In vitro Modeling of Early Donor-Recipient Immune Interactions for Liver Graft tolerance: Evaluation of Soluble CD83 Modulation

by

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To MOM and DAD, for all their love, support and sacrifices…

ABSTRACT

In vitro Modeling of Early Donor-Recipient Immune Interactions for Liver Graft tolerance: Evaluation of Soluble CD83 Modulation

Farinaz Nazmi

Doctor of Philosophy in Immunology May 05, 2025

Lifelong immunosuppression remains a major challenge in pediatric liver transplantation, underscoring the need for strategies that promote immune tolerance. This study aimed to model early donor–recipient immune interactions in vitro and assess the immunomodulatory potential of soluble CD83 (sCD83). Peripheral blood mononuclear cells from 18 liver transplant recipients were stimulated with anti-CD3/CD28 and donor liver homogenates, followed by co-culture of activated T cells with naïve B cells and T follicular helper cells in a transwell system, with or without sCD83 treatment. After 9 days, gene expression of CIITA and FOXP3 was analyzed by qPCR, and cytokine levels (IL-6, IL-10, IL-21) were measured via ELISA. While sCD83 did not suppress T cell activation, co-cultures with activated T cells resulted in significant downregulation of CIITA in B cells, suggesting early plasma cell differentiation, with no change in FOXP3 expression. Notably, cytokine analysis revealed reduced IL-6 and elevated IL-10 levels, indicating a shift toward a regulatory immune phenotype. This patient-derived co-culture model effectively simulates early alloimmune responses and highlights a context-dependent immunomodulatory role of sCD83, warranting further investigation using diverse cellular and organoid systems.

OZETCE

Karaciğer Greft Toleransı İçin Erken Donör-Resipiyent İmmün Etkileşimlerinin İn Vitro Modellemesi: Çözünür CD83 Modülasyonunun Değerlendirilmesi

Farinaz Nazmi

İmmünoloji, doktora
Mayis 05, 2025

Karaciğer naklinde ömür boyu immünosupresyon gereksinimi, özellikle pediatrik hastalarda, en önemli zorluklardan biri olmaya devam etmektedir. Bu çalışma, erken donör-alıcı immün etkileşimlerini in vitro ortamda modelleyerek çözünür CD83 (sCD83) molekülünün immün düzenleyici potansiyelini değerlendirmeyi amaçlamıştır. Toplam 18 karaciğer nakli alıcısından elde edilen PBMC'ler anti-CD3/CD28 ve donör karaciğer homojenatları ile uyarılmış, ardından aktive T hücreleri naif B hücreleri ve TFH hücreleriyle birlikte transwell sistemi içinde, sCD83 varlığında ya da yokluğunda ko-kültüre edilmiştir. Dokuz gün sonunda, CIITA ve FOXP3 gen ekspresyonları qPCR ile, IL-6, IL-10 ve IL-21 düzeyleri ise ELISA yöntemiyle analiz edilmiştir. sCD83 tedavisi T hücre aktivasyonunu baskılamamış olsa da, aktive T hücreleriyle yapılan ko-kültürler B hücrelerinde CIITA ekspresyonunun anlamlı şekilde azalmasına neden olmuş, bu da erken plazma hücresi farklılaşmasını düşündürmüştür. FOXP3 düzeyleri değişmeden kalırken, sitokin analizi IL-6'da azalma ve IL-10'da artış göstermiş, bu da düzenleyici bir immün ortamın geliştiğine işaret etmiştir. Bu hasta kaynaklı model, erken alloimmün yanıtların etkili bir simülasyonunu sunmakta ve sCD83'ün bağlama özgü etkilerini ortaya koymaktadır; molekülün greft toleransındaki rolünün daha kapsamlı anlaşılabilmesi için ileri hücre ve organoid temelli çalışmalara ihtiyaç vardır.

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ABBREVIATIONS

AIH	Auto-Immune Hepatitis
ALF	Acute Liver Failure
AMR	Antibody Mediated Rejection
APC	Antigen Presenting Cells
BA	Biliary Atresia
BREGs	Regulatory B cells
CMV	Cytomegalovirus
CNIs	Calcineurin Inhibitors
DCs	Dendritic Cells
DILI	Drug Induced Liver Injury
FOXP3	Fork head Box P3
GVHD	Graft Versus Host Disease
IRP	Ischemia-Reperfusion Injury
IS	Immunosuppressant
KC	Kupffer Cells
LSECs	Liver Sinusoidal Endothelial Cells
MHC	Major Histocompatibility
MDSC	Myeloid Derived Suppressor Cells
MMIC	Mesenchymal-Mediated Immune Control
mTOR	Mammalian Target Of Rapamycin
MMF	Mycophenolate Mofetil
PD-1	Programmed Death-1
PD-L1	Programmed Death-1 Ligand
PFIC	Progressive Familial Intrahepatic Cholestasis
PTLD	Post-Tranplant Limphoproliferative Disprder
RES	Reticuloendothelial
RSV	Respiratory Virus
TCR	T cell Receptor
TGFB-1	Transforming Growth Factor
TFH	T follicular Helper Cells

TFR	T Follicular Regulatory cells
TCMR	T Cell Mediated Rejection
TREGs	Regulatory T cells

Chapter 1

INTRODUCTION

1- Liver transplantation

1-1 Background on liver transplantation

End-stage liver disease remains a leading cause of morbidity and mortality in pediatric populations, and liver transplantation is currently the only definitive treatment for many children with progressive hepatic failure. Timely referral to specialized transplant centers is essential for patients with a high risk of liver-related mortality. Over the last four decades, pediatric liver transplantation has undergone transformative advances, with reported long-term survival rates now exceeding 85% in high-volume centers (Lauterio, Di Sandro et al., 2015). Moreover, improvements in donor allocation and surgical techniques have made transplantation increasingly accessible, with nearly 90% of children listed for transplant eventually receiving a liver graft (Yazigi, 2013).

Indications for pediatric liver transplantation differ significantly from those in adults, reflecting the unique disease spectrum in children. The most common indication is biliary atresia**,** accounting for nearly half of all pediatric liver transplants. Other cholestatic diseases such as Alagille syndrome**,** progressive familial intrahepatic cholestasis (PFIC), and alpha-1 antitrypsin deficiency also constitute major indications. In addition, metabolic liver diseases including urea cycle disorders**,** maple syrup urine disease**,** Crigler-Najjar syndrome**,** and glycogen storage diseases are increasingly recognized indications for transplant, even in the absence of end-stage cirrhosis, due to the potential for metabolic correction post-transplantation.

Acute liver failure (ALF) is another critical indication, often requiring urgent transplantation due to the rapid progression and unpredictability of the disease course. Pediatric patients with

ALF frequently present diagnostic and prognostic challenges, and decisions regarding transplant eligibility must be made swiftly. In selected cases, hepatic malignancies such as hepatoblastoma may also warrant liver transplantation, particularly when tumors are unresectable but confined to the liver.

Despite the increasing success of pediatric liver transplantation, significant challenges remain. Short-term complications include perioperative mortality, ischemia–reperfusion injury, and primary graft dysfunction. However, the most pressing issues in long-term care relate to the immune response particularly acute and chronic rejection and the systemic effects related to lifelong immunosuppression**,** including infections, renal dysfunction, cardiovascular disease, and impaired growth and neurodevelopment.

These limitations underscore the urgent need for strategies that promote immune tolerance and reduce reliance on chronic immunosuppressive therapy. Achieving a state of operational tolerance—defined as stable graft function without ongoing immunosuppression—has been documented in a small subset of pediatric recipients, particularly those who received living-donor grafts at an early age, suggesting a developmental window of enhanced immune adaptability.

1-2 Tolerance and liver

Transplantation tolerance refers to a state in which the recipient's immune system accepts a donor allograft without requiring ongoing immunosuppressive therapy, while retaining the ability to respond to infectious and neoplastic challenges. To induce and sustain the tolerance is the ultimate goal of transplantation immunology, as it would eliminate the need for chronic immunosuppression and its associated toxicities. Tolerance may arise spontaneously in some organ systems, particularly the liver, or may be induced through specific immune interventions.

Understanding the mechanisms by which tolerance develops is essential for designing targeted therapies that promote durable graft acceptance.

1-2-1 Central tolerance

The process of central tolerance takes place as lymphocytes mature, occurring in the thymus for T cells and in the bone marrow for B cells, where self-reactive clones are eliminated or inactivated. In the thymus, T cells expressing T cell receptors (TCRs) with high affinity for self-peptides presented by major histocompatibility complex (MHC) molecules undergo negative selection and apoptosis. A similar process occurs in the bone marrow for B cells, where autoreactive cells undergo receptor editing, deletion, or anergy.

In transplantation, central tolerance can be therapeutically induced via the establishment of **mixed chimerism** a state in which both donor and recipient hematopoietic cells coexist. This is typically achieved through transplantation of donor hematopoietic stem cells alongside the organ graft, allowing the thymus to present donor-derived antigens during T cell maturation. This strategy can lead to deletion of donor-reactive T cells. However, the induction of central tolerance in clinical settings remains limited due to the need for preconditioning regimens, risks of graft-versus-host disease, and difficulty in achieving stable engraftment.

1-2-2 Peripheral tolerance

Peripheral tolerance mechanisms function to control mature lymphocytes in the periphery that have escaped central deletion. These include:

1-2-3-1 Clonal anergy

T cells need to receive two signals to be fully activated: antigen recognition through the TCR and a co-stimulatory signal. When T cells encounter antigen in the lack of appropriate co-stimulation, they enter a state of anergy, in which they become functionally inactive and unresponsive to subsequent stimulation. This mechanism is important in preventing immune responses to non-threatening antigens, including donor alloantigens under certain tolerogenic conditions.

1-2-3-2 Activation induced cell death

Prolonged antigenic stimulation, particularly in the presence of high inflammatory signals, can lead to activation-induced cell death via apoptotic pathways. Fas–FasL interactions are a primary mechanism for this process. Clonal deletion eliminates donor-reactive T cells and reduces the size of the alloreactive T cell pool, contributing to long-term tolerance.

1-2-3-3 Regulatory T cells

Regulatory T cells, especially the $CD4^+CD25^+FOXP3^+$ subset, are central to the induction and maintenance of peripheral tolerance. Tregs function through:

- Secretion of anti-inflammatory cytokines such as IL-10 and TGF-β
- Direct suppression of effector T cells
- Inhibition of B cell antibody production
- Competition for IL-2, which limits effector T cell proliferation

In murine and human models, the expansion of Tregs has been associated with spontaneous or induced tolerance. Clinical trials exploring the adoptive transfer of ex vivo expanded autologous Tregs have shown safety also potential efficacy in kidney and liver transplant recipients. Tregs not only suppress acute rejection but may also modulate long-term fibrotic and chronic rejection pathways.

1-2-3-4 Regulatory B cells

B regulatory cells (Bregs) are appeared as pivotal modulators of immune responses in both liver immunobiology and transplantation contexts. Bregs support immune homeostasis through the production of IL-10, IL-35, and TGF-β, which dampen inflammatory T cell responses and restrain dendritic cell function. Their role extends beyond mere suppression, as they actively promote the expansion of regulatory T cells, thereby reinforcing the liver's intrinsic tolerance mechanisms. Notably, hepatic Bregs can interact with other resident immune cells, modulating both innate and adaptive arms of the immune system, which is particularly important in preventing excessive immune-mediated liver injury (Kimura, Rickert et al. 2020).

In the context of transplantation, Bregs are increasingly recognized as key players in the induction and maintenance of graft tolerance. Experimental and clinical data indicate that Bregs can prolong allograft survival by suppressing effector T cell proliferation, inhibiting pro-inflammatory cytokine production, and promoting a tolerogenic milieu within the graft

microenvironment(Liao, Yang et al. 2025). Importantly, their regulatory functions depend on both antigen-specific recognition and interaction with co-stimulatory pathways, as demonstrated in murine models where Breg-dependent tolerance required antigen engagement and TGF-β signaling (Kimura et al., 2020). Additionally, human studies have shown an enriched B cell–related gene signature in operationally tolerant transplant recipients, suggesting a potential biomarker role for Bregs in identifying patients who may benefit from immunosuppressive minimization (Chong and Khiew 2017) Collectively, these findings highlight Bregs not only as immune suppressors but also as active orchestrators of tolerance in liver and transplantation settings.

1-2-4 The role of cytokines in tolerance

The cytokine environment plays a pivotal role in determining whether an immune response results in graft rejection or tolerance. Pro-inflammatory cytokines such as IFN-γ IL-12 and IL-6, and promote Th1 and Th17 responses, which are associated with acute rejection as well as chronic rejection. and chronic rejection. In contrast, anti-inflammatory cytokines including IL-10, TGF-β, and IL-35 support regulatory cell development and suppress effector T cell activity.

An important element of the tolerogenic cytokine milieu is the balance between T follicular helper (TFH) cells and T follicular regulatory (TFR) cells. TFH cells promote B cell maturation and antibody production, including donor-specific antibodies (DSAs), which are associated with antibody-mediated rejection (AMR). TFR cells, by contrast, limit excessive B cell activation and help maintain tolerance.

Chronic exposure to tolerogenic cytokines can alter the transcriptional programming of T cells, reducing their capacity for cytotoxicity and enhancing regulatory phenotypes. This cytokine balance is therefore both a marker and a mediator of tolerance.

1-3 Liver-Specific Mechanisms of Tolerance

The liver is considered a uniquely tolerogenic organ and exhibits several features that distinguish it from other transplant sites. These characteristics contribute to the relatively high frequency of operational tolerance observed in liver transplant recipients, particularly children. The liver's mechanisms of tolerance involve several key features, including its unique anatomical and vascular characteristics, as well as its inherently tolerogenic microenvironment.

1-3-1 Anatomical and Vascular Features

The liver is uniquely supplied with blood from two sources: the hepatic artery and the portal vein. The portal vein delivers blood from the gastrointestinal tract, carrying a continuous influx of gut-derived antigens, including dietary proteins and microbial products, directly to the liver. This constant exposure necessitates a finely regulated immunological tolerance to prevent excessive immune activation and maintain systemic homeostasis (Biswas and Lopez-Collazo, 2009). The anatomical and microenvironmental features of the liver are fundamental to its tolerogenic capacity. Structurally, the liver is organized into functional units composed of liver sinusoidal endothelial cells (LSECs), which form capillary networks connecting to the hepatic vein, alongside non-parenchymal cells—including immune cells—that make up the hepatic reticuloendothelial system (RES). While these cells play essential roles in sensing inflammatory stimuli, their interactions are equally critical in promoting the liver's immunotolerant environment (Huang, Lu et al., 2018). As a result, the liver microenvironment is conditioned to favor immune adaptation and tolerance rather than immune activation.

1-3-2 Tolerogenic Microenvironment

The hepatic immune environment includes specialized resident immune cells such as:

- Kupffer cells (liver-resident macrophages), which produce IL-10 and prostaglandin E2
- Liver sinusoidal endothelial cells (LSECs), which present antigen in a non-inflammatory context

- Hepatic stellate cells, which may also have immunosuppressive functions

These cell types contribute to a milieu that favors Treg induction, deletion of effector T cells, and limited co-stimulation.

1-4 Liver immune system

Liver transplantation was first acknowledged in the 1960s when Roy Calne conducted liver transplants in outbred pigs. Their research demonstrated that 12 out of 55 allografts achieved long-term survival without the need for immunosuppression (Calne, Sells et al. 1969). Over the years, numerous similar studies have been carried out, and by 2009, approximately 22% of patients worldwide were able to maintain survival without immunosuppressive therapy (Orlando, Soker et al. 2009).

The liver has a distinctive ability to suppress immune responses, largely due to the fact that it receives a significant volume of blood from the gastrointestinal tract through the portal vein. This blood contains various nutrients and commensal microbial products, contributing to the liver's tolerance to foreign antigens (Doherty 2016). While this immune-regulatory property benefits liver transplant recipients by preventing graft rejection, it can also be detrimental in certain conditions, such as liver infections caused by viruses or the development of tumors within the liver (Koshiba, Li et al. 2007).

Several factors contribute to the liver's unique ability to suppress immune responses. Some of these include the local cytokine milieu, the presence of regulatory immune cells, and the contributions of both parenchymal and non-parenchymal liver cells.

1-4-2 Cytokines

A study conducted on IFN-gamma knockout allograft recipient mice revealed that acute rejection occurred, and the grafts did not survive beyond two weeks. In contrast, wild-type recipients exhibited long-term allograft survival. IFN-gamma, an inflammatory cytokine produced by effector T cells, is generally known to promote graft rejection. However, this

study found that in wild-type recipients, there was a notable decrease in cytotoxic CD8 T cells among the infiltrating immune cells, while CD4 T cells became predominant.

One possible explanation is that IFN-gamma production facilitates the apoptosis of infiltrating effector T cells, thereby shifting the immune response toward tolerance. In IFN-gamma knockout grafts, there was a significant increase in the CD8/CD4 ratio, but this imbalance was not observed in the draining lymph nodes or spleen. This suggests that IFN-gamma-mediated immune regulation primarily occurs within the graft itself (Li, Kuhr et al. 2008). This study suggests that tolerance in transplanted liver is initiated by the host immune response during the rejection process.

1-4-3 Regulatory cells

After transplantation, the liver's inflammatory environment encourages the accumulation of myeloid-derived suppressor cells (MDSCs) within the graft. These cells help promote immune tolerance through several pathways, including suppressing $CD4^+$ and $CD8^+$ T cell proliferation and enhancing the development of regulatory T cells (Tregs) (Li, Kuhr et al., 2008). Research has shown that in rejected allografts, there is often an increase in Th17 cells accompanied by a decrease in Treg numbers, highlighting the importance of maintaining a balance between these immune cell subsets for graft survival (Zhou, Yang et al., 2015).

1-4-4 Liver nonparenchymal cells

Liver non-parenchymal cells play a crucial role in determining the fate of liver allografts. This is evident from studies showing that while solid organ transplantation has been successfully performed for decades, cell-based transplants, such as hepatocyte transplantation, have had limited success. In fact, allogeneic hepatocyte transplants are acutely rejected (Bumgardner, Heininger et al. 1998), whereas whole liver allografts are more readily accepted. This contrast highlights the significant immunoregulatory influence of non-parenchymal cells in promoting graft tolerance. Following transplantation, the graft's CD45+ non-parenchymal cells are quickly replaced by recipient-derived cells, whereas CD45- cells retain their donor origin even in the long term. When these CD45- cells were isolated and introduced into a mixed lymphocyte reaction culture, they demonstrated the ability to suppress T cell proliferation. This immunosuppressive effect was mediated by enhancing the apoptotic death of

proliferating CD8 T cells, suggesting a crucial role for these donor-derived cells in promoting graft tolerance (Morita, Joyce et al. 2015). Hepatic progenitor/stem cells (HpSCs) are key non-parenchymal cells in the liver with strong immunoregulatory properties. Their tolerogenic potential has been demonstrated in islet transplantation, where co-transplantation with allogeneic islets effectively protected the grafts from rejection (Charles, Chou et al. 2013). This protective effect is linked to the expression of B7-homolog 1 (B7-H1), also known as programmed death ligand-1 (PDL-1), which induces apoptosis in effector T cells. Additionally, HpSCs contribute to immune tolerance by increasing the production of soluble factors such as inducible complement-3, which enhances the infiltration of myeloid-derived suppressor cells (MDSCs) into the graft (Hsieh, Chou et al. 2013). HpSCs also promote the expansion of regulatory T cells (Tregs) in an IL-2-dependent manner, further suppressing the activity of activated T cells. These immunoregulatory functions are dependent on an intact IFN-gamma signaling pathway in HpSCs. Moreover, recent findings indicate that HpSCs can directly suppress B cell responses both in vitro and in vivo in the presence of anti-CD40 and IL-4. This suppression is achieved by inhibiting B cell proliferation, reducing IL-6 production, and decreasing immunoglobulin secretion. The suppressive effects on B cells occur through direct cell-to-cell contact, involving surface molecules on HpSCs and activation of the B7-H1 pathways (Li, Lu et al. 2016).

1-4-4-1 Liver Kupffer cells

Almost 80% of total macrophages in the human body reside in the liver, known as Kupffer cells (KCs). Positioned in the liver sinusoids, KC is critical in filtering blood from the portal vein. As antigen-presenting cells (APCs), they can mediate both anti-inflammatory and pro-inflammatory responses. Their functional phenotype can shift between M1 and M2 depending on the environmental state. The anti-inflammatory effects of KCs are driven by the production of IL-10 as well as their interactions with regulatory T cells (Tregs) (Breous, Somanathan et al. 2009). In this context, because of the presence of IL-10, liver dendritic cells (DCs) also exhibit reduced immunostimulatory capacity compared to their counterparts in other organs (Pillarisetty, Shah et al. 2004). Considering the innate lymphoid cells in the liver, including Natural Killer cells (NKs), they can both have role in tolerance and rejection as

well. However, donor NK cells can have cytotoxic activity on the recipient's T effector cells (Cerboni, Zingoni et al. 2007). Within the liver resident T cells, CD8 T cells are more abundant compared to CD4 cells. The immune regulation of these cells is dependent on quantity of antigen. While low amount of antigen can lead to robust immune activation, abundance of antigen, like what happens in the transplantation, results in T cell depletion. This depletion occurs due to the insufficient growth factors, inadequate expression of co-stimulatory molecules and the upregulation pf programmed death-1 (PD-1) ligand, which collectively suppress function of T cells (Tay, Wong et al. 2014).

Studies conducted both *in vitro* and *in vivo* have shown that their interaction with CD8 T cells induces proliferation and IL-10 secretion, which in turn suppresses CD8 T cell function. Additionally, Kupffer cells exhibit high surface expression of B7-H1 (PD-L1) and PD-1 while displaying low levels of MHC-II. This unique expression profile allows them to act as central cellular scavengers, efficiently capturing and processing circulating antigens, thereby contributing to immune tolerance in the liver (Li, Zhu et al. 2015). In the presence of pre-existing liver damage, the tolerogenic function of Kupffer cells is weakened or even reversed, leading to antigen-specific cytotoxicity. This shift is accompanied by a phenotypic change in Kupffer cells from a tolerogenic M2 profile to a pro-inflammatory M1 state, characterized by high expression of CD80 and the accumulation of inflammatory macrophages. This phenotypic shift in Kupffer cells is regulated by their interaction with mesenchymal cells. In other words, while Kupffer cells secrete pro-inflammatory cytokines in response to antigens, their proinflammatory cytokine production is significantly reduced in the presence of mesenchymal cells. Additionally, the secretion of immunosuppressive cytokines, such as IL-10 and transforming growth factor-beta (TGF-β), is markedly increased, further promoting an immunoregulatory environment (Zhao, Sun et al. 2016).

The liver allograft is not merely a passive recipient of host immune attacks; rather, it actively mounts a counter-response. The dependence of mesenchymal-mediated immune control (MMIC) on IFN-gamma suggests that a pro-inflammatory microenvironment is a prerequisite for inducing liver transplantation tolerance. MMIC functions as an intrinsic negative feedback loop, in which mesenchymal cells interact with effector lymphocytes to regulate the inflammatory response. Unlike graft-versus-host disease (GVHD), which is driven by graft-

derived lymphocytes attacking various recipient cells, MMIC is mediated by graft mesenchymal cells targeting recipient effector T cells. For MMIC to function successfully, several requirements must be fulfilled, including the presence of enough active mesenchymal cells, a functional IFN-gamma/B7-H1 signaling pathway, and a supportive microenvironment that enables effective cell-to-cell communication. While MMIC has been most commonly described in the liver, evidence suggests it is not limited to this organ, as spontaneous acceptance of kidney allografts has also been reported, though it occurs less often compared to the liver. The presence of stellate cells in other organs, such as the kidney and pancreas, suggests that MMIC may be a broader phenomenon. However, the liver's distinct anatomy and high abundance of hepatic progenitor/stem cells (HpSCs) likely enhance its MMIC capacity, contributing to the varying outcomes of liver allograft acceptance versus rejection (Moris, Lu et al. 2017).

1-4-4-2 Liver regulatory Dendritic cells

In murine models of liver transplant tolerance, donor-derived dendritic cells (DCs) have been shown to play a pivotal role in modulating alloreactive T cell responses and promoting immune tolerance. Experimental depletion of these cells, achieved through the administration of CD11c-targeted diphtheria toxin in recipient mice, results in a breakdown of tolerance and the onset of acute rejection, underscoring their critical regulatory function (Lu, Rudert et al. 1995, Yokota, Yoshida et al. 2016).

In addition to donor-derived DCs, recipient dendritic cells also contribute significantly to the establishment of liver transplant tolerance. Notably, recipient CD11c+ DCs infiltrating the graft acquire donor MHC class I molecules through a process termed "cross-dressing." This phenomenon occurs early after transplantation, with the highest frequency of cross-dressed DCs detected within the graft around day 7 post-transplant. These cross-dressed DCs display a tolerogenic phenotype, marked by elevated expression of PD-L1, secretion of the anti-inflammatory cytokine IL-10, and a functional capacity to suppress donor-specific T cell proliferation. Concurrently, an increase in PD-1hi TIM-3+ CD8+ T cells, often associated with T cell exhaustion or deletion, is observed within the graft, further indicating the induction of a tolerogenic immune environment (Ono, Perez-Gutierrez et al. 2018).

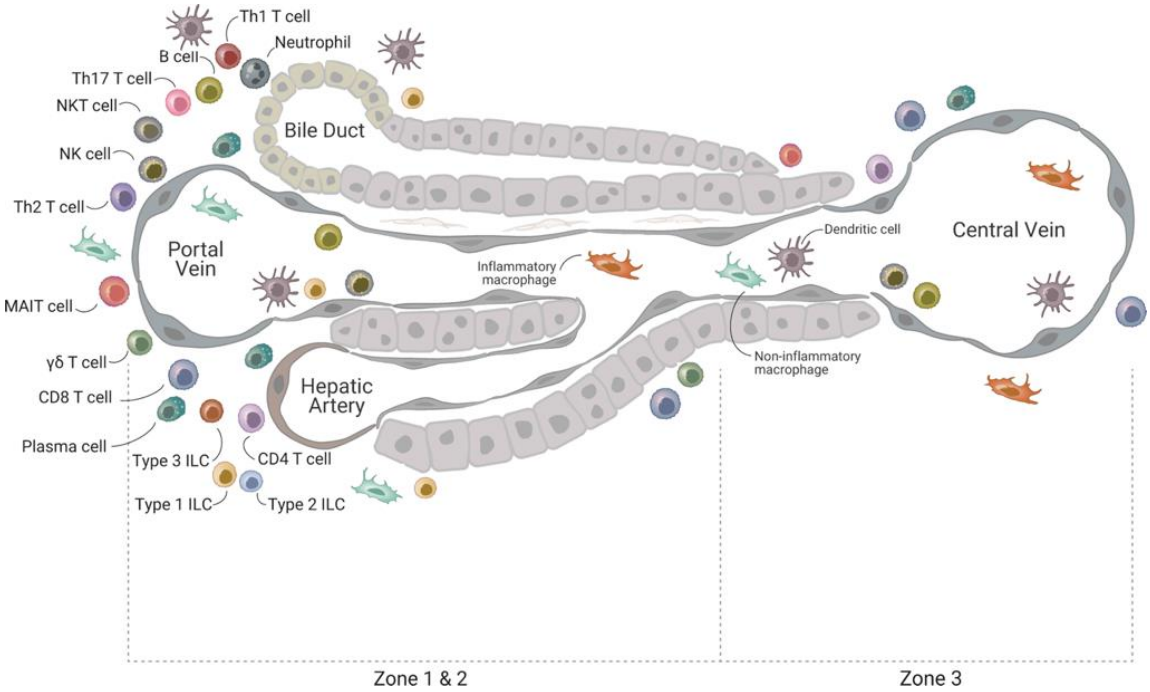


Figure 1.1: The immune niche of the liver (Cheng, Nakib et al. 2021).

1-5 Operational tolerance in the liver

The tolerance induction after liver transplantation is mediated through three main mechanisms. These include 1) The activity of passenger leukocytes 2) The modulation of inflammatory responses and 3) the balance between effector T cell deletion and regulatory T cell proliferation. The concept of "rejection induces tolerance" highlights the mechanism where inflammatory responses after LT lead to the establishment of immune tolerance. As an inflammatory cytokine, the secretion of IFN-γ triggers the upregulation of PD-L1 on the LSECs and MDSCs. This, in return, induces the apoptosis of the effector T cells (Mele, Kneteman et al. 2003, Morita, Joyce et al. 2015). Moreover, the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-dependent pathways also play a complementary role. The resulting reduction in the population of effector T cells activity, promotes the proliferation of

the Tregs. Liver grafts trigger an allo-reactive immune response in the recipient that is inherently flawed due to the activation caused by passenger leukocytes (PL) (Li, Zheng et al. 2005). Mechanisms driven by IFN-γ gradually tempers this immune response that the immune system initiates. These include the activation of LSECs and hepatic stellate cells (hSCs), the recruitment of myeloid-derived suppressor cells (MDSCs), the polarization of Kupffer cells to an M2 phenotype, PD-L1-induced T cell apoptosis, and the proliferation of regulatory T cells (Tregs). Additionally, there is direct cytotoxicity from NK cells and the promotion of a tolerogenic phenotype in DCs. Together, these processes lead to the deletion of effector T cells and the establishment of immune tolerance in the liver graft.

Previously, it was hypothesized that clonal deletion of T cells occurs in the liver, similar to the process in the thymus (Kamada 1985). However, this hypothesis was directly tested by analyzing T cell receptor usage in a mouse liver transplantation model. The study's findings revealed no evidence of T cell deletion (Dahmen, Qian et al. 1994). Despite this, another study examining cytotoxic T cell activation through antigen-presenting cells (APCs) demonstrated that these cells become fully activated within the liver (Klein and Crispe 2006). In a study conducted by Qian et al. (Qian, Lu et al. 1997) shortly after transplantation, there was a significant infiltration of lymphocytes, accompanied by high donor-specific T cell activity. However, these infiltrating lymphocytes gradually disappeared due to locally induced apoptotic cell death. This study suggested that long-term graft survival was achieved through the elimination of activated T cells via apoptosis.

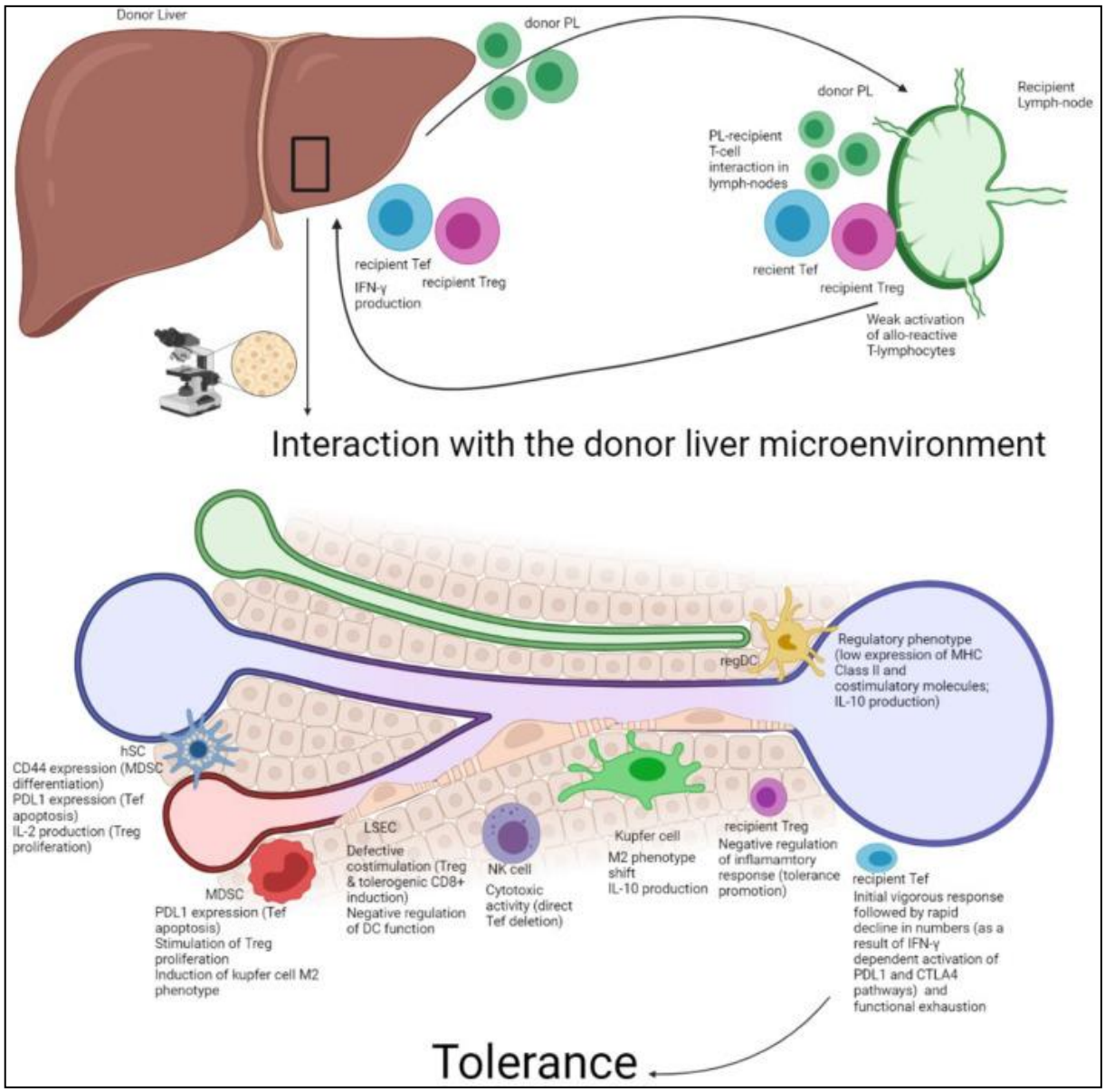


Figure 1.2: Putative mechanisms of tolerance (Toti, Manzia et al. 2021).

1-5-1 Clinical Evidence of Operational Tolerance

Spontaneous or operational tolerance, defined as long-term graft function without immunosuppression, has been observed in a subset of liver transplant recipients. Pediatric recipients are particularly prone to this phenomenon, possibly due to greater immunological

plasticity, reduced memory T cell populations, and early exposure to donor antigens. Immunological profiling of tolerant patients has revealed increased Treg numbers, altered B cell signatures, and specific gene expression profiles associated with regulation.

These observations suggest that the liver's intrinsic features can be harnessed or mimicked in therapeutic strategies aiming for tolerance induction in other organ systems.

1-6 Opposite of tolerance: rejection

Graft rejection following solid organ transplantation is a complex process involving numerous immune mediators. It is characterized by the recognition of donor alloantigens by the recipient's immune system, which subsequently triggers both innate and adaptive immune responses directed against the graft. Over time, these processes can culminate in vascular injury, tissue necrosis, fibrosis, and ultimately, graft failure. Understanding the cellular and molecular mechanisms underlying allograft rejection is essential for developing targeted immunosuppressive therapies aimed at preserving long-term graft function and improving transplant outcomes.

Although the liver possesses distinctive immunological characteristics that grant it a degree of immune privilege relative to other organs, immunological rejection remains a significant challenge for transplant recipients. Rejection is most commonly observed within the first month after the transplant procedure. and are typically managed with high-dose corticosteroids. Acute rejection may also arise later in the post-transplant period, often presenting with less typical clinical features. While historically more prevalent within the initial months following transplantation, the incidence of chronic rejection has markedly declined in the modern era of immunosuppressive therapy and is now estimated to affect only 2–3% of recipients, often manifesting several years after the procedure. Liver allograft rejection involves multiple pathways, including both cellular and antibody-mediated mechanisms.

1-6-1-1 Acute cellular rejection

Acute cellular rejection, also referred to as T cell-mediated rejection (TCMR), commonly arises during the early post-transplant period and is generally manageable with immunosuppressive therapy. It is frequently associated with a cholestatic pattern in liver function tests. To confirm the diagnosis, a liver biopsy is essential, allowing for the histological evaluation of inflammatory cell infiltration and injury to both the biliary epithelium and hepatocytes (Figure 1-A-B) (Shetty, Adams et al. 2012). Although not always clinically urgent, if left untreated or unresolved, this form of rejection may persist and lead to long-term complications.

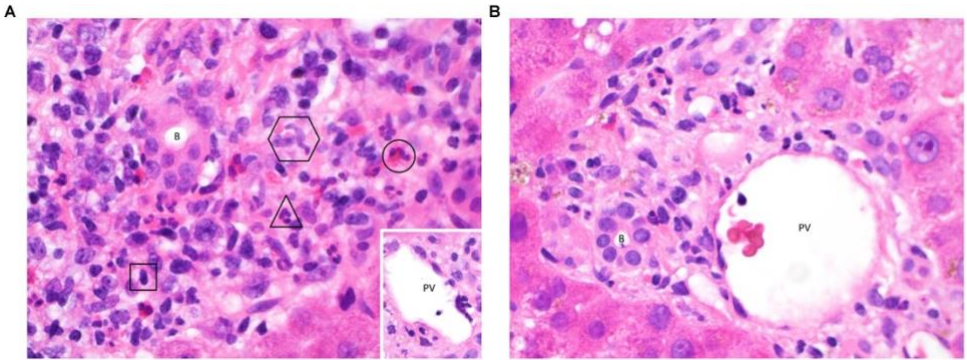


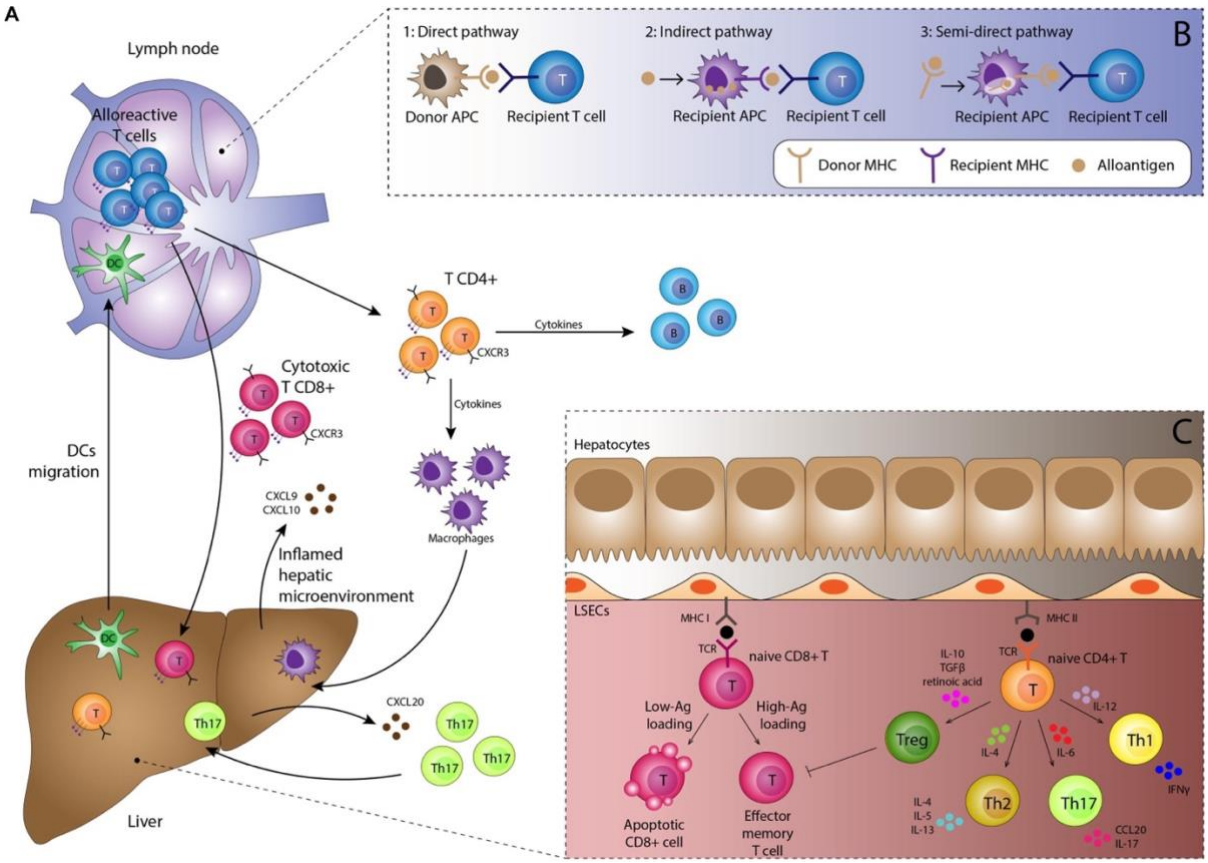
Figure 1.3: (A) Infiltrated lymphocytes in hepatic inflammation (B) Normal liver with rare lymphocyte infiltration.

Rejection is largely driven by alloantigen presentation by activated donor derived DCs, which are transferred with the liver and reside in portal and perivascular areas. Inflammatory stimuli such as ischemia-reperfusion injury enhance their activation and migration to lymphoid tissues. In lymph nodes, these DCs engage recipient naïve T cells via the direct antigen presentation pathway, where both the foreign MHC molecule and bound peptide are recognized as non-self. T-cell activation requires three key signals: TCR recognition of the peptide-MHC complex, integrin-mediated adhesion, and co-stimulatory signaling (e.g., CD28-B7 interactions). Once activated, T cells initiate a signaling cascade involving calcineurin and NFAT, ultimately leading to IL-2 production—a key driver of T-cell proliferation and clonal expansion. The **indirect** and **semi-indirect** pathways of allorecognition

are predominantly mediated by recipient-derived dendritic cells (DCs) that gradually accumulate within the allograft. This accumulation is facilitated through mechanisms such as direct cell-to-cell interactions and the uptake of donor-derived exosomes. In the indirect pathway**,** donor alloantigens are internalized, processed, and presented by recipient DCs or other antigen-presenting cells (APCs) via self-MHC molecules to naïve T cells. In contrast, the semi-indirect pathway involves the presentation of intact donor MHC-peptide complexes by recipient APCs, without processing (Demetris, Bellamy et al. 2016, Lei, Reinke et al. 2019).

When stimulated through these pathways, $CD8^+$ T cells mature into cytotoxic T lymphocytes (CTLs), which are directly involved in causing graft damage. At the same time, $CD4^+$ T cells differentiate into multiple effector subsets, such as Th1, Th2, Th17 cells, and regulatory Tregs, each contributing uniquely to shaping the immune response toward either graft rejection or tolerance (Figure 1. 2A-C).

Figure 1.4: (A) Pathways of T cell mediated rejection (Ronca, Vincenzo, et al. 2020).



In the context of TCMR, activated CD8+ cytotoxic T cells contribute to allograft injury by recognizing donor MHC class I molecules expressed on biliary epithelial cells, LSECs, and hepatocytes. The resulting tissue damage is primarily executed through the granzyme-perforin pathway or via Fas-FasL interactions, where Fas ligand (FasL) on T cells engages Fas receptors on target cells, leading to apoptosis. CD4+ T cells contribute to rejection by secreting pro-inflammatory cytokines such as interferon-gamma (IFN-γ) and interleukin-2 (IL-2), which activate macrophages and further amplify the immune response. Th2 cells, through interactions with B cells, promote the generation of donor-specific antibodies, while

Th17 cells exacerbate tissue damage by producing IL-17, a cytokine that facilitates neutrophil recruitment to the graft site (Goddard, Williams et al. 2001, Benechet and Iannacone 2017).

Notably, memory T cells also play a significant role in acute rejection. Their rapid responsiveness and lo and increased production of donor-specific antibodies, thereby intensifying graft injury (Hartigan, Sun et al. 2019).

Macrophages exhibit functional plasticity and can adopt either pro-inflammatory or immunosuppressive phenotypes, depending on the cytokine milieu. Stimulation with IFN-γ typically drives macrophages toward a pro-inflammatory phenotype, while IL-13 and IL-4 promote an immunosuppressive profile. During acute rejection, macrophages are predominantly polarized into a pro-inflammatory state, characterized by the secretion of cytokines such as IL-6, TNF-α, IL-18, and the production of ROS. This activation is triggered by signals from damaged tissues, which engage pattern recognition receptors like Toll-like receptors (TLRs) on the macrophage surface (Azad, Donato et al. 2018).

Similarly, neutrophils are rapidly recruited to the site of inflammation during TCMR, particularly in response to PRI and the secretion of Th17-associated cytokines such as IL-17. In contrast, eosinophils**,** which are hallmarks of TCMR in the liver, are recruited and activated under the influence of Th2 cytokines, particularly IL-4 and IL-5. Once activated, eosinophils release cytotoxic granules that disrupt cell membranes and contribute to tissue damage (Rodríguez-Perálvarez, Germani et al. 2012, Huang, Tohme et al. 2015).

Additionally, natural killer (NK) cells may be activated during rejection through interactions with MHC class I chain-related molecules A and B (MIC-A/B) expressed on the allograft. Under normal conditions, NK cell activity is suppressed through engagement of killer immunoglobulin-like receptors (KIRs) with self-MHC class I molecules. However, during rejection, non-self MHC I molecules may fail to adequately engage KIRs, leading to a loss of inhibitory signaling and subsequent NK cell activation (Ware and Kumar 2017).

1-6-1-2 Antibody mediated rejection

Hyperacute rejection, the most severe and immediate form of allograft rejection, is mediated by pre-existing donor-specific antibodies (DSAs) and classified under antibody-mediated rejection (AMR). Although this phenomenon is extremely rare in liver transplantation especially in the context of ABO-incompatible grafts—it remains a critical consideration. Diagnosing AMR is particularly challenging, as histological findings from liver biopsy alone are often insufficient. A comprehensive evaluation incorporating clinical presentation, serological evidence, and immunohistochemical staining is necessary for accurate diagnosis (Heidt, Hester et al. 2012).

DSAs may be pre-formed (present prior to transplantation) or develop de novo post-transplantation. Their formation begins when naïve B cells encounter alloantigens within an inflamed microenvironment. Under the influence of co-stimulatory signals—such as CD28-B7 and CD40-CD40L interactions—B cells undergo proliferation and differentiation. Some of these activated B cells become antibody-secreting plasma cells, producing DSAs that target donor antigens. Others migrate to lymphoid follicles, where they form germinal centers and undergo somatic hypermutation and affinity maturation, enhancing the specificity and potency of the antibody response (O'Leary, Kaneku et al. 2014). The binding of these antibodies to non-self MHC molecules on the graft surface initiates a cascade of events, including complement activation, ultimately contributing to graft injury (Figure 1-3 A–B). Although the precise role of these mediators in antibody-mediated rejection (AMR) within the liver remains incompletely defined, chemotactic molecules such as C3a and C5a, known as anaphylatoxins, are recognized as potent pro-inflammatory agents. These complement fragments are believed to contribute significantly to AMR by activating mast cells and basophils, enhancing macrophage activity, promoting the recruitment of granulocytes, including eosinophils, and increasing vascular permeability, thereby amplifying local inflammation and tissue injury (Valenzuela, McNamara et al. 2014).

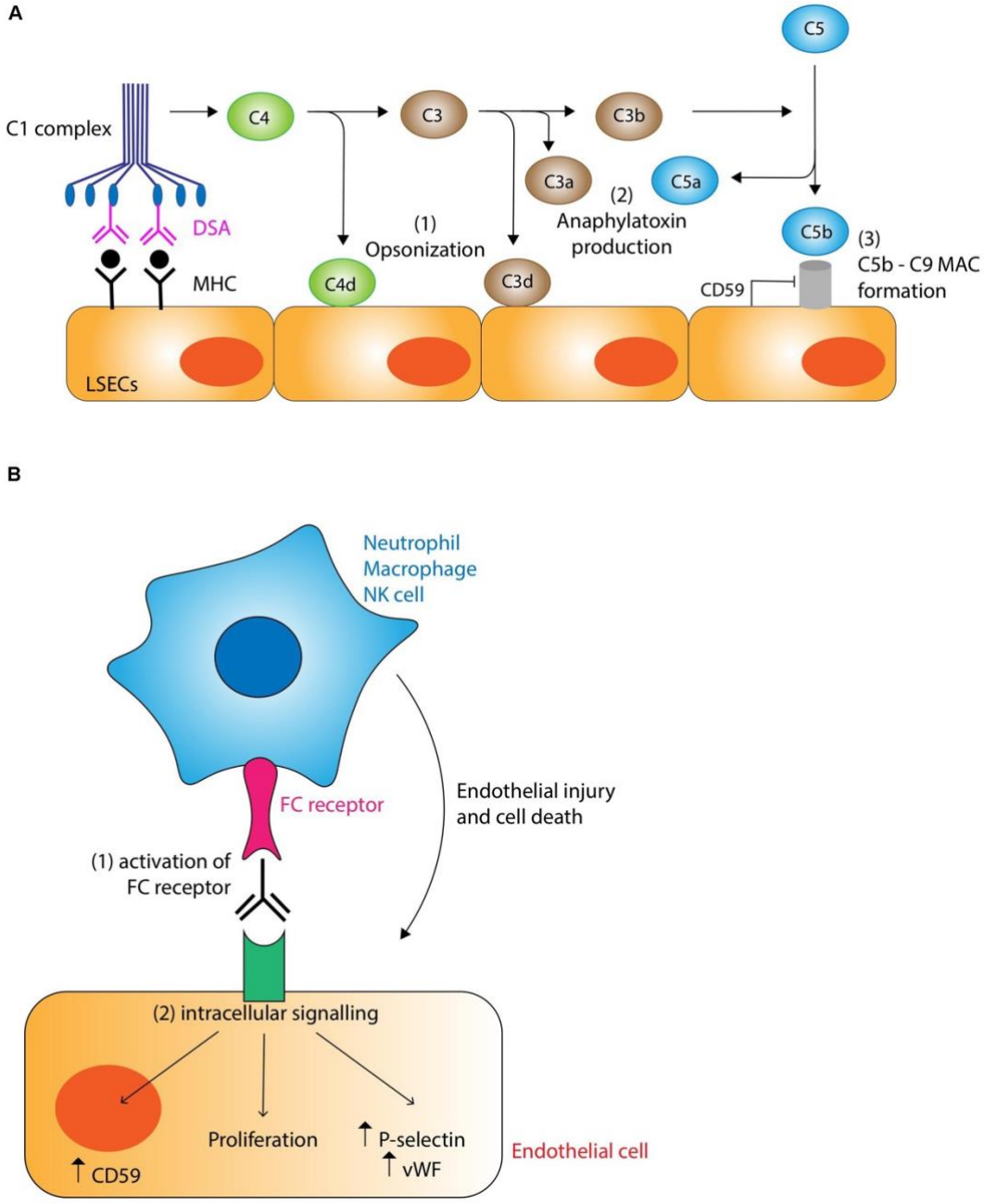


Figure 1.5: Pathways of antibody mediated rejection (Ronca, Vincenzo, et al. 2020).

1-7 Immunosuppression in Liver Transplantation

Immunosuppressive therapy is the cornerstone of modern liver transplantation (LT), enabling the prevention of acute and chronic allograft rejection by inhibiting recipient immune responses to donor antigens. Despite the liver's relatively tolerogenic nature compared to other solid organs, immunosuppression is still essential, particularly during the early post-transplant period. However, long-term use of immunosuppressive agents is associated with significant adverse effects, especially in pediatric recipients, necessitating careful monitoring and individualized regimens.

The objectives of immunosuppression in the context of liver transplantation include:

a. Prevention of acute cellular and antibody-mediated rejection
b. Maintenance of stable long-term graft function
c. Minimizing drug-related toxicities
d. Preservation of immune defense against infections and malignancies
e. Facilitation of immune adaptation or operational tolerance in selected cases

Achieving a balance between adequate immunosuppression and avoidance of over-immunosuppression is a dynamic process that often requires adjustment over time.

1-7-1 Common Immunosuppressive Agents in Liver Transplantation

Immunosuppressive regimens typically involve a combination of agents targeting different pathways of the immune response. The main classes include corticosteroids, calcineurin inhibitors, inhibitors of mammalian target of rapamycin (mTOR), antimetabolites and antibody therapies.

1-7-1-1 Corticosteroids

These drugs, commonly administered in the form of hydrocortisone, prednisone, prednisolone, and methylprednisolone, exert their immunosuppressive effects by inhibiting antibody production, upregulating anti-inflammatory cytokines such as IL-10, and downregulating pro-inflammatory mediators like IL-6 and IL-2. However, due to their significant adverse effects, corticosteroids are typically tapered and discontinued within 3 to 6 months following liver transplantation to minimize long-term complications. Common complications following the withdrawal of immunosuppressive medications include acute rejection, the development of steroid-resistant rejection, and renal impairment (Fairfield, Penninga et al. 2018).

1-7-1-2 Calcineurin inhibitors

Calcineurin inhibitors (CNIs) represent a key class of immunosuppressive agents used in transplantation, with two principal drugs in this category: cyclosporine and tacrolimus. Cyclosporine functions by binding to cyclophilin, an intracellular protein, thereby inhibiting the activation of calcineurin. This inhibition prevents the transcription of interleukin-2 (IL-2), ultimately suppressing T cell activation and proliferation. Tacrolimus, the primary immunosuppressant used in liver transplantation, exerts a similar effect by binding to a different intracellular protein, FK-binding protein (FKBP). This complex also inhibits calcineurin phosphatase activity, thereby blocking T lymphocyte activation and reducing the risk of allograft rejection (Patel, Cook et al. 2016).

1-7-1-3 Inhibitors of mammalian target of rapamycin

Mammalian target of rapamycin inhibitors (mTOR inhibitors) function by blocking the mTOR complex, a critical regulator of cellular metabolism, growth, and proliferation. One widely used mTOR inhibitor is sirolimus. Like tacrolimus, sirolimus binds to the FK-binding protein

(FKBP); however, it acts synergistically with tacrolimus by inhibiting downstream signaling from interleukin-2 (IL-2) receptors, thereby suppressing both T and B cell proliferation. Everolimus, a derivative of sirolimus, shares a similar mechanism of action but also has the added benefit of reducing angiogenesis. Additionally, everolimus has a shorter half-life compared to sirolimus, which may influence dosing strategies and therapeutic monitoring (Yatscoff, Wang et al. 1995, De Simone, Fagiuoli et al. 2017).

1-7-1-4 Inhibitors of purine and pyrimidine synthesis

Mycophenolic acid (MPA) and azathioprine (AZT) are the principal antimetabolite agents used in immunosuppressive therapy. As a purine synthesis inhibitor, MPA interferes with the synthesis of guanosine monophosphate (GMP), a critical component for DNA replication. Mycophenolate mofetil (MMF), a prodrug of MPA, is commonly used due to its improved oral bioavailability. By inhibiting GMP production, these agents effectively suppress the proliferation of both B and T lymphocytes, thereby reducing the risk of immune-mediated graft rejection (Germani, Pleguezuelo et al. 2009, De Simone, Fagiuoli et al. 2017).

1-7-1-5 Antibody therapy

Antibody-based therapies are primarily employed during the induction phase of immunosuppression or in cases of steroid-resistant rejection. These agents are classified into two main subgroups: monoclonal and polyclonal antibodies. Monoclonal antibodies such as basiliximab and daclizumab are humanized antibodies directed against the interleukin-2 receptor alpha chain (IL-2RA), thereby inhibiting T cell activation. Polyclonal preparations, including anti-thymocyte globulin (ATG) and anti-lymphocyte globulin (ALG), consist of antibodies targeting multiple T cell surface antigens such as CD2, CD3, CD4, and CD8. These are typically administered via a central venous catheter and are used for both induction therapy and the treatment of steroid-resistant rejection, although they are employed less frequently than monoclonal antibodies due to their broader immunosuppressive effects and higher risk of adverse events (Lupo, Panzera et al. 2008, Penninga, Wettergren et al. 2014).

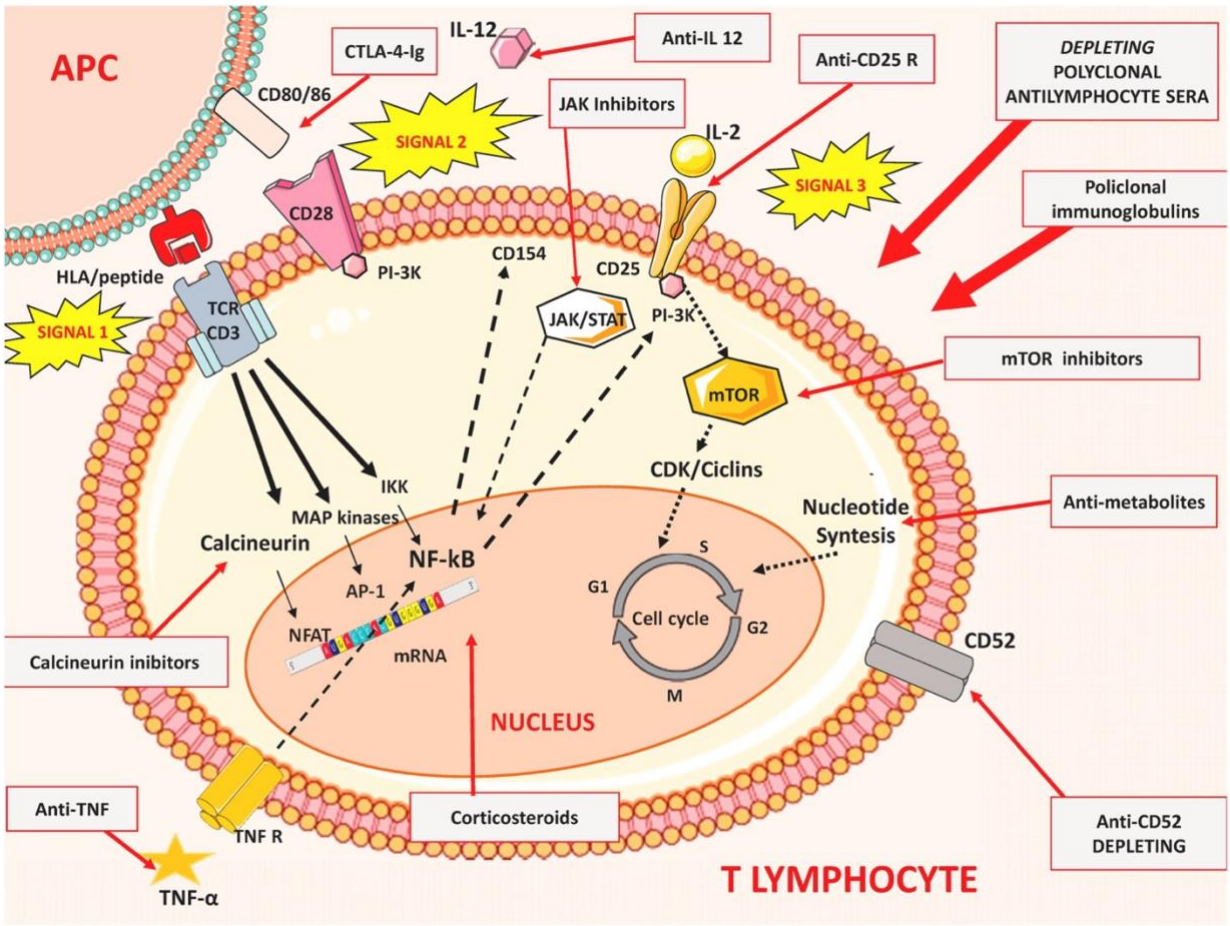


Figure 1.6: Mechanisms of action in IS treatments (Meneghini, Bestard et al. 2021).

1-7-2 Immunosuppression in Pediatric Liver Transplantation

Children have distinct pharmacokinetics and immune profiles compared to adults, necessitating age-appropriate immunosuppressive protocols. Key considerations include:

- Greater susceptibility to infections (e.g., CMV, EBV, opportunistic fungi)
- Heightened sensitivity to drug toxicities, including growth suppression, nephrotoxicity, and neurocognitive effects

- Higher rates of nonadherence during adolescence, which is a leading cause of late rejection and graft loss
- Potential for immune plasticity and operational tolerance, making children ideal candidates for future weaning studies

1-7-3 Adverse effects of IS

Immunosuppressive therapy plays a vital role in the success of liver transplantation by preventing immune-mediated graft rejection and promoting long-term graft survival. However, while these drugs are essential for maintaining tolerance to the transplanted organ, their prolonged use can lead to a range of complications. Although calcineurin inhibitors (CNIs) remain the cornerstone of immunosuppressive therapy due to their high efficacy, their long-term use is associated with significant adverse effects. One of the most notable complications is nephrotoxicity, primarily resulting from endothelial injury and arteriolar vasoconstriction, which contribute to chronic renal ischemia. In addition, post-transplant patients receiving CNIs frequently develop new-onset diabetes mellitus (NODM), hyperlipidemia, hypertension, and neurotoxicity. There is also an increased risk of malignancies, including skin cancers and post-transplant lymphoproliferative disorders (PTLD), further highlighting the importance of careful monitoring and individualized immunosuppressive regimens (Wilkinson and Pham 2005, Farouk and Rein 2020).

Compared to calcineurin inhibitors, mTOR inhibitors such as sirolimus and everolimus are associated with a distinct side effect profile. Hematological complications, including anemia and leukopenia, are relatively common. These agents may also contribute to hyperlipidemia by disrupting insulin signaling pathways. Although less frequently than with CNIs, mTOR inhibitors can also cause proteinuria. Pulmonary toxicity, though rare, has been reported and represents a serious potential adverse effect. Additionally, mTOR inhibitors are known to impair wound healing and may cause mucosal irritation, such as stomatitis (Silva Jr, Cibrik et al. 2010, Wadei, Zaky et al. 2012). Similar to CNIs, their use is also linked to an increased risk of malignancies. Antimetabolites such as mycophenolate mofetil (MMF) are commonly

associated with gastrointestinal side effects, including nausea, diarrhea, and abdominal discomfort. In addition to these, MMF has been linked to hepatotoxicity, which may present as cholestatic liver injury, nodular regenerative hyperplasia, or sinusoidal obstructive syndrome. Though less common, acute pancreatitis is another potential complication observed in patients receiving MMF (Wagner, Earley et al. 2015). Corticosteroids, which modulate both pro-inflammatory and anti-inflammatory signaling pathways, are associated with a broad range of adverse effects, including a heightened risk of infections (Ramamoorthy and Cidlowski 2016). In solid organ transplant recipients, studies have reported a 1.3- to 5.9-fold increase in infections caused by multidrug-resistant bacteria and opportunistic fungi, such as Nocardia, Aspergillus, and Pneumocystis jirovecii. Additionally, the incidence of viral infections including cytomegalovirus (CMV), adenovirus, and respiratory syncytial virus (RSV) is significantly elevated in this population. Corticosteroid therapy has also been linked to an increased risk of tuberculosis and strongyloidiasis, particularly in individuals receiving long-term glucocorticoid treatment, emphasizing the importance of infection surveillance and prophylactic strategies in transplant recipients (Penninga, Wettergren et al. 2014).

In our previous study, we investigated how immunosuppressive (IS) treatments affect B regulatory cell (Breg) frequencies and broader immune dynamics in pediatric patients. We assessed total B cells, IL-10$^+$ B cells, Bregs, and IL-10$^+$ Bregs in peripheral blood mononuclear cells (PBMCs) after incubation with various IS drugs followed by cytokine stimulation. While overall B cell and Breg frequencies remained similar to baseline under *in vitro* conditions, treatment with 6-mercaptopurine (6MP) and tacrolimus (Tac) notably increased the proportion of IL-10$^+$ total B cells. In the clinical cohorts, we recruited pediatric patients across eight groups, including autoimmune hepatitis (AIH) patients under standard IS regimens, those with drug-induced liver injury (DILI), liver transplant recipients on Tac, and healthy controls. Longitudinal immune monitoring over 24 months revealed that total T cells, B cells, and CD4$^+$/CD8$^+$ T cell frequencies remained stable, as did naïve CD4$^+$ and CD8$^+$ T cells. However, memory B cells increased significantly during the first year and remained elevated. While total and naïve regulatory T cells (Tregs) gradually declined over time, activated Tregs increased in long-term IS recipients, though their expression of CTLA-4

diminished. Additionally, the ratio of effector T cells to Tregs remained elevated in AIH patients compared to healthy controls, suggesting persistent immune dysregulation despite treatment. Together, these findings highlight the nuanced impact of IS therapy, with selective modulation of B and T cell subsets, underscoring the need for further investigation into immune balance under long-term IS regimens (Yuksel, Nazmi et al. 2023).

Table 1-1 Summary of the Complications after long-term Immunosuppressants Following Liver Transplantation

Immunosuppressant	Associated Complications
Calcineurin Inhibitors (CNIs)(Tacrolimus, Cyclosporine)	Nephrotoxicity/chronic kidney disease1, cardiovascular disease, Neurologic disease2, Thrombotic microangiopathy, Chronic nephrotoxicity (defined as elevated serum creatinine for 3+ months, progressive), Hyperuricemia (can impair kidney's ability to excrete uric acid), Malignancy (chronic use associated with increased risk, including chronic kidney disease, diabetes, hyperlipidemia, hypertension), Thrombotic microangiopathy/hemolytic uremic syndrome (associated with tacrolimus), Malignancy (increase risk, decrease genome stability, impair DNA repair), De novo solid tumors (2–3 fold higher risk), Non-melanoma skin cancers (10–30 fold higher risk), Post lymphoproliferative disorder (PTLD) (10–30 fold higher risk), Higher risk of nonmelanoma skin cancers compared to other IS10 , PTLD, Cyclosporine associated with increased risk of breast cancer, Cumulative exposure to tacrolimus associated with increased risk of de novo post-LT malignancies, Neurotoxicity (long-term effects on CNS limited data, cognitive dysfunction, brain atrophy), Proposed mechanisms for cerebral atrophy: premature atherosclerosis, microangiopathy, White matter hypodensities on MRI associated with increased tacrolimus and cyclosporine trough levels, Posterior reversible encephalopathy syndrome (PRES), Neurologic complications potentially more common with tacrolimus, Tremor, Gingival hyperplasia (Cyclosporine), Hirsutism (Cyclosporine), Cardiac effects (Cyclosporine accelerates arteriosclerosis)
Antimetabolites, (Mycophenolate mofetil - MMF, Azathioprine)	Gastrointestinal side effects such as nausea, vomiting, abdominal discomfort, and diarrhea are common concerns. Myelosuppression, which results from impaired purine synthesis and impacts both white and red blood cell production, can occur with these agents. Azathioprine use has been linked to hepatotoxicity, manifesting as mild, temporary increases in aminotransferase levels, cholestatic liver injury, nodular regenerative hyperplasia, and sinusoidal obstructive syndrome, as well as an elevated risk of acute pancreatitis (though this has not been consistently observed in kidney transplant studies). MMF tends to carry a greater risk of diarrhea compared to either MMF monotherapy or azathioprine. Additionally, MMF is classified as Category X due to its association with pregnancy loss and birth defects. Regarding blood cell effects, leukopenia is more frequent with MMF than azathioprine during the first post-transplant year, while anemia appears more commonly with azathioprine. Both drugs can contribute to neutropenia. Long-term use of azathioprine, particularly beyond five years, has also been associated with an increased risk of nonmelanoma skin cancers. Notably, MMF has not been linked to a heightened risk of posttransplant lymphoproliferative disorder (PTLD).
Mammalian Target of Rapamycin (mTOR) inhibitors (Sirolimus, Everolimus)	Myelosuppression, Proteinuria, Impaired wound healing, Stomatitis, Myelosuppression, Hyperlipidemia (significantly higher rates than CNIs, increased total cholesterol and triglycerides), Proteinuria (higher rates than CNIs, risk of progressive renal disease), Renal failure rates potentially lower for everolimus compared to CNIs, Pulmonary toxicity (rare but potentially fatal, interstitial lung disease, bronchiolitis obliterans organizing pneumonia, pulmonary alveolar hemorrhage), May reduce the risk of malignancy compared to CNIs28, Impaired wound healing (by inhibiting cellular proliferation and angiogenesis; especially associated with sirolimus loading doses), Stomatitis (associated with sirolimus and everolimus, can be severe)
Corticosteroids (Glucocorticoids - GC)	Numerous adverse effects, Infectious complications (higher risk of bacterial, fungal, viral infections, multidrug-resistant bacteria, Nocardia, Aspergillosis, PCJ, CMV, adenovirus, RSV), Increased risk of tuberculosis and Strongyloidiasis, Bone disease, New Onset Diabetes Mellitus (NODM) or exacerbation of preexisting DM, Hypertension, Dyslipidemia (increased total cholesterol, triglycerides, decreased HDL), Obesity, Neuropsychiatric adverse effects, Gastrointestinal adverse, Increased risk of thrombosis, Adrenal insufficiency, Exposure for 1+ month associated with increased risk of basal cell carcinoma and squamous cell carcinoma, Steroid-free regimens associated with reduced risk of DM and CMV infection
Agents for Induction or Steroid-Refractory Rejection	Complications similar to OKT3, May be an independent risk factor for CMV disease and malignancy, May result in significantly higher rate of leukopenia when used with tacrolimus, MMF, and steroids compared to no rATG induction, Infusion reactions (cytokine release syndrome, SIRS - fever, chills, oxygen desaturation, tachycardia, hypo/hypertension, urticaria), Increased risk for infections (PCJ, CMV). Severe lymphocyte depletion results in increased risk for infections, Associated with severe recurrent hepatitis, Increased risk for infections (PCJ, CMV).

1-7-4 Weaning and Minimization Strategies

In light of the long-term risks of immunosuppressive therapy, clinical interest has grown in weaning protocols aimed at reducing or even discontinuing immunosuppression in selected patients. Studies have identified pediatric liver recipients—particularly those with stable graft function, living donor grafts, and early transplantation—as potential candidates for gradual immunosuppressive withdrawal under close immunological monitoring.

Operational tolerance is characterized by:

Sustained graft function without rejection

Absence of DSAs

Enrichment of regulatory immune cell populations (e.g., Tregs, transitional B cells)

Although promising, such strategies are not yet standard of care and are currently limited to controlled research settings.

1-8 Current graft monitoring tools and limitations

1-8-1 Liver function tests

After liver transplantation, monitoring graft health is critical to detect early signs of dysfunction or rejection. Routine liver function tests (LFTs) are among the primary tools used for this purpose. LFTs measure serum levels of enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and bilirubin, providing an indirect assessment of hepatocellular integrity and biliary function. While abnormal LFT results can raise suspicion for complications such as acute rejection, biliary obstruction, infection, or recurrent disease, they often lack specificity and sensitivity in differentiating between causes of graft injury. Studies have shown that LFTs perform poorly in accurately assessing the state of the liver allograft after transplantation. As a result, when LFTs are persistently abnormal without a clear clinical

explanation, further diagnostic procedures, such as liver biopsy, are frequently required to confirm the underlying pathology.

Table 1- 2 Liver Function Test (LFTs)

Alanine aminotransferase (ALT)	Alanine aminotransferase (ALT) is an enzyme primarily located in the liver, though smaller amounts are present in the kidneys, heart, and muscles. Under normal conditions, ALT levels in the blood remain low; however, when liver cells are damaged, they release ALT into the circulation. Conditions such as bile duct obstruction, cirrhosis, or liver cancer may result in ALT levels that are only slightly elevated or sometimes within the normal range.
Alkaline phosphatase (ALP)	Alkaline phosphatase (ALP) is an enzyme present throughout the body, playing a role in various physiological processes. The primary contributors of ALP in the bloodstream are the liver and bones. Elevated ALP levels can suggest the presence of liver conditions or specific bone disorders.
Gamma-glutamyl transferase (GGT)	Gamma-glutamyl transferase (GGT) is an enzyme predominantly found in the liver, with normally low concentrations in the bloodstream. When liver damage occurs or bile flow is obstructed, GGT levels typically rise, making it a valuable indicator for identifying bile duct abnormalities. Testing GGT can also help clarify the cause of elevated alkaline phosphatase (ALP) levels, as both GGT and ALP increase in liver and bile duct conditions, while ALP alone is elevated in bone disorders. Elevated GGT levels may also be observed in individuals who consume excessive alcohol or those taking medications metabolized by the liver, such as carbamazepine and phenobarbitone.
Aspartate aminotransferase (AST)	Aspartate aminotransferase (AST) is an enzyme primarily located in the liver but is also present in red blood cells, the heart, and other muscles. When cells in these tissues are damaged, AST is released into the bloodstream. Very high AST levels are commonly seen in acute hepatitis, while chronic hepatitis usually results in normal or slightly elevated AST levels. Conditions such as bile duct obstruction, cirrhosis, or liver cancer can lead to mild or near-normal AST elevations.
Bilirubin	Bilirubin is a yellow pigment produced as part of the body's natural process of breaking down aging red blood cells. The liver plays a key role in processing and eliminating bilirubin from the body. Under normal conditions, only minimal amounts circulate in the blood, but when liver function is impaired, bilirubin levels can rise. Elevated bilirubin in the bloodstream may result from excessive production, impaired removal, or bile duct blockages. High bilirubin levels lead to jaundice, a condition characterized by yellowing of the skin and the whites of the eyes.
Albumin	Albumin is the primary protein produced by the liver and plays a crucial role in transporting hormones, vitamins, medications, and minerals like calcium through the bloodstream. One of its key functions is maintaining the body's fluid balance by preventing fluid from leaking out of blood vessels into surrounding tissues. Individuals with chronic liver conditions or kidney disease are at risk for low albumin levels, either due to reduced production by the liver or excessive loss through impaired kidneys. When albumin levels are insufficient, fluid can accumulate in the lungs and other areas of the body. While albumin concentrations may remain normal in the early stages of liver disease, they typically decline as liver damage progresses, making albumin measurement a useful marker for assessing liver disease severity.
Total Protein	The total protein test evaluates the combined levels of albumin and other blood proteins, including antibodies. In many cases, total protein levels remain within the normal range even in the presence of liver disease.
Prothrombin Time (PT)	Prothrombin is a protein produced by the liver that plays a key role in blood clotting and helps prevent excessive bleeding. The prothrombin time (PT) test measures how quickly blood clots. Prolonged clotting time indicated by this test can signal potential liver dysfunction.

1-8-2 Protocol liver biopsy

A liver biopsy is still considered the most definitive method for making a diagnosis for acute rejection and evaluating graft health in transplant recipients. In both transplanted and non-transplanted patients, biopsy allows for definitive diagnosis, assessment of disease severity, and monitoring of treatment responses. In cases of unexplained abnormal liver function tests, liver biopsy can establish a diagnosis in approximately 90% of patients.

In transplant medicine, protocol liver biopsies—performed at predetermined time points rather than in response to clinical deterioration—have historically been advocated, especially during the early and late phases post-transplantation. Early protocol biopsies, often conducted around seven days after transplantation, are recommended because LFTs alone are insufficient to diagnose or determine the severity of acute rejection. Early histological evaluation enables timely adjustment of immunosuppressive therapy, preserving graft function. Late protocol biopsies usually performed after six months, help distinguish between normal and pathological changes, adjust immunosuppressive regimens, and detect subclinical disease that may not yet be reflected in biochemical tests.

In our previous study, we emphasized that liver biopsy remains the gold standard for investigating the pathophysiology of liver diseases, both before and after transplantation. However, despite its value, liver biopsy—as well as peripheral blood sampling—offers limited opportunity for comprehensive profiling of the hepatic immune landscape. To address this limitation, we explored whether liver fine needle aspirates (FNAs), as a less invasive alternative, could be used to characterize the hepatic immune microenvironment in pediatric patients. For the first time, in a large cohort of pediatric transplant recipients, we demonstrated that FNAs are a practical and reliable tool for sampling the liver immune system. Notably, FNA-derived data showed stronger correlations with several liver function parameters compared to both blood and liver tissue, highlighting the unique potential of this approach in advancing the understanding of pediatric liver immunology (Yuksel, Demirbaş et al. 2022).

1-8-3 Limitations

The routine use of protocol biopsies has declined due to several limitations. The procedure is invasive and carries risks such as pain, bleeding, and, in rare cases, more severe complications. Additionally, liver biopsies are costly, with expenses varying based on the healthcare setting and region. Interpretation of biopsy results can also be subjective, with potential for sampling errors and inter-observer variability among pathologists. Complications, although relatively rare, can be serious. Pain at the biopsy site is the most common, affecting up to 30% of patients. Bleeding occurs in about 1–2% of cases and can necessitate interventions such as blood transfusions or surgery. Infections, while uncommon, are possible, and there's also a risk of injury to adjacent organs. Furthermore, sampling errors can occur, as only a minuscule portion of the liver is examined, potentially missing focal lesions or providing an inaccurate assessment of disease extent.
Given the limitations associated with liver biopsies, there is a growing emphasis on developing and utilizing non-invasive diagnostic methods thus, there is an urgent need for reliable, non-invasive **biomarkers** that can predict or reflect allograft acceptance or rejection.

1-9 Rational for deep immunologic monitoring

Despite significant advances in liver transplantation outcomes, predicting and managing immune responses to the allograft remain major clinical challenges. Standard clinical tests give limited insights into the underlying immunological mechanisms driving graft acceptance or rejection in addition to other limitations. Given the complex and dynamic nature of the immune response post-transplantation, **deep immunologic monitoring** is increasingly recognized as a critical tool to better understand graft status, tailor immunosuppressive therapies, and potentially predict long-term outcomes.

The integration of systemic and tissue-level immune profiling offers a unique opportunity to uncover cohort-specific immune signatures, characterize early immunological events that precede clinical rejection or tolerance, and ultimately contribute to the development of more personalized, biomarker-driven post-transplant care strategies.

1-10 Introduction to CD83

CD83, located on chromosome 6p23, is a member of the immunoglobulin superfamily. In humans, it encodes a 45 kDa protein consisting of 205 amino acids and is highly glycosylated (Dudziak, Nimmerjahn et al. 2005). The most stable form of CD83 is expressed on dendritic cells from various tissues in both humans and mice (Zhou, Schwarting et al. 1992). However, it is also found on other cell types, including B cells, neutrophils, monocytes, macrophages, and natural killer (NK) cells (Li, Ju et al. 2019). Additionally, CD83 is expressed on light zone B cells within germinal centers (Victora, Dominguez-Sola et al. 2012). Studies have identified the membrane-bound form of CD83 on the surface of non-regulatory human T cells. In contrast, $CD4^+$ regulatory T cells in mice exhibit high levels of CD83 expression (Kreiser, Eckhardt et al. 2015). Following activation of Toll-like receptors (TLRs) on the cell surface or in response to TNF secretion, CD83 is transported from the Golgi apparatus to the surface and endosomal compartments in dendritic cells (DCs), macrophages, and B cells. CD83 protein is found in two forms, membrane bound and soluble form.

1-10-1 mCD83 functions

The transmembrane region of the CD83 protein interacts with and inhibits the membrane-associated RING-CH8 (MARCH-8) ubiquitin ligase, which normally promotes the internalization and degradation of MHC class II molecules. Similarly, membrane-bound CD83 (mCD83) enhances the expression of MHC-II and CD86 on activated antigen-presenting cells, such as B cells and dendritic cells, by modulating the activity of another ubiquitin ligase, MARCH-1. Through this signaling, mCD83 plays a regulatory role by suppressing the function of various immune cell populations. For instance, when mCD83 is engaged by specific antibodies or binds to neighboring cells also expressing CD83 (homotypic interaction) *in vitro*, it reduces the cells' ability to mature and secrete pro-inflammatory cytokines. This effect is mediated via the MAPK signaling pathway (Bates, Flanagan et al. 2015).

Studies have shown that CD83 expression on B or T cells in mice promotes increased proliferation and enhanced survival of these cells *in vivo*. However, transgenic overexpression

of CD83 in these cells has the opposite effect, resulting in reduced proliferation, diminished immunoglobulin production and class-switch recombination, along with elevated secretion of regulatory cytokines—particularly IL-10—from marginal zone B cells (Kretschmer, Lüthje et al. 2007).

1-10-2 sCD83 functions

The mechanisms underlying the immunosuppressive functions of soluble CD83 (sCD83) are still under investigation. To explore this, several studies have utilized recombinant sCD83 (rsCD83) constructs, in which the extracellular domain of CD83 is fused to an immunoglobulin Fc region. These constructs have consistently demonstrated immunosuppressive properties (Lechmann, Krooshoop et al. 2001). For instance, interaction of rsCD83 with dendritic cells (DCs) expressing mCD83 leads to reduced secretion of pro-inflammatory cytokines. Additionally, rsCD83 disrupts F-actin–dependent calcium signaling in DCs by preventing the co-localization of ORAI1 channels and mitochondria at the DC–T cell immunological synapse. Furthermore, binding of rsCD83 to the TLR4/MD-2 complex on monocytes promotes the production of anti-inflammatory mediators such as indoleamine 2,3-dioxygenase (IDO), interleukin-10 (IL-10), and prostaglandin E2 (PGE2), in a cyclooxygenase-2 (COX-2)–dependent manner. This cascade ultimately suppresses T cell proliferation and IL-2 secretion (Chen, Zhu et al. 2011, Horvatinovich, Grogan et al. 2017).

1-10-3 Soluble CD83 versus mDC83 signaling pathways

While the expression pattern of CD83 has been clearly established through the use of reporter mouse models and detailed cellular phenotyping, the identification of its receptor or binding partner (CD83L) remained uncertain for an extended period. Many investigations seeking to define CD83's interaction partners focused on the binding dynamics of soluble CD83 (sCD83) with potential receptors. It has been shown that sCD83 can bind to murine B cells, human immature and mature dendritic cells (DCs), and activated $CD8^+$ T cells. However, none of these studies definitively confirmed a specific binding partner. Notably, it was discovered that sCD83 must form higher-order multimers, specifically dodecamers, to

successfully bind activated human T cells and T cell leukemia lines—an interaction not seen with the conventional dimeric form of sCD83 (Hirano, Butler et al. 2006).

Interestingly, although studies using dimeric sCD83 revealed that human monocytes and DCs express a putative CD83 ligand (CD83L), the data suggest that sCD83 may engage different ligands with varying binding affinities. Several CD83 interaction partners have been proposed. For instance, the transmembrane domain of CD83 has been shown to bind MARCH-family E3 ubiquitin ligases, contributing to the stabilization of immune molecules such as CD86 and MHC class II. Additionally, in human mature DCs, CD83 interacts with GRASP55—a protein crucial for Golgi structure and CD83 surface transport (Stein, Blume et al. 2015). Regarding sCD83 specifically, recent research by Bates et al. introduced the possibility of homotypic CD83 interactions—that is, CD83 binding to itself. This hypothesis is supported by the observation that many cell types capable of binding sCD83 also express membrane-bound CD83 (mbCD83). For example, human T cells require prior activation (via anti-CD3/CD28 stimulation) to bind sCD83, a process that simultaneously induces mbCD83 expression. This homotypic interaction may integrate both the regulatory role of sCD83 and the modulatory effects of mbCD83. If either binding partner is absent, the immune response may become dysregulated. Since CD83 lacks classic intracellular signaling motifs, its homotypic binding could serve as a scaffold for recruiting other signaling proteins. This could also explain the distinct distribution patterns of CD83L depending on whether sCD83 is in dimeric or multimeric form. However, homotypic interaction alone does not fully account for sCD83's effects on monocytes, which, unlike DCs, do not express mbCD83 under resting conditions. A more recent study addressed this by showing that sCD83 directly binds to the MD-2/TLR4 complex on monocytes. This interaction initiates an anti-inflammatory signaling cascade, leading to sustained downregulation of IRAK-1, thereby rendering the cells unresponsive to further TLR stimulation. Interestingly, regulatory T cells (Tregs) lacking CD83 exhibit increased IRAK-1 levels and show abnormal activation, a phenotype also seen in CD83-deficient DCs. These cells are more likely to promote autoimmunity due to their impaired regulatory function (Wild, Krzyzak et al. 2019).

In addition, a very recent study proposed a new role for sCD83 within the regulatory immune network. It was found that sCD83 binds to CD154 on Th2 cells, suppressing expression of the anti-apoptotic protein Bcl2L12 and promoting apoptosis in this T cell subset. *In vivo*, administration of sCD83 to mice with allergic rhinitis significantly reduced disease symptoms by dampening the Th2 response. Consistently, patients with allergic rhinitis displayed lower serum levels of sCD83, which negatively correlated with IgE concentrations. Since B cells are thought to be the primary producers of natural sCD83, these findings align with the observed Th2 bias in mice lacking CD83 in B cells (Packhäuser, Roman-Sosa et al. 2017). Although it is possible that mbCD83 interacts with some of the same partners as sCD83, this has not yet been experimentally validated. Future studies aimed at identifying additional interaction partners across various cell types will be crucial for fully understanding the extensive regulatory network controlled by CD83. Collectively, the idea that mbCD83 can act in trans to modulate immune responses, similarly to sCD83, may help explain the altered immune phenotypes observed in CD83 conditional knockout models.

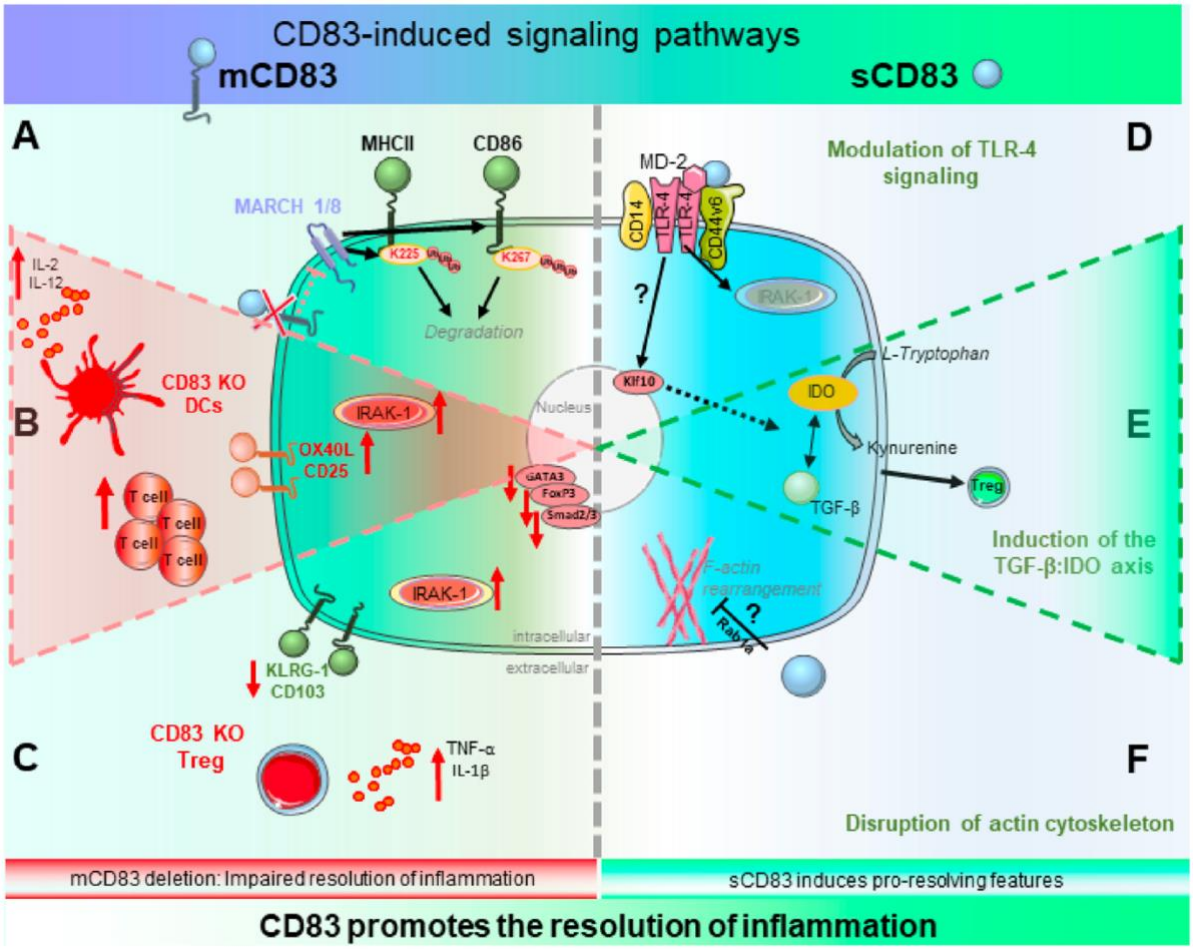


Figure 1.7: Signaling pathways of mCD83 and SCD83 (Peckert-Maier, Royzman et al. 2022)

1-10-4 Role of sCD83 in autoimmunity

Soluble CD83 (sCD83) proteins have demonstrated promising therapeutic effects in several mouse models of autoimmune and inflammatory conditions. In the experimental autoimmune encephalomyelitis (EAE) model, which serves as an analog for multiple sclerosis (MS), intraperitoneal injection of recombinant sCD83 (rsCD83) successfully prevented disease development and was linked to decreased production of T cell-associated cytokines, such as IFN-γ, IL-2, IL-4, and IL-10. In a mouse model of inflammatory bowel disease, rsCD83 treatment helped ease colitis symptoms by reducing the infiltration of inflammatory cells and maintaining the structural integrity of the colon, largely by inducing sustained expression of indoleamine 2,3-dioxygenase (IDO) in dendritic cells, which promoted immune tolerance by influencing T cell responses. Similarly, in a model of experimental autoimmune uveitis (EAU), applying rsCD83 topically provided protection by promoting the formation of IDO-expressing tolerogenic DCs and suppressing $CD4^+$ T cell activity both in eye tissues and the spleen. Additionally, rsCD83 therapy reduced the expression of mCD83 on $CD83^+CD3^-NK1.1^+$ cells, a subset known to infiltrate inflamed ocular tissue. Transfer of rsCD83-treated NK cells into EAU mice resulted in marked reductions in retinal inflammation and tissue injury. In a systemic lupus erythematosus (SLE) mouse model, rsCD83 administration notably delayed the production of disease-associated anti-dsDNA autoantibodies and lowered anti-histone IgG levels when compared to untreated mice. (Starke, Steinkasserer et al. 2013, Lin, Man et al. 2017).

1-10-5 Role of sCD83 in transplantation

Beyond its role in modulating autoimmunity, sCD83 also holds promise as a therapeutic agent for reducing solid organ transplant rejection. In murine models, administration of rsCD83 significantly delayed acute cellular rejection of major histocompatibility complex (MHC)-mismatched skin grafts by suppressing recipient T cell responses. Similar protective effects were observed in renal and corneal transplant models, where rsCD83 treatment prevented graft rejection through the induction of tolerogenic mediators such as indoleamine 2,3-dioxygenase (IDO) and TGF-β (Lan, Ge et al. 2010, Bock, Rössner et al. 2013). In a cardiac allograft setting, rsCD83 not only extended graft survival but also promoted donor-specific

tolerance, which was associated with diminished activation markers on dendritic cells and a reduced capacity to stimulate allogeneic responses. Interestingly, despite its immunosuppressive effects in animal models, elevated levels of endogenous sCD83 have been detected in various human autoimmune and inflammatory conditions. For example, increased concentrations of sCD83 have been found in the synovial fluid and serum of patients with rheumatoid arthritis, as well as in the serum of individuals with multiple sclerosis. The functional significance of these elevated sCD83 levels remains unclear, but it is hypothesized that they may represent a compensatory mechanism by which the immune system attempts to restore homeostasis. Targeting CD83 with antibodies presents a promising strategy to selectively eliminate activated APCs capable of triggering allogeneic T cell responses, while preserving non-activated APCs that contribute to immune tolerance and maintaining memory T cells essential for protection against infections and malignancies. This approach was first tested in preclinical models using a polyclonal rabbit-derived anti-human CD83 antibody, and later refined with the development of a high-affinity human IgG1 monoclonal antibody (mAb), 3C12C. Both antibodies effectively induced antibody-dependent cell-mediated cytotoxicity (ADCC) against CD83-expressing cells, especially activated DCs, thereby suppressing allogeneic T cell proliferation in mixed leukocyte reactions without impairing memory T cell responses to viral antigens such as cytomegalovirus and influenza. In a preclinical xenogeneic graft-versus-host disease (GVHD) model, where human peripheral blood mononuclear cells (PBMCs) were introduced into SCID mice, a single 125 μg dose of either antibody prevented the onset of acute GVHD. Importantly, this was achieved without significantly depleting donor-derived total T cells, regulatory T cells (Tregs), or compromising memory T cell responses to viral and tumor antigens. Additionally, administration of up to 10 mg/kg of 3C12C in non-human primates was well tolerated, showing no major clinical side effects or changes in overall blood cell counts. Nonetheless, reductions were observed in specific $CD83^+$ cell populations, including $CD1c^+$ DCs and B cells (Lan, Ge et al. 2010). Furthermore, anti-CD83 antibody therapy has demonstrated efficacy in other inflammatory contexts. In a xenogeneic mouse model of giant cell arteritis (GCA), treatment with a murine anti-human CD83 mAb resulted in depletion of activated

DCs within human artery grafts, preventing both immune cell infiltration and activation of co-transferred human T cells (Ma-Krupa, Jeon et al. 2004).

This study aims to provide valuable insights into how the immune system interacts with transplanted livers and how we might be able to improve transplant acceptance. If our model successfully identifies markers of immune tolerance and demonstrates that sCD83 can enhance tolerance, this could open the door to new treatments that reduce reliance on lifelong immunosuppressive drugs. Ultimately, our goal is to improve long-term outcomes for children undergoing liver transplantation, making the process safer and more effective for future patients.

1-11 Aims and Hypothesis

The primary aim of this study is to develop a robust and reproducible *in vitro* model that simulates the early immunological interactions between donor liver tissue and recipient immune cells. This model will be used to investigate cellular activation, migration, and functional responses relevant to liver allograft tolerance and rejection. Additionally, longitudinal immune profiling of liver transplant recipients will be conducted to correlate *in vitro* findings with *ex vivo* immune signatures observed post-transplantation. The study also aims to identify surrogate biomarkers in blood or tissue that reflect early immune dynamics and can predict graft outcomes.

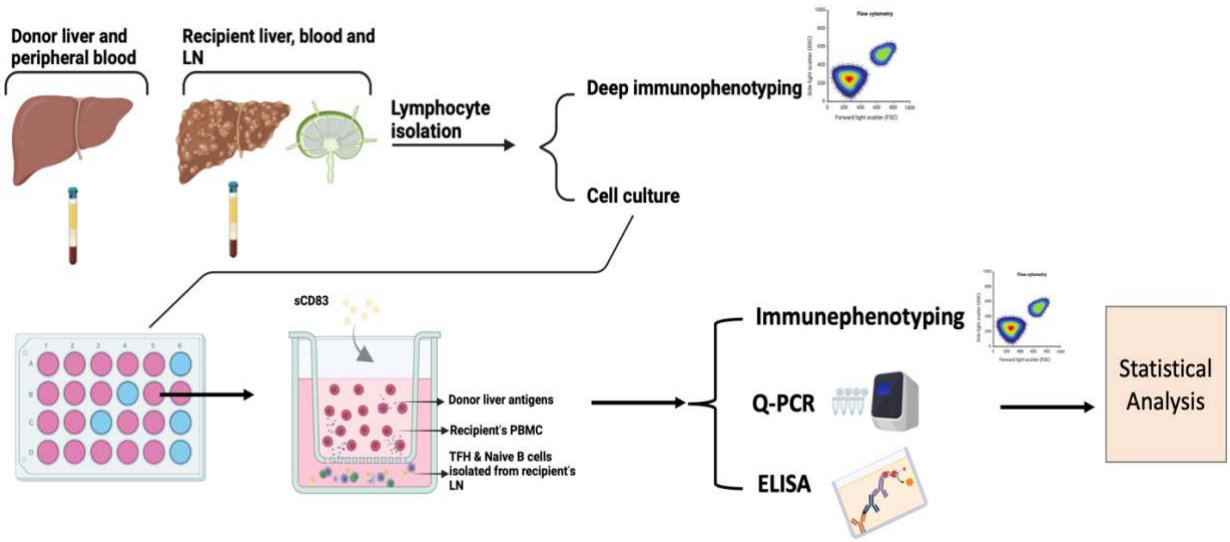
Secondary aim is to **evaluate the immunomodulatory effects of sCD83** when administered during early immune activation, focusing on its potential to promote regulatory pathways.

We hypothesize that:

1. A patient-derived *in vitro* model replicating early donor–recipient immune interactions will reveal critical cellular and molecular mechanisms regulating liver allograft tolerance or rejection.

2. The immune activation and modulation patterns observed in the *in vitro* system will mirror post-transplant immunological signatures and correlate with clinical outcomes.
3. Early administration of sCD83 will enhance regulatory immune pathways, reduce inflammatory responses, and result in measurable changes in key immunological markers that could serve as surrogate indicators for long-term graft prognosis.

Figure 1.8: Graphical abstract of study.



Chapter 2

MATERIALS AND METHODS

2.1 Study population

In this study, our primary objective is to develop a system that replicates the physiological conditions following transplantation. The study population consists of two main groups.

1) Patients with end-stage liver diseases undergoing liver transplantation

2) Healthy controls.

The inclusion criteria for the transplantation group are:

For patients:

1- Patients undergoing their first organ transplantation.
2- Patients without any active infection at the time of surgery
3- Patients not receiving any form of immunosuppressive therapy prior to transplantation.

For healthy donors:

1- Individuals aged between 18 and 55 years.
2- Individuals with no known acute or chronic medical conditions particularly those affecting immune system.

The exclusion criteria

For patients:

1- History of any previous organ transplantation
2- Presence of active infection at the time of recruitment, due to its potential to interfere with immune parameters and study outcomes.
3- Use of immunosuppressive medications prior to transplantation

For healthy donors:

1- Any prior diagnosis of chronic immunological or autoimmune disease
2- Current use of any medications, including non-steroidal anti-inflammatory drugs (NSAID) or immunomodulatory agents.

2.1.2 Data collection

Upon enrollment, demographic data including age, gender, relevant medical history as well as baseline clinical information such as liver disease etiology and any prior treatments were obtained from patient's medical records. These data provide essential context for the interpretation of both immunological and clinical outcomes throughout the study.

2.1.3 Clinical assessments

Post-transplant follow-up includes routine clinical evaluations, which encompass standard laboratory tests (e.g., liver function tests) and imaging studies. These assessments are conducted to monitor graft function and to identify any early signs of transplant rejection, in accordance with standard clinical care protocols.

2.1.4 Sample collection

Biological samples are collected at defined time points throughout the transplantation process. These include:

1- Intraoperative period: Blood and liver tissue samples collected during the transplantation procedure.
2- Post-transplant follow-up: Blood samples obtained during routine follow-up visits, and additional liver tissue samples in cases where rejection is suspected.

This staged sampling strategy is designed to facilitate longitudinal analysis of immunological markers and to correlate them with clinical events across the transplantation timeline.

2.1.5 Ethical considerations

This study received approval from the institutional review board of Koç University (2025.006.IRB2.002). Informed written consent was obtained from the patients/patient's parents of all participants, and the study adhered to the principles outlined in the Declaration of Helsinki.

2.2 Samples collection

From each patient, during the surgery and routine follow up liver biopsy, 3 ml of peripheral blood was collected in Heparin coated tubes (Vacutainer® Heparin Tubes -Sodium Heparin) and kept at room temperature (rt). During the surgery 3*3 mm of donor liver and explanted liver from recipient was taken. Also, liver draining lymph node of the recipient was taken. During the routine liver biopsy follow up small part of the biopsy was taken put in separate and accurately labeled dry tubes (VACUSERA 9 ml Z No Additive) containing 1 ml saline solution. These biopsy samples were kept on ice and were then quickly transported to the lab.

2.3 lymphocytes (peripheral blood mononuclear cell-PBMC) isolation from blood

The peripheral blood was diluted at a ratio of 1:1 with room temperature RPMI-1640 medium (Cat no: 12-702F, Lonza, Switzerland) and was then carefully layered onto 3 ml of Lymphoprep™ Density Gradient Medium (Cat no: 07801, Alere Technologies, Norway), present in a 15 ml Falcon tube (Falcon™ 15 mL Conical Centrifuge Tubes, Thermo Fisher Scientific). This solution was quickly centrifuged in soft acceleration and off deceleration mode, at a speed of 2000 rpm for 15 minutes at room temperature. The resulting, separated

and whitish buffy coat lymphocyte layer was carefully isolated and transferred to a sterile 15 ml falcon tube. This was then washed with 1:3 ratio of RPMI and centrifuged again at a speed of 2000 rpm, acceleration max, deceleration max, for 7 minutes. Following this, the supernatant was discarded, and the cell pellet was resuspended in 2 ml fetal bovine serum (FBS, Cat no:16000044 , Thermo Fisher Scientific, USA) and the cells were counted using hemocytometer. 200 ul of this resultant PBMC solution was then transferred to a sterile 5 ml FACS tube (SARSTEDT) and kept for FACS (Fluorescence-activated cell sorting) staining.

2.4 Lymphocyte isolation from biopsies

For each type of biopsy (esophageal or duodenal), an enzyme solution was prepared by adding 0.5 mg of Collagenase IV (Sigma-Aldrich) for each ml of complete media (RPMI + 5% FBS) and vortexed thoroughly to mix completely. 1 ml of this enzyme solution was put in each accurately labeled sterile 15 ml falcon tube for each gastro-intestinal (GI) sample. These tubes containing the solution were then incubated in a water-bath at 37°C until further use. Each sample was placed in a separate and accurately labeled petri-dish and chopped into <1 mm pieces using a sterile scalpel. The minced tissue was then collected using a micropipette and transferred into their respective enzymatic solution containing falcon tubes, which were then vortexed and placed in the water-bath at 37°C for 5 mins. Once the first incubation ended, the sample tubes were taken out from the water-bath and vortexed for 30 seconds and put back in the water-bath again for another 5 minutes. At the end of the incubation, the tubes were taken out and over-diluted with complete media (CM) with a ratio of 1:4, and then centrifuged at a speed of 2000 rpm, acceleration max, deceleration max, for 5 mins at 4°C. The resulting supernatant was then discarded, and the cell pellet was resuspended in 1 ml of phosphate buffered saline solution (PBS, 1X, Cat no. L0616, Biowest). Using a 70-um cell strainer (SARSTEDT), the cell solution was carefully transferred to labeled FACS tubes and centrifuged for 2 mins at 2000 rpm, to remove any remaining CM. Following the centrifugation, the FACS tubes were carefully decanted to remove the supernatant and the cell pellet was resuspended in 100 ul of PBS, and the cells were counted.

2.5 FACS (Fluorescence Activated Cell Sorting) staining

The PBMCs were washed with PBS and resuspended in 100 ul of PBS. 0.2 ul of viability dye (Zombie NIR™ Fixable Viability Kit, BioLegend) was added to each PBMC and biopsy FACS tube and the cells were incubated in the dark at rt for 15-20 minutes. The cells were then over-diluted with PBS and centrifuged at 2000 rpm for 2 minutes. This step was called the washing step. Following this washing, the FACS tubes were carefully decanted to remove the supernatant and the cell pellet was resuspended in PBS. Based on the cell count, from each FACS tube, the cell volume was split into two FACS tubes (40 ul of cell solution in each FACS tube) for two different panels. Panel 1 consists of T cell subsets, whereas panel 2 consists of B cell subsets, NK, NKT and MAIT cells. Based on the different panels, 0.75 ul of each antibody (based on our optimized protocol) (Table 2.1) was added to each tube and the cells incubated in the dark at rt for 15-20 minutes. After incubation, the cells were washed, as described before in the washing step, and resuspended again in 50 ul PBS, following with fixation.

2.6 Intracellular staining

For staining of intracellular markers including FOXP3 (Forkhead Box P3), a transcription factor, and CTLA-4, Cells were incubated in 300 µL of FOXP3 fixation/permeabilization buffer (eBioscience), followed by incubation with 300 µL of intracellular permeabilization buffer (eBioscience). After washing with PBS, the cell pellet was resuspended in 300 µL of PBS.

2.7 Fixation of cells

After the final antibody staining incubation, the cells were washed and fixed using 200 µL of PBS and 100 µL of 4% paraformaldehyde (PFA), at 4°C for 20-25 minutes. Following this, the cells were washed and resuspended in 300 µL of PBS, making them ready for flow cytometry.

2.9. Cell staining and sorting

The lymphocytes isolated from lymph nodes were washed with PBS and resuspended in 100 µL of PBS. 0.2 µL of viability dye (Zombie NIR™ Fixable Viability Kit, BioLegend) was added to each PBMC and biopsy FACS tube and the cells were incubated in the dark at rt for 15-20 minutes. The cells were then over-diluted with PBS and centrifuged at 2000 rpm for 2 minutes. This step was called the washing step. Following this washing, the FACS tubes were carefully decanted to remove the supernatant and the cell pellet was resuspended in PBS. Then CD45, CD20, CD27, IgM, CD4, CXCR5 and PD-1 antibodies were added to the cell suspension. Following this, the cells were washed and resuspended in 300 ul of PBS, making them ready for flow cytometry.

The gating strategy is shown in figure 2.1.

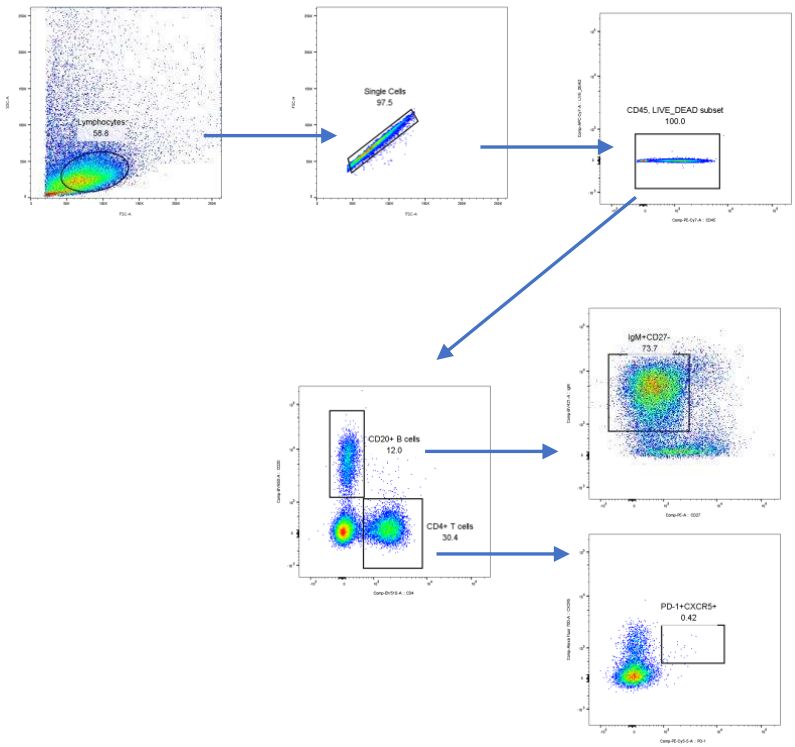


Figure 2.1: Gating strategy of lymph node isolated cells

2.10 T cell activation/proliferation assay

2.10.1 Sample preparation

PBMCs from recipient blood were isolated as mentioned above. Then cells were maintained under sterile conditions using RPMI 1640 supplemented with 10% FBS, 1% antibiotics.

2.10.2 Loading of Anti-Biotin MACSiBeadTM Particles

To obtain a homogenous suspension, Anti-Biotin MACSiBeadTM Particles were resuspended thoroughly by vortexing. 100 µL of CD2-Biotin, 100 µL and CD28-Biotin and 100 µL CD3-Biotin was edded to tube. Vortexed again. Then 500µL Anti-Biotin MACSiBead Particles was added antibody mix and adjusted to 1 ml. This suspension was then incubated in 2–8 °C under constant, gentle rotation for 2 hours.

2.10.3 T cell activation protocol

PBMCs were resuspended in 1 mL of phosphate-buffered saline (PBS). To label the cells for tracking cell proliferation, 1 µL/mL of CFSE (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen™) was added to the suspension. The cells were incubated at 37°C for 15 minutes in the dark. Following incubation, the cells were washed to remove excess CFSE and resuspended in complete medium.

A total of 2.5×10^4 loaded Anti-Biotin MACSiBead Particles were added to an equivalent number of PBMCs (2.5×10^4 cells) in a 1:1 ratio. The cell suspension was incubated at 37°C in a humidified incubator with 5% CO_2 for 96 hours. After the incubation period, cells were harvested and processed for subsequent analyses.

2.11. Tissue homogenate preparation

Healthy donor tissue was processed under sterile conditions using a Petri dish. The tissue was first rinsed with phosphate-buffered saline (PBS) and then transferred into a 1.5 mL sterile Eppendorf tube containing 500 µL of PBS. A stain-free metal bead was added to the tube, and

mechanical disruption was performed using the Qiagen TissueLyser II at 20 Hz for 3 minutes. The resulting suspension was transferred to a fresh Eppendorf tube and centrifuged at 16,000 × g for 10 minutes at 4 °C. The supernatant was collected and used in subsequent cell culture experiments.

2.12. Migration Assay

The migration assay was conducted using a 24-well plate setup. Cells isolated from lymph nodes, including naïve B cells and T follicular helper (Tfh) cells, were resuspended in 600 µL of complete medium and seeded into each well. A chemoattractant, CCL22, was added at a final concentration of 25 ng/mL to each well. Subsequently, 0.5 µm transwell inserts were carefully placed in each well. Activated and non-activated PBMCs were prepared by washing and resuspending 1 × 10^6 cells in 100 µL of complete medium, with the presence and absence o 10% fetal bovine serum (FBS). These cells were then added to the upper chamber of the transwell insert. After an incubation period of 24 hours at 37°C the inserts were removed, and cell migration was assessed under a light microscope. Quantitative or qualitative data were collected for further analysis.

2.13. Co-culture assay

For the co-culture assay, both stimulated and unstimulated peripheral blood mononuclear cells (PBMCs) were stained with anti-CD45 and anti-CD20 antibodies. $CD45^+CD20^-$ cells were sorted and subsequently co-cultured with naïve B cells and T follicular helper (TFH) cells, which were isolated from lymph node-derived lymphocytes. The co-cultures were maintained for 9 days. After that, the supernatants were collected for ELISA and the cells were collected for subsequent gene expression analysis.

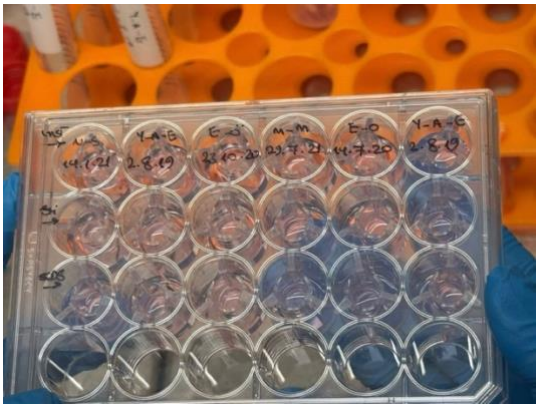
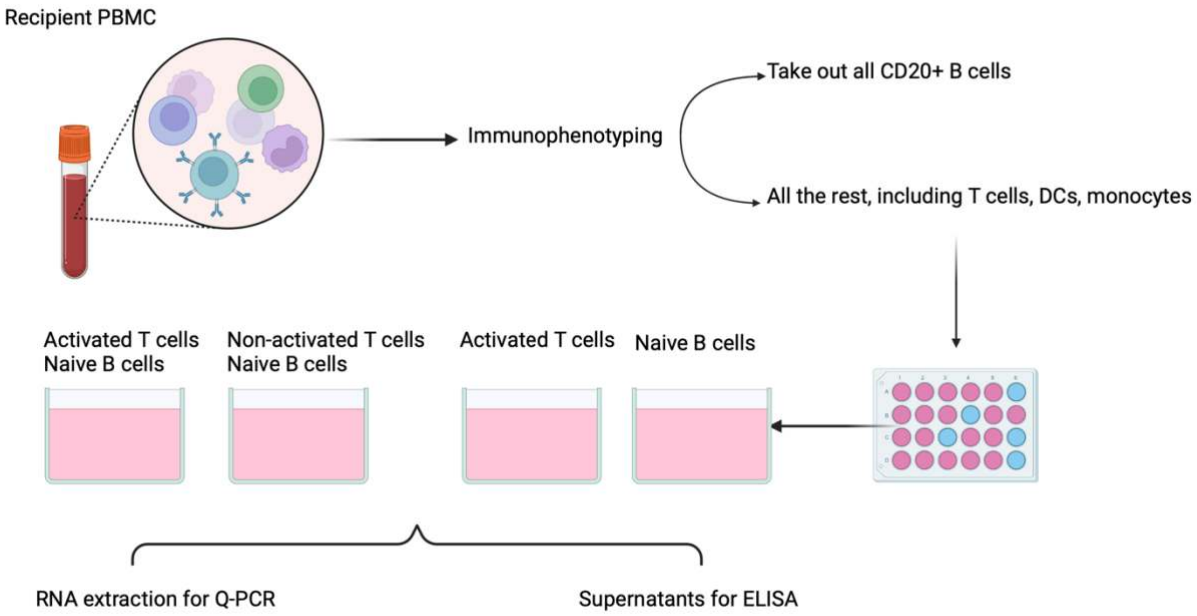


Figure 2.2: Illustration of 24-well plate with transwell inserts.

Figure 2.3: Graphical Design for Sample Preparation to Gene Expression Analysis



2.14. RNA extraction

To investigate gene expression patterns, total RNA was extracted from cell lysates of cultured cells, using Rneasy Mini Kit (Cat. No. 74104, Qiagen, Germany). Samples were collected according to standard procedures before RNA extraction. Samples were gently mixed in buffer RLT in an appropriate volume to ensure complete cell lysis. 1 volume of 70% ethanol was added to the lysate and mixed well by pipetting. The lysate was then transferred to a RNeasy Mini Spin Column, where RNA molecules selectively bound to the column membrane upon centrifugation. To remove impurities and contaminants, then buffer RW1 was used to wash the column. Additional washing steps with buffer RPE were optionally performed to enhance RNA purity. Subsequently, the spin column was placed in a new microcentrifuge tube, and Rnase-free water was added to elute the RNA. Centrifugation was applied to facilitate and ensure RNA separation from the column membrane and collection of

eluted RNA. NanodropTM 1000 was used to measure the amount and purity of RNA (Thermo, USA).

2.15. cDNA synthesis

For cDNA synthesis, iScript cDNA Synthesis Kit (BIORAD, USA) was used. All steps were performed as described in the manufacturer's instructions. The RNA extracted in the previous steps was used for cDNA synthesis. For each sample, the reaction mixture was prepared in a PCR microtube and spun (Table 2.2). Complete reaction mix was incubated in a thermal cycler using the protocol provided in Table 2.3. The synthesized cDNAs were kept at -20oCto be used for real-time PCR.

Table 2.1 cDNA synthesis components and volumes per reaction

Components	**Volume per Reaction**
5x iScript Reaction Mix: 4 µl	4 µl
iScript Reverse Transcriptase: 1 µl	1 µl
RNA template (100 fg-1µg RNA) Variable	Variable
Nuclease-free water	Variable
Total	**20 µl**

Table 2.2 cDNA synthesis thermal protocol

Steps	**Period (minutes)**	**Temperature (ºC)**
Priming	5	25
cDNA synthesis	20	46
Inactivation	1	95
Termination	Hold	4

2.16 Gradient PCR

Gradient PCR was performed to identify the ideal annealing temperature for the primers. The reactions were set up as described in Table 2.4. A range of five temperatures (56°C, 58°C, 60°C, 62°C, and 64°C) was tested using the The Applied Biosystems Veriti Thermal Cycler, with the cycling conditions outlined in Table 2.4. After completion, the products were analyzed on a 1.5% agarose gel. Bio Rad Gel electrophoresis was used to visualize the PCR products and determine the temperature yielding the most specific and intense amplification.

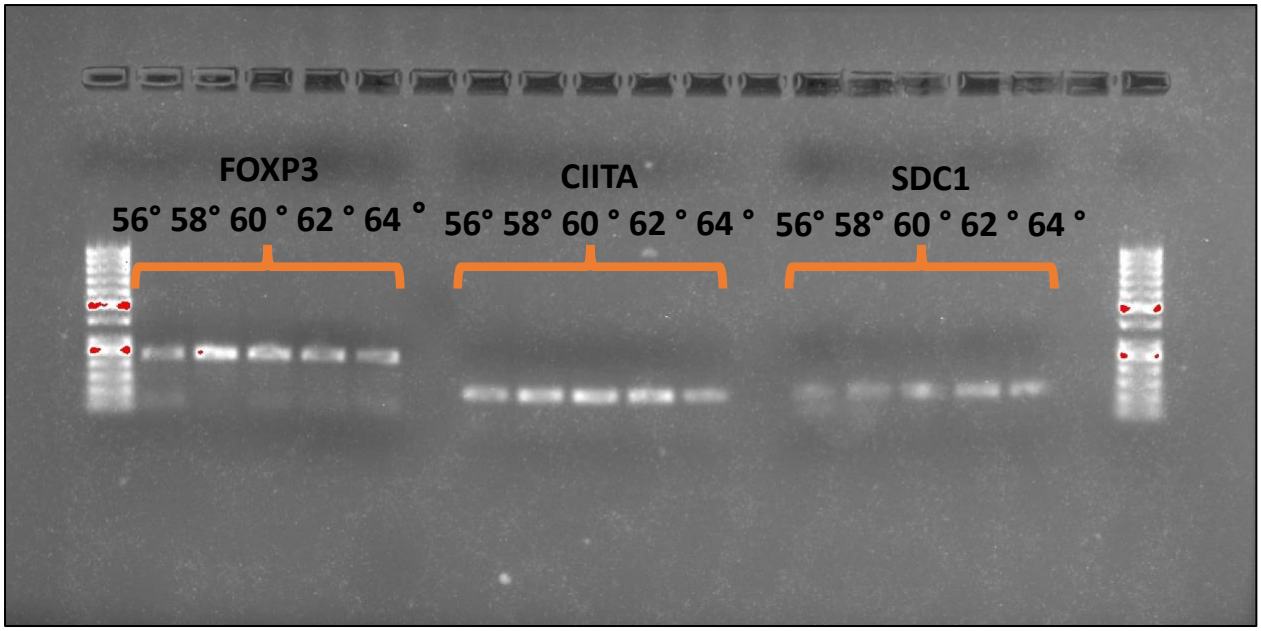


Figure 2.4: Gradient PCR products on agarose gel.

Table 2.3 Gradient PCR thermal protocol.

Steps	Period	Temperature (ºC)	Cycle
Initial denaturation	2-3 minutes	94-95	
Denaturation	30 seconds	94-95	
Annealing	30 seconds	Gradient range	
Extension	30 seconds	72	
Final extension	5 minutes	72	
Termination	Hold	4	

2.17 Q-PCR

2.17.1 Q-PCR protocol

Real-time PCR analysis of the transcription factor FOXP3 as a marker for regulatory T cells, Class II Major Histocompatibility Complex Trasnactivator (CIITA) as marker for differentiation of naïve B cells to plasma cells, was conducted using the SYBR green and Relative Quantification (RQ) technique. This method relies on SYBR green dye binding to double-stranded DNA.

Greater reproduction rates lead to increased SYBR green binding. Consequently, fluorescent emissions are directly proportional to the reproduction rate. PCR reactions were performed using RealQ Plus Master Mix Green (Amplicon, Denmark) in accordance with the manufacturer's instructions. Real-time PCR reaction was conducted as per the conditions in Table 2.5 using the QuantStudio 7 Flex System device (Applied BiosystemsTM, USA) following the conditions in Table 2.6. Subsequently, gene expression was evaluated using the

2- ΔΔCt method (Livak & Schmittgen, 2001).The primer sequences employed in the study are detailed in Table 2.7.

Table 2.4 qRT-PCR amplification mix content.

Content Name	**Volume**	**Final Concentration**
Roche SYBR Green (2x)	5 µl	1x
Primer (10 mM)	0.33 µl	0.33 mM
DNA (25 ng)	1 µl	-
Nuclease free water	Adjust to 10 µl	-

Table 2.5 SYBR green qRT-PCR thermal protocol.

Steps	**Temperature (ºC)**	**Period**	**Cycle**
Pre incubation	95	5 minutes	1
Amplification	95	19 seconds	40
o Annealing	60	30 seconds	
o Extension	72	30 seconds	
Melting curve	95	5 seconds	
	65	1 minute	
	97	5 minutes	

Table 2.6 Sequences of primers

NCBI Gene Name (Homo sapiens)	**Gene Symbol**	**Forward primer (5'→'3')**	**Reverse primer (5'→3')**
Forkhead Box P3	*FOXP3*	GTGGCCCGGATGTGAGAAG	GGAGCCCTTGTCGGATGATG
Class II Major Histocompatibility Complex Trasnactivator	*CIITA*	CCTGGAGCTTCTTAACAGCGA	TGTGTCGGGTTCTGAGTAGAG

2.17.2 Q-PCR optimization and standard curve analysis

To determine the optimal cDNA concentration for each primer pair, a serial dilution of a representative cDNA sample was prepared. The tested concentrations included 500 ng, 250 ng, 125 ng, 15 ng, and 0.4 ng per reaction, each diluted tenfold and added as 1 µL to individual reaction tubes to reach a total volume of 10 µL. To have standard curves for each primer set we used dilutions to assess amplification efficiency and linearity. Based on the standard curve analysis, a final input of 25 ng cDNA per reaction was selected for the CIITA and FOXP3 primers (Figure 2.17.2. a-b), as this concentration yielded optimal efficiency and a high coefficient of determination (R^2). In contrast, amplification with the SCDN1 primers demonstrated non-specific binding and poor amplification efficiency, as reflected by a low R^2 value. Consequently, this target was excluded from further analysis.

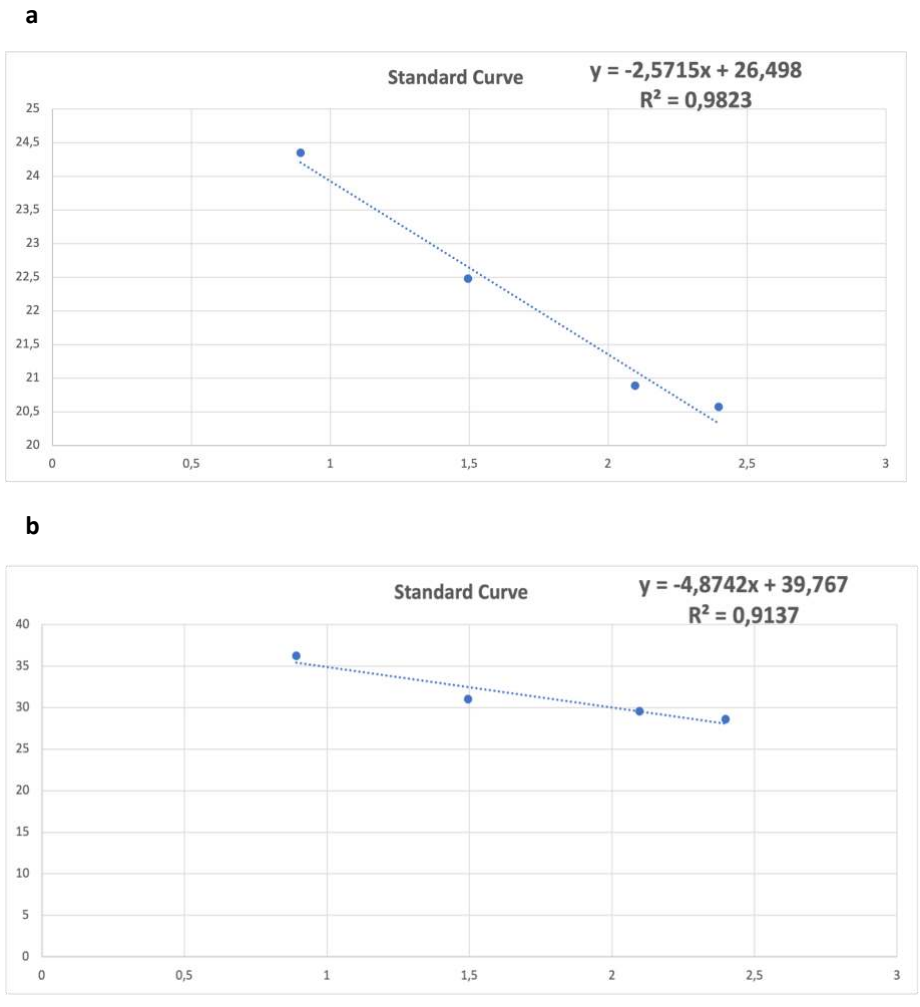


Figure 2.5: Standard curve analysis of primers.

2.18 Measurement of IL-10, IFN-gamma and IL-21 in cell culture supernatants using ELISA

2.18.1 Reagents and Standards:

Human IL-10 Quantikine Kit (D1000B) and Human IFN-gamma Quantikine QuickKit (QK285) was purchased from Biotechne R&D systems. Standards and detection antibodies were prepared according to the manufacturer's instructions.

2.18.2 Assay procedure

Following the preparation of reagents, working standards, and samples as outlined in the manufacturer's protocol, 200 µL of standards, controls, and samples were dispensed into each well. The plate was incubated at room temperature (RT) for 2 hours. Afterward, wells were aspirated and washed three times with 400 µL of Wash Buffer. Next, 200 µL of Human IL-10 or IFN-gamma conjugate was added to the respective wells, followed by another 2-hour incubation at RT. Following a wash step, 200 µL of Substrate Solution was added to each well and incubated for 20 minutes at RT. Finally, 50 µL of Stop Solution was added to each well, and the optical density was measured within 30 minutes using a microplate reader.

2.18 Statistical analysis

Statistical analyses were carried out using GraphPad PRISM version 8 (GraphPad Software Inc, San Diego, CA, USA). Given the limited sample size, non-parametric methods were employed to evaluate statistical significance. The Mann-Whitney U test was used for comparisons between two groups. Spearman's rank correlation was applied for assessing relationships between variables, while Fisher's Exact test was used for categorical data comparisons. Results were reported as medians along with interquartile ranges (IQR). A p-value below 0.05 was considered statistically significant.

Chapter 4

RESULTS

3.1 Study population

This study population consisted of a total number of 23 patients who underwent ABO compatible living donor liver transplantation. (PBMC n=11, IHL n=8).

3.1.1 Demographic and clinical characteristics:

In this study, a total of 18 patients were recruited, comprising 16 pediatric and 2 adult liver transplant recipients. The primary diagnoses included immune-mediated chronic liver disease (CLD; n = 7), biliary atresia (BA; n = 3), progressive familial intrahepatic cholestasis (PFIC type 3, n=2, type 2, n = 1), and metabolic liver diseases (n = 5). Detailed demographic information, including age and gender distribution, is presented in Tables 1–3.

Table 3-1 Patients demographics

Groups	**CLD (n=7)**	**BA (n=3)**	**PFIC (n=3)**	**Metabolic liver disease (n= 5)**
Age in years (median, [IQR])	**3 (1-7)**	**2 (1-12)**	**5 (5-16)**	**1.5 (1-13)**
Gender (M/F)	**4/3**	**1/2**	**2/1**	**2/3**

3.3. Protocol optimization

In this study, several critical steps required optimization to establish a robust experimental system. First, preparation of the donor tissue homogenate needed to preserve surface antigens

to ensure they retained their capacity to stimulate recipient immune cells, while also being compatible with placement on the transwell membrane. Importantly, this antigenic

stimulation was performed in parallel with anti-CD3/anti-CD28 bead stimulation, as tissue antigens alone were insufficient to robustly activate immune cells. Second, the isolation of lymphocytes from lymph nodes posed challenges, as factors such as patient age, primary disease, and lymph node size and quality directly impacted both the yield and viability of isolated cells. Third, selecting the appropriate transwell pore size was essential to allow activated cells to migrate toward the chemoattractant without simply settling by gravity. Optimization also included the composition of media in both upper and lower compartments, the concentration of chemoattractants, and the timing required to achieve maximal cell migration. Fourth, setting up the co-culture system proved particularly challenging, as maintaining the viability of naïve B cells was difficult, even with optimized culture conditions, including the addition of B cell-supporting cytokines. However, to preserve physiological relevance, no additional supplements were added, allowing the study to assess the response of naïve B cells and TFH cells solely to activated recipient PBMCs. Finally, the experimental design had to account for the identification and separation of cells that migrated from the upper to the lower compartment, which was essential for subsequent functional assays. Once these methodological challenges were addressed, the study proceeded with the analysis of the recruited patient samples.

3.3. T cell activation assay using recipients' PBMCs

In the next phase of the study, PBMCs isolated from liver transplant recipients were subjected to an *in vitro* T cell activation protocol. The cells were stimulated using anti-CD3/anti-CD28 T cell activation and expansion beads to assess their activation potential. In parallel, tissue homogenates prepared from donor liver samples were added to evaluate whether they could exert a synergistic effect on T cell activation.

However, no additive or synergistic effect was observed with the inclusion of donor tissue homogenates, as the activation beads alone were sufficient to induce maximal T cell activation ($p= 0.0001$). As shown in the corresponding figure, nearly 100% activation was achieved following 96 hours of stimulation, as indicated by the robust upregulation of CD25 on both $CD4^+$ and $CD8^+$ T cells. This activation profile was markedly elevated compared to the unstimulated control samples (Figure 3.3.1a-c). Additionally, light microscopy images taken at the end of the stimulation period revealed distinct colonies of proliferating cells, further confirming the robust activation and expansion of lymphocytes in response to bead stimulation.

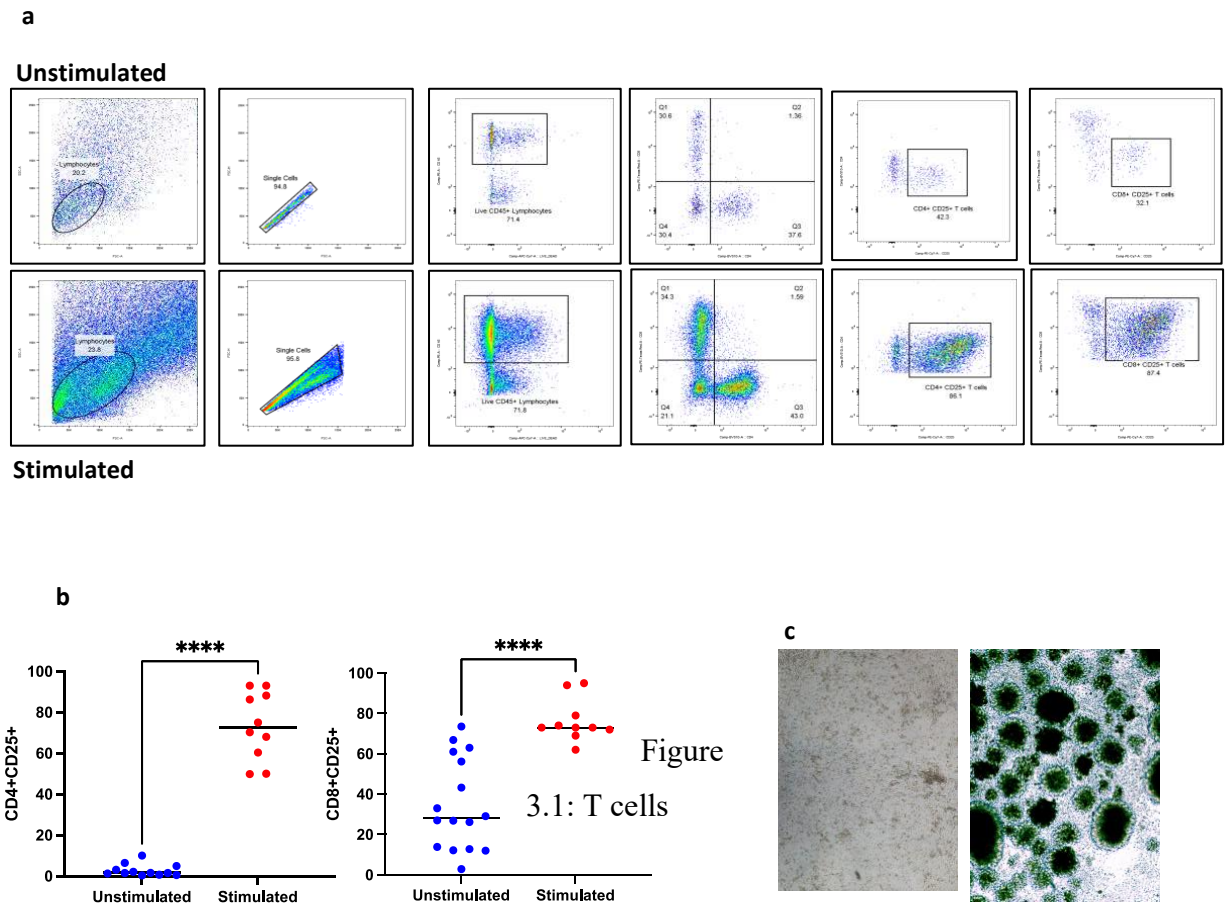


Figure 3.1: T cells efficiently activated with anti-CD3/anti-CD28 beads.

3.3.2. Evaluation of soluble CD83 (sCD83) on T cell activation

To investigate the potential immunomodulatory effect of sCD83, we next examined whether its addition to the culture medium could attenuate T cell activation. sCD83 was added to PBMC cultures stimulated with anti-CD3/anti-CD28 beads, and activation levels were assessed after 96 hours.

As shown in the Figure 3.3.2., the addition of soluble CD83 did not result in a reduction in T cell activation. The expression of the activation marker CD25 on both $CD4^+$ and $CD8^+$ T cells remained comparable to levels observed in cultures stimulated without CD83. These findings suggest that, under the current experimental conditions, soluble CD83 alone is insufficient to suppress bead-induced T cell activation.

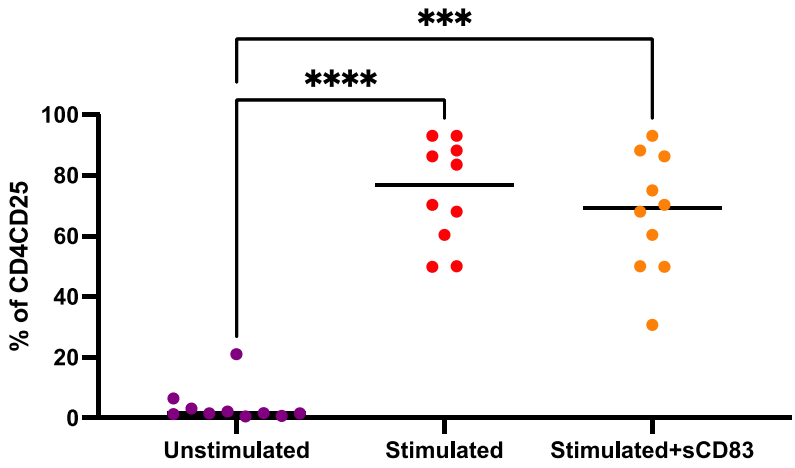


Figure 3.2: sCD83 treatment.

3.4. Isolation and sorting of naïve B cells and follicular helper T cells from lymph nodes

In the following phase of the study, lymph node samples obtained from transplant recipients during surgery were used to isolate specific lymphocyte subsets. Single-cell suspensions were first prepared from the lymph node tissues, followed by flow cytometric sorting to purify naïve B cells ($CD20^+IgM^+CD27^-$) and follicular helper T (TFH) cells ($CD4^+CXCR5^+PD\text{-}1^+$) (Figure 3.4.a)

Following cell sorting from lymph node-derived lymphocytes, the number of recovered cells in each population was assessed. As shown in the corresponding figure, a significant difference was observed in the number of $CD4^+$ T cells isolated from patients with CLD (p=0.04) compared to those with metabolic liver as well as among BA and metabolic liver disease (p=0.004). Significant variations in the frequency of naïve B cells ($CD20^+IgM^+CD127^-$) were observed between the BA and metabolic groups (p = 0.01), as well as between the metabolic and PFIC groups (p = 0.009).

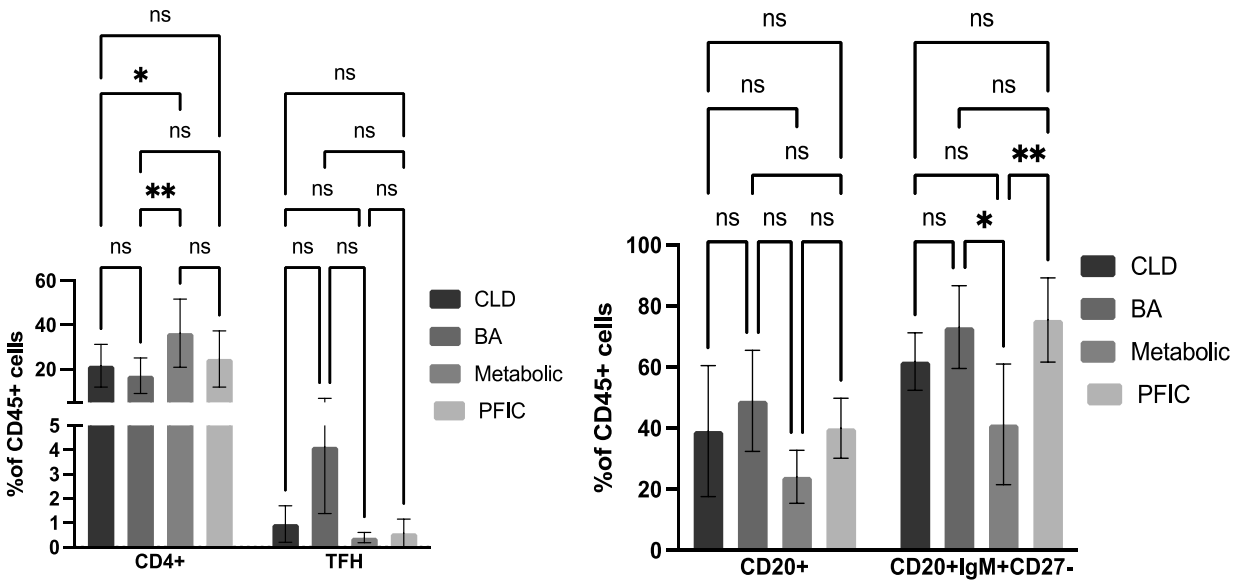


Figure 3.3: Immunophenotype of lymph-node isolated cells.

3.5 Transwell Co-culture assay to assess T cell-mediated B cell activation

To investigate the interaction between activated T cells and lymph node-derived B and TFH cells, a transwell co-culture system was established. PBMCs isolated from liver transplant recipients were first stimulated or left unstimulated, and the activated cells treated with sCD83, were stained and sorted to isolate $CD45^+CD20^-$ cells, effectively depleting the B cell

population (Figure 3.5.a). These $CD45^{+}CD20^{-}$ cells were placed in the upper chamber of 5 µm pore transwell inserts in complete culture medium.

In the lower chamber, naïve B cells and TFH cells—previously isolated and sorted from recipient lymph nodes—were cultured in the presence of the chemokine CCL22, used as a chemoattractant to facilitate cellular migration.

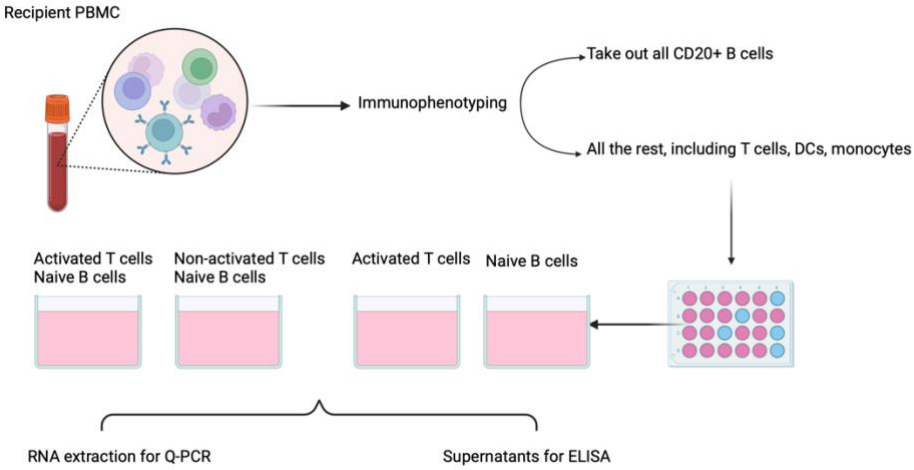


Figure 3.4: Graphical design of sample preparation for q-PCR.

After 24 hours, the number of cells that had migrated from the upper to the lower chamber was quantified. A significant increase in migration was observed in cultures with stimulated PBMCs compared to those with unstimulated cells ($p = 0.02$), indicating that T cell activation enhances the migratory response and potential cellular interaction within this co-culture system. However, there was no significant difference in migration between the activated group and the sCD83-treate group, indicating that sCD83 did not alter the migratory behavior of activated T cells under these experimental conditions (Figure 3.5 a-b).

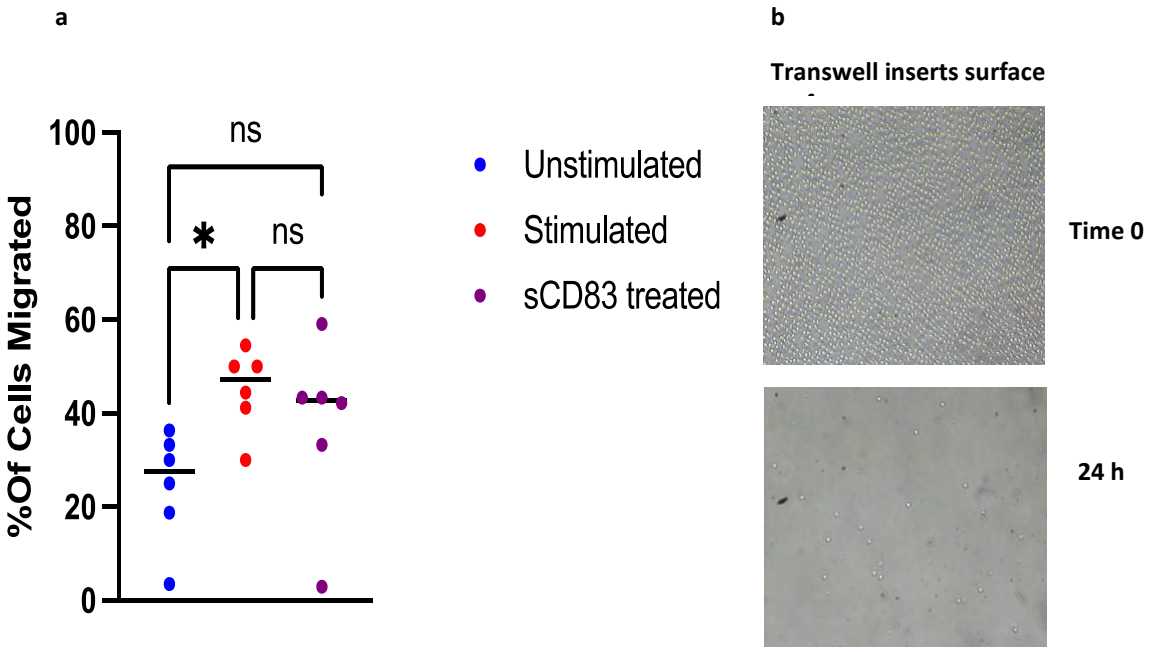


Figure 3.5: Cell migration assay in the presence and absence of sCD83.

3.6. Gene expression analysis following

3.6.1 *CIITA* and *FOXP3* expression in PBMC-derived cells

An initial analysis was performed on stimulated and unstimulated $CD45^{+}CD20^{-}$ PBMCs cultured alone for 96 hours. This served as a reference to evaluate the baseline expression of regulatory genes within these cells, independent of lymph node-derived B or TFH cell influence. Quantitative qPCR was conducted to measure the expression of *FOXP3* and *CIITA*, representing markers of regulatory T cell activity and B cell antigen presentation, respectively. The results revealed a significant downregulation of *FOXP3* in the stimulated PBMC-$CD20^{-}$ group compared to the unstimulated control ($p = 0.03$), suggesting an decrease in regulatory T cell-associated transcriptional activity upon T cell activation. In contrast, *CIITA* expression showed no significant difference between the two groups (Figure 3.6.1.a-b).

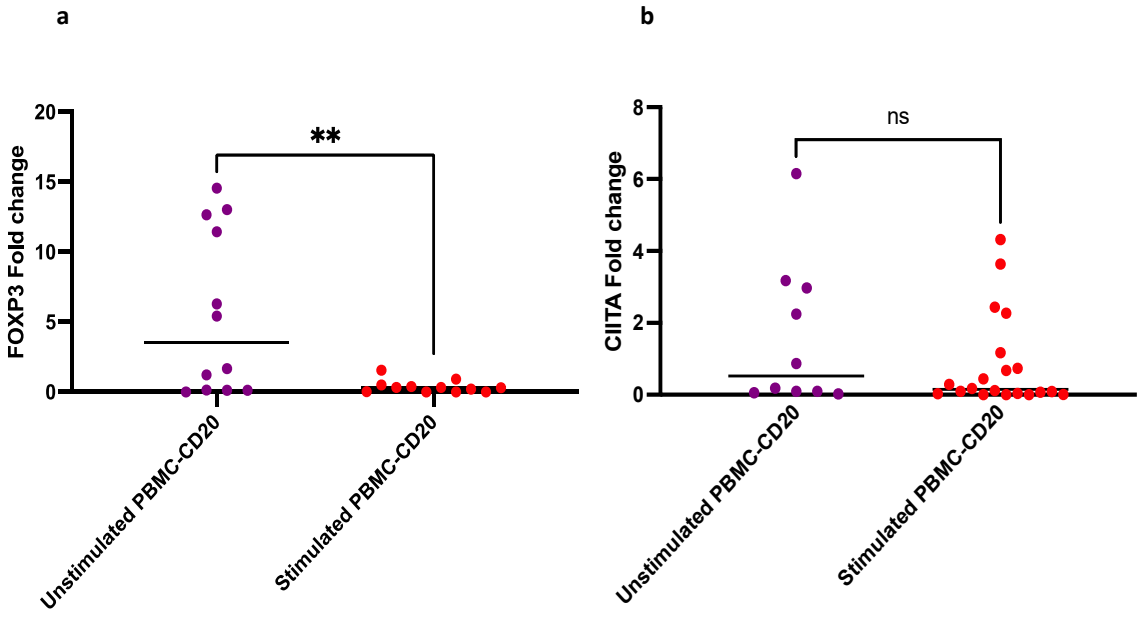


Figure 3.6: FOXP3 and CIITA expression in stimulated/unstimulated PBMC-CD20.

3.6.2 Gene expression assessment in the co-culture system

To assess the functional outcome of the co-culture system, particularly the activation status of B and T cells, naïve B cells and TFH cells were co-cultured with stimulated or unstimulated $CD45^{+}CD20^{-}$ cells for a period of 9 days. Following the co-culture period, the expression of *CIITA* and *FOXP3 was assessed to* investigate potential immunological changes induced by co-culture conditions and to determine whether T cell stimulation influenced transcriptional activation of key regulatory genes in B and T cells present in the system.

Interestingly, *FOXP3* expression remained unchanged between co-cultures involving stimulated and unstimulated $CD45^{+}CD20^{-}$ PBMCs, suggesting no significant alteration in regulatory T cell-associated transcriptional activity under these conditions. However, a significant downregulation of *CIITA* was observed in the stimulated co-culture group compared to the unstimulated group ($p = 0.01$) (Figure 3.6.2.a-b).

This reduction in *CIITA* expression may indicate a shift in the phenotype of naïve B cells toward a more differentiated state, potentially reflecting early plasma cell development. Previous studies have reported that *CIITA*, a master regulator of MHC class II expression, is

notably downregulated during plasma cell differentiation. Thus, the observed decrease in *CIITA* may reflect the initiation of terminal B cell differentiation within the stimulated co-culture environment.

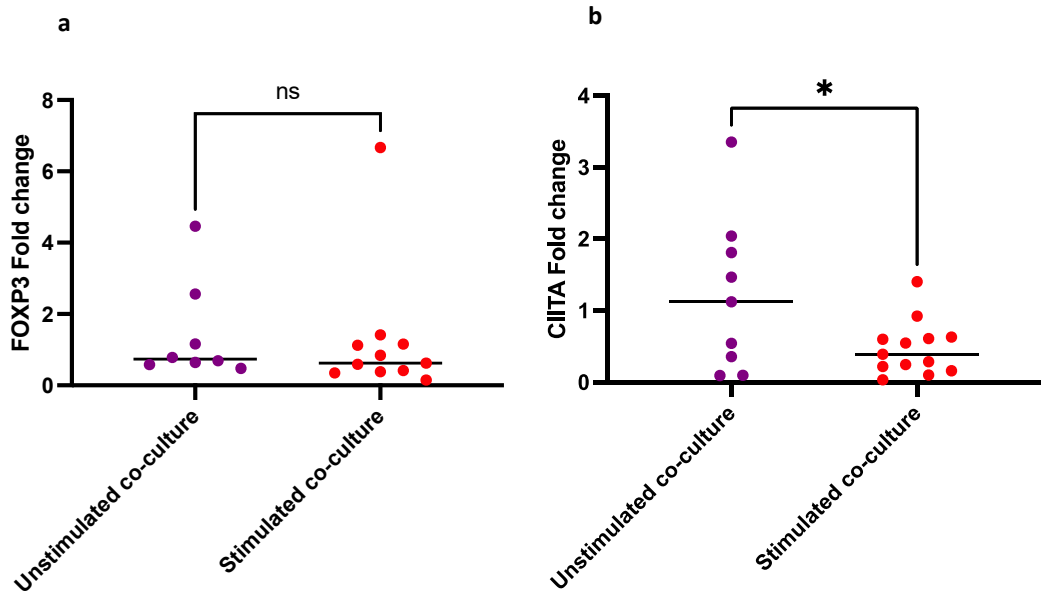


Figure 3.7: FOXP3 and CIITA expression in stimulated/unstimulated co-cultures.

3.7. Cytokine profiling of co-culture supernatants

To further confirm the immunological impact of the co-culture system on cellular behavior, cytokine levels in the culture supernatants were measured on day 9 using ELISA. The selected cytokines included IL-6, a pro-inflammatory marker; IL-10, a regulatory cytokine; and IL-21, a cytokine primarily secreted by TFH cells and important for B cell help.

The results revealed a significant reduction in IL-6 secretion in the stimulated co-culture condition compared to the unstimulated group ($p=0.03$), indicating a diminished

inflammatory profile within the system. This shift was further supported by a significant increase in IL-10 levels ($p=0.04$), suggesting an enhanced regulatory environment and

reinforcing the hypothesis of a phenotypic transition from an inflammatory to a more regulatory state (Figure 3.7.a-b).

In contrast, IL-21 levels remained unchanged between the two groups. This stability may reflect a consistent presence or functional status of TFH cells across both conditions, or it may suggest that the activation stimulus applied did not further modulate IL-21 production, which is known to be tightly regulated and context-dependent in lymphoid tissue environments (Figure 3.7.c).

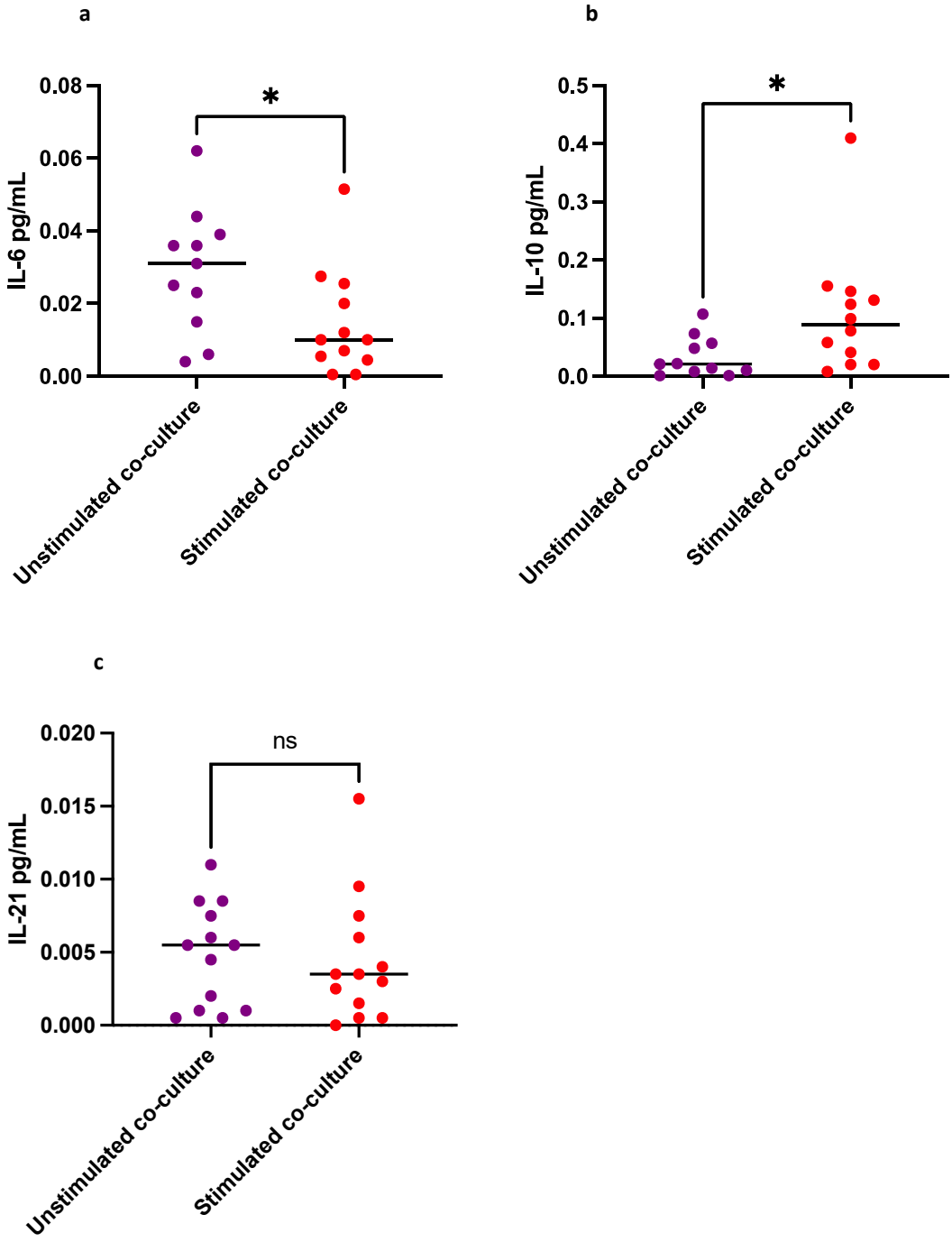


Figure 3.8: ELISA results.

Chapter 4

DISCUSSION

In this study, we aimed to model the early immune cell interactions that occur following liver transplantation by mimicking the proposed immunological mechanisms within the liver and its draining lymph nodes.

4-2 T Cell activation and soluble CD83 modulation

To explore potential avenues for modulating early donor–recipient immune interactions in liver transplantation, we established an *in vitro* system using PBMCs from liver transplant recipients stimulated with anti-CD3/CD28 beads, with the presence or absence of soluble CD83 (sCD83). This approach was designed to mimic alloactivation while evaluating the immunomodulatory capacity of sCD83, a molecule previously described to have tolerogenic properties in various immune contexts. Inclusion of donor tissue homogenates in the cultures failed to further augment activation, suggesting that bead stimulation alone provides a maximal signal sufficient to bypass the need for additional antigenic complexity. Although donor antigens may play a role in shaping specificity, the current system effectively isolates TCR-mediated activation from alloantigenic variability.

Soluble CD83, an immunoregulatory molecule derived from the membrane-bound form expressed on mature dendritic cells, has been shown in several studies to suppress immune responses and promote tolerance by acting on dendritic cells and inducing regulatory cell subsets (Prechtel et al., 2007; Breloer & Fleischer, 2008). However, in the present model, addition of sCD83 to bead-stimulated cultures did not reduce the expression of CD25 on either $CD4^+$ or $CD8^+$ T cells, suggesting that sCD83 was insufficient to modulate direct TCR-driven T cell activation.

This result is consistent with literature indicating that the tolerogenic effects of sCD83 are primarily mediated via myeloid cells such as dendritic cells or macrophages, which are not the primary drivers in our T cell–focused assay (Zöller et al., 2011). Additionally, it is possible that the absence of additional inflammatory or co-inhibitory signals (e.g., IL-10, TGF-β, PD-L1 engagement) limited the capacity of sCD83 to induce downstream regulatory effects. Other studies have shown that sCD83 is more effective in reducing alloimmune responses *in vivo*, where complex cell–cell interactions and tissue-derived cues shape the immune response (Lechmann et al., 2001; Lin et al., 2020).

These findings highlight the context-dependent nature of sCD83 activity. While sCD83 shows promise as a tolerance-inducing agent, its effects are unlikely to manifest in simplified T cell–only systems, underscoring the need for models that incorporate dendritic cells or three-dimensional tissue environments for future mechanistic exploration.

4-3 B-T cell co-culture: tolerogenic Shifts

To simulate early cellular crosstalk within secondary lymphoid environments relevant to liver transplantation, we established a transwell co-culture system using stimulated or unstimulated $CD45^{+}CD20^{-}$ PBMCs from transplant recipients and lymph node–derived naive B cells and T follicular helper (TFH) cells. This system aimed to model migratory signaling, transcriptional reprogramming, and cytokine-mediated interactions that may contribute to tolerogenic or immunogenic outcomes post-transplant.

Initial migration assays showed that activated $CD45^{+}CD20^{-}$ PBMCs significantly enhanced the migration of B and TFH cells across the transwell membrane, compared to their unstimulated counterparts. This observation supports the hypothesis that activated T cells secrete chemokines or alter the microenvironment in ways that promote interaction with B cells a fundamental step in lymphoid architecture and germinal center (GC) formation (Walters & Vinuesa, 2016).

Interestingly, treatment with sCD83 did not alter this migratory behavior, again suggesting that while sCD83 may modulate certain APC-mediated functions, it does not directly impair

the chemokine-driven recruitment of lymphocytes under the current *in vitro* conditions. Future studies incorporating dendritic cells or stromal cues may clarify whether sCD83 alters chemokine gradients in more complex settings.

4-3-1 Transcriptional shifts indicate B cell differentiation and immune modulation

Following 9-day co-culture, we observed a significant downregulation of CIITA in naive B cells exposed to activated T cells. CIITA, the master regulator of MHC class II gene expression, is typically downregulated during plasma cell differentiation, as antigen presentation becomes less relevant once B cells commit to antibody production (Reimold et al., 2001; Sci Immunol, 2017). Thus, the observed reduction may indicate early B cell maturation and a shift away from APC function.

This is particularly significant in the context of the hepatic lymph node environment, which favors tolerance over activation. Studies have shown that dendritic cells in hepatic LNs exhibit a mature phenotype yet secrete anti-inflammatory cytokines and promote regulatory responses (Boor et al., 2019). Moreover, hepatic LNs contain lower numbers of cross-presenting cDC1 cells and an abundance of regulatory APCs, potentially influencing B cell fate toward non-inflammatory or antibody-secreting phenotypes without driving autoimmunity. Liao et al. (Liao, Yang et al. 2025) highlight the pivotal role of Bregs in improving graft survival across multiple solid organ transplantation models, including the liver, where they help establish an anti-inflammatory microenvironment that supports long-term tolerance. This is consistent with our observation of increased IL-10 production and reduced pro-inflammatory cytokine secretion in our *in vitro* model, suggesting that Breg-mediated mechanisms may contribute to the tolerogenic shift induced by this system.

Recent research also underscores the critical involvement of Bregs, dendritic cells, and innate lymphoid cells (ILCs) in shaping transplant outcomes. Bregs have been shown to promote tolerance through IL-10, IL-35, and TGF-β, which suppress effector T cells and support the expansion of regulatory subsets. DCs play a pivotal role in immune equilibrium, with immature DCs inducing tolerance and mature DCs driving immune activation.

Our finding that sCD83 had no effect on migration or cytokine changes may reflect insufficient DC activation in our system. ILCs have also emerged as key players, with ILC3s supporting epithelial integrity in intestinal grafts and IL-10–producing ILC2s dampening inflammatory responses. Although we did not assess ILCs directly, the regulatory environment observed in our study raises the possibility that ILCs contribute to B-T cell interactions.

Furthermore, Wang et al. (2024) recently used single-cell multi-omics to map immune recovery after liver transplantation, identifying distinct phases from innate immune activation to adaptive immune remodeling and eventual stabilization. They identified NK cells and specific monocyte populations as key players in acute rejection, emphasizing the interplay between adaptive and innate immunity in transplant tolerance.

Contrary to expectations, FOXP3 expression remained unchanged in these co-cultures. FOXP3 is the canonical transcription factor for regulatory T cells (Tregs), and its stability suggests that the 9-day culture period and isolated conditions did not suffice to expand or induce $FOXP3^+$ Tregs. Notably, the absence of stromal or tolerogenic dendritic cues may have limited the potential for Treg induction, which is typically driven by TGF-β, retinoic acid, or tolerogenic antigen presentation (Kim et al., 2007; Boor et al., 2019).

4-3-2 Cytokine profile supports a regulatory shift in the co-culture environment

Analysis of cytokines in the supernatants revealed a compelling immune reprogramming: IL-6, a key pro-inflammatory cytokine associated with B cell activation, plasma cell differentiation, and Treg instability, was significantly reduced in the stimulated co-culture condition. This reduction may indicate a dampening of inflammatory responses or an early transition to a more quiescent B cell state (Dienz & Rincon, 2009).

Conversely, IL-10 levels were significantly increased, aligning with a more regulatory or tolerogenic immune environment. IL-10 production is a hallmark of both Tregs and regulatory B cells (Bregs), suggesting that B cells or TFH cells within this co-culture system may adopt a regulatory phenotype. This finding is consistent with reports that hepatic-draining LNs favor immune tolerance, and that TFH-B cell interactions in lymphoid organs

can skew toward IL-10-producing phenotypes in the presence of regulatory cues (Walters & Vinuesa, 2016; Boor et al., 2019). Interestingly, IL-21 levels remained unchanged between the groups. As a signature cytokine of TFH cells, IL-21 is critical for B cell help, GC formation, and class switching. Its stability across conditions may reflect preserved TFH identity, or it may indicate that TFH function was not further modulated by T cell activation state in this simplified co-culture. Additionally, studies have shown that IL-21 production by TFH cells can remain stable even under regulatory conditions, possibly maintaining baseline support for B cell survival (Crotty, 2011).

Multiple studies have underscored the liver's tolerogenic tendencies, attributed in part to its constant exposure to gut-derived antigens and its rich composition of immunomodulatory cells (Crispe, 2014; Boor et al., 2019). Hepatic-draining lymph nodes (HLNs) play a critical role in this process, serving as the primary site for T cell priming in response to liver-derived antigens (Boor et al., 2019). Compared to peripheral lymph nodes, HLNs harbor lower numbers of pro-inflammatory dendritic cells (e.g., cDC1s), a higher proportion of IL-10-producing APCs, and exhibit an overall environment that supports immune tolerance rather than activation.

Our results mirror this paradigm: despite T cell activation *in vitro*, downstream B cell differentiation favored reduced antigen presentation (via CIITA downregulation) and enhanced IL-10 secretion, a profile consistent with regulatory skewing. Although FOXP3 expression was stable and not significantly induced, the broader cytokine and transcriptional milieu supports a model of partial immune reprogramming favoring tolerogenic adaptation. These data are particularly relevant in pediatric liver transplantation, where natural tolerance has been observed more frequently than in adults (Chandok & Watt, 2012).

4-4 Limitations and future directions

While the present study offers valuable insights into the early immune dynamics relevant to liver transplant tolerance, several limitations should be acknowledged to contextualize the findings and guide future research.

4-4-1 Limitations

First, the *in vitro* model, though informative, lacks the full complexity of the liver and lymph node microenvironment. The simplified nature of the co-culture system, composed primarily of sorted T cells, B cells, and TFH cells, does not recapitulate the cellular diversity of secondary lymphoid tissues or HLNs. As such, critical components such as dendritic cells, stromal cells, and macrophages, and likely ILCs, all of which are known to play pivotal roles in tolerance induction were absent from the system. This limitation may have also affected the apparent lack of response to soluble CD83, which in other studies has shown significant tolerogenic effects primarily through myeloid or dendritic cell targets (Zöller et al., 2011; Lin et al., 2020).Second, the reliance on a limited cytokine panel (IL-6, IL-10, IL-21) constrains the ability to comprehensively characterize the cytokine milieu. Broader multiplex cytokine profiling could reveal additional changes in the immune landscape.
Third, while transcriptional changes in CIITA and FOXP3 were assessed, the study did not measure downstream functional consequences, such as antigen presentation capacity, antibody production, or memory B cell formation, which are critical to fully understand the impact of T cell activation.

Lastly, the effect of sCD83 might require alternative experimental conditions to be adequately captured, and the lack of its impact in this study should be interpreted cautiously, pending further investigation under different immunological settings.

4-4-2 Future Directions

Future investigations should aim to extend the co-culture duration to examine whether the observed trends — particularly CIITA downregulation and IL-10 upregulation — are sustained or amplified over time. Longer cultures may better replicate the kinetics of germinal center responses and regulatory T cell differentiation.

To further elucidate B cell fate decisions, subsequent experiments should assess surface markers such as CD138 and CD38, which indicate plasma cell differentiation, as well as immunoglobulin secretions. Functional assays would add depth to the transcriptional data and clarify whether B cells in the stimulated co-cultures progress toward antibody-secreting phenotypes or regulatory B cell subsets.

The use of single-cell RNA sequencing could also provide high-resolution mapping of transcriptional states across T, B, and TFH cell subsets, revealing subtle changes in gene expression and uncovering rare or transitional states not captured by bulk analysis. This would be especially useful in dissecting the interplay between B cell differentiation and TFH functional plasticity within the co-culture.

This study will be compared with the recipient lymph node (LN) of an ABO-incompatible graft, which is associated with a high risk of rejection.
By first conducting this comparison with the recipient lymph node (LN) of an ABO-incompatible graft—characterized by a high risk of rejection—the system setup will be completed. This approach will not only enable the identification of key differences between rejection and tolerance but also allow for the systematic planning and execution of subsequent experimental phases.

Lastly, incorporating *in vivo* models or three-dimensional lymphoid organoid systems may better replicate the complex tissue environment necessary for complete TFH-B cell interactions and allow validation of the findings in a physiologically relevant setting.

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