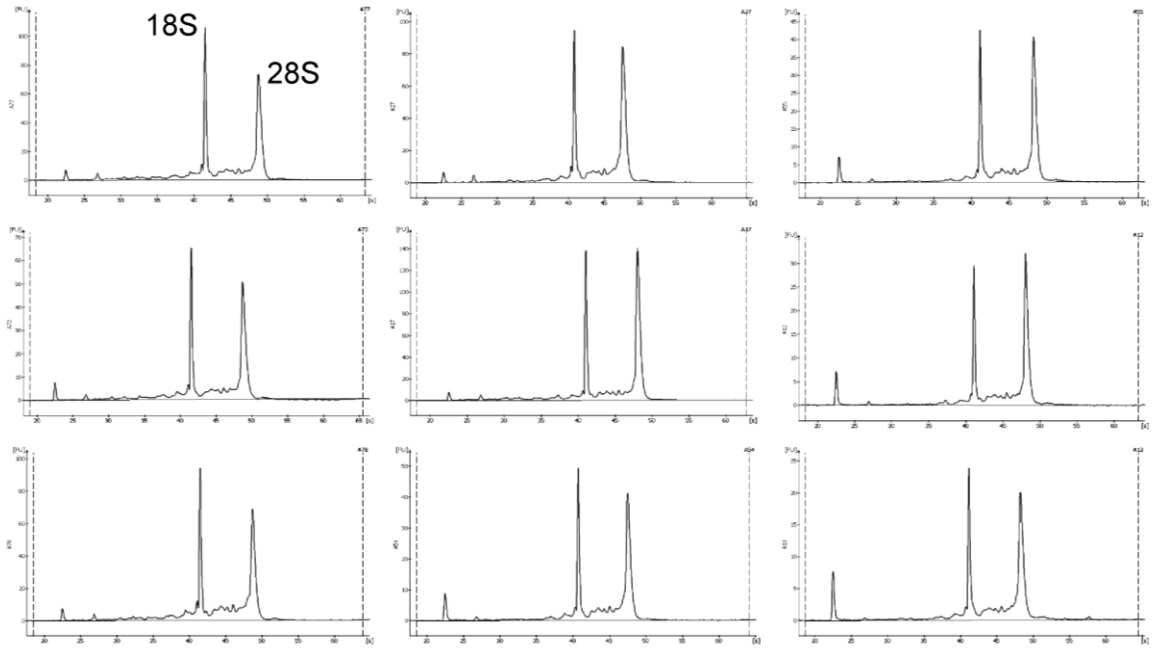

Figure 6. RNA quality in 9 biopsies collected from patients. An electropherogram shows the characteristic signature of high quality RNA, where the 18S and 28S ribosomal RNA peaks are clearly visible, with no smear between the peaks. In partly degraded RNA the 18S and 28S peaks are lower, the RNA size distribution is shifted toward smaller fragments with a smeared appearance.

IDO MEASUREMENTS

Tryptophan is an essential amino acid. It is degraded by two enzymes, the constitutively expressed tryptophan 2,3-dioxygenase (TDO) and inflammation-induced indoleamine 2,3-dioxygenase (IDO).[101, 102] Activation of IDO is regulated posttranslationally. Thus IDO mRNA or protein expression does not always agree with IDO activity.[103, 104] IDO converts tryptophan to N-formyl kynurenine, which is rapidly converted to kynurenine.[102] Reduced tryptophan levels and excess kynurenine intermediates both have immunomodulatory

effects, including the suppression of T cells.[105, 106] IDO activity leads to local depletion of tryptophan, and influences the levels of tryptophan and kynurenine in serum. The ratio of kynurenine/tryptophan in serum is considered a reliable marker for IDO-induced tryptophan degradation and thus IDO activity.[107, 108] In our study, the level of kynurenine and tryptophan was measured by high performance liquid chromatography. To increase the sensitivity of the kynurenine measurements, we used a fluorescence detector, instead of UV-absorbance as previously described.[108] Kynurenine has a maximum λ excitation at 365 nm and λ emission at 480 nm[109], whereas tryptophan λ excitation is 285 nm and λ emission 365 nm. Peak sizes were measured as area under curve. Quantitation was performed with external calibrators of tryptophan and kynurenine (both Sigma Aldrich) that were run with each assay.

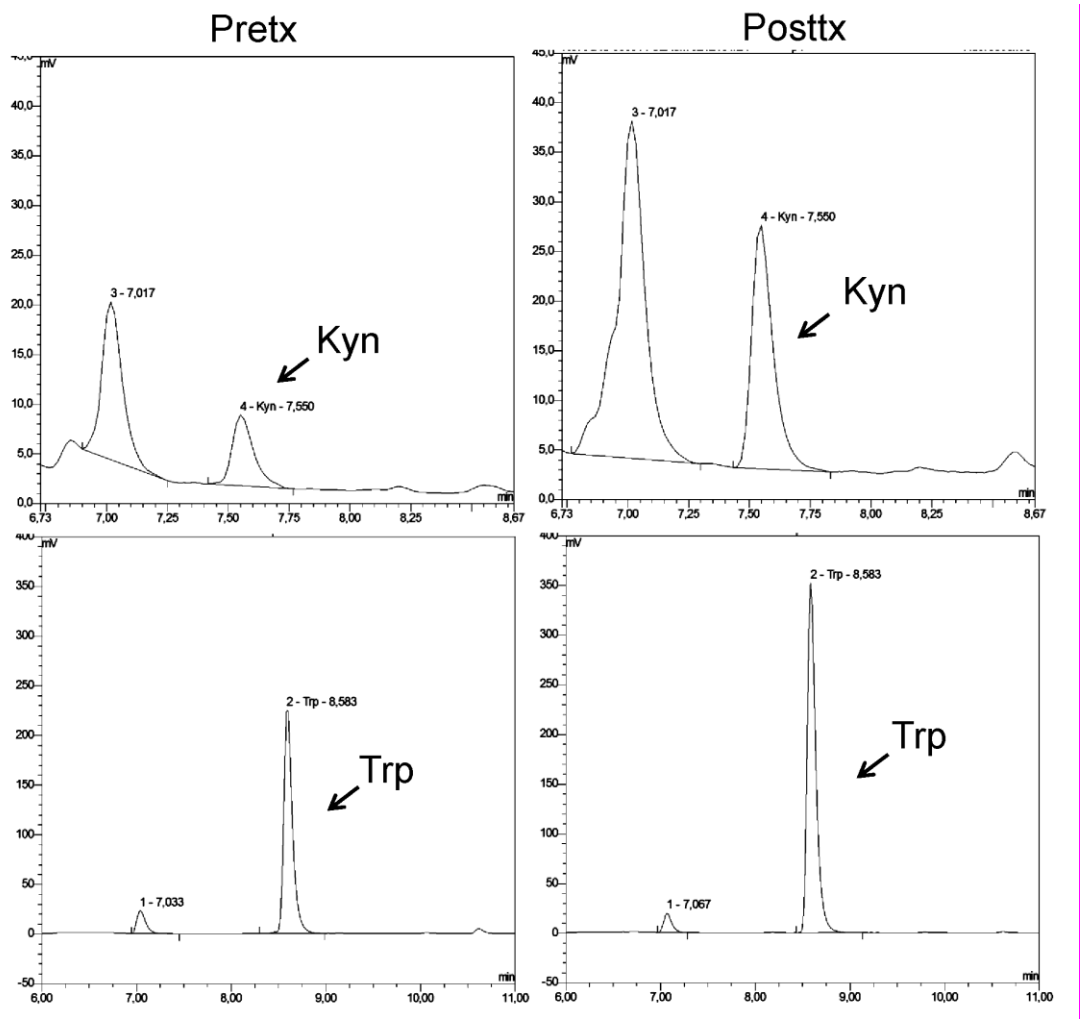

Figure 7. High performance liquid chromatography analysis of tryptophan degradation. Kynurenine and Tryptophan peaks detected in patient sera collected before and after transplantation.

DENDRITIC CELL CULTURE

Dendritic cells are found in almost all tissues, but in very low concentrations, making it very difficult to use tissue-derived DCs directly for in vitro studies. A similar dilemma is true for blood DCs and CD34+ precursors. Since the demonstration by Sallusto and Lanzavecchia in 1994, that DCs can be generated from human blood monocytes using GM-CSF and IL-4, this has become the most widely accepted protocol for in vitro studies on DCs.[110] Peripheral blood mononuclear cells (PBMC) from buffy coats (obtained from the hospital blood bank) were isolated on density gradients with Ficoll-Paque (Amersham). Monocytes were selected either by plastic adherence, where non-adherent cells were removed after 1.5 hours, or purified with positive selection for $CD14^+$ cells using magnetic activated cell sorting (Miltenyi Biotech). Dendritic cells were differentiated by culture in the presence of recombinant human GM-CSF and IL-4 both at 1,000 U/mL (R&D Systems) for 5 days, giving rise to immature DCs. Maturation of DCs is commonly achieved by exposure to inflammatory signals. In our studies we matured the cells with cytokine cocktails. To simulate the activation of DCs in a liver and a kidney graft after ischemia/reperfusion we matured the cells with factors we previously had found to be expressed in the grafts after reperfusion, namely IFN-γ, TNF-α and IL-1β (paper 2 and 4). To further improve the *in vitro* model we also added HMGB1 in the last experiments in paper 4. This is a TLR4-ligand, known to be induced in liver and kidney grafts after ischemia/reperfusion.[70] In paper 3 two different cytokine cocktails were used and compared; the gold standard maturation cocktail for DC-vaccines (TNF-α, IL-1β, IL-6 and PGE2), and the αDC1-cocktail (IFN-α, IFN-γ, TNF-α, IL-1β and polyinosinic:polycytidylic acid (poly-I:C)). The gold standard maturation cocktail is considered efficient in priming of Th1 CD4+ cells as well as CD8+ CTLs, but results in impaired production of IL-12p70.[111] As IL-12 has proved essential for direct recognition of tumor cells by CTLs[112], Maillard and colleagues introduced the αDC1-maturation cocktail, which induces the development of Th1-deviating DCs with IL-12 producing ability.[44]

MIXED LEUKOCYTE REACTIONS

Mixed leukocyte reactions (MLR) are used to determine the stimulatory function of DCs, when incubated together with allogeneic leukocytes. As stimulator cells, we used DCs derived from CD14+ monocytes, and they were activated for 24 hours in presence of different maturation-cocktails as described above. In vivo, DCs are activated in the periphery and migrate to the lymphoid organs where they initiate an immune response. Thus, to investigate the cells' stimulatory ability after leaving the inflammatory environment in the graft, we used a model where the cells were introduced into fresh medium for 24 hours following cytokine stimulation. Then, after another wash, they were used as stimulator cells. When indicated, DCs were further stimulated with 200 ng/mL histidine-

tagged CD40-ligand (CD40L) followed by the addition of anti-polyhistidine monoclonal antibody (4 μg/mL) for 20 min. Stimulation of DCs with CD40L mimics the DC interaction with CD40L-expressing cells such as T cells in the lymph node that enable DCs to activate CTLs.

Allogeneic PBMC were used as responder cells in 1:5 DC:PBMC ratio and incubated for 24 hours or 6 days before harvest. To investigate the impact of NK cells in the MLR, CD56+ cells were removed from the PBMC culture in some settings (paper IV). CD56+ cells are mainly NK and natural killer T (NKT) cells. In paper III, purified NK cells were used as responder cells in the MLR. These were isolated with the NK cell isolation kit (Miltenyi Biotec) by negative immunomagnetic cell separation and used in 2:5 DC:NK ratio in the MLR.

IFN-γ levels were measured in the cell culture supernatants after 24 hours as an indicator of NK cell activation since NK cells are important providers of IFN-γ in the early phase of an immune response.[65] We also studied the MLR production of IFN-γ after 6 days as an estimate of Th1-deviation of the cultured cells.

REVIEW OF RESULTS

GLOBAL GENE ANALYSIS (PAPER I)

As an initial approach, microarrays were used to investigate global changes in the grafts during auxiliary liver-kidney transplantation. We found that the liver underwent major changes in global gene expression after reperfusion. In a correspondence analysis, the liver graft and native liver started at distant points from each other, but after one week the liver graft had adapted and the global gene expression was similar to that of the native liver. In contrast, the kidney gene expression was more stable before, during and one week after transplantation. This may indicate that the kidney was more resistant towards ischemia than the liver graft, or that the liver graft adapts more easily to the new environment. The early changes in the liver graft were mainly immunological, with increased expression of several biological pathways, including macrophage-mediated immunity, cytokine- and chemokine-mediated immunity and the NFκB-signaling cascade. The tolerogenic properties of the liver are thus unlikely caused by the liver being more resistant towards ischemia/reperfusion injury than a kidney graft.

EXPRESSION OF INFLAMMATORY MEDIATORS IN GRAFTS AFTER TRANSPLANTATION (PAPER I & II)

The expression of a number of inflammatory mediators was increased in the grafted organs shortly after transplantation shown by real-time PCR in ten patients. Expression of proinflammatory cytokines was upregulated in similar ways in the liver and kidney grafts; one hour after reperfusion the mean levels of IFN-γ increased 16-fold compared to pretransplant levels, while TNF-α and IL-1β levels increased 4-fold. The cytokine expression was still elevated in the grafts one week after transplantation. The NFκB signaling pathway, which plays a central role in the ischemia/reperfusion response, was also upregulated in both grafts early after transplantation, as indicated by an elevation in a number of NFκB-regulated genes shortly after reperfusion; NFKB1, NFκB-inhibitor A (NFKBIA) and A20. The expression levels of these genes were higher in the liver than in the kidney graft one week after transplantation.

A number of inflammatory chemokines were also induced after reperfusion. Chemokines known to recruit monocytes as well as immature DCs and T cells, CCL2, CCL3 and CCL4, were all enhanced in both liver and kidney one hour after reperfusion. The mRNA levels of CCL3 and CCL4 were still high in the kidney one week later, whereas the expression in the liver graft was normalized at that point. CXCL2, a potent neutrophil recruiting chemokine, was only upregulated in kidney (8-fold increase). Interestingly, the liver expressed exceedingly high levels of the DC and memory T cell recruiting chemokine

CCL20 (60-fold increase five hours after reperfusion). Thus, different populations of inflammatory cells may infiltrate the grafts after reperfusion, so that neutrophils are preferentially recruited to the kidney whereas the liver mainly attracts DC precursors and memory T cells.

CORRELATION OF GENE DATA TO GRAFT OUTCOME (PAPER I)

Patients with an early kidney rejection episode, within the first month, demonstrated a deviating gene expression pattern in the liver graft shortly after transplantation compared with patients not experiencing acute rejection episodes during the first three months. Out of the 45 investigated genes, 14 were expressed at a significantly higher levels in patients without early acute rejection, whereas no genes were significantly lower in this group. The genes that were elevated in patients without rejection episodes included the previously mentioned NFκB-associated genes; NFKB1, NKFBIA, A20; and several chemokines, including the monocyte, DC and T cell recruiting chemokines mentioned above, CCL2, CCL3, CCL4 and CCL20. The neutrophil-recruiting CXCL2 was not significantly different between the groups. Also, CX3CL1, a monocyte- and T cell-attracting molecule, increased 6-fold (geo. mean 5.82; +2.11, -1.55) in patients without rejection, but only 1.3-fold (geo. mean 1.34; +0.64, -0.43) in patients with early rejection.

Other genes that were significantly higher expressed in the liver graft in patients without rejection included intercellular adhesion molecule (ICAM) 1, TNF-α, the transcription factor early growth response 2 (EGR2) and the tolerogenic enzyme IDO. Two molecules of the apoptosis pathway; FAS and BID, were also elevated in this patient group.

In total, the stronger expression of these genes indicates an increased inflammatory response in the liver graft in patients that avoid rejection, in particular along pathways that relate to recruitment and activation of inflammatory cells in the graft.

ACTIVATION OF IDO AFTER LIVER TRANSPLANTATION (PAPER II)

In the global gene analysis performed in samples from one patient undergoing auxiliary liver-kidney transplantation, we detected strong signals from the immunoregulatory enzyme IDO in the auxiliary liver graft after transplantation. The expression was confirmed by real-time PCR in ten patients undergoing the same procedure, where the mean mRNA levels of IDO increased 12-fold in the auxiliary liver graft 4 hours after liver reperfusion compared to the pre-transplant levels (n=8). One week later the expression had increased nearly 100-fold in both liver and kidney grafts.

To confirm that the IDO gene expression gave rise to an active IDO enzyme, IDO activity was investigated in these patients. We found that the IDO activity,

estimated from the kynurenine/tryptophan ratio in serum, was elevated; 24 hours after transplantation, the mean levels increased with 57% compared to pre-transplant levels (n=3). A similar change in IDO activity was found in patients after standard liver transplantation. In contrast, the IDO activity in patients after standard kidney transplantation was reduced. This disparity between the patient groups was significant at 1, 3 and 7 days after transplantation, suggesting that IDO induction is a unique feature of the liver graft.

As previous findings suggest that IDO-positive DCs have a tolerogenic profile, and inhibits allogeneic T cell responses, we studied IDO production in DCs exposed to serum from liver transplanted patients. Interestingly, DCs matured in the presence of patient sera collected after liver or auxiliary liver-kidney transplantation had higher IDO mRNA and IDO activity levels compared with cells treated with pre-transplant sera from the same patients. This was not the case with cells treated with sera collected from kidney transplanted patients, indicating that liver transplantation uniquely leads to the release of soluble factors that are able to induce IDO in dendritic cells.

Thus, the tolerogenic enzyme IDO is activated after liver but not kidney transplantation, and may be one factor that mediates the liver tolerogenic effect in combined auxiliary liver-kidney transplanted patients.

NK RECRUITING ABILITY OF DENDRITIC CELLS (PAPER III AND IV)

Recent publications have demonstrated that NK cells play an important role in the initiation of a Th1-deviated immune response by providing an early source of IFN-γ during the DC/T cell cross-talk that takes place in the lymphoid organs after migration of antigen-presenting DCs from the periphery.[65, 113, 114] We therefore investigated the ability of DCs to recruit and activate NK cells after maturation. In paper III we found that DCs matured in the presence of IFN-γ, TNF-α, IL-1β, IFN-α and poly I:C (αDC1) were superior in the recruitment and activation of NK cells compared with DCs treated by the gold standard maturation cocktail for DC-vaccines consisting of TNF-α, IL-1β, IL-6 and PGE2 (PGE2-DC). In the next paper (paper IV) we investigated this ability in DCs activated with maturation-factors produced during transplantation induced ischemia/reperfusion. IFN-γ, TNF-α and IL-1β mRNAs were all found to be induced in the early events after both liver and kidney reperfusion (paper II), whereas only liver reperfusion led to a profound rise in IL-10 protein levels (as reviewed below). DCs matured with IFN-γ, TNF-α and IL-1β in the presence of IL-10 produced significantly lower levels of NK-recruiting chemokines CXCL9/MIG, CXCL10/IP-10 and CXCL11/I-TAC) compared with DCs matured without IL-10. Moreover, IL-10 reduced the DC ability to initiate a Th1-deviated immune response, estimated by the IFN-γ production by allogeneic T cells in a six day mixed leukocyte reaction (MLR). Finally, we studied the effect of IL-10 on DC capacity to activate allogeneic NK cells in a

MLR. Stimulation of DCs with CD40L before co-culture with allogeneic PBMC, as a source of NK cells, led to IFN-γ production within 24 hours. CD40L was added to imitate the cross-talk between mature DCs and recipient T cells that leads to licensing of the DCs in the secondary lymphoid organs. Addition of IL-10 during DC maturation reduced the IFN-γ level and removal of NK cells abolished the production. This indicates that NK cells are responsible for the early production of IFN-γ and that IL-10 DCs are less able to activate NK cells compared with DCs matured in the absence of IL-10.

IL-10 RELEASE AFTER AUXILIARY LIVER TRANSPLANTATION (PAPER IV)

To further clarify differences between liver and kidney transplantation that may explain the liver tolerogenic effect, we studied the release of cytokines to serum. This was done in patients undergoing combined auxiliary liver-kidney transplantation, standard liver transplantation and kidney transplantation. Starting with a study of three patients undergoing auxiliary liver-kidney transplantation, we found an elevation in IL-10 levels as early as five minutes after liver reperfusion. One hour later IL-10 levels reached 155 (+30.0, -27.8) pg/mL. The subsequent kidney reperfusion did not affect the concentration, and IL-10 returned to background levels 24 hours later. These results were confirmed in three other auxiliary liver-kidney patients, and a similar pattern was established in liver-transplanted patients. The profound release of IL-10 was liver-specific, as renal transplantation only induced a minor elevation of IL-10 in serum. Thus, anti-inflammatory IL-10 may play an important role in the liver tolerogenic effect.

DISCUSSION

A new possibility to receive a kidney transplant has brought new hope to highly sensitized patients. In combined auxiliary liver-kidney transplantation a liver graft is transplanted a few hours before the kidney graft with the sole purpose of protecting the kidney from rejection.[30, 115] This procedure is based on previous findings that a liver graft is less sensitive to preformed antibodies and liver allograft survival is not affected by a positive cross-match.[6] The liver can even protect another organ transplanted from the same donor against preformed lymphocytotoxic antibodies and improve the long-term allograft survival.[19, 20, 25]

The recruitment and activation of inflammatory cells in a graft shortly after transplantation has a direct effect on allograft survival as both passenger DCs and recipient APCs are important mediators of T cell activation and initiation of an immune response. The aim of this thesis was to elucidate mechanisms associated with activation of passenger leukocytes and recruitment of inflammatory cells in the liver graft, and how these may contribute to the liver-induced acceptance of a subsequently grafted kidney in highly sensitized patients. Focus lies on how the expression of IDO and IL-10 in the liver may modulate the immune stimulatory ability of passenger DCs, and how the early cross-talk between passenger DCs and NK cells may play a role in the initiation of an immune response. I will also discuss how recruitment of leukocytes to the graft in the early post-transplantation period may play a role in this. Finally, the correlation between inflammatory response in the auxiliary liver graft and acceptance of a kidney graft is discussed.

ACTIVATION OF PASSENGER DCS IN A LIVER COMPARED TO A RENAL GRAFT

Ischemia/reperfusion injury is an important determinant of allograft survival as it contributes to local inflammation and stimulates passenger DCs, leading to T cell activation and initiation of a donor-specific immune response. Investigation of transcriptional changes of inflammatory genes may therefore aid in the understanding of the early events that takes place in a graft after reperfusion. We first hypothesized that the liver and kidney differ in terms of ischemia/reperfusion initiated inflammation, leading to more aggressive immune response and a stronger activation of passenger DCs in a kidney graft compared with a liver graft. This was contradicted by our finding that in a global gene analysis the liver underwent major changes after reperfusion whereas the kidney gene expression was more stable during transplantation, indicating that the kidney was less affected by ischemia/reperfusion injury compared to the liver (paper I). Also, the expression of several classical proinflammatory mediators, e.g. IFN-γ, TNF-α and IL-1β, which are potent activators of DCs, was similar in both liver and kidney shortly after auxiliary liver-kidney transplantations (paper II).

We found that several NFκB-associated genes were upregulated in both liver and kidney grafts, indicating an activation of the NFκB-signaling pathway (paper I). NFκB is a central transcription factor in ischemia/reperfusion injury, and its activation induces a cascade of inflammatory mediators, such as cytokines and chemokines.[116] Activation of NFκB may therefore contribute to the maturation of passenger DCs within both liver and kidney grafts. There are studies that suggest a role for NFκB in protection of liver grafts after transplantation.[117, 118] However, other studies indicate that NFκB activation may enhance ischemic damage during surgery.[119, 120]

Certain other immune suppressive factors are produced solely after liver but not kidney transplantation. These factors, IDO, and IL-10, may also affect the ischemia/reperfusion induced maturation of passenger DCs.

IDO

Since the publication by Munn and Mellor in 1998 that IDO is necessary to avoid rejection of semi-allogeneic fetuses in pregnant mice[121], the role for IDO in immune regulation have started to unravel. Several publications have shown that overexpression of IDO prolongs graft survival in experimental transplantation of various organs.[122, 123]

Spontaneous tolerance induction of a liver allograft is a well-know phenomenon in experimental transplantation. In 2000 Pan et al published their findings that IDO mRNA is induced after allogeneic but not syngeneic liver transplantation.[124] Miki et al confirmed IDO expression in a mouse model of liver transplantation, peaking at day 7 after transplantation.[125] Interestingly, the spontaneous tolerance was abrogated by the administration of the IDO-inhibitor 1-methyl-tryptophan (1-MT), indicating that IDO is an important mediator of liver tolerance, as seen in fig 8.

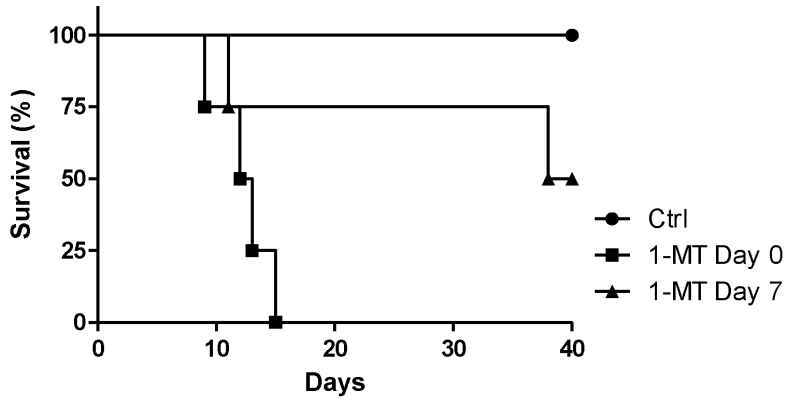

Figure 8. Survival of mouse liver graft recipients treated with IDO inhibitor 1-MT at different time points. Modified from Miki et al. 2001.

These data are in agreement with our finding that IDO activity increased 24 hours after auxiliary liver-kidney as well as standard liver transplantations, but not in patients undergoing kidney transplantation (paper II). IDO mRNA levels were increased in the liver graft as early as a few hours after auxiliary liver-kidney transplantation, and the gene expression level at this point correlated with the clinical outcome. Thus, patients without an early rejection episode expressed higher levels of IDO in the liver graft five hours after reperfusion than patients that were to experience an acute rejection within the first month after transplantation.

In contrast, Brandacher and colleagues found that in kidney transplanted patients, IDO activity was significantly higher in those that were later to undergo a rejection than in patients with an uncomplicated follow up, and concluded that IDO activity may function as a marker to predict rejection.[126] Using immunohistochemistry they found that IDO was mainly expressed by renal tubular epithelial cells in the grafts. As IDO is induced by IFN-γ in these cells, they speculate that the expression of IDO may be a protective response to the inflammatory process in the graft during rejection, but unable to prevent the alloresponse.

In the auxiliary liver-kidney transplanted patients, we were unable to conclude from our data in what cell type IDO was induced in the grafts, but when sera collected from these patients were added to DCs *in vitro*, they increased the expression and activity of IDO, suggesting that DCs activated in the presence of liver derived factors (i.e. within a liver graft) may be IDO positive. Several publications have demonstrated that IDO-producing DCs have a tolerogenic profile, and suppress the activation of interacting T cells in several ways. Thus, a liver-specific induction of IDO in DCs may very well be one factor that contributes to a tolerogenic profile of passenger DCs derived from a liver graft.

It is still uncertain whether it is the deprivation of tryptophan or the toxicity of its metabolites that mediates the immune regulatory function of IDO, and it might be a combination of both pathways.[127] As reviewed by Mellor and Munn, numerous studies have investigated the effect of IDO-positive DCs on T cell function.[128] IDO-expressing DCs inhibit T cell proliferation, and induce regulatory T cells as well as T cell anergy. Other studies show contradictory results, indicating that the timing and circumstances for IDO activation are crucial for its function as an immune inhibitory enzyme.

IL-10

IL-10 is a classical anti-inflammatory cytokine that suppresses pro-inflammatory responses and reduces T cell proliferation.[129] However, the anti-inflammatory function of IL-10 on T cell responses is mediated mainly via its effects on macrophage and DC functions, e.g. by reducing the MHCII expression and

limiting their production of proinflammatory cytokines.[130-133] We found that serum levels of IL-10 increased rapidly after auxiliary liver-kidney transplantation (paper IV). This is in agreement with previous studies on experimental and clinical standard liver transplantation.[134-136] Renal transplantation also increased the levels of IL-10 in serum, but significantly less than after liver transplantation, indicating that the high IL-10 levels are induced in a liver-specific way. IL-10 is known to be produced by many different myeloid and lymphoid cells, mainly T cells, macrophages and DCs.[137] We found that the IL-10 levels increased as early as five minutes after reperfusion, indicating that IL-10 expression is not induced by reperfusion, but possibly post-transcriptionally regulated or accumulated in the graft during the ischemia-period. Kupffer cells, a liver resident macrophage subtype, are often suggested to be responsible for the IL-10 production in the liver. In line with this, Kupffer cells produce profuse amounts of IL-10 after LPS stimulation.[138] However, a publication on liver reperfusion in rats showed that removal of Kupffer cells did not reduce the IL-10 release seen one hour after liver reperfusion.[139] Interestingly, hypoxia has been proved to induce the production of IL-10 in DCs[77], indicating that liver resident DCs may be responsible for the fast IL-10 release that occurs after liver reperfusion.

THE ROLE OF NK CELLS IN INITIATION OF AN IMMUNE RESPONSE

Since the discovery in 1999 that DCs are able to stimulate NK cell effector functions[63], the interactions between NK cells and DCs have been widely investigated, as reviewed by Waltzer et al.[140] NK cell effector functions are enhanced by interaction with activated DCs, leading to NK cell IFN-γ production, proliferation and cytotoxicity. On the other hand, activated NK cells induce DC maturation by secretion of TNF-α and IFN-γ and cell-cell contact involving NKp30.[141, 142] Thus, a bidirectional cross-talk between DCs and NK cells takes place in the lymphoid organs, as well as in the periphery, that help to coordinate innate and adaptive immune responses. By providing an early source of IFN-γ, NK cells aid DCs in skewing of a T cell response, favoring the induction of a Th1-profile.[65, 113] In our study on DC activation with two different cytokine cocktails (paper III) we found that properly activated human DCs are indeed able to recruit NK cells by the production of CXCR3-ligands, and activate these cells as demonstrated by their expression of CD69 and intracellular IFN-γ production.

In transplantation, NK cells have repeatedly been shown to enhance the adaptive immune responses leading to rejection, as reviewed by Kitchens et al. [143] Human individuals with killer cell immunoglobulin-like receptors that are inhibited by donor MHC are less likely to undergo a rejection episode.[144] In animal studies, where T cell-mediated responses were repressed experimentally by depletion of T cells[145] or lack of costimulatory molecules[146, 147], NK cells proved to play a

role in allograft survival. NK cells may not be sufficient to induce rejection directly, but facilitate the actions of alloreactive T cells. The exact mechanisms are still unclear, but suggested to include the ability of NK cells to promote DC maturation and the subsequent activation and Th1-deviation of alloreactive T cells.[148] In paper IV, we found that DCs activated with ischemia/reperfusion associated maturation factors produce copious amounts of NK-recruiting chemokines, and are able to induce IFN-γ production in the NK cells, indicating activation. When the liver transplantation-associated cytokine IL-10 was added during DC maturation, the cells secreted less NK-recruiting chemokines and were unable to induce an early IFN-γ production from the NK cells.

This suggests that passenger DCs and/or recruited recipient DCs that are matured in a liver graft shortly after reperfusion (that is in the presence of IL-10) are less able to recruit and activate NK cells compared with passenger DCs in a kidney graft. Hence, one to our knowledge previously unrecognized aspect of the immune inhibitory impact of IL-10 on DCs in the transplantation setting, may involve their impaired ability to recruit NK cells, which consequently may reduce the Th1 deviation of alloreactive T cells.

RECRUITMENT OF LEUKOCYTES TO THE GRAFT IN THE EARLY POST-TRANSPLANTATION PERIOD

Chemokines play an important role in the recruitment of host inflammatory cells into the graft after transplantation.[149, 150] CXC chemokines are responsible for neutrophil infiltration and activation, whereas CC chemokines preferentially recruit mononuclear cells, such as monocytes and lymphocytes. Their expression is enhanced by ischemia/reperfusion-induced upregulation of reactive oxygen species, TLR stimulation and NFκB activation.[151] Thus, chemokines function as important mediators of alloantigen-independent injury in the early post-transplantation period. Moreover, the alloantigen-independent recruitment of leukocytes into the grafts allows APCs to infiltrate the grafts, capture and process alloantigens before migration to the secondary lymphoid organs, where they may initiate an allospecific immune response.[149]

We found an early expression of leukocyte-recruiting chemokines CCL2, CCL3 and CCL4 in both liver and kidney grafts after reperfusion (paper I). In contrast, the expression of CCL20 and CXCL2 differed substantially between the organs. CXCL2 is a potent neutrophil-recruiting chemokine, and its expression in the kidney shortly after reperfusion predicts an infiltration of neutrophils into the kidney after transplantation. The liver, on the other hand, displayed abundant levels of CCL20, a chemokine attracting dendritic cells as well as memory T cells, as discussed below.

Recruitment of recipient DCs to the liver graft

CCL20 is the most powerful chemokine for inducing migration of certain subtypes of DCs and their precursors.[152-154] The DC population responding to CCL20 in vivo has not yet been fully identified as each DC subtype responds to a unique spectrum of chemokines that varies depending on their maturation grade.[152] Langerhans precursors and CD14-derived DCs are attracted to CCL20, whereas immature CD11c+ blood DCs and monocyte-derived DCs have proved unresponsive in *in vitro* migration assays.[153, 155] However, the latter cell type does respond to CCL20 in the presence of transforming growth factor (TGF) β[156] or IL-10[157], two factors known to be constitutively produced in the liver. Thus, the high level of CCL20 in the liver grafts suggests that DCs may be recruited to the liver after transplantation.

As mentioned previously, recruitment of antigen presenting cells to a graft permits loading of antigens and migration to the secondary lymphoid organs where they may initiate an immune response through the indirect pathway of allopresentation. Notably, a previous publication demonstrated that presence of host APCs was indispensable for the induction of spontaneous tolerance towards liver grafts in a rat model.[158] It may thus be suggested that in the tolerogenic environment in the liver, with expression of IDO and IL-10, host antigen presenting cells may contribute to allograft tolerance by induction of Treg that suppress an alloresponse.

In highly sensitized patients, with preformed antibodies that reflect sensitization of the indirect pathway, a suppression of the indirect pathway is likely to be involved in the liver induced acceptance of a kidney graft. Thus, a preferential recruitment of immature host DCs to the liver compared to the kidney, as suggested by the profound expression of CCL20 in the liver graft after transplantation, may aid in the suppression of the indirect pathway of allorecognition in these patients.

Recruitment of T cells to the liver graft

In addition to DCs, several subsets of T cells, including effector T cells and memory T cells migrate towards CCL20. Thus, the high expression of CCL20 that we found in the liver graft after transplantation indicates that T cells are recruited to the liver, including presensitized donor-specific memory T cells. Spontaneous tolerance induction in experimental liver transplantation has previously been shown to be preceded by an early graft infiltration of T cells, similar to a rejection episode, which resolves as the infiltrating T cells undergo apoptosis in the liver graft.[100, 159] A similar phenomenon was also found in a model of combined liver and small bowel transplantation resulting in liver-induced acceptance of the small bowel graft.[160] The authors found a heavy T cell infiltration in the liver, compared to the small bowel graft. Within weeks the T cells became apoptotic, leading to donor specific tolerance. As apoptotic T cells

were found in the liver but not small bowel graft, it was suggested that T cell apoptosis was induced by local liver mediators, such as hepatocyte secreted Fas ligands or soluble MHC. Also, the liver produces TGF-β, which is known to induce T cell apoptosis.[161] Co-culture of hepatocytes and T cells has further been shown to induce T cell apoptosis, in a hepatic lectin and ICAM dependent way.[162]

Other studies suggest that after liver transplantation T cells are activated in an inappropriate way in lymphoid tissues, destining them to apoptosis within a few days. The high number of infiltrating T cells in the liver graft, rather than the bowel, in the experimental study discussed above may also reflect their preferential migration towards CCL20, which is expressed exclusively in the liver according to our findings.

CORRELATION BETWEEN INFLAMMATORY RESPONSE IN THE AUXILIARY LIVER AND ACCEPTANCE OF THE KIDNEY GRAFT

Correlation of gene expression data with the early acute rejection episodes that occurred in 50% of the patients after auxiliary liver-kidney transplantation revealed that the expression levels of several of the investigated genes were associated with the clinical outcome (paper I). Genes that had an increased expression in patients without early rejection were associated with an inflammatory reaction, e.g. chemokines and NFκB-associated genes. This was surprising as one would expect a more intense immune response to be detrimental for the graft survival.

However, previous research on spontaneous tolerance of a liver allograft in experimental transplantation has clearly established that the phenomenon is not caused by immunological ignorance of the donor antigens. Rather it is an active process involving T cell activation, proliferation and infiltration of the allograft.[163] A rapid and transient increase in the expression of IFN-γ and IL-2 in the lymphoid tissues, accompanied by the migration of donor leukocytes to these sites, is found in tolerant animals, whereas animals subsequently rejecting their grafts have a slower progression. Similar findings have been presented in other studies, suggesting that an early immune activation is required to induce spontaneous acceptance of a liver allograft in experimental transplantation.[164, 165] This is in line with our data on a rapid increase of inflammatory genes in the auxiliary liver of patients with a better acceptance of their grafts. This is also in agreement with the hypothesis suggested above; that the liver is a tolerogenic environment, and host APCs recruited to the liver promote acceptance. An increased inflammatory response in the liver would lead to a larger number of host APCs infiltrating the liver and subsequently more tolerogenic APCs in the lymphoid organs, promoting a tolerogenic T-cell response.

As the gene expression profile in the auxiliary liver graft predicted rejection of the renal graft, further studies are needed to establish whether an increased inflammatory response is indeed protective in all patients. It is possible that the increased recruitment of inflammatory cells to the liver graft is protective specifically in pre-sensitized patients undergoing combined liver-kidney transplantation since these patients have high levels of donor-specific memory B and T cells. Infiltration of these cells into the auxiliary liver may protect the sensitive kidney from them. The patients have a functional native liver; therefore any immune damage that takes place in the auxiliary liver graft would be of less consequence for them.

The clear difference between patients at risk for early rejection and those that avoid such response suggests that high- and low-risk patients can be identified as early as a few hours after graft reperfusion. This information could be used to adapt the immune suppressive therapy and thereby reduce the rate of rejections in the high risk group, and avoid adverse effects caused by overuse of immunosuppression in low risk patients.

CONCLUDING REMARKS

The aim of this thesis was to establish molecular mechanisms that contribute to the liver tolerogenic function in patients after combined auxiliary liver-kidney transplantation. We focused on dendritic cells as these cells transferred with the graft to the allogeneic recipient, are important initiators of an alloresponse.

This thesis demonstrates that liver transplantation is associated with an increased activity of the immunoregulatory enzyme IDO that does not occur after kidney transplantation. In addition, IDO was induced in DCs treated with patient sera collected after liver reperfusion, and a positive correlation was found between IDO-expression in the liver graft and clinical outcome. Previous publications suggest a role for IDO in spontaneous tolerance induction of liver grafts in rodents, but our data indicate that IDO may also be important for the tolerogenic function of a liver in humans. Thus IDO may be one factor that contributes to the liver-induced acceptance of a kidney in presensitized patients, possibly via the induction of tolerogenic, IDO-positive DCs in the liver graft.

The release of high levels of IL-10 was also associated with liver reperfusion but not kidney reperfusion. IL-10 is a classical anti-inflammatory cytokine, and it reduces the T cell stimulatory ability of DCs. We found that DCs activated in the presence of IL-10 are less able to recruit NK cells, as estimated by their reduced ability to secrete the NK-recruiting chemokines MIG, IP-10 and I-TAC. As NK cells are important for the DC ability to induce a Th1-deviated immune response, this may be another factor that hampers the immune stimulatory function of passenger DCs from a liver graft.

We also found that the protective role of the liver graft was associated with a pro-inflammatory response within the graft after reperfusion, as higher expression levels of several inflammatory genes correlated with a reduced risk of kidney rejection. In particular, a number of leukocyte recruiting chemokines were expressed at higher levels in the liver graft of patients without early rejection. The liver and kidney grafts responded differently to reperfusion in terms of chemokine expression, where the liver strongly upregulated the DC and memory T cell recruiting chemokine CCL20, whereas the kidney upregulated the expression of CXCL2 which attracts neutrophils to the graft. Thus, the higher tolerance towards liver transplants, compared to renal transplants, when it comes to acceptance of an allogeneic graft may be associated with the infiltration of different populations of inflammatory cells into the organs after reperfusion.

The liver is unique among transplanted organs in the sense that it provides a tolerogenic environment comprising IDO and IL-10 induction after reperfusion as well as a constitutive expression of both IL-10 and TGF-β as demonstrated in other publications. Therefore the maturation of passenger DCs within the organ, as well as the preferential recruitment of immature DCs and DC precursors to

the liver graft, may give rise to tolerogenic DCs that are able to suppress the donor-specific alloresponse that otherwise would be expected in pre-sensitized patients.

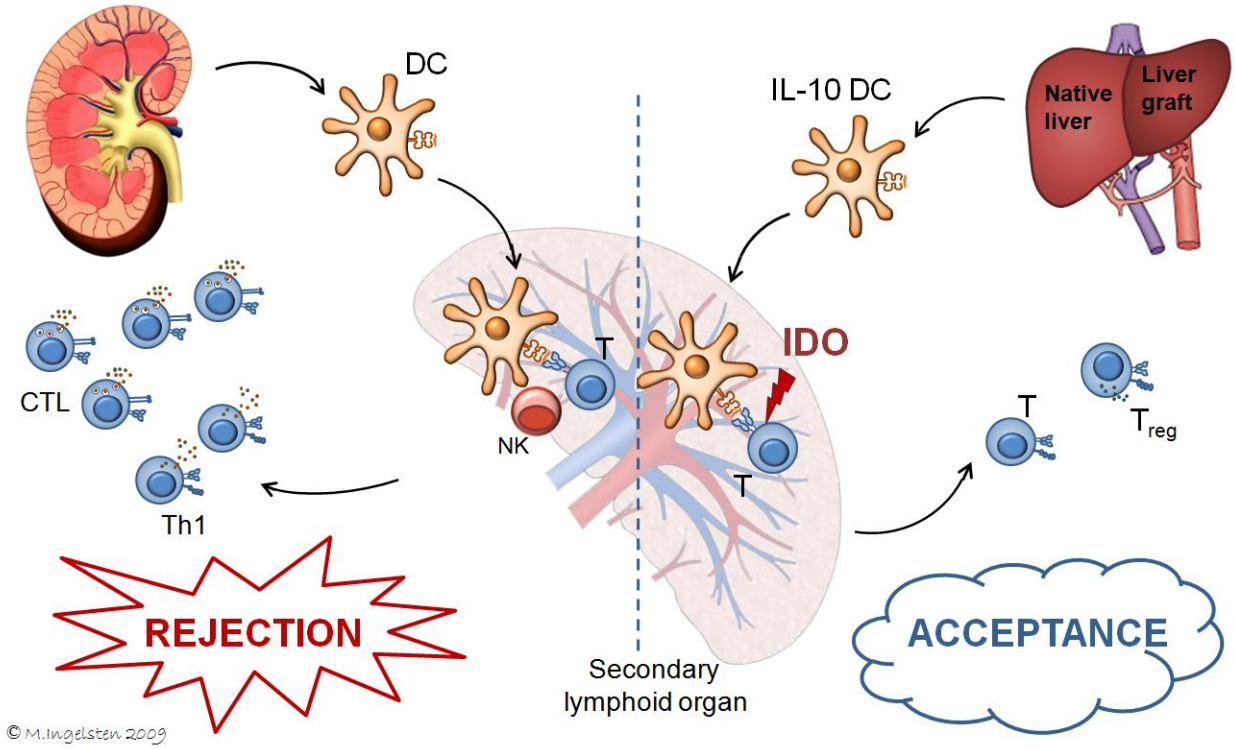

Figure 9. Suggested model for differences in DC stimulatory ability after maturation in kidney and liver graft respectively. DCs activated in a kidney recruit NK cells to the secondary lymphoid organ and initiate a Th1-deviated alloresponse leading to activation of cytotoxic T cells (CTL). Liver-derived DCs are less able to attract NK cells, and initiate a Th1-response. Moreover they may be IDO-positive, leading to T cell anergy and development of Treg.

FUTURE PERSPECTIVES

Further knowledge of the tolerogenic mechanisms that operate in the liver may be useful to improve the outcome in clinical transplantation. This could be approached from several angels:

- Identification of new drug targets to induce tolerance rather than reduce host defense systemically as today's immune suppressants. IDO is one promising molecule in this aspect. Further studies on the manipulation of IDO activation is needed, as the timing and localization of IDO have proved essential for its tolerogenic function. The anti-inflammatory cytokine IL-10 is another potential target for tolerance induction as IL-10 treated DCs have a tolerogenic phenotype. IL-10 treatment is currently used in 5 clinical trials to treat inflammatory diseases, e.g. ulcerative colitis.[166] Recombinant IL-10 treatments have shown variable results as high systemic levels of IL-10 may function in a Th2-stimulatory rather than anti-inflammatory way. Thus, as for IDO, the timing, level and localization of IL-10 are essential for its tolerogenic function.
- Design of new protocols for desensitizing therapy. DCs are widely investigated as immune regulators in cell therapy, e.g. cancer vaccines. DCs are not only initiators of an immune response, but may also suppress and regulate immunity. Thus, if recipient DCs were manipulated to become tolerogenic, and thereafter pulsed with donor antigens *in vitro*, they could potentially be used for desensitization in the transplantation setting.
- Identification of high- and low-risk patients by gene expression profiling. This may be utilized in the post-transplantation phase for selection of appropriate levels of immunosuppression, thereby alleviating the side effects of immunosuppressive medication in low-risk patients, and increase graft survival in high-risk patients, by lowering the incidence of acute rejection.

POPULÄRVETENSKAPLIG SAMMANFATTNING

För att undvika avstötning efter en njurtransplantation är det av största vikt att den transplanterade vävnaden är kompatibel med mottagaren och att patienten inte har antikroppar mot donatorns vävnadstyp, HLA. Dessa antikroppar kan ha bildats när patienten tidigare exponerats för främmande vävnadstyper i samband med en tidigare organtransplantation, blodtransfusion eller graviditet. För patienter som har utvecklat HLA-antikroppar är chansen idag minimal att finna en perfekt matchad njure för transplantation, och livslång dialys är enda alternativet. Vid levertransplantation däremot har förekomsten av antikroppar ingen betydelse för resultatet efter transplantationen. Fallbeskrivningar har publicerats där även njuren undgått avstötning vid kombinerad lever-njurtransplantation trots närvaro av donatorspecifika antikroppar.

På Sahlgrenska Sjukhuset har man utifrån denna bakgrund börjat genomföra transplantationer där en del av patientens egen fungerande lever ersätts av donatorns lever innan njuren transplanteras, så kallad kombinerad auxiliär lever-njurtransplantation. Levern transplanteras alltså enbart för att skydda njuren från avstötning. Detta har visat sig vara mycket framgångsrikt, och den nya njuren accepteras trots patienternas höga nivåer av antikroppar mot donator-HLA.

Målet med denna avhandling var att skaffa kunskap om mekanismerna bakom leverns skyddande förmåga. Vi har upptäckt att genen som bildar toleransenzymet IDO aktiveras kraftigt hos patienter som genomgår auxiliär lever-njurtransplantation. Detta beror sannolikt på levertransplantatet, då en ökad IDO-aktivitet bekräftats i serum hos våra auxiliära patienter, samt levertransplanterade patienter, men inte hos personer som genomgår vanlig njurtransplantation. IDO är sedan tidigare känt för att i djurstudier skydda fostret från avstötning vid en graviditet. Ett foster bär på en kombination av moderns och faderns vävnadstyp och är alltså delvis främmande för moderns kropp och måste skyddas från hennes immunförsvar under graviditeten. IDO kan bland annat produceras av immunförsvarets dendritiska celler, som kan styra balansen mellan aktivering av ett immunsvar och nedreglering av immunsvaret, dvs. toleransinduktion. Dendritiska celler som producerar IDO kan skapa tolerans.

Vi har vidare upptäckt en ökning av den immunreglerande faktorn IL-10 i serum hos patienter omedelbart efter auxiliär lever-njurtransplantation. Även detta kan tillskrivas levertransplantatet då patienter som genomgått vanlig njurtransplantation utsöndrar mycket lägre nivåer av IL-10. Dendritiska celler som aktiveras i närvaro av IL-10 är mindre benägna att aktivera immunförsvaret och anses toleransinducerande. Både IL-10 och IDO kan alltså ha betydelse för leverns förmåga att skydda njuren från avstötning vid auxiliär lever-njurtransplantation.

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