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Organ transplantation and the liver tolerance effect: history, mechanisms, and potential implications for the future of transplant care

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ORGAN TRANSPLANTATION AND THE LIVER TOLERANCE EFFECT:
HISTORY, MECHANISMS, AND POTENTIAL IMPLICATIONS FOR THE
FUTURE OF TRANSPLANT CARE

by

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ORGAN TRANSPLANTATION AND THE LIVER TOLERANCE EFFECT: HISTORY, MECHANISMS, AND POTENTIAL IMPLICATIONS FOR THE FUTURE OF TRANSPLANT CARE

ANDREW J. KIM

ABSTRACT

Chronic immune insult and immunosuppressant-related toxicities have remained an enduring challenge in organ transplantation. Long-term survival of transplant patients has improved marginally in recent decades due to these challenges. To circumvent these issues, transplant investigators have researched immune tolerance mechanisms that demonstrate potential to induce immunosuppression and rejection-free survival in the clinic. One mechanism in particular, the liver tolerance effect, has already demonstrated this experimentally and clinically. Liver transplants in experimental models and human patients have exhibited the ability to become spontaneously accepted without being rejected by the recipient’s immune system. Research in recent decades has revealed that the liver parenchymal and non-parenchymal cell populations harbor potent immunomodulatory properties. In the context of liver transplantation, it has been found that two cell populations in particular, the mesenchyme-derived liver sinusoidal endothelial cells and hepatic stellate cells, mediate the induction of liver transplant tolerance through a mechanism known as mesenchyme-mediated immune control.
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>ATRA</td>
<td>all-trans retinoic acid</td>
</tr>
<tr>
<td>B7-H1</td>
<td>B7-homolog 1 (also PD-L1)</td>
</tr>
<tr>
<td>B7-H4</td>
<td>B7-homolog 4</td>
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<tr>
<td>CNI</td>
<td>calcineurin inhibitor</td>
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<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HSC</td>
<td>hepatic stellate cell</td>
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<tr>
<td>ICAM-3</td>
<td>intercellular adhesion molecule-3</td>
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<td>IFN-γ</td>
<td>interferon-γ</td>
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<tr>
<td>IFN-γR</td>
<td>interferon-γ receptor</td>
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<tr>
<td>IL-4</td>
<td>interleukin-4</td>
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<tr>
<td>IL-10</td>
<td>interleukin-10</td>
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<td>IL-12</td>
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<td>IL-17</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<td>Kupffer cell</td>
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<td>KO</td>
<td>knockout</td>
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<td>LSEC</td>
<td>liver sinusoidal endothelial cell</td>
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LSECtin........................................... liver sinusoidal endothelial cell lectin
LPS........................................................ lipopolysaccharide
MDSC ........................................... myeloid-derived suppressor cell
MHC ...................................................... major histocompatibility complex
MHC-I ........................................... major histocompatibility complex class I
MHC-II ........................................... major histocompatibility complex class II
MLR .................................................. mixed-leukocyte reaction
MPA ................................................... mycophenolic acid
mTOR ................................................... mechanistic target of rapamycin
NPC ................................................... non-parenchymal cell
PC ........................................................ parenchymal cell
PD-1 .................................................. programmed cell-death protein-1
PD-L1 ........................................... programmed death-ligand 1 (also B7-H1)
PGE\textsubscript{2} ........................................... prostaglandin E\textsubscript{2}
TCR ........................................................ T-cell receptor
Teff. ...................................................... effector T cell
TGF\textbeta ................................................ transforming growth factor \textbeta
Th ........................................................ helper T cell
Th1 ........................................................ helper T cell 1
Th2 ........................................................ helper T cell 2
Th17 ....................................................... helper T cell 17
TRAIL .............................................. tumor necrosis factor-related apoptosis-inducing ligand
Treg .......................................................... regulatory T cell
TUNEL ........................................ terminal deoxynucleotidyl transferase dUTP nick end labeling
WT .......................................................... wild type
INTRODUCTION

The modern history of organ transplantation, spanning over the last century, has been woefully characterized by extensive trial and error, but this has allowed the transplant patients of today to enjoy a vast improvement in short-term survival and quality of life. However, in recent decades, the long-term survival of patients has shown minimal improvement due to progressive donor organ dysfunction caused either by chronic immune insult or immunosuppressant-mediated side effects. For this reason transplant investigators have been pursuing an understanding of immune tolerance mechanisms. Of particular interest has been the liver tolerance effect, a strange immunological phenomenon first observed in pigs in 1969 in which liver transplants were spontaneously accepted without the need for immunosuppressant drugs.

The following thesis is divided into two parts. The first part of this thesis—the following subsections of this introduction—will provide historical context for organ transplantation with respect to the development of immunosuppression as well as the events that sparked the discovery of immune tolerance mechanisms, including the liver tolerance effect. This will set the stage for the second part of the thesis, which will focus on describing the current evidence gathered on specific cellular mediators of liver tolerance as well as a discussion of a relatively recent theory that attempts to explain spontaneous liver transplant acceptance and how the implications of this theory may affect the future of transplant care.
December 23, 1954 marked a watershed moment in the history of organ transplantation. At 23-years-old, Ronald Herrick donated one of his kidneys to his identical twin brother, Richard, and a surgical team led by Joseph Murray at Peter Bent Brigham Hospital (Figure 1) would go on to perform the world’s first successful human organ transplant procedure that resulted in long-term survival (Guild, Harrison, Merrill, & Murray, 1955). Decades-long research and experimentation elaborating upon Alexis Carrel’s pioneering techniques in vascular anastomosis made it technically possible for Murray’s team to perform such an operation (Annales de la Société de médecine de Lyon, 1902; Doyle, Lechler, & Turka, 2004; Sade, 2005). Although the clinical transplantation of other organs proceeded to success at different rates, after 1954 it no longer seemed to be a question of whether organ transplantation could be done in humans, but rather how effective would it be as a treatment for end-stage organ failure and other indications (Starzl, 2000).

The nature of the operation performed by Murray and his team was reflective of the deeper troubles that confounded early transplant clinicians and investigators. In this unique case the donor and recipient were genetically identical individuals—this is known as syngeneic transplantation, whereas transplantation involving two genetically disparate individuals is allogeneic; the caveat in both situations being that the donor and recipient are members of the same species. During the first several decades of the 1900’s, the prevailing wisdom from animal experimentation was that autotransplantation (transplantation within the same individual) and syngeneic transplantation resulted in
organ or graft acceptance, while allogeneic transplantation resulted in graft rejection (Doyle et al., 2004; Sade, 2005). However, it was hardly understood why this was the case or how rejection was even mediated, for that matter.

The nature of graft rejection remained largely a mystery until the 1940’s when studies by Peter Medawar suggested an immune mechanism of graft rejection. Medawar had performed 625 skin grafting operations in outbred rabbits, which distinctly demonstrated that rejection was mediated by a recipient’s own immune cells (Medawar, 1944, 1945). Allogeneic skin grafts were marked by extensive lymphocytic infiltration and other rejection characteristics, whereas autografted skin remained intact and healed with no evidence of major immune involvement. Medawar also observed that larger
allogeneic skin grafts were rejected faster than smaller ones and that repeated transplantation of allogeneic grafts from the same donor resulted in faster rejection as well. In regards to the observation on graft size, the accelerated kinetics of rejection in the larger skin grafts appeared to be counterintuitive, as one might expect that a larger graft would take a longer time to destroy. However, this made sense from an immune perspective, as a large foreign “threat” would trigger a proportionately sized response by the immune system, thereby destroying the graft faster than one might expect (Davis, 2014). The observation of accelerated rejection in repeated transplantation also seemed to confirm immune involvement since it mimicked a memory phenotype that was commonly observed in the immune system’s response to repeatedly encountered pathogens (Davis, 2014). From this evidence it was clear that organ rejection in allogeneic transplantation was mediated by a recipient’s immune system, and further, in the special circumstances of autotransplantation or syngeneic transplantation, the genetic identity of the transplanted tissue conferred protection from rejection.

Murray’s success in 1954 was therefore heavily dependent on the genetic identify of the Herrick twins. As most individuals lack an identical twin or duplicate organs, it was clear that the future of organ transplantation in humans would have to heavily rely on allogeneic grafts (i.e. organs from genetically dissimilar donors), but this presented clinicians and investigators with a perplexing quandary: how does one control or prevent the immune response in graft rejection? It is interesting to note here that an equally significant question spurred by Medawar’s observations was, how does the immune system know to recognize self from non-self? This eventually led to the discovery of the
major histocompatibility complex (MHC) genes, which play a significant role in lymphocyte physiology as well as the tissue typing that is used to match transplant recipients with appropriate donors.

One of the first solutions to this predicament would stem from the anti-folate research performed by Sidney Farber and his collaborators during the 1940’s. Farber understood that the crucial vitamin, folate (B₉), stimulated the proliferation of leukemia cells *in vitro* (Miller, 2006). It stood to reason that folate analogues that antagonized endogenous folate could potentially be anti-leukemic, which Farber would eventually demonstrate through the clinical use of aminopterin (Farber, Diamond, Mercer, Sylvester, & Wolff, 1948). By the 1950’s, investigators in other fields were adopting a similar practice in rational drug design to discover other potential anti-cancer drugs. In the field of purine metabolism, Gertrude Elion and George Hitchings were developing purine analogues after early studies showed that these compounds could inhibit the growth of bacterial cells (Elion, 1989). This ultimately led to the chemical synthesis of 6-mercaptopurine (6-MP) (Elion, Hitchings, & Vanderwerff, 1951) and subsequently, azathioprine, an immunosuppressant anti-proliferative agent used today under certain circumstances of transplant care (Schumacher & Gajarski, 2011). The discovery of azathioprine was the result of alterations to 6-MP, which at the time showed promise as an anti-leukemic and immunosuppressant drug—azathioprine is the prodrug of 6-MP and is converted to 6-MP in the bloodstream by erythrocyte glutathione (De Miranda, Beacham, Creagh, & Elion, 1973; Elion, 1989). Subsequent comparison studies of the two drugs demonstrated azathioprine to be less toxic than 6-MP (Elion, Bieber, &
Hitchings, 1960) and better at suppressing the immune response to allogeneic renal grafts in canine transplant models (Calne, 1961). As a result, azathioprine was tested in the clinic in combination with the corticosteroid, prednisone, which allowed transplantation between genetically unrelated humans for the first time in 1962—one patient out of 8 treated with azathioprine survived past 6 months (Murray, Merrill, Harrison, Wilson, & Dammin, 1963).

Following the clinical implementation of azathioprine, newer and more potent immunosuppressant drugs were being discovered in various bacterial and fungal sources, and they remain in use as the standard of care in a majority of today’s transplant treatment protocols (Schumacher & Gajarski, 2011). Importantly, the discovery of these so-called, “modern immunosuppressants,” coincided with immense leaps in our understanding of lymphocyte physiology and the myriad biochemical signaling pathways that regulate cellular activity.

The first modern immunosuppressant to be developed was the calcineurin inhibitor (CNI), cyclosporine—discovered in the fungi *Cylindrocarpon lucidum* and *Trichoderma polysporum* (Dreyfus et al., 1976; Ruegger et al., 1976). Studies done by Jean Borel and colleagues during the 1970’s characterized the immunosuppressive properties of cyclosporine in various animal models (Borel, 1976; Borel, Feurer, Gubler & Stahelin, 1976; Borel, Feurer, Magnee & Stahelin, 1977). In contrast to its anti-proliferative predecessors, azathioprine and cyclophosphamide, cyclosporine demonstrated a unique ability to specifically and reversibly suppress both cell-mediated and humoral immunity without compromising bone marrow function (Starzl, 2000).
Further testing in models of allogeneic heart, kidney, liver, and pancreas transplantation revealed that cyclosporine had a significant effect in delaying graft rejection (Calne & White, 1977; Calne et al., 1979; Calne, White, Rolles, Smith & Herbertson, 1978; Green & Allison, 1978; Kostakis, White & Calne, 1977). By the 1980’s, cyclosporine had become a mainstay in clinical transplant immunosuppression. It improved outcomes for renal transplantation (Starzl et al., 1980) and elevated the endeavors in heart, liver, and lung transplantation to the level of clinical reality (Cooper, 1990; Griffith, Hardesty, Deeb, Starzl, & Bahnson, 1982; Reitz et al., 1982; Starzl, Klintmalm, Porter, Iwatsuki, & Schröter, 1981).

Cyclosporine remained the top-of-the-line immunosuppressant for transplantation until 1989, when it was discovered that certain liver transplants could not be protected from rejection by cyclosporine alone, but rather by a new kind of CNI, tacrolimus or FK 506 (Starzl et al., 1989). Unlike cyclosporine, tacrolimus was discovered in a bacterial source, *Streptomyces tsukubaensis* (Kino et al., 1987). The clinical investigation of tacrolimus found that it was particularly useful in cases of intractable organ rejection, where cyclosporine and other immunosuppressants failed to work. In initial clinical liver transplant studies, it was found that approximately 75% of intractably rejecting livers could be saved using tacrolimus (Fung et al., 1990). Subsequent clinical trials revealed that a similar percentage of intractably rejecting organs could be rescued in kidney and thoracic organ transplantation (Armitage, Fricker, Del Nido, Cipriani, & Starzl, 1991; Starzl et al., 1989, 1990).
Other effective immunosuppressants would also come to be discovered in the same time frame as the development of cyclosporine and tacrolimus. The most prominent of these drugs are the mechanistic target of rapamycin (mTOR) inhibitors, everolimus and sirolimus (rapamycin) (Calne et al., 1989; Schuurman et al., 1997; Thomson & Woo, 1989). Additionally, an older drug, the antibiotic mycophenolic acid (MPA)—a de novo guanine nucleotide synthesis inhibitor—was repurposed as an anti-proliferative immunosuppressant (Allison & Eugui, 1993). The immunosuppressant drugs discussed up to this point represent the bulwark of modern day immunosuppression: anti-proliferatives (azathioprine and MPA), CNI’s (cyclosporine and tacrolimus), mTOR inhibitors (everolimus and sirolimus), and corticosteroids (prednisone) (Schumacher & Gajarski, 2011). The combination use of these immunosuppressants are responsible for the remarkable increase in short-term survival of transplant recipients—extending survival once measured in days, weeks, and months during the early years of transplantation to survival measured in years (1-, 3-, 5-, 10-year time frames and for the lucky few, several decades).

Though transplant recipients have enjoyed a remarkable increase in short-term survival since the start of clinical organ transplantation, long-term survival has seen little to no improvement in recent decades and remains one of transplantations enduring challenges. This challenge stems from the difficulties associated with immunosuppressant administration. A crucial aspect of transplant care is the ability of clinicians to mitigate the potential adverse effects associated with transplantation. This requires dosing with just enough immunosuppressant to prevent acute or chronic graft rejection, while
simultaneously aiming to minimize drug-specific toxicities and the long-term risks of chronically depressing the immune system. However, many of these problems are difficult to avoid despite employing the safest drug regimens.

In the earliest clinical studies with immunosuppressants, particularly the CNI’s, severe toxicities were reported, which included cosmetic changes (e.g. facial brutalization, gingival hyperplasia, and hirsuitism), hypertension, metabolic derangements (e.g. diabetogenesis and hyperlipidemia), nephrotoxicity, neurotoxicity, and pneumonitis (Starzl, 2000; Watson & Dark, 2012). Despite the trial and error processes used to determine the dose ceilings and floors for each of the immunosuppressants, transplant patients today still suffer from some of these toxicities (Starzl, 2000). Additionally, cardiovascular mortality in relation to long-term immunosuppression is the leading cause of death amongst transplant patients (Jacques Dantal & Soulillou, 2005). Other complications related to long-term immunosuppression may arise as well. For instance, patients are at an increased risk of acquiring bacterial and fungal infections as well as developing malignancies (Dantal et al., 1998; Dantal & Soulillou, 2005; Dharnidharka, Stablein, & Harmon, 2004). Coupled with the high cost of immunosuppressant medications, these side effects can lead to patient non-compliance with the drug protocol, which is the primary cause of organ failure—due to immune rejection—in transplant recipients (Orlando, Soker, Stratta, & Atala, 2013). These patients eventually become re-listed for organ transplantation, thus further burdening an already strained organ donation system.
While immunosuppressants have largely helped to legitimize organ transplantation as a practical means of treating end-stage organ failure, it is clear that improvements in long-term survival and elimination of immunosuppressant-mediated complications and toxicities are a requirement for transplantation to progress beyond its current state. For this reason, transplant immunologists have been pursuing an understanding of the mechanisms that underlie immune tolerance. By being able to induce the acceptance of an organ graft, clinicians may avoid the use of immunosuppressant medications that limit long-term survival. The source of inspiration for much of the knowledge regarding immune tolerance in organ transplantation would come from an unlikely source, English pigs.
**Immune Tolerance: The Golden Goal of Transplantation**

The concept of immune tolerance was born of the endeavor to understand an enigma that had long-plagued cattle farmers since ancient times, the freemartin cattle. A freemartin is the reproductively sterile female that manifests *in a majority* of heterosexual dizygotic twinning events, i.e. male-female fraternal twinning. The mechanism by which this occurred was poorly understood and much less was known about how to distinguish whether the female twin was developmentally normal or a freemartin. However, by 1916, Frank Lillie had demonstrated evidence of fused vascular beds that existed *in utero* between the placenta of the male (bull) twin and that of the female freemartin twin—in the birth of a normal bull and normal female, the placental circulation was shown to be separate (Lillie, 1916). Thus, freemartinism could be explained by the vascular exchange of male-determining “hormonic” factors, which suppress the normal development of the female twin’s reproductive system. The implications of shared placental vasculature between bull and freemartin twins would have a significant impact on the establishment of the concept of immune tolerance.

During the early 1940’s, Ray Owen was investigating cattle in a quasi-commercial context. At the time, American purebred cattle associations in partnership with the lab Owen worked for routinely submitted cattle blood samples in order to identify purebred individuals (Martin, 2015). To Owen’s surprise, blood tested from a bull and freemartin twin pair—they were calves at this point—revealed identical blood group characteristics. In cattle breeding, fraternal twinning often resulted from female insemination by two different bull fathers. It was therefore reasoned that each fraternal
twin, the bull and freemartin, should express different and separate blood group antigens corresponding to those that they inherited from their respective bull fathers. The identity in blood characteristics that Owen observed between these twin calves upended this logic and suggested something of deeper consequence. Subsequent to his initial observations, Owen devised a technique by which he could separate erythrocytes by blood group antigen (Martin, 2015). In testing hundreds of other bull-freemartin twin pairs, Owen revealed that in each case, both twins always shared the same two-erythrocyte populations, one population originating from self and the other from the twin. Owing to Lillie’s 1916 insight regarding shared in utero vasculature between bull-freemartin twins, Owen reasoned that erythrocyte progenitors (described by Owen as “ancestral cells”) from each twin must be exchanged during embryonic development so that each twin could produce a mixed population of erythrocytes that continues into adulthood (Owen, 1945). The persistence of a foreign erythrocyte population in each twin and the absence of an immunological response to those foreign cells, thus beckoned a new avenue of understanding in immunology: that the concept of biological “self” is learned by the developing embryo’s immune system. As a consequence, an individual could be made immunologically tolerant to any foreign tissue so long as exposure occurs during embryological development, but this would remain to be explained conceptually and demonstrated experimentally at a later time.

Owen’s observations remained dormant until Frank Burnet and Frank Fenner conceptualized their “self-marker” hypothesis in their monograph, “The Production of Antibodies” (Burnet & Fenner, 1949). Burnet and Fenner attempted to explain how the
body’s immune cells could distinguish self from non-self through the employment of unique molecular tags—what we now know of as MHC molecules. Armed with the observations made by Owen just four years earlier, they also deduced that the antibody producing cells of the body could develop a “tolerance” to foreign tissues during embryonic life. Burnet and Fenner’s derivation of tolerance from Owen’s observations inspired Peter Medawar—who previously investigated the immune nature of graft rejection—Rupert Billingham, and Leslie Brent to perform cell transfer experiments in mouse embryos (Billingham, Brent, & Medawar, 1953). Suspensions of cells from an adult mouse donor were injected into several mouse embryos of a different genetic constitution, an allogeneic transplant of sorts. Once those embryos developed into young mice, the trio transferred skin grafts from the same adult donor to each young mouse. The skin grafts remained intact without showing any evidence of rejection. Additionally, skin grafts from a third-party donor were transplanted onto these mice, but these grafts eventually demonstrated the characteristic features of rejection. This indicated that the tolerance acquired by these mice was specific to the original donor. This “actively acquired tolerance” to foreign tissues during embryonic development inspired individuals in the transplant field, particularly Roy Calne. In the words of Calne, “[actively acquired tolerance] raised an important question not yet answered: could an adult immune system be temporarily returned to the fetal state while the organ graft was inserted, and could the immune system then regain its protective role, having accepted the foreign graft?” (Calne, 2012).
While immunologists worked further to understand the mechanisms of tolerance, the transplant field pushed onward finding other means of manipulating the immune system to make transplantation possible, e.g. testing immunosuppressant drugs. The next significant revelation in immune tolerance would not be uncovered until the technical challenges of liver transplantation were overcome by the experimental and clinical work of Thomas Starzl (Calne, 2012). Owing to the large size of the liver and the considerable coagulopathies that are often associated with liver disease, the first clinical liver transplantation attempts made by Starzl in 1963 were so unsuccessful that there was an agreed moratorium on clinical liver transplantation until further experimental work could be done (Calne, 2012; Starzl et al., 1964). However, by 1967, the outcomes of liver transplantation were beginning to improve, and thereafter, liver transplantation could be done practically in both the experimental and clinical settings.

This set the stage for Calne to begin his own experimentation with liver transplantation, an interest based off of earlier observations that liver allografts in pigs experienced mild and prolonged rejection in the absence of immunosuppression—other solid organs experienced acute rejection (Calne et al., 1967). In 1969, Calne and colleagues found that 12 out of 55 liver transplants in pigs resulted in long-term, immunosuppression-free survival (Calne et al., 1969). These liver allografts displayed early characteristics of rejection that spontaneously resolved on their own, evidence of active immune suppression by the liver. Additionally, these allografts were able to induce tolerance to skin and kidney transplants from the same donor, a clear demonstration of specific and actively acquired tolerance, but in this case, without the need to revert to an
embryonic state as previously postulated by Calne. The results of the 1969 study were so astonishing to transplant investigators that it even prompted the publication of an article in the Lancet tilted, “Strange English Pigs” (“Strange English Pigs,” 1969), and so it was that the liver tolerance effect was established.

The induction of tolerance in liver allografts was subsequently shown to be possible in other species, including mice and rats (Kamada, Brons, & Davies, 1980; Qian et al., 1994; Zimmermann, Davies, Knoll, Gokel, & Schmidt, 1984). Starzl also reported evidence of liver transplant tolerance in humans during the early 1990’s (Reyes et al., 1993). Eleven patients displayed stable liver function with no evidence of rejection after being taken off of immunosuppressant medications due to non-compliance or lymphoproliferative disorder. Since then, the clinical transplantation of the liver has clearly recorded lower rates of rejection compared to other transplantable organs, with as much as 20% of all liver transplant patients living immunosuppression free (Bishop, Bertolino, Bowen, & McCaughan, 2012; Lerut & Sanchez-Fueyo, 2006). Additionally, multiple simultaneous-transplant studies in which the liver is transplanted along with another solid organ (e.g. the intestines, kidneys, or lungs) have shown that the liver can protect these organs from rejection as well—just as was preliminarily shown in Calne’s 1969 study (Abu-Elmagd et al., 2009; Benseler et al., 2007; Creput et al., 2003; Opelz, Margreiter, & Döhler, 2002; Rasmussen, Davies, Jamieson, Evans, & Calne, 1995).

The questions that remain are how does the liver establish tolerance for itself and other organs? From where does it derive its immunomodulatory properties? The liver’s intrinsic immunomodulatory properties are derived from its parenchymal cell (PC) and
non-parenchymal cell (NPC) populations. The remainder of this thesis will focus on the contributions that certain NPC populations make to the establishment of local and systemic immune tolerance in general and in the context of liver transplantation. The thesis will then conclude with a discussion of a recent theory of liver transplant tolerance induction based on these NPC populations.
SPECIFIC AIMS

The objectives of this thesis include the following:

1. Introduce the liver in a seldom-considered context, its immune function.

2. Highlight the evidence gathered on specific NPC populations in the liver, which contribute to the liver’s ability to skew the immune response in favor of tolerance.

3. Discuss the recent mechanistic hypothesis explaining liver transplant acceptance, known as mesenchyme-mediated immune control, and how this new perspective of organ-resident mesenchyme cells may affect the future of transplant care.
THE LIVER AS A LYMPHOID ORGAN

As the of the largest solid-organ in the abdominal cavity, the liver is unsurprisingly responsible for numerous vital physiological functions, which include synthesizing and clearing a multitude of plasma proteins, producing fat-emulsifying bile for digestion, regulating glucose storage, and detoxifying and excreting harmful compounds of both endogenous and exogenous origin (e.g. metabolic neo-adducts.neo-antigens and pharmaceutical drugs). What has become clear over the last 50 years, since Calne’s discovery of the liver tolerance effect, is that the liver posses additional functional roles, bona fide immunomodulatory properties that are as vital to normal physiological function as the rest of the liver’s well-characterized roles. These immunomodulatory properties are made possible, in part, due to the liver’s location, architecture, and PC/NPC populations (Crispe, 2009).

The liver is positioned at a unique vascular confluence where it receives large volumes blood from systemic circulation, approximately 20% of the cardiac output. The nutrient-rich blood of the portal vein mixes with the oxygen-rich blood of the hepatic artery upon entering the liver. This nutrient rich blood, low in oxygen tension, then sluggishly percolates through the maze-like microvasculature of the liver known as the hepatic sinusoids. The sheer volume of the liver and its numerous parallel sinusoidal vessels, coupled with occasional microvascular occlusions by intraluminal Kupffer cells (KC, the resident liver macrophage), account for the low perfusion pressure and the slow flow of blood that is characteristic of the liver—in some cases, the flow of blood can
even be completely stopped or reversed due to the presence of occluding KCs (MacPhee, Schmidt, & Groom, 1995). This hemodynamic characteristic of the liver is what allows its PC and NPC populations to effectively act as immune regulators. The slow flow of blood gives scavenging cells ample time to not only clear the blood of gut-derived nutrients, but also of innocuous food antigens, bacterial metabolites, bacterial cell components (e.g. lipopolysaccharide (LPS), flagellin, peptidoglycans, etc.), and other debris, self or non-self, originating from the gut (Horst, Neumann, Diehl, & Tiegs, 2016; Thomson & Knolle, 2010). In a non-hepatic context, challenge by an antigenic load similar to that found in the portal vein would normally result in a powerful inflammatory immune response. Despite a large influx of antigens coming from the portal vein and the persistent stimulation of innate immune receptors on both PC and NPC populations, the liver responds by active immune suppression and the production of soluble immune-regulatory cytokines like interleukin-10 (IL-10), transforming growth β (TGFβ), and prostanoids (prostaglandin E₂ (PGE₂)), thus favoring a state of tolerance as opposed to a state of immunity (Crispe, 2009; Gao, Jeong, & Tian, 2008; Knolle & Gerken, 2000). These mechanisms presumably evolved to prevent the liver, the source of so many vital physiological functions, from inflicting immune-mediated damage to itself, as can be seen in cases of autoimmune hepatitis (Crispe, 2009).

In contrast to other immune privileged organs like the eye, which rely on compartmentalization to avoid the immune system (Niederkorn, 2013), the liver is unique in that it uses its PC and NPC populations to actively suppress immune responses in order to achieve immune tolerance (Horst et al., 2016; Wohlleber & Knolle, 2016). These cells
are far more than just simple scavengers. They act via antigen-dependent and independent mechanisms to skew the immune response towards tolerance. As is the case with antigen clearance, the slow flow of blood provides PCs and NPCs with ample time to make extensive interactions with passing leukocytes, T cells in particular. While multiple cell populations of the liver contribute to immune tolerance in varying contexts, the next couple of subsections will be dedicated to describing the general immune suppressive capabilities of two NPC populations of mesenchymal origin that have drawn significant interest for their function in the induction of liver transplant tolerance, the liver sinusoidal endothelial cells (LSEC) and hepatic stellate cells (HSC).
Liver Sinusoidal Endothelial Cells

The LSEC is the most prominent NPC population in the liver, comprising upwards of 50% of all NPCs (Demetris et al., 2016). They are positioned at the interface between the sinusoid lumen and the subendothelial space of Dissé, regulating the two-way exchange of materials by altering the pore size of their fenestrations. Due to the lack of proteinaceous diaphragms that span individual fenestrations and a proper basement membrane beneath the LSECs, the fenestrations can accommodate the passage of entire cells or cellular processes, which is critical to the interaction of passing leukocytes with subendothelial HSCs and hepatocytes (Demetris et al., 2016). Beyond acting as a semi-permeable barrier between the blood and liver parenchyma, LSECs are also efficient scavenging and antigen-presenting cells (APC) (Ebrahimkhani, Mohar, & Crispe, 2011; Limmer et al., 2000; Schurich et al., 2009)

Due to the remarkably efficient scavenging capability of LSECs, they compete with liver dendritic cells (DC) for exogenous antigen that can be processed and presented on MHC class II (MHC-II) molecules, which are recognized by passing naïve and effector CD4+ T helper (Th) cells (Limmer et al., 2000; Schurich et al., 2009). Under steady-state conditions, LSECs express only low levels of MHC-II and T-cell co-stimulatory ligands, CD80/86 (see “2-Signal Model” in Appendix I) (Knolle et al., 1998). The LSEC expression of MHC-II and CD80/86 are kept low by immunosuppressive IL-10, which is expressed by various liver cells in response to the physiological levels of LPS arriving from the gut. Despite the low levels of MHC-II and CD80/86, LSECs are still able to activate both naïve and effector Th cells (Cambahia et
al., 2013, 2014; Kruse et al., 2009). However, the outcomes of LSEC-mediated activation diverge with normal Th-cell activation due to the LSEC’s lack of co-stimulatory ligand expression and the necessary T cell-activating cytokine, IL-12.

In the context of naïve Th-cell activation, LSECs have been shown induce a Th-cell phenotype that resembles regulatory T cells (Treg) in function, but lacks the classical Treg markers CD25 and Foxp3 (Kruse et al., 2009). These non-conventional CD25^{low}Foxp3^{-} Tregs were shown to express the immunosuppressive cytokines, IL-4 and IL-10, resulting in the suppression of T-cell proliferation and function in vitro and the suppression of autoimmunity in a murine model of autoimmune hepatitis (Kruse et al., 2009). LSECs may also induce the production of conventional Tregs (CD25^{high}Foxp3^{+}), but in a TGFβ-contact-dependent manner (Carambia et al., 2014). These LSEC-induced conventional Tregs effectively suppress T-cell immunity and induce systemic tolerance, as evidenced by a murine model of experimental autoimmune encephalomyelitis (Carambia et al., 2015). In this model, auto-antigen (i.e. the antigen causing autoimmunity) was delivered specifically to LSECs via nano-particles. The LSECs subsequently induced the differentiation of auto-antigen-specific conventional Tregs that suppressed the autoimmune condition, generating systemic tolerance to the auto-antigen.

In the context of effector Th-cell activation, LSECs are able to modulate the functions of these Th cells so as to induce tolerance. Th1 and Th17 effector cells normally differentiate in response to intracellular and extracellular pathogens, respectively, and they secrete the inflammatory cytokines interferon-γ (IFN-γ) and IL-17 to aid in pathogen elimination (Parham, 2014). When Th1 and Th17 cells were co-
cultured with LSECs, their secretion of inflammatory cytokines was inhibited (Carambia et al., 2013). This inhibition is accomplished by LSEC secretion of immunosuppressive IL-10, as well as the increased expression of the co-inhibitory ligand, programmed death-ligand 1 (PD-L1) (see “2-Signal Model” in Appendix I). Additionally, Th1 cells have been found to secrete their own IL-10 in response to Notch receptor engagement by Notch ligands expressed on LSECs (Neumann et al., 2015). LSECs may also modulate the function of Th2 effector cells. Th2 cells normally differentiate in response to extracellular parasitic infection and secrete anti-inflammatory cytokines that initiate repair and recovery of the tissues that have been damaged by the parasite (Parham, 2014). It has been shown that LSEC activation of Th2 cells leads to their proliferation, resulting in increased levels of anti-inflammatory cytokine IL-4, thus contributing further to tolerance (Klugewitz et al., 2002).

LSECs are also capable of activating naïve CD8⁺ cytotoxic T lymphocytes (CTL) through the expression of MHC class I (MHC-I) molecules. Similar to liver DCs, LSECs harbor cross-presentation mechanisms (see “Cross-Presentation” in Appendix II) (Ebrahimkhani et al., 2011; Limmer et al., 2000; Schurich et al., 2009), which allow exogenously derived antigens that are normally presented by MHC-II molecules to be diverted towards presentation on MHC-I molecules—MHC-I is recognized exclusively by CTLs. This allows LSECs to use antigens of exogenous origin to trap and modulate the function of naïve CTLs specific for these antigens. Upon cognate interaction between LSEC MHC-I and naïve CTL T-cell receptor (TCR), naïve CTLs become non-responsive or anergic (Limmer et al., 2000) and transform into long-lived, memory-like T cells that
express high levels of the anti-apoptotic protein, bcl-2 (von Oppen et al., 2009). The continued suppression of LSEC-activated CTLs is dependent on the upregulation of the co-inhibitory signaling via PD-L1/PD-1, which occurs after initial cognate interactions between the LSEC and naïve CTL. It is a similar suppressive mechanism to that seen in LSEC modulation of the Th1 and Th17 effector cells. This “tolerized” state of the CTL, however, is reversible upon sufficient stimulation of CD28 co-stimulatory receptor on the anergic CTL (Diehl et al., 2008). The induction of this non-responsive state is presumably to save pathogen-specific naïve CTLs from clonal deletion mediated by DCs under non-inflammatory conditions. In non-inflammatory conditions, DCs do not express the necessary co-stimulatory ligands to fully activate naïve CTLs, thus resulting in their elimination by induced apoptosis (Horst et al., 2016). The anergic phenotype of LSEC-activated CTLs is thus beneficial in the context of inducing systemic tolerance to organ transplants and saving unique T-cell clones that are pathogen-specific from clonal deletion. However, it is interesting to note that this mechanism may be harmful in the context of immunity to cancer, since cancer-specific CTLs maybe tolerized and prevented from enacting necessary cytolytic functions on cancer cells.

A remaining aspect of LSEC-mediated immune suppression to consider is antigen-independent (non-APC) function as an immune “bystander” (Horst et al., 2016; Schildberg, Sharpe, & Turley, 2015). As has been discussed up to this point, LSECs are particularly efficient at directly modulating the immune responses of Th cells and CTLs by interacting with these cells in an antigen-dependent context. However, LSECs express a unique adhesion molecule known as liver sinusoidal endothelial cell lectin (LSECTin),
which binds to intercellular adhesion molecule 3 (ICAM-3) on activated T cells (Liu et al., 2004). The ability to non-specifically adhere to activated T cells is what allows the liver to sequester large amounts of these cells and suppress their function. It has been shown in a murine model of T cell-mediated acute hepatitis, that the absence of LSECtin can exacerbate the disease, while exogenous addition of recombinant LSECtin can lead to partial recovery (Tang et al., 2009). In addition to non-specific trapping of activated T cells, LSECs are capable of preventing DCs from activating naïve CTLs, thus abrogating DC-mediated T-cell immunity (Schildberg et al., 2008). This “vetoing” capacity of LSECs is achieved through contact-dependent mechanisms, which downregulate the expression of DC co-stimulatory ligands and the T cell-activating cytokine, IL-12. The permanence of this mode of T-cell suppression was highlighted by the fact that supplementation of exogenous stimulatory signals could not rescue these cells from suppression. This contact-dependency in vetoing APC function has also been observed in HSCs, suggesting that this is a broad mechanism employed by liver NPCs to alter the immune function of certain APCs (Horst et al., 2016; Schildberg, Wojtalla, et al., 2011).

LSECs are efficient immunosuppressive cells that operate through antigen-dependent and independent mechanisms. The culmination of these mechanisms allows LSECs to effectively induce immune tolerance rather than immunity, thus providing a partial picture of how tolerance may be induced in liver transplantation and co-transplantation, among other contexts.
Hepatic Stellate Cells

The HSC is the second largest stromal cell population in the liver after LSECs and comprises 10-15% of the total liver cell population (Demetris et al., 2016). They function as liver-specific pericytes that occupy the subendothelial space of Dissé, between the fenestrated LSECs and plates of hepatocytes. Under steady state non-inflammatory conditions in the liver, HSCs have numerous quiescent functions of which the most prominent are vitamin A storage, the deposition and turnover of extracellular matrix (ECM) in the space of Dissé, and the control of microvascular blood flow by regulation of sinusoidal vascular tone (Hellerbrand, 2013). However, during states of inflammation or liver damage, HSCs become activated and assume new functions. They may trans-differentiate into contractile myofibroblasts, which are responsible for the collagen and ECM deposition seen in liver fibrosis. Additionally, activated HSCs may assume various immunomodulatory functions.

Activated HSCs have been reported to interact with CD4\(^+\) Th cells in a possible antigen-presenting context. In a murine hepatic fibrosis model, activated HSCs were found to be in close proximity to Th cells in periportal regions, suggesting that the HSCs may be modulating T-cell activity by antigen presentation (Muhanna, Horani, Doron, & Safadi, 2007). These purely qualitative \textit{in vivo} observations, however, have been challenged by \textit{in vitro} studies, which cast doubt on the antigen-presenting capacity of activated HSCs. These studies have demonstrated that purified HSCs express low levels of MHC-II molecules and lack co-stimulatory ligand expression (Ichikawa, Mucida, Tyznik, Kronenberg, & Cheroutre, 2011). Additionally, they exhibit poor antigen uptake
abilities in comparison with LSECs and DCs (Schildberg, Kurts, & Knolle, 2011). However, the APC function of activated HSCs may be context dependent, as the addition of exogenous IFN-γ to HSC culture increased the expression of molecules required for antigen processing and presenting (Ichikawa et al., 2011). Ascertaining the precise degree to which HSCs function as APCs has been difficult, especially since obtaining highly pure HSC populations for in vitro culture and replicating the exact in vivo conditions of the liver remain technically challenging. Further studies will be required to clarify whether HSCs are sufficiently competent to be considered as an APC population in the liver.

In terms of antigen-presentation to CD8+ CTLs, it has been found that HSCs lack the ability to cross-present antigens as do LSECs and DCs (Ichikawa et al., 2011). While this result may suggest an inability of HSCs to activate CTLs using exogenously derived antigens on MHC-I, HSCs may still be able to acquire this APC function since it has been shown that HSCs engage in a process known as trogocytosis (Ichikawa et al., 2011). During trogocytosis, certain cell populations are able to exchange pieces of cell membrane that contain MHC molecules as well as co-stimulatory or co-inhibitory ligands (Huang et al., 1999). By this mechanism, it is possible for HSCs to acquire the needed cell surface molecules to participate in antigen-presentation and subsequent activation of both Th cells and CTLs, however, further study is required to understand the extent to which this happens.

Though the immunomodulatory function of HSCs as APCs has been largely questioned, their functional role as immunosuppressive bystander cells acting in an
antigen-independent (non-APC) manner is well characterized. Upon activation in liver inflammation or injury, HSCs acquire robust T-cell suppressive capabilities in addition to their pro-fibrotic capacity. The ability to inhibit T cells is brought about by increased expression of co-inhibitory ligand PD-L1 and immunosuppressive cytokines IL-6, IL-10, and TGFβ, after exposure to inflammatory cytokine IFN-γ—shown to be dose dependent (Charles et al., 2013). Co-culture of activated HSCs with activated T cells demonstrated HSC-dependent initiation of T-cell apoptosis, presumably by contact-dependent ligation of HSC PD-L1 with T-cell PD-1. Other studies have shown that HSC PD-L1 does not act alone and that increased expression of B7-homolog 4 (B7-H4, another co-inhibitory ligand related to PD-L1) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) also resulted in T-cell suppression and apoptosis (Chinnadurai & Grakoui, 2010; Yang et al., 2010). HSCs have also been observed to indirectly suppress T-cell function by induction of Treg differentiation from naïve Th cells in an antigen-independent manner. When HSCs were co-cultured with naïve Th cells in the presence of liver DCs (the stand-in APCs), HSC-derived all-trans retinoic acid (ATRA) and TGFβ were able to bias naïve Th cell differentiation towards conventional CD25^{high}Foxp3^{+} Tregs (Dunham et al., 2013). Additional studies are needed to identify the mechanism by which HSC-derived ATRA induces Treg differentiation.

In addition to directly suppressing T-cell function and inducing Treg differentiation, HSCs have also been observed to induce other immunosuppressive cell populations. Activated HSCs are able to interact with peripheral blood monocytes in the sinusoid lumen via long cytoplasmic extensions that traverse LSEC fenestrations
(Demetris et al., 2016; Höchst et al., 2013). These peripheral blood monocytes are induced by HSCs to differentiate into myeloid-derived suppressor cells (MDSC), which effectively inhibit T-cell proliferation and function. Other bystander functions of HSCs include a similar APC vetoing capacity as seen in LSECs. Through contact-dependent mechanisms and expression of ATRA and IL-10, HSCs can depress the APC function of liver DCs, thus preventing DC-mediated T-cell activation (Bhatt et al., 2014; Lee et al., 2005).

HSCs are potent immunosuppressive cells that most likely act primarily in an antigen-independent fashion. Through robust contact-dependent T-cell suppression, induction of suppressor cell populations like MDSCs and conventional Tregs, and modulating DC APC function, these cells can work collectively with LSECs to induce formidable local and systemic immune tolerance that allow for the induction of liver transplant tolerance.
IFN-γ SIGNALING AND MESENCHYME-DERIVED NPCs MEDIATE TOLERANCE INDUCTION IN LIVER TRANSPLANTATION

In 2003, Philip Halloran’s group demonstrated that IFN-γ signaling is absolutely required for spontaneous liver transplant acceptance (Mele et al., 2003). Wild type (WT) liver allografts were transplanted into mice of WT and IFN-γ knockout (KO) backgrounds. It was found that allografts in IFN-γ KO mice succumbed to acute rejection with none of the grafts surviving beyond 14 days. On the other hand, grafts in WT recipients demonstrated long-term survival with nearly half of the allografts surviving past 100 days. These results were somewhat counterintuitive, as IFN-γ was understood to be an inflammatory cytokine expressed by various effector T (Teff) cell populations that mediate graft rejection. This raised several important questions: Why is IFN-γ required for spontaneous graft acceptance? What cellular populations does IFN-γ act through? What are the downstream mediators of IFN-γ signaling that bring about acceptance? Etc.

These results are striking to say the least, however, it would take another 12 years before the questions raised by this study would begin to get answered in full. During the early 2000’s, the individual roles of different liver cell populations in tolerance induction were still being parsed out, which may in part explain the delayed investigation into IFN-γ signaling in the context of transplantation. In 2015, Shiguang Qian’s group determined that two mesenchyme-derived cell populations, the LSECs and HSCs, are instrumental in inducing spontaneous liver allograft acceptance via IFN-γ signaling (Morita et al., 2015). The following sub-section is a brief summary of key results from that study.
Rejection is Necessary to Induce Spontaneous Liver Allograft Acceptance

In the study performed by Morita et al., donor liver allografts were derived from both WT and IFN-γ receptor (IFN-γR) KO mice. Both types of allografts were transplanted into WT recipients of a different MHC constitution (Figure 2) in order to recapitulate and corroborate the findings of Mele et al. All WT allografts demonstrated long-term survival (>80 days), while none of the IFN-γR KO allografts survived past 15 days, thus confirming that IFN-γ signaling is indispensible to spontaneous liver allograft acceptance.

Figure 2. A schema of the initial transplant experiments used to corroborate the finding that IFN-γ signaling is indispensible to spontaneous liver allograft acceptance. Donor liver allografts originated from mice of two different backgrounds, WT C57BL/6 and IFN-γR KO, but both mice were similar in MHC constitution, H-2^b. These livers were subsequently transplanted into mice of WT C3H background with a different MHC constitution, H-2^k. WT C3H mice that received WT allografts demonstrated long-term survival (>80 days), while WT C3H mice that received IFN-γR KO allografts succumbed to acute rejection, none surviving past 15 days. This confirmed that IFN-γ signaling is indispensible for spontaneous allograft acceptance. Figure was created by the author (represents methodology in Morita et al., 2015).
Qualitative analysis of WT and IFN-γR KO liver allografts by hematoxylin and eosin (H&E) stain and immunohistochemistry (IHC) for CD4 and CD8 T-cell markers revealed that IFN-γR KO allografts were characterized by dense lymphocytic infiltrate containing an excess of CD8⁺ T cells in comparison to WT allografts (Figure 3). The WT allografts contained only marginal levels of lymphocytic infiltrate and contained lower levels of CD8⁺ T cells. The levels of CD4⁺ T cells were similar between WT and IFN-γR KO allografts suggesting that IFN-γ signaling is somehow involved in the clearance of CD8⁺ T cells. Additionally, long-term follow up of WT allografts revealed that the number of CD8⁺ T cells decreased over time with CD4⁺ T cells becoming predominant. These results were confirmed by quantitative flow analysis.

![Figure 3. IFN-γR KO allografts are characterized by dense lymphocytic infiltrate.](image)

The two panels on the left are H&E stains demonstrating panacinar lymphocytic infiltration of IFN-γR KO allografts and marginal periportal infiltration of WT allografts. IHC for CD4 (red) and CD8 (green) in the two right-hand panels shows the excess build up of CD8⁺ T cells in IFN-γR KO allografts, suggesting IFN-γ signaling is required for clearance of CD8⁺ T cells. Figure taken from (Morita et al., 2015).
To determine if the defective clearance of CD8$^+$ T cells in IFN-$\gamma$R KO allografts was in relation to inadequate induction of apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining for evidence of apoptotic cells and Western blot for the apoptotic protein, procaspase 3, was performed on lymphocytic infiltrates from both grafts. It was found that WT allografts contained far higher levels of TUNEL$^+$ cells and procaspase 3 in comparison to IFN-$\gamma$R KO allografts, thus demonstrating that intact IFN-$\gamma$ signaling is required for the induction of T-cell apoptosis. To take things one step further, Morita et al., performed adoptive cell transfer of donor-specific CD8$^+$ Teff cells (T cells specific for donor MHC, H-2$^b$) into WT and IFN-$\gamma$R KO allografts. Flow analysis of subsequent graft-infiltrating T cells for evidence of proliferation and apoptosis revealed that WT allografts could effectively clear these donor-specific CD8$^+$ Teff cells while IFN-$\gamma$R KO allografts could not, thus indicating that IFN-$\gamma$ signaling is a requirement for induction of apoptosis in CD8$^+$ Teff cells, importantly, donor-specific CD8$^+$ Teff cells.

In the next phase of experiments, Morita et al. tested the effect of IFN-$\gamma$R deficiency on the response of different cell populations in order to deduce which cells were acting through IFN-$\gamma$ signaling to bring about allograft acceptance. In examining the suppressor cell populations, namely Tregs and MDSCs, it was found that counts of these cells were equally elevated in both WT and IFN-$\gamma$R KO allografts compared to syngeneic transplant controls (these controls were liver transplants from one WT C3H mouse to another genetically identical WT C3H mouse, which should not elicit an immune response). This suggested that suppressor cell populations are mobilized early in the graft
rejection response and do not rely on IFN-γ signaling for induction. Since the induction of Tregs and MDSCs was unaffected by IFN-γR deficiency, this indicated that these cells are not sufficient to induce allograft acceptance—because the end result is still acute rejection in IFN-γR KO allografts.

Liver NPCs were also considered due to their known protective and tolerogenic roles in the liver. NPCs were particularly appealing since it was known that hepatocyte cellular transplantation (which lacks NPCs) invariably suffers rejection (Bumgardner et al., 1998). This hinted that allograft NPCs were critical for tolerance induction and acceptance in whole-liver transplantation. Upon examining NPC populations in the liver, Morita et al. discovered that NPCs of hematopoietic origin (CD45+) are rapidly replaced by recipient CD45+ NPCs following transplantation. On the other hand, non-hematopoietic (CD45-) NPCs remain intact, therefore it was reasoned that CD45+ NPCs likely do not contribute to establishing tolerance induction and allograft acceptance. In one-way mixed leukocyte reaction (MLR) for T-cell proliferation, WT allograft-derived CD45- NPCs exhibited effective suppression of the T-cell proliferative response while IFN-γR KO allograft-derived CD45- NPCs exhibited near complete loss of their inhibitory function. Additional MLR experiments with activated donor-specific CD8+ Teff cells demonstrated that IFN-γR KO allograft-derived CD45- NPCs were unable to inhibit CD8+ Teff cell proliferation or induce apoptosis, thus signifying that CD45- NPCs are reliant on intact IFN-γ signaling to perform these functions.

In identifying the downstream mediators of IFN-γ signaling in CD45- NPCs, Morita et al. compared the cell surface expression profiles of WT and IFN-γR KO
allograft-derived CD45\(^{-}\) NPCs. It was noted that B7-H1 expression was reduced on IFN-\(\gamma\)R KO allograft-derived CD45\(^{-}\) NPCs—B7-H1 is the co-inhibitory ligand, PD-L1. The ligation of PD-L1 on CD45\(^{-}\) NPC with co-inhibitory receptor PD-1 on CD8\(^{+}\) Teff cells is likely what mediated the induction of Teff-cell apoptosis and the inhibition of the T-cell proliferative response. This was confirmed by B7-H1 KO studies. IFN-\(\gamma\) signaling is therefore critical to the expression of B7-H1 on allograft-derived CD45\(^{-}\) NPCs, which mediates the aforementioned effects on T cells.

Cell-specific monoclonal antibodies were used to reveal the precise identity of the CD45\(^{-}\) NPCs. It was found that the expression of B7-H1 tightly correlated with cells that were positive for CD105 (LSECs) and desmin (HSCs)—cells of mesenchymal origin (Figure 4). To further test the immunomodulatory properties of these cells, isolated LSECs and HSCs were co-transplanted (separately) with pancreatic islet allografts into type I diabetic mice. Both LSECs and HSCs allowed for prolonged survival of islet allografts, though HSCs demonstrated more potent immunosuppressive abilities. Additional experiments were conducted to confirm that these cells also possessed immunosuppressive capabilities in a human context. Isolated human liver CD45\(^{-}\) NPCs were found to similarly respond to IFN-\(\gamma\) by upregulating B7-H1 expression in a dose-dependent manner. Lastly, when co-cultured with activated T cells in one-way MLR, the CD45\(^{-}\) NPCs were able to inhibit the T-cell proliferative response.

The results of this study suggest that the pro-inflammatory environment (high local concentrations of IFN-\(\gamma\)) of the liver generated by the initiation of a T cell-mediated rejection (TCMR) response by the recipient, allows liver graft CD45\(^{-}\) NPCs to upregulate
the expression of B7-H1 co-inhibitory ligand and thereby suppress the proliferation of CD8$^+$ Teff cells and induce their apoptosis. The elimination of donor-specific CD8$^+$ Teff cells thus generates a state of specific immune tolerance to the donor liver allograft, thus explaining spontaneous allograft acceptance in the liver.

Figure 4. CD45$^-$ NPCs are LSECs and HSCs. To reveal the precise identity of allograft CD45$^-$ NPCs, monoclonal antibodies were used to identify specific cell populations. By identifying overlapping expression of cell-surface markers with B7-H1 expression, the identities of the CD45$^-$ NPCs were revealed to be LSECs and HSCs. In the top row of panels, DAPI (nuclear expression), CD105 (LSEC cell-surface marker), and B7-H1 expression are shown separately and in unison at the far right. A similar display is made for desmin-expressing HSCs in the bottom row of panels. Figure taken from (Morita et al., 2015).
DISCUSSION

The study recently performed by Morita et al. suggests that rejection is a necessary precondition to liver transplant acceptance, a counterintuitive conclusion made clear by a mechanistic explanation derived from their results. After reperfusion of a liver transplant, donor antigen floods the liver-draining lymph nodes and spleen of the recipient, where APCs present this antigen to activate donor-specific T cells into Teff cells—the beginnings of the graft rejection response. Due to the efficient trapping of Teff cells in the liver via LSEC-expressed LSECtin as well as other antigen-dependent and independent mechanisms, large amounts of Teff cells begin to accumulate in the liver where they secrete the inflammatory cytokine, IFN-γ. The greater the local concentration of IFN-γ, the greater the upregulation of B7-H1 co-inhibitory ligand on liver mesenchymal NPCs like LSECs and HSCs. The ligand B7-H1 expressed by LSECs and HSCs with PD-1 co-inhibitory receptor expressed by Teff cells results in Teff cell proliferative inhibition and subsequent apoptosis, thus eliminating large amounts of donor-specific Teff cells that would have otherwise continued to cause rejection. The recipient has thereby actively acquired specific tolerance to the liver allograft. This negative feedback loop between Teff cells and graft mesenchymal cells is the basis for the novel hypothesis, mesenchyme-mediated immune control (MMIC), which was proposed by Morita et al.

Though the study conducted Morita et al. considers one aspect of graft mesenchyme-mediated immune suppression (i.e. IFN-γ-stimulated Teff cell elimination),
the MMIC hypothesis suggests that graft mesenchyme-derived cells use various mechanisms that work in concert to effectively suppress the recipient’s donor-specific immune response. As was discussed in previous sections, the mesenchyme-derived LSECs and HSCs are competent and potent immunosuppressive cells that work through differing antigen-dependent and independent mechanisms to accomplish tolerance. Surely in the context of inducing liver transplant tolerance, LSEC and HSC-mediated induction of Tregs and MDSCs, modulation and suppression of Teff function, and expression of immunosuppressive cytokines work in unison with IFN-γ-mediated clonal deletion of donor-specific Teff cells to accomplish formidable specific tolerance for liver allografts.

As mesenchyme-derived cells are common to all organs, it is likely that MMIC is not a singular characteristic of the liver, but a potential mechanism shared by many organs. The liver is not the only organ to have recorded instances of spontaneous acceptance. In fact, there have been instances of spontaneous acceptance in experimental kidney transplantation, though this occurs at a far lower rate than what is observed in the liver (Russell, Chase, Colvin, & Plate, 1978). Regardless, certain organs contain homologous cells to that of the LSEC and HSC in the liver. Endothelial cells are ever present in all organs and stellate cells/pericytes are seen in other organs as well, e.g. the intraglomerular mesangial cell in the kidneys. The vastly differing capabilities of MMIC between other organs and the liver may be due to several liver-intrinsic factors. The liver is a large solid organ, and by virtue of its mass it contains more mesenchyme-derived cells from which to perform MMIC with. Additionally, the liver has a specialized tissue
architecture and microenvironment, characterized by slow blood flow and soluble immunosuppressive mediators, which likely contribute to the ability to perform MMIC. Lastly, the liver contains evolved mechanisms for immune suppression in order to deal with the high antigenic load coming from the portal vein that other organs just simply lack. In any case, the concept of MMIC as a universal mechanism of tolerance induction shared by all organs may be clinically useful. There is potential for co-transplantation of purified mesenchyme-derived graft cells or even in situ enhancement of graft mesenchyme cells. As it stands, the effort to improve transplantation by using mesenchyme-derived cells has already begun in experimental models, notably, by co-transplanting pancreatic islet allografts with HSCs, Sertoli cells, and mesenchymal stem cells (Figliuzzi et al., 2009; Morita et al., 2015; Suarez-Pinzon et al., 2000).

There are other significant clinical implications for MMIC as well. In returning to the discussion of LSEC and HSC-mediated clonal deletion of donor-specific Teff cells, it is exceptional that the precondition to tolerance induction is rejection and inflammation. The significance of rejection-mediated tolerance has to do with current clinical practices in transplantation. Most transplant centers globally (50-70%) start patients on immunosuppression during the peri- and post-operative period (Schumacher & Gajarski, 2011). By starting patients on immunosuppression, particularly as it pertains to liver transplantation, the recipient is prevented from mounting a graft rejection response that is required for tolerance induction. By suppressing T-cell activation and proliferation through immunosuppression, there cannot be sufficient graft accumulation of Teff cells that secrete IFN-γ, thus abrogating the negative feedback loop enforced by graft
mesenchyme-derived cells. Though this is purely speculating based off of the preliminary experimental results from the study by Morita et al., it will be interesting to see how transplant centers might integrate rejection-mediated tolerance, at least with liver transplant patients, should this concept gain more traction in the clinic.

Inducing liver transplant tolerance in the clinic may be aided by identifying predictive biomarkers. A recent review canvassed efforts to identify useful biomarkers, which have focused on recipient characteristics of peripheral blood including the ratio of monocytoid dendritic cells to plasmacytoid dendritic cells, the relative numbers of Tregs, or ratios of other T-cell subsets (Baroja-Mazo et al., 2016). While these characteristics may turn up as useful diagnostic tools, it has become clear from MMIC that the effort to find useful biomarkers should also include the donor/allograft. It may be possible to identify elements of the IFN-γ/B7-H1 pathway that may serve as useful predictors of tolerance induction.

The induction of immunosuppression-free transplant tolerance, like that seen in liver, has been the goal of transplant investigators for decades. MMIC is a unique hypothesis that offers a fresh perspective on the possibility of inducing transplant tolerance not only in the liver, but also in the other transplantable organs. With a renewed focus on graft mesenchyme-derived cells, further studies should continue to investigate the liver and its unique cellular populations, while also delving into the potential immunomodulatory properties of other organ mesenchyme-derived cells. Additionally, clinical applications of MMIC should be evaluated to identify new therapeutic targets and strategies for transplant care.
APPENDIX I

2-Signal Model

During the activation of a naïve T cell, 2 essential signals are required for full activation into an Teff cell (1) engagement of the T-cell receptor (TCR) by its cognate peptide:MHC complex [the antigen-specific signal] and (2) engagement of the co-stimulatory receptor, CD28, by its ligands CD80/86 [the co-stimulatory signal]. Under steady state conditions, professional antigen-presenting cells (APC) like dendritic cells (DC) do not express co-stimulatory ligand. If a naïve T cell were to engage a DC in the absence of co-stimulatory ligand, these cells would be signaled to undergo apoptosis or anergy (induction of functional un-responsiveness). However, during an inflammatory or infectious state, DCs can acquire co-stimulatory ligand expression. As a result, DCs can provide a Signal 1 (so long as that they present the proper peptide:MHC) and a Signal 2 (now that they express co-stimulatory ligand). APCs can also express co-inhibitory ligands like PD-L1 (B7-H1) that interact with T-cell co-inhibitory receptor PD-1. Depending on the balance of co-stimulatory and co-inhibitory ligands expressed on APCs a T cell can be either activated or suppressed.
APPENDIX II

*Cross-Presentation*

Peptide antigens are presented to T cells either by major histocompatibility complex class I (MHC-I/recognized by CD8\(^+\) T cells) or class II (MHC-II/recognized by CD4\(^+\) T cells) molecules. MHC-II molecules are loaded with peptide fragments that derive from exogenous protein antigens (proteins that were endocytosed by the cell), while MHC-I molecules are traditionally loaded with peptide fragments that derive from endogenous protein antigens (cytosolic proteins). Cross-presentation is a useful mechanism that certain antigen-presenting cells (APC) use in order to present antigens of exogenous origin to CD8\(^+\) T cells. The mechanisms by which endocytosed proteins are processed and transferred to the MHC-I loading pathway are still unclear, but the end result is that exogenous peptide antigen is presented on MHC-I that can be recognized by CD8\(^+\) T cells. The practicality of cross-presentation is readily appreciated by the example of a non-virally infected cell. If a non-virally infected cell endocytoses exogenous viral particles, the normal chain of events would lead to presentation of viral peptide on MHC-II molecules, which are only recognized by CD4\(^+\) T cells that cannot directly kill virally infected cells. By cross-presentation, processed viral-peptides are diverted to the MHC-I loading pathway so that viral peptides are presented on MHC-I to virus-specific CD8\(^+\) T cells capable of killing virally infected cells. Thus cross-presentation is an effective mechanism by which APCs activate CD8\(^+\) T cells using exogenously derived antigen.
REFERENCES


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