Tolerance and chimerism in liver transplantation

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INTRODUCTION

Advances in immunosuppressive therapy have greatly impacted on the successful outcome of organ –and most particularly liver– transplantation. With the introduction of new immunosuppressants the incidence of acute rejection has considerably decreased, and transplanted patient survival is now 83% and 70% after 1 and 5 years, respectively (1). However, toxicity associated with these drugs is significant, and induces the development of blood hypertension, hyperlipidemia, diabetes, renal failure, and de novo tumors in transplanted patients (2,3). As a result, and to improve immunosuppressive therapy efficacy and specificity, a greater insight into the mechanisms of primary allogeneic response to foreign antigens, and into the mechanisms of antigen-specific tolerance is crucial to help avoid or decrease immunosuppressant use as much as possible.

It is a well known fact that liver transplants may be performed with no immunosuppression among animal species such as pigs, and selected rat and mouse combinations (4-6). This fact, and the possibility of completely avoiding immunosuppressants in selected patients with liver transplant (7-10), lead both to consider the liver an immunologically privileged organ (11) that may be tolerated with less immunosuppression after transplantation; immunosuppressants can be even completely withdrawn on occasion (operational tolerance) (12). Experimental data support the notion of induced tolerance in humans undergoing organ transplantation. While tolerance is an ideal goal, heterogeneity in potential donor-receiver combinations, the receiver’s immune status and underlying disease, and the unforeseeable consequences of infection render stable tolerance an extremely challenging objective for all patients (13).

Tolerance in transplantation would be the lack of immune response to alloantigens (foreign antigens) in the transplanted organ in the absence of immunosuppression (14). Immune tolerance is the absence of response to an encountered antigen, and is therefore an active phenomenon (15). Tolerance to self antigens, as is the case with tolerance to alloantigens, is a function of the immune system. Mechanisms involved in tolerance to transplanted organs are complex and partly unknown. The goal of this review is to describe mechanisms involved in tolerance, particularly in the setting of liver transplantation, and to highlight the role of cell chimerism and immunoregulation phenomena in tolerance development.

ALLORECOGNITION AND REJECTION

The immune system differentiates self from foreign. Specifically, the term allorecognition refers to the capability of T cells to recognize, among members of the same species, genetically-encoded polymorphisms in antigenic molecules, including those making up the major histocompatibility complex (MHC). Graft rejection begins when an MHC foreign antigen expressed in the transplanted organ is recognized by the receiver’s T cells. In the presence of inflammatory damage, as a result of transplantation-related surgery and ischemia,
donor antigen presenting cells (APCs), and most particularly dendritic cells (DCs—the most significant antigen presenting cells) included in the graft, maturate and migrate through afferents or the blood to graft-draining lymphoid organs. There they present donor allogeneic peptides and MHC to the host’s pre-extant alloreactive T cells through the so-called direct antigen presentation pathway (16,17) (Fig. 1). These host T cells are mostly—but not all of them—CD4 (helper) lymphocytes responsive to class II MHC antigens (18). Rejection may also occur through the so-called indirect antigen presentation pathway. In this case host APCs capture and process donor MHC antigens, and present them in the context of host MHC molecules to host T cells (19) (Fig. 1). The indirect antigen presentation pathway possibly represents a weak stimulus for rejection, since donor DC depletion—which deletes the direct presentation pathway—suffices to prevent rejection (16). The direct presentation pathway possibly predominates during the immediate post-transplant period, and is the most relevant factor for acute rejection, given the fast migration of donor antigen-expressing graft DCs to secondary lymphoid organs, where they meet allospecific T cells (20). The indirect presentation pathway is likely triggered by antigens leaving the transplanted organ or—in liver transplants—by class I MHC molecules that are captures and presented by DCs themselves. Thus, while the direct presentation pathway is important to initiate acute rejection, the indirect pathway may be relevant for sustained, persistent response to alloantigens, and may play a role in chronic rejection (21). At the same time, the indirect pathway may be involved in immunoregulation phenomena, since allospecific T cells exhibit regulatory properties by inhibiting interferon (IFN)-γ production in patients with renal transplant (22).

T cells can receive, process, and relay stimuli to the nucleus using intracellular activation transduction signals. These signals modify intracellular lipids that initiate the activation and nuclear translocation of transcription factors regulating gene activation and expression (23).

T lymphocytes require three types of coordinated signals to become activated (Fig. 2). The initial signal or signal 1 results from contact between the MHC/allopeptide complex in APCs and T-cell receptor (TCR) (11). The second signal or signal 2 (costimulatory signal) results from the interaction of costimulatory molecules in T cells with their ligands on APCs, which allows differentiation of T cells into effector CD4 (proinflammatory cytokine release) or CD8 (cytotoxic activity) cells (24). Tertiary signals (signal 3) mediated by cytokines such as interleukin 2 (IL-2) are then essential for cell proliferation and differentiation into effector cells, and the development of memory cells; chemokines direct their migration to secondary lymphoid organs (spleen and lymph nodes) (25). Signal 1 in the absence of costimulatory signal 2, as is commonly the case in the liver, results in a state of unresponsiveness (anergia) where T cells recognize (via the TCR) but cannot respond to newly-encountered antigens. This is why multiple studies were performed in an attempt to block the costimulatory signal, and thus prolong graft survival.

Costimulatory signals between T cells and APCs are primarily mediated by two molecular classes: class I: superfamiliy of immunoglobulins (CD28/CD80-CD86, CTLA4/CD80-CD86, ICOS/ICOS-L, PD1/PD1); class II: superfamiliy of TNF receptors (CD40/CD40L, OX40/OX40L, 4-1BB/4-1BB-L, RANKL/TRANCE y LIGHT/LIGHT-L).

![Fig. 1. Direct and indirect antigen presentation pathways.](image1)

![Fig. 2. T-cell activation signals.](image2)
The most important and better known costimulatory signals are mediated by CD28 and CD40. The CD28 molecule is expressed by CD4 and CD8 lymphocytes, and their ligands B7-1 (CD80) and B7-2 (CD86) are expressed by various APCs (DCs, T cells, and macrophages). This signaling increases IL-2 production, lymphocyte proliferation, and antiapoptotic protein induction (26). CTLA-4 (cytotoxic T-lymphocyte associated antigen 4) is a protein related to CD28 that is expressed by activated T cells, and –just as CD28– binds CD80 and CD86, which results in a negative signal that lessens T-cell function (26).

Costimulation by CD40, expressed by APCs, and CD40-L or CD154, expressed by T cells, B-cells, and NK (natural killer) cells, is critical for immune response to alloantigens. This type of CD40/CD154 costimulation increases APC activity, with increased expression of class II MHC, CD80, and CD86, and a greater production of cytokines such as IL-12 (27).

When T cells receive stimulation and costimulation signals graft injury ensues at the expense of mainly cytotoxic (CD8+) T cells, which induce cellular apoptosis in the graft. In liver graft rejection apoptosis through the perforin/granzyme pathway (28) is a major rejection mechanism, together with the Fas/Fas ligand pathway (29), both induced by cytotoxic T cells.

THE LIVER AS A PRIVILEGED IMMUNE ORGAN

Liver structure has deep implications for hepatic immune function. The liver is continuously exposed to huge numbers of antigens mainly from the portal system. Around 30% of total blood flow goes through the liver every minute, providing this organ with nearly 10^8 peripheral lymphocytes every 24 hours (30).

The liver, due to its anatomical and immune properties, is a place where gastrointestinal tract antigens and alloantigens—in transplanted livers—are presented to lymphocytes through a complex network of sinusoidal cells and APCs. The liver’s lymphocyte population is rich in NK and NKT (natural killer T) cells, and plays a crucial role in first-line defense against pathogens, thus modulating liver damage and circulating lymphocyte recruitment. The presence of a high percentage of non-conventional lymphocytes—which only rarely are found in peripheral blood—in the liver has been documented of late, including NK cells, TCR γδ-expressing NKT cells (γδ and Vγ24JαQ TCR cells), and DCs. NKT γδ cells possess immunoregulatory properties and seemingly recognize CD1d as expressed on the surface of APCs (Kupffer cells) and hepatocytes (31). NK cells (CD3-CD16+CD56+) represent up to 45% of liver lymphocytes, but only 5 to 15% of peripheral mononuclear cells. NK cells seem to emit negative signals to the host’s T cells upon their migration into the liver following liver transplant, thus contributing to liver graft tolerance.

The context wherein liver-resident antigens are presented to T cells favors a tolerogenic environment. This includes the nature of donor liver APCs, presence or absence of costimulatory molecules, and cytokine microenvironment. Specifically, liver APCs are constitutionally capable of inducing tolerogenic responses in T cells (32).

Liver sinusoidal endothelial cells (LSECs) have a unique phenotype expressing myeloid cell markers (CD1, CD4, CD11c). These LSECs are similar to immature DCs, and make up a new type of organ-specific APCs. LSEC-activated CD4+ lymphocytes cannot differentiate into Th1 (IL-2-producing) cells, and express high immunosuppressive IL-10 levels (33). In addition, LSEC-stimulated CD8+ lymphocytes cannot respond to new antigenic stimuli (32).

A number of studies have discussed endothelial cell renewal in transplanted organs, and its relation to tolerance or rejection. Endothelial renewal has been demonstrated in transplanted hearts (34), kidneys (35), and livers (36). Laggij et al. (35) demonstrated that following a renal transplant the replacement of donor endothelial cells by host endothelial cells was correlated to vascular rejection. In the transplanted liver, it has been suggested that the replacement of donor endothelial cells by donor endothelial cells derived from bone marrow cells may favor graft tolerance (36,37). However, it was subsequently demonstrated that endothelial chimerism in the liver graft is not associated with tolerance induction in liver transplant patients after complete immunosuppression withdrawal (9).

It was recently demonstrated that the liver capacity to induce tolerance partly results from in situ T-cell activation. The power of liver cells as tolerogenic antigen-presenting cells is well known. Contrary to the dogma that naïve T cells cannot interact with parenchymal cells outside lymphoid organs, intrahepatic lymphocytes and circulating naïve CD8+ cells have been seen to interact with hepatocytes by means of cytoplasmic extensions capable of going through LSEC fenestrations (38) (Fig. 3). This local activation of T cells by hepatocytes provides the latter with a significant role as APCs and induces tolerance development in the liver (39).

THE PHENOMENON OF LIVER GRAFT TOLERANCE

Several experimental and clinical observations suggest that the liver is “less immunogenic” than other vascularized organs, and that liver grafts may induce tolerogenic properties in other organs.

Un unusual phenomenon in transplanted organs is spontaneous liver graft tolerance in selected species, including the mouse, rat, and pig in the absence of im-
munosuppression (40-42), despite an initial stimulation of the immune system (15). The transplanted liver may also behave as an immunosuppressor when transplanted to animals with rejection after a pancreas or heart transplant, and revert said rejection (43-44).

Liver grafts in humans are less susceptible to humoral rejection (45) and requires no MHC compatibility between donor and recipient (46). A liver transplant usually protects transplanted kidneys from the same donor against humoral and cell-mediated acute rejection (47). In selected liver transplant patients, immunosuppression can be definitely withdrawn in up to 25-30% of patients (7-10), who occasionally remain well for up to 30 years following said discontinuation, which is considered an “operational” tolerance (12).

The presence of tolerance in both animal and human models following a liver transplant has led to the use of such models in research on tolerance intrinsic mechanisms.

TOLERANCE MECHANISMS IN LIVER TRANSPLANTATION

While the precise mechanisms that establish and maintain tolerance to transplanted organs are poorly understood, the various tolerance studies performed in animals – less in humans – show that tolerance is an active, highly-regulated process. An immune activation process (lymphocyte proliferation and differentiation) usually occurs as immune response to liver grafts. This natural responsive process may result in two distinct behavior pathways (Fig. 4): (1) an immune pathway (or group of pathways) for graft destruction (rejection pathways) and (2) a pathway (or group of pathways) for tolerance (tolerogenic pathway). This dualistic paradigm posits the presence of liver graft-induced mechanisms that result in rejection or tolerance depending on the quantitative balance between these two pathways (15). Precise immune mechanisms underlying the tolerance pathway are not fully understood, but may include the following phenomena (48): (1) Clonal apoptosis-mediated deletion of donor-specific T cells (central or peripheral tolerance); (2) peripheral tolerance (regulation, anergy from deficient costimulation, deletion from repeat activation).

CENTRAL TOLERANCE

Central tolerance phenomena occur primarily in the thymus. T cell precursors derived from bone marrow stem cells mature in the thymus, where they learn to tell self from foreing. During T-cell differentiation CD4-CD8- lymphoid precursors become CD4+CD8+ T cells and then CD4+TCR+ T cells (if class II HLA molecules were presented) or CD8+TCR+ T cells (if class I HLA molecules were presented). When a T cell binds the peptide-HLA complex presented by thymic epithelial cells through a low-affinity TCR a so-called “positive selection” occurs, which allows the survival of lymphocytes that subsequently enter the circulation to ultimately colonize secondary lymphoid organs. In contrast, lymphocytes binding these complexes through high-affinity receptors experience apoptosis (negative selection) (49). Some self-reactive lymphocytes escape towards peripheral sites, and thus the presence of natural peripheral regulating mechanisms to control this kind of self-reactivity should be contemplated. These regulatory mechanisms have become fundamental for transplantation and autoimmunity research, and are mediated by regulatory T cells and tolerogenic dendritic cells.

Two experimental approaches exist in murine models that may generate highly stable central tolerance – allo-
geneic thymus transplantation or bone marrow transplantation, with both strategies being difficult to apply in the clinical setting.

With fetal or neonatal allogeneic thymus transplantation in a recipient thymectomized and depleted of CD4 and CD8 T cells, the host will remain at the mercy of de novo generated T cells able to recognize self from foreign, and rejection of an allogeneic graft with the same haplotype as that of the thymus is thus prevented (50).

Tolerance induction through bone marrow transplantation is based on the establishment of a mixed hematopoietic chimerism. When the recipient’s thymus is colonized by donor DCs central chimerism ensues. During T-cell differentiation in the thymus donor DCs may play a role in the purging of alloreactive T cells by negative selection or clonal deletion, thus giving rise to a collection of T cells tolerant to self and of donor alloantigens (51). However, if the deletion of alloreactive lymphocytes by mixed chimerism is not complete following bone marrow transplantation the potential for rejection remains, and tolerance is only possible when immunoregulating mechanisms for the control of these lymphocytes are developed (52,53).

The clinical application of bone marrow transplantation animal models using conditioning protocols with total lymphoid irradiation, or non-myeloablative protocols with depleting anti-CD4 and anti-CD40L antibodies, or CTLA4-IG fusion proteins inhibiting the binding of CD28 by CD80 or CD86, is difficult to establish. Since early experiences in renal transplantation by Monaco et al. (54) a number of studies have been carried out with liver, pancreas, heart, and lung transplants (55), but complete tolerance was never achieved. Many problems remain unsolved, particularly the deletion of peripheral alloreactive cells escaping central deletion after central chimerism is established with bone marrow cell transfusion (56).

**PERIPHERAL TOLERANCE. THE ROLE OF REGULATORY CELLS**

In liver graft spontaneous tolerance models there is significant early activation of host lymphocytes at secondary lymphoid organs, and their subsequent deletion from exhaustion (15). The stimulus is provided by the huge amount of donor leukocytes that rapidly migrate to lymphoid tissues and stimulate production of huge amounts of IL-2 and IFN-γ by host CD4 cells, that become depleted and subsequently undergo apoptosis (57).

In addition to the above activation-induced cell death mechanisms and other tolerance mechanisms including anergy and immunological ignorance, the presence of regulatory cells seems to play a central role in tolerance to transplanted organs (58). Various regulatory T-cell subtypes have been identified: suppressor CD4+CD25+

T cells, IL-10-producing T regulatory type 1 (Tr1) cells, transforming growth factor-β (TGF-β)-producing Th3 cells, natural killer (NKT) cells (CD8+CD28-CD27+ and CD4-CD8-)(12,59).

CD4+CD25+ T regulatory cells, together with NKT cells, develop spontaneously in the thymus, innately exert their immunoregulatory activity, and their effect does not seem to be antigen-dependent (60). CD4+CD25+ T regulatory cells were described by Sakaguchi et al. in 1990 (61), and were seen to represent 5-15% of peripheral CD4+ cells. This type of T regulatory cells also express memory (CD45RA-CD45RO+CD45RB+), CTLA-4 or CD152, and GITR (glucocorticoid-induced tumor necrosis factor receptor) phenotypes. It was recently demonstrated that a discriminating marker of CD4+CD25+ regulatory T cells was transcription factor Foxp3 (62). CD8+CD25+ T regulatory cells reduce immune response by altering allogeneic T response, antibody production, cytokine secretion, and antigen presentation (63). This type of regulatory cells, aside from inhibiting effector T cell cytolytic capacity, by intercellular contact convert other responsive CD4+CD25- T cells to a regulatory phenotype, thus amplifying the suppressing effect of regulatory cells (infectious tolerance) (64). This type of regulatory T cells, induced in peripheral blood, suppress allogeneic response through soluble mediators such as IL-10 and/or TGF-β.

The fact that human peripheral-blood CD4+CD25+ T regulatory cells can suppress response to alloantigens (65) has awaken interest in the generation of this type of cells as a therapeutic tool to induce tolerance in transplants. An example would be repeated naïve T cell stimulation with immature DCs, which would induce the production of CD4+CD25+ T regulatory cells (66).

It was recently seen that peripheral-blood CD4+CD5+-foxp3 cells increase in patients with liver transplant and operational tolerance (tolerance without immunosuppression after several years) (67,68). This finding, together with an increased expression of genes coding for Tγδ cells, NK receptors, and proteins involved in proliferation termination (68), seems to represent a characteristic of liver transplant patients who can tolerate immunosuppression withdrawal.

**DENDRITIC CELLS AND TOLERANCE**

Dendritic cells play a crucial role in the establishment of both central and peripheral tolerance. In peripheral sites DCs induce tolerance by neutralizing reactive T cells (deletion, anergy, or inactivation). In addition, immature DCs can induce CD4+CD25+ T regulatory cells in an antigen-specific manner (69). Tolerogenic DC generation by various strategies, including use of rapamycin (70), may induce tolerance, as has been demonstrated in animal models. The existence of both monocytoid DCs...
cytes, with their subsequent deletion from exhaustion. A liver. Migration within the recipient’s lymphoid system dependant upon the transfer of donor leukocytes in the species. Such spontaneous tolerance is an active process low its being spontaneously accepted in some animal.

**SUMMARY AND CONCLUSIONS**

The liver has particular tolerogenic properties that allow its being spontaneously accepted in some animal species. Such spontaneous tolerance is an active process dependant upon the transfer of donor leukocytes in the liver. Migration within the recipient’s lymphoid system results in early immune activation for recipient lymphocytes, with their subsequent deletion from exhaustion. A most significant mechanism in tolerance induction is the suppression of allogeneic responsiveness by regulatory T cells. Leukocyte-related microchimerism has been questioned as a primary mechanism for sustained tolerance.

In the clinical setting, systematically monitoring the various immunological parameters after a transplant will be most relevant to identify phenotypes, and maybe genetic polymorphisms associated with graft tolerance. Ongoing studies are currently analyzing this issue in an attempt to identify patient parameters that may predict tolerance to immunosuppression withdrawal after a period of adjustment to the new organ.

**REFERENCES**


65. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4qCD25q T cells with regulatory properties by repetitive stimulation with allogeneic immu-