Transplantation Immunology

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1.1 Introduction

The twentieth century began with uniformly unsuccessful endeavors at investigational allotransplantation, and the next half-century was marked by repeated failure. The recognition of histocompatibility antigens [1] and the primary role of lymphocytes [2] in allorecognition were the two innovations that laid the foundation of transplant immunology that was to eventually form the basis for strategies leading to successful solid organ transplantation.

In the era preceding the development of dialysis, where end-stage renal disease meant imminent death, numerous attempts at renal transplantation failed to yield long-term survivors. The first successful renal transplant, performed between identical twins at the Peter Bent Brigham Hospital in Boston on December 23, 1954, by Moore, Murray, Merrill, and Harrison, energized the transplant community with passion – the barrier of histoincompatibility overcome by virtue of transplantation of a kidney between identical twins, nevertheless demonstrating the utility of organ replacement. Subsequently, Starzl performed the first successful kidney transplant between histoincompatible individuals, under azathioprine-based immunosuppression, 6 years following the twin transplant [3].

"The Relation of Immunology to Tissue Homotransplantation" was the title for the first international transplant conference sponsored by the New York Academy of

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A.D. Pinna, G. Ercolani (eds.), *Abdominal Solid Organ Transplantation: Immunology, Indications, Techniques, and Early Complications*,
DOI 10.1007/978-3-319-16997-2_1

Sciences in 1954 [4]. Current strategies in solid organ transplantation are based upon a comprehensive understanding of immunology and the use of potent immunosuppressive agents, which have led to high rates of success. Investigations in transplantation immunology have led to paradigm shifts in immunology and have greatly contributed to novel approaches to enhance allograft survival while minimizing morbidity.

In this chapter, we will provide an overview of transplant immunology including the principles of allorecognition, the immunological basis of organ rejection (including the immunology of xenotransplantation), and the rationale for historical and current immunosuppressive therapy. Focus will be placed on clinical solid organ transplantation with emphasis on fundamental concepts related to the up-to-date practice of transplant medicine.

1.2 Allorecognition

1.2.1 Major Histocompatibility Complex

The major histocompatibility complex (MHC) refers to a collection of genes, or genetic region, whose fundamental function is the production of proteins that present self and foreign antigens (peptide fragments) to immunocytes as part of normal immunologic surveillance. These complexes of peptide fragments in conjunction with MHC molecules are recognized by specific receptors on immunocytes and under specific circumstances initiate an immunologic cascade with the intent of creating a regulated and targeted response.

Human leukocyte antigen (HLA) is the term used for human MHC molecules. Two distinct classes of MHC antigens, Class I (HLA-A, HLA-B, HLA-C) and Class II (HLA-DR, HLA-DP, HLA-DQ), are located on chromosome 6. HLA Class I molecules are expressed on all nucleated cells as well as platelets, while HLA Class II are associated with antigen-presenting cells (APCs), including lymphocytes, monocytes, macrophages, and dendritic cells. A limiting factor in the exchange of organs between nonidentical individuals is the high degree of polymorphisms within the HLA loci, with resultant large numbers of allelic combinations resulting in low probabilities of complete matching in a random setting. The difference in MHC molecules between the donor and recipient is a primary cause of graft rejection.

1.2.2 Mechanisms of Allorecognition

In the thymus, T lymphocytes are selected for their ability to differentiate self from nonself, i.e., those T lymphocytes with excessive affinity for self-MHC are deleted (negative selection), whereas those with appropriate affinity are designated for maturation and export to the peripheral immune system (positive selection). This process is designed to deal with altered self (both viral- and tumor-related changes) as well as foreign antigens from bacterial and parasitic infections. Physiologic antigen processing involves peptide fragment generation via proteasome degradation of

cytosolic proteins and presentation on Class I MHC and via proteolytic degradation of proteins within phagosomes and subsequent presentation with Class II MHC antigens. This process entails indirect antigen presentation, meaning that the immunocyte receptor recognizes the peptide fragments in context within the MHC binding groove.

Conceptually, the immune system would never naturally encounter alloantigens – the sole exception being in the context of pregnancy. Although the placenta provides a barrier between the mother and fetus during pregnancy, low levels of maternal and fetal cells can be found circulating in the fetus and the mother, respectively. Persistence of fetal cells in the mother has been associated with late autoimmune disorders [5], while the presence of maternal cells in the fetus is associated with tolerance to maternal antigens [6] and may also be associated with autoimmune disorders in offspring [7]. Since all pregnant women have detectable fetal cells in their blood by 36 weeks of gestation, elimination of these cells may be mediated by peripherally circulating T cells with high affinity for nonself HLA and would entail direct allorecognition rather than generating alloantibody responses through indirect antigen presentation, which may adversely affect subsequent pregnancies.

In allotransplantation, recognition of foreign MHC likely involves both indirect and direct allorecognition. Indirect allorecognition utilizes a mechanism similar to the one involved in recognition of foreign antigens – specifically, fragments of foreign MHC molecules are processed through the phagosome and presented as antigenic peptides bound in the groove of self-MHC Class II molecules on APC. In direct allorecognition, foreign MHC molecules on donor cells that migrate out of the allograft are directly recognized by T lymphocytes, perhaps secondary to the innate affinity of T-cell receptors (TCR) for MHC molecules [8]. The binding of these TCR in direct antigen presentation is not thought to be specifically to the MHC binding groove; in fact, the recognition of donor MHC does not require antigen processing through APC [9].

It is possible that the direct allorecognition pathway predominates in the early phases of alloimmune responses and accounts for the strength of the alloimmune response related to a high T-cell precursor frequency, estimated to be as high as one in ten circulating T cells. It has been speculated that indirect alloantigen presentation may be important in chronic transplant rejection, which is likely to be mediated through various cytokines and chemokines released by T helper cells, as well as the effects of alloantibody generated by B cells stimulated via an indirect antigen presentation pathway.

1.2.3 Transplant Rejection

1.2.3.1 Hyperacute Rejection

Patients, who have had prior exposure to MHC antigens via previous transplant procedures, blood transfusions, or pregnancies, are at risk for developing antibodies reactive with alloantigens. When preexisting antibodies to blood groups, HLA, or other polymorphic antigens expressed on the graft are present in the recipient, they

can immediately bind to the graft and activate complement or arm cytolytic cells via antibody-dependent cellular cytotoxicity (ADCC) pathways. When the B-cell surface immunoglobulin receptor binds specific noncarbohydrate antigens in the context of soluble T helper cytokines, B cells are activated. CD4+ helper T-cell cytokines are responsible for the activation of B cells and thus indirectly for the majority of antibody production. B cells undergo differentiation, divide, and become plasma cells, which secrete soluble forms of the antigen-specific antibodies displayed on their cell surface. Plasma cells are long-lived and migrate to the bone marrow, where low levels of antibodies are secreted throughout the life of the plasma cell. Both IgM and IgG alloantibodies can be detected in the serum as well as in the graft of animals and humans undergoing allograft rejection. Preformed anti-HLA Class I antibodies, and occasionally anti-endothelial antibodies, play an important role in hyperacute rejection and accelerated vascular rejection seen in previously sensitized transplant recipients [9].

Events culminating in hyperacute rejection include binding of complement components, which themselves can cause direct damage through the membrane attack complex (MAC), and indirectly through chemokine properties of complement breakdown products, C3a and C5a, as well as deposition of platelets and fibrin, infiltration by granulocytes and monocytes, and fibrinoid necrosis of the vessel wall. This form of rejection manifests within minutes to hours after transplant, leading to graft failure as well as systemic manifestations such as disseminated intravascular coagulopathy. Fortunately, the incidence of hyperacute rejection has decreased significantly by employing routine HLA cross-matching screening, as well as avoiding ABO incompatilbility, prior to transplantation [10].

1.2.3.2 Acute Rejection

HLA differences activate a variety of events that result in acute cellular rejection and also set the stage for the development of chronic rejection. Recent advances in molecular and cellular immunology have further unraveled interactions between APC and T and B cells. These include elucidation of pathways involved in T-cell activation and apoptosis; identification of novel regulatory cells, including T-regulatory cells, B-regulatory cells, and suppressive APCs; as well as greater appreciation of the complex interactions between innate and adaptive immunity. Furthermore, elucidation of triggers of B-cell activation and antibody synthesis have allowed for the development of B-cell-specific immunosuppression.

Since T cells serve as the central hub in the cascade of alloimmunity, a brief overview of the current understanding of T-cell activation and proliferation is warranted. Optimal activation of naïve T cells requires coordinated signal transduction through three pathways: (1) nuclear factor-κB (NF-κB) pathway, (2) mitogen-activated protein (MAP) kinase-induced activator protein-1 (AP-1) activation, and (3) calcium-dependent calcineurin dephosphorylation of nuclear factor of activated T cells (NFAT) [11, 13]. Antigen-specific T cells interact with APC through the T-cell receptor (TCR)/MHC Class II molecule (signal 1) and CD28 costimulatory molecule/B7 (CD80 and CD86) molecules (signal 2) within the contact area, also

known as the "immunological synapse." Subsequently, phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) on the CD3 cytoplasmic tail results in downstream activation of protein kinase C (PKC) and MAP kinase with resultant activation of transcription factors, in particular NF-κB and AP-1 [12]. In addition, PKC appears to synergize with the serine/threonine phosphatase, calcineurin, to dephosphorylate NFAT to bind to a nuclear translocating protein. These cytoplasmic factors translocate to the nucleus, where they bind to their respective response elements, leading to gene transcription and synthesis of a variety of proteins, including interleukin-2 (IL-2), the α-chain of the IL-2 receptor (CD25), the CD40 ligand (CD154), interferon gamma, tumor necrosis factor α and β (TNF-α and TNF-β), and stem cell growth factors (e.g., granulocyte colony stimulatory factor) [12]. Secreted factors, in particular IL-2, bind in an autocrine or paracrine manner to their corresponding receptors on the T-cell surface to deliver signal 3 by activation of Janus kinase (JAK), in particular the JAK-3 isoform. This in turn leads to activation of other key downstream regulatory proteins, including FRAP, also known as mTOR ("mammalian target of rapamycin"). mTOR plays a key role in the signal transduction pathways downstream to many growth factor receptors (including the IL-2 receptor). This results in DNA synthesis and initiation of T-cell clonal proliferation as well as generation of effector T cells [14]. Other cytokines, such as IL-4, induce B-cell maturation and antibody synthesis, while other cytokines have pleomorphic effects, such as smooth muscle proliferation, and induce fibroblast proliferation, all hallmarks of chronic rejection [15–18]. Fortunately, the incidence of acute rejection is more frequent in the initial 6 weeks after engraftment and declines in incidence and severity after this period [19]. Nevertheless, for memory T cells, the need for costimulation is eliminated, and TCR-CD3/antigen-MHC engagement is all that is required for subsequent T-cell proliferation.

Once the antigen is consumed or removed, the process downregulates by virtue of several events. APC presents a negative costimulatory signal, CTLA4, which opposes the positive action of CD28. In addition, T-regulatory cells (Tregs), which bear CD25 and CTLA4, are generated. These cells inhibit T-cell proliferation in both animals and humans. Both CTLA4 and Tregs appear to induce activated T-cell apoptosis, also known as programmed cell death, a process of DNA fragmentation [20, 21].

1.2.3.3 Chronic Rejection

Progressive decline in allograft function, months or years after transplantation, is manifested by gradual vascular obliteration (a hallmark in all types of allografts), eventually leading to fibrosis and allograft dysfunction [22]. In addition, each organ system may have specific manifestations of chronic allograft rejection, such as "vanishing bile duct syndrome" in the livers, accelerated diffuse coronary artery allograft vasculopathy in hearts, bronchiolitis obliterans in the lungs, recurrent diabetes mellitus in the pancreas, and chronic allograft nephropathy in the kidneys. Immunological factors, such as episodes of acute rejections, degree of histoincompatibility, and level of pre-sensitization, as well as other non-immunological factors, such as: ischemia reperfusion injury, hyperlipidemia, immunosuppressive drug toxicity, and infection-related allograft inflammation, contribute to chronic allograft

dysfunction. Occurrences of episodes of acute rejection predispose to the development of chronic rejection in several studies [23]. Alternatively, indirect allorecognition may result in chronic rejection. Experimental evidence suggests that infiltration of host APC into the graft, which is depleted of APC, makes it susceptible to chronic rejection [24].

1.3 Immunosuppression

1.3.1 Introduction

Prior to the advent of immunosuppression, transplant recipients received total body irradiation to suppress the immune response, and all ended with poor outcomes. The first breakthrough in immunosuppression occurred when Dameshek and Schwartz reported the development of 6-mercaptopurine (6-MP) [25]. Calne reported on the successful use of 6-MP for renal transplants in dogs [26]. This was followed by the report that azathioprine (AZA), a derivative of 6-MP, was as effective and less toxic, facilitating its widespread use in 1962. Starzl combined AZA and steroids, which became the standard regimen for renal transplantation prior to the advent of T-cell-specific immunosuppression. The ability to minimize hyperacute rejection with the introduction of crossmatching resulted in improved early survival after kidney transplantation. Subsequently, the application of the more targeted immunosuppressive agents, cyclosporine and then tacrolimus, further reduced the risk of immunologic loss after transplantation and greatly contributed to making transplantation routinely successful.

Optimal immunosuppression as it relates to transplantation is defined as the level of drug therapy that achieves graft acceptance with the least suppression of systemic immunity. In so doing, the amount of systemic toxicity, namely, infection and malignancy, in addition to drug-specific side effects, is minimized, although not entirely eliminated [27]. Therapeutic drug monitoring and titration of immunosuppression is limited to only a few immunosuppressive agents, and, in practice, over- or under-immunosuppression almost invariably becomes apparent only in retrospect.

The use of immunosuppressive agents in transplantation can be categorized into three settings: (1) initial induction therapy with potent suppression of the immune response, (2) maintenance therapy to minimize the risk of acute and chronic rejection while maximizing immune competency and minimizing toxicity, and (3) reversal of acute and/or stabilization of chronic rejection episodes. A fine balance is required between the potency and the toxicity of these agents, in some cases requiring therapeutic drug monitoring. As mentioned earlier, occurrence of acute rejection episode(s) may predispose to chronic rejection, even though the acute rejection episode is treated adequately. Although this underscores the traditional dogma "prevention is better than cure," it is now appreciated that in order to achieve the Holy Grail of transplantation, namely, tolerance, some immune activation is necessary. Whether newer immunosuppressive agents are able to allow selective activation of pro-tolerant regulatory pathways remains to be seen in the clinical setting.

In order to better understand the use of immunosuppressive agents, it is helpful to categorize their mechanisms of action, such as antimetabolite, depleting, anti-inflammatory, inhibition of cytokine synthesis, and inhibition of growth factor proliferation. Most immunosuppressive regimens utilize combinations of these agents to obtain additive or synergistic effects of the various classes of agents while minimizing their toxicity. As the T-cell response is the hub of activation of other downstream effector mechanisms of alloimmunity, it is not surprising that most approved immunosuppressive agents are targeted to directly or indirectly control the T-cell response. Nevertheless, several drugs have been utilized in transplantation targeted to these downstream effector pathways, such as the alloantibody response and cytokine-driven myofibroblast proliferation.

1.3.1.1 Antimetabolites

Azathioprine (AZA)

AZA is a purine nucleoside analogue and an inactive prodrug. It is well absorbed after oral administration and is metabolized in the liver to the active drug 6-MP, which in turn is converted to active metabolite thioinosinic acid (TIMP). 6-MP is catabolized via the thiopurine methyltransferase and the xanthine oxidase pathways, with the final metabolites excreted in the urine. Hence, the main immunosuppressive activity of AZA depends on the metabolism to thioguanine nucleotides [28]. Thioguanine nucleotides are incorporated into and damage DNA and RNA, causing inhibition of transcription and arrest of cell proliferation [29]. TIMP inhibits the enzymes adenylosuccinate synthetase, adenylosuccinate lyase, and inosine monophosphate dehydrogenase, thus interfering with guanylic and adenylic acid synthesis from inosinic acid. TIMP is converted to thioguanylic acid, which is incorporated into DNA, thus interfering with DNA synthesis. AZA suppresses proliferation of activated B and T lymphocytes and also decreases the number of circulating monocytes by arresting bone marrow promyelocyte cell cycle.

AZA use has fallen with the availability of mycophenolic acid derivatives (see "mycophenolic acid") and is now largely used in treatment of autoimmune diseases. The major nonimmune toxic side effect of AZA is dose-limiting bone marrow suppression (BMS), leading to pancytopenia. Hence, monitoring of blood counts is used to guide dosing. The hematological side effects are generally reversible, when AZA dosage is reduced or when it is temporarily discontinued. As with other antiproliferative immunosuppressants, nausea, vomiting, and reversible hair loss may occur with AZA. AZA may also cause reversible cholestasis and infrequently results in severe veno-occlusive liver disease [30] and interstitial pneumonitis. It can be also associated with pancreatitis, assumed to be secondary to a hypersensitivity reaction [31]. It appears to be safer to use in pregnancy than other antiproliferative agents, as fetal cells lack the enzyme necessary to produce potentially toxic thioguanine nucleotides.

The interaction between AZA and allopurinol deserves special attention. Allopurinol inhibits xanthine oxidase, which is an important route of drug elimination for AZA – inhibiting the breakdown of AZA and its metabolites. This results in enhanced toxicity of AZA, thus necessitating dramatic dose reduction. Severe and

prolonged neutropenia with sepsis has been reported in patients treated concomitantly with both drugs [32]. A safer alternative for patients requiring allopurinol is to substitute AZA with mycophenolate derivatives.

Mycophenolic Acid

Mycophenolate mofetil (MMF) is an immunosuppressant introduced for use in transplantation [33–39]. MMF is a prodrug form of mycophenolic acid (MPA) and was developed as a replacement for AZA and currently is a cornerstone in a number of maintenance regimens. After absorption, it is rapidly converted to its active metabolite MPA via hydrolysis. MPA is a reversible noncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), which is a key enzyme in the de novo synthesis of guanosine monophosphate (purine synthesis pathway). This inhibition of IMPDH causes a deficiency of guanosine and deoxyguanosine nucleotides (preventing DNA and RNA synthesis) and a relative excess of adenosine nucleotides (inhibits 5-phosphoribosyl-1-pyrophosphate synthase, which further halts the purine synthesis). Hence, MPA selectively inhibits lymphocyte proliferation as lymphocytes are relatively dependent on the de novo purine synthesis pathway. In vitro, MPA suppresses antibody formation, inhibits cytotoxic T-cell production, and reduces the expression of certain adhesion molecules.

MPA is 90 % bound to plasma proteins. The main pathway of elimination is by hepatic glucuronidation and excretion in both the stool and urine. Of note is that the biliary excreted form of MPA glucuronide metabolites can undergo bacterial breakdown and reabsorption, also known as the enterohepatic circulation where inactive MPA is converted back to active MPA by glucuronidases from gut flora.

The results from various trials related to renal transplant, including multicenter double-blinded placebo-controlled trials, have shown that MMF-treated patients have a significant decrease in the incidence of acute rejection when compared to patients treated with placebo or AZA without an increase in the adverse events [35].

MMF is usually well tolerated. The major side effects are related to gastrointestinal symptoms such as nausea, vomiting, and diarrhea, which subside with a decrease in the dose of MMF. The gastrointestinal symptoms are believed to be secondary to the dependency of the gastrointestinal tract epithelial cells on IMPDH. MMF does not cause nephrotoxicity, hepatotoxicity, or significant bone marrow suppression, but patients treated with MMF may be more likely to develop invasive CMV disease [38]. In addition, MPA/MMF use during pregnancy has been associated with microtia and facial dysmorphic features in the offspring [39] and recommendations include discontinuing MMF/MPA at least 6 weeks prior to becoming pregnant.

1.3.1.2 Anti-inflammatory

Corticosteroids

Synthetic glucocorticoids have been used for all phases of transplant immunosuppression including induction and maintenance immunosuppression, as well as for treatment of acute rejection episodes. Prednisone, prednisolone, methylprednisolone,

and hydrocortisone are the main compounds used in transplantation. Oral absorption of glucocorticoids is otherwise very high, and they are 90 % bound to plasma proteins. Hepatic p450 enzymes metabolize the glucocorticoids, but rarely do inducers or inhibitors of the p450 enzymes require a dose adjustment of glucocorticoids.

Corticosteroids possess both immunosuppressive and anti-inflammatory properties [40]. The pharmacologic effects include suppression of macrophage function, prevention on T lymphocyte proliferation, inhibition of cytokine production (in particular IL-1), reduction in adhesion molecule expression, induction of lymphocyte apoptosis, alteration of leukocyte trafficking, inhibition of leukocyte transmigration through blood vessels, and reduction of MHC expression. Other effects also include suppression of prostaglandin synthesis, decreasing capillary permeability, inhibiting histamine and bradykinin release, as well as reducing the absolute number of neutrophils and eosinophils. Corticosteroids bind to the intracytoplasmic receptors within target cells to form an active corticosteroid receptor complex (CRC), which binds to the DNA in the nucleus at the corticosteroid response element (CRE) of the promoters of target genes. The CRC and the CRE interaction results in induction or suppression of the transcription of the target genes. It activates the gene that inhibits the activity of NF-kB (an important transcriptional activator for many proinflammatory cytokines) [41].

The administration of corticosteroids is typically recommended as a single morning dose to resemble the standard physiologic rhythm of the pituitary-adrenal axis. High-dose intravenous methylprednisolone is the standard therapy for acute rejection (250–500 mg/day for 3 days) and effectively reverses 85–90 % of acute rejection episodes. Oral prednisone in equivalent doses can also be used with comparable results.

Frequent side effects of corticosteroids include hypertension, hyperlipidemia, diabetes mellitus, peptic ulceration, poor wound healing (impaired fibroblast growth and collagen synthesis), proximal muscle weakness, osteoporosis, and growth retardation in children (suppression of pituitary-adrenal axis). Less common side effects include pancreatitis, psychosis, posterior subcapsular cataract, and avascular necrosis of the femoral head. The classic "Cushingoid" features are secondary to soft tissue and dermatologic changes (fat redistribution, skin atrophy, striae, and acne). Acute adrenal insufficiency can develop, even up to 12 months after cessation of steroids, when the patient is stressed. Hence, corticosteroids used in transplantation carry considerable potential for morbidity [40, 42]. Protocols that minimize or avoid the use of glucocorticoids have been advocated in a variety of solid organ transplant trials, but given the higher rates of rejection seen, this practice often requires the use of other adjuvants or induction immunosuppression.

1.3.1.3 Inhibition of Cytokine Synthesis

Cyclosporine (CsA)

CsA is a natural lipid-soluble cyclic 11-amino-acid peptide isolated from the fungus *Tolypocladium inflatum*. It is insoluble in water and has a variable oral bioavailability of approximately 30 % [43]. Complex preparations are essential to ensure absorption in the oral formulation due to its insolubility. The original oral formulation

(Sandimmune™) was bile dependent, and its incomplete emulsification yielded marked interindividual variations in bioavailability. The microemulsion formulation has more predictable pharmacokinetics, i.e., better absorbed, bile independent, and provides more consistent blood levels. The relative bioavailability is increased between 74 and 139 % [44], and the total area under the concentration-time curve (AUC) is increased by 30 % [45], when compared to the original conventional preparation. Once absorbed, it is extensively bound to red blood cells and plasma proteins, with only 5 % of the drug free in plasma. It is eliminated mainly via hepatic metabolism, and the drug metabolism is via cytochrome P450 IIIA enzymes [46].

CsA functions to prevent antigen-specific T-cell activation, and this immunosuppressive effect is well established [28, 47]. CsA acts by binding to cyclophilin, which is a cytoplasmic protein that belongs to the immunophilin family. The CsA-cyclophilin complex inhibits calcineurin (CN), which is a calcium-dependent serine phosphatase, preventing the activation of several CN-dependent transcription factors, including NFAT (nuclear factor of activated T cells). This results in the inhibition of the expression of T-cell cytokines such as IL-2, IL-3, IL-4, IFN-γ, TNF-α, and GM-CSF, exerting an effective immunosuppressive effect. On the other hand, the expression of TGFβ is promoted in the presence of CsA, which inhibits T-cell activation, but may promote renal fibrosis, a result of long-term CsA therapy [48].

Nephrotoxicity is the most important nonimmune side effect of CsA and is mediated by afferent arteriolar vasoconstriction, believed to be caused by CN inhibition [49–51]. Characteristic biopsy findings in advanced chronic nephrotoxicity are "striped" interstitial fibrosis and arteriolar hyalinization. CsA levels are usually high in acute nephrotoxicity, but not necessarily so in chronic nephrotoxicity. CsA is diabetogenic, although simultaneous use of corticosteroids muddles the cause of diabetes. Other side effects include neurotoxicity, hypertension [51], hyperuricemia, hyperkalemia, hyperlipidemia, hypertrichosis, gingival hypertrophy, coarsening of facial features [52], and transient hepatotoxicity. Although CsA trough monitoring is not a good predictor of its immunosuppressive effect, it is a common practice to measure blood levels and make dose adjustments because of its narrow therapeutic window.

Tacrolimus (FK506)

Perhaps the most studied and most commonly utilized immunosuppressive drug is tacrolimus (FK). FK is a macrolide antibiotic isolated from the bacterium *Streptomyces tsukubaenis*. FK is superior to CsA in terms of immunosuppressive efficacy, as validated by several clinical trials [53–64], and the relative resistance of FK to p-glycoprotein countertransport, which decreases the intracellular drug levels, may be contributing to its enhanced efficacy. Patients treated with FK have less frequent and less severe rejection episodes compared to CsA-based immunosuppressive protocols.

FK binds to an immunophilin, FK-binding protein (FKBP), in the cell cytoplasm. FK-FKBP complex inhibits CN activity, similar to the mechanism of CsA. FK has additional immunosuppressive effects in vitro, independent of NFAT inhibition [28, 47]. FK is much more potent than CsA on a mg-to-mg comparison. FK is also

variably absorbed, and thus therapeutic drug monitoring is essential – to achieve efficacy while minimizing the risk of toxicity [66–68]. FK has a half-life of 12 h; hence, it is administered 12 h apart (two daily doses) – although rarely indicated, FK can also be given intravenously at 30 % of the oral dose. Goal levels vary by organ type, duration posttransplant, and other factors. Recently, a once daily formulation has been investigated with promising results, particularly related to compliance and quality of life measures [69].

The degree of nephrotoxicity caused by FK appears to be equivalent to CsA [70–72]. FK is associated with neurotoxicity [72] and is also diabetogenic [62, 63] but is not associated with hypertension, gingival hypertrophy, hypertrichosis, or hypercholesterolemia. Gastrointestinal side effects such as diarrhea and anorexia are comparable to those seen with other macrolide antibiotics, like erythromycin.

1.3.1.4 Depleting Antibodies

OKT3

OKT3 is a murine monoclonal antibody directed against 20,000 Da CD3 complex of molecules on the surface of thymocytes or mature human T cells [73]. As CD3 associates with the T-cell receptor (TCR), essential for antigen recognition and function [74–76], the immunosuppressive effects of OKT3 are in part mediated by modulation or removal of CD3/TCR complex from the T-cell surface via shedding or endocytosis, thus rendering the T cells dysfunctional and immunologically incompetent [77–81].

OKT3 was the first monoclonal antibody to be used in mainstream clinical medicine. In transplantation, OKT3 was shown to be very effective for the treatment of severe acute rejection and was also widely used for induction immunosuppression. Concerns of early lymphoproliferative disease in OKT3-treated patients limited its prophylactic use, and as other antilymphocyte antibody preparations became available, OKT3 was subsequently phased out of production. Nevertheless, many of the lessons learned with the use of OKT3 apply to these newer agents and are worth discussing.

The most common side effect of OKT3 is the cytokine-release syndrome (CRS), which typically began 45–60 min after the initial dose and lasted up to several hours. It resulted in fever, chills, myalgia, weakness, and gastrointestinal (nausea, vomiting, diarrhea) and pulmonary (bronchial spasms) symptoms. This symptom complex was attributable to a variety of mediators, including cytokines, released from T cells activated by the binding of OKT3 to the CD3-TCR complex [84]. Premedication with steroids, antihistamine, and antipyretics, especially indomethacin, generally reduced the severity of the first dose effect. Significant suppression of the cell-mediated immunity predisposes to opportunistic infections and malignancies. Neurological adverse effects include headache, convulsion, and aseptic meningitis. Lastly, the use of OKT3 was associated with a high incidence of the development of host antibodies to the murine immunoglobulin, both to the idiotype and structural epitopes [82, 83]; thus, it is generally advisable to measure the human anti-mouse antibody (HAMA) titers in patients prior to retreatment with a second course of any murine monoclonal antibody.

Antithymocyte Globulin (ALG)

Antithymocyte globulin (ATG) is a member of a class of agents of polyclonal antibodies directed against immunocytes. While ATG's mechanism of action is incompletely understood, ATG has been shown to cause T-cell depletion by inducing apoptosis as well as complement- and antibody-mediated pathways [84]. The current FDA-approved ATG formulation (Thymoglobulin) is a polyclonal rabbit anti-human thymocyte globulin (RATG) obtained by immunization of rabbits with human thymocytes. It was approved by the FDA in 1999 for the treatment of acute renal graft rejection in conjunction with concomitant immunosuppression. ATG contains cytotoxic antibodies directed against a variety of antigens expressed on human T lymphocytes. ATG includes antibodies against T-cell markers such as CD2, CD3, CD4, CD8, CD11a, CD18, CD25, CD44, CD45, HLA-DR, HLA Class I heavy chains, and ß2-microglobulin.

Currently ATG remains widely used in a variety of settings including induction regiment and patients with concern for renal insufficiency, (in order to minimize early exposure to calcineurin inhibitors), and in cases of acute rejection [85], ATG is associated with a variety of side effects in both the acute and delayed settings. In the acute setting ATG can be associated with a cytokine-release syndrome characterized by fevers, chills, shortness of breath, tachycardia, hypotension, and, in the extreme setting, cardiovascular collapse. Additionally, cross-reactivity of the serum can result in pancytopenia and thrombosis [86].

1.3.1.5 Anti-growth Factor-Induced Proliferation

Anti-IL-2 Receptor Alpha-Chain Antibody: Basiliximab

Basiliximab (Simulect, Novartis Pharma) is a chimeric monoclonal antibody developed to target the alpha chain of the IL-2 receptor (CD25). As mentioned earlier, IL-2 plays an important role in expansion of T cells as well as T-cell cytokine production. Basiliximab has been used as induction therapy in solid organ transplant as well as in the treatment of episodes of acute rejection. One study comparing basiliximab to ATG induction therapy in renal transplant recipients at high risk of delayed graft function or acute cellular rejection observed no difference in overall or graft survival at 1 year, while there were significantly fewer episodes of acute rejection [87]. These findings were found to be similar on 5-year follow-up [88]. In liver transplant recipients, basiliximab has been shown to be efficacious as well as to decrease rates of rejection, to improve 2-year graft, and to improve overall survival compared to regiments without induction therapy [89]. In liver recipients, basiliximab is most often used in patients with renal dysfunction at time of transplant in order to delay CNI initiation [90]. In the pediatric transplant population, basiliximab remains the most often utilized induction agent. The drug is typically dosed at 20 mg dose for adults and pediatric patients over 35 kg and at 10 mg/dose for those <35 kg [91]. A mechanistically similar humanized monoclonal antibody against CD-25, daclizumab (Roche), has been used and studied with similar effects including decreased incidence of acute rejection and as an induction agent to allow delayed CNI initiation for patients with some renal insufficiency. It has been removed for marketing reasons and is no longer available in the USA or Europe [92].

1.3.1.6 Mammalian Target of Rapamycin (mTOR) Inhibitors

Rapamycin (Sirolimus, RAPA)

Rapamycin is a macrolide antibiotic structurally related to tacrolimus. It binds to FKBP-12 but does not inhibit cytokine gene transcription in T cells. It blocks signals transduced from the IL-2 receptor and other growth factors to the nucleus by acting on phosphatidylinositol kinases, also known as "mammalian target of rapamycin" (mTOR). It also inactivates p70S6 kinase resulting in selective inhibition of the synthesis of new ribosomal proteins and prolonging cell cycle progression from G1 to G2 [93]. Thus, the mechanism of action differs significantly from either Tac or CsA in that RAPA inhibits both B- and T-cell responses to alloantigen [94].

RAPA has a poor bioavailability after oral administration and the dosing frequency is once daily. Therapeutic monitoring of sirolimus should be based on whole blood concentrations because of the high sequestration of sirolimus by erythrocytes [95]. The adverse effect profile of sirolimus is unique compared to other immunosuppressants. Unlike cyclosporine and tacrolimus, nephrotoxicity and neurotoxicity are rarely seen with sirolimus. The side effects with rapamycin include GI disturbances, diabetes mellitus, myocardial necrosis, and testicular atrophy. Because RAPA acts to suppress growth factor-driven proliferation, dose-dependent myelosuppression can be seen following initiation of sirolimus therapy in particular at higher drug concentrations [96, 97]. In addition, fibroblast proliferation is suppressed, leading to a higher rate of wound complications, such as lymphocele formation, wound disruption, and hernia formation [98, 99]. Hyperlipidemia is commonly seen in patients receiving sirolimus, manifesting as hypercholesterolemia and hypertriglyceridemia. This effect has been reported in virtually all clinical trials.

1.3.1.7 SDZ-Rapamycin (Everolimus, EVR)

Everolimus (EVR) is an analogue of rapamycin, acting in a similar fashion to sirolimus (Fig. 1.1). It differs from sirolimus in several pharmacologic aspects (Table 1.1), which alters dosing frequency (usually twice-a-day dosing) and use of concomitant dosing with other immunosuppressive medications.

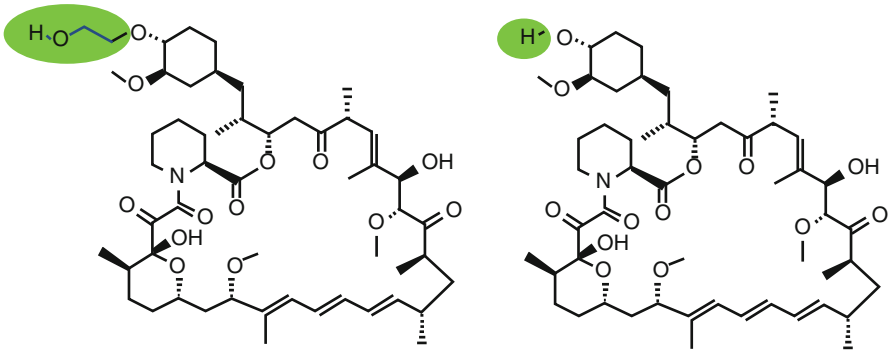

Fig. 1.1 Structure of mTOR inhibitors, everolimus (*left*), and sirolimus (*right*). Everolimus differs from sirolimus due to the addition of a 2-hydroxyethyl group at C_{40}

Table 1.1 mTOR inhibitors – key clinical differences

	Sirolimus	Everolimus
Oral bioavailability	14 %	20 %
Time to T_{max}	1–2 h	1–2 h
Half-life	62 h	28 h
Loading dose	6.0 mg	No
Time to steady state	5–7 days	4 days
Plasma protein binding	92 %	74 %
Dosing interval	Once daily	Twice daily
Target trough levels	4–12 ng/mL	3–8 ng/mL
Concomitant dosing with CsA	4 h post-CsA dose	Yes

During the initial trials in de novo renal transplant patients, EVR was utilized with full-dose CsA but was quickly adjusted to utilize low-dose CsA due to increased nephrotoxicity. In the ZEUS trial, an open-label, randomized control study evaluating the use of EVR with CNI withdrawal for renal transplant recipients, patients received usual dosing of mycophenolate sodium, corticosteroids, and cyclosporine for the first 4.5 months at which point the study patients were randomized to either continued cyclosporine-based therapy or conversion to EVR with withdrawal of CsA. The EVR group was shown to have a significantly improved eGFR compared to the cyclosporine group (71.8 vs 61.9 mL/min per 1.73 m^2). Twelve-month BPAR was similar between the groups; however, there was an increased rate of BPAR during the period of CNI withdrawal compared to continued CNI group. Graft loss and death were similar among the groups at 12 months post-kidney transplant [100].

A phase 3 multicenter, randomized, controlled study to evaluate the efficacy and safety of concentration-controlled EVR to minimize and/or eliminate tacrolimus in de novo LTX recipients recently completed enrollment with 2-year follow-up. A total of 719 adult LTX recipients were initially treated with tacrolimus and steroids with/without MMF during a 30-day immediate post-LTX period. At that point, screened patients were then randomized into one of three treatment arms (everolimus with tacrolimus elimination, low-dose everolimus with low-dose tacrolimus, or standard tacrolimus). The primary objective was modified to a standard efficacy failure (biopsy-proven acute rejection, graft loss, death) with a key secondary endpoint to demonstrate superior renal function in the everolimus treatment groups, compared to tacrolimus control at month 24. In this study, there was a significantly increased incidence of rejection in the group of patients where tacrolimus was eliminated. The incidence occurred during the weaning of tacrolimus. Although patient survival and graft survival were not affected, there was an early increase in BPAR in groups that had tacrolimus elimination. At the end of 24 months, there was no difference in the primary endpoint of efficacy failure; however, the incidence of biopsy-proven acute rejection was statistically lower in the low-dose everolimus/tacrolimus group than the control group. Of great interest was the finding that the eGFR was significantly higher in the low-dose everolimus/tacrolimus group

compared to the control group [101]. This study has been extended to evaluate 36- and 48-month outcomes with no further increased incidence of rejection with continued improvement in eGFR of the low-dose everolimus/tacrolimus group in early reports [102].

1.3.1.8 New Immunosuppressive Agents

Novel therapeutic strategies are currently being employed either clinically or are undergoing clinical testing in solid organ transplantation and may have promise as new or adjunctive immunosuppressive agents. They can be categorized into:

1. Inhibitors of signal 1: TCR blockade
2. Inhibitors of signal 2: costimulatory blockade
3. Inhibitors of signal 3: inhibition of growth factor-driven proliferation

1.3.1.9 Signal 1 Inhibition

TOL101

TOL101 (T10B9, MEDI-500) is a murine IgM k chain mAb directed against the alpha and beta subunits of the TCR and appears to lead to internalization of the TCR rather than T-cell depletion. The predecessor antibodies for TOL101, T10B9, and MEDI-500 have been administered to approximately 135 patients across 13 studies from 1986 to 2000 – over 100 of these patients were recipients of solid organ transplants. The largest of these studies was a 76-patient phase 2 trial investigating T10B9 vs. OKT3 (at that time, considered standard of care) for the treatment of acute renal transplant rejection. Graft survival and subject survival were high (>80 %) over 4 years and similar between the two treatment groups. The incidence and severity of adverse events (including fever, respiratory symptoms, gastrointestinal complaints, and neurological symptoms) were substantially higher in the OKT3 group than in the T10B9 recipients [103]. TOL101 is currently in a phase 1/2 study as part of an immunosuppressive regimen that includes tacrolimus, MMF, and steroids in patients undergoing primary kidney transplantation. Because of the large size of this molecule, the pharmacokinetic profile of this agent may be more favorable (due to longer intravascular retention) in LTX patients, where pharmacokinetics demonstrated higher clearance of IgG preparations.

Sotrastaurin

Sotrastaurin (AEB071) is a novel immunosuppressant that blocks early T-cell activation via inhibition of PKC, integrating in particular signaling pathways downstream of the T-cell receptor (TCR) and the CD28 co-receptor. Sotrastaurin has been shown to specifically inhibit early T-cell activation through signals 1 and 2 but not T-cell proliferation (signal 3) by selectively blocking the calcineurin-independent pathway signaling through NF-kB resulting in inhibition of cytokine gene transcription. AEB071 is being developed for the prevention of acute rejection in solid organ allotransplantation in combination with or without a calcineurin inhibitor (CNI). In

contrast to CNIs, AEB071 potently and selectively blocks a calcineurin-independent pathway pointing toward a clear differentiation in mode of action and possibly the side effect profile between AEB071 and CNIs.

Thus far, sotrastaurin has been used in two phase 2 de novo renal transplant trials. In one study, recipients were randomized to sotrastaurin (200 mg b.i.d.) + standard-exposure tacrolimus (SET) or reduced-exposure tacrolimus (RET) (SET: $n=76$; RET: $n=66$) or control (SET + MPA, 720 mg b.i.d.; $n=74$) [104]. In both sotrastaurin groups, patients were converted from tacrolimus to MPA after month 3, achieving CNI-free immunosuppression. The primary endpoint was composite efficacy failure (treated biopsy-proven acute rejection, graft loss, death, or loss to follow-up), while the key secondary endpoint was GFR. Composite efficacy failure rates were 4.1, 5.4, and 1.5 % at month 3 (pre-conversion) and 7.8, 44.8, and 34.1 % at study end in the control, sotrastaurin + SET, and sotrastaurin + RET groups, respectively. In addition, the median GFR at month 6 was 57.0, 53.0, and 60.0 mL/min/1.73 m^2, respectively. Based on the primary endpoint, the Data Safety Monitoring Board (DSMB) recommended premature study discontinuation. Although the initial sotrastaurin + tacrolimus regimen was efficacious and well tolerated and the postconversion sotrastaurin + MPA regimen showed inadequate efficacy, longer-term evaluation of sotrastaurin + tacrolimus appears warranted.

In another study, de novo renal transplant recipients with immediate graft function were randomized 1:2 to tacrolimus (control, $n=44$) or sotrastaurin (300 mg b.i.d.; $n=81$) [5]. All patients received anti-IL2-RA, MPA, and steroids. The endpoints were similar to that noted in the previous trial. In this trial, the composite efficacy failure at month 3 was higher for the sotrastaurin versus control regimen (25.7 % vs. 4.5 %, $p=0.001$) with rejection rates higher in the sotrastaurin group compared to control, 23.6 % vs. 4.5 %, respectively ($p=0.003$), which led to early study termination by the DSMB. Of great interest was the finding that the median estimated GFR was higher for sotrastaurin versus control at month 3: 59.0 vs. 49.5 mL/min/1.73 m^2 ($p=0.006$) [105]. Further follow-up studies have been recently completed showing reasonable efficacy but decreased tolerability compared to current standard regiments [106].

1.3.1.10 Signal 2 Inhibition

Abatacept (CTLA4-Ig)/Belatacept (LEA29Y)

Abatacept is a chimeric fusion protein that consists of the extracellular domain of CTLA-4, and the Fc domain of IgG blocks the B7 (CD80, CD86)/CD28 pathway. This agent is approved for use in moderate to severe psoriasis. Belatacept is a molecular mutation of abatacept, differing from abatacept in two amino acid positions in the binding domain to B7, associated with a higher binding avidity and slower dissociation rate, with resultant inhibition of T-cell activation greater than that of abatacept. With both molecules, blockade of the B7/CD28 interaction leads to inhibition of T-cell proliferation. Belatacept was investigated in a phase 2 de novo kidney transplant trial with a CsA regimen as control. This trial consisted of belatacept injections every 2 weeks for 1 year. There was an improvement in the GFR in

the belatacept-treated group compared to the CsA group, but there was no difference in biopsy-proven acute rejection [107]. In follow-up pivotal phase 3 trials, belatacept demonstrated 1-year subject and graft survival that was comparable to CsA, with improved renal function and less metabolic complications such as incidence of new-onset diabetes mellitus, blood pressure, and abnormal lipid profile [108]. An increased risk of posttransplant lymphoproliferative disorder (PTLD), particularly among Epstein-Barr virus-negative recipients, was a notable adverse event. However, belatacept was associated with an increased frequency of early acute rejection compared with CsA. With longer-term follow-up, the impact of these rejections appeared to be limited. Extended follow-up over 3 years demonstrated evidence of ongoing efficacy, which did not differ between the two belatacept dose regimens (LI and MI) evaluated, and in particular, GFR was better preserved in both belatacept groups, even in those that experienced an acute rejection, compared to CsA-treated recipients. In addition, the incidence of chronic allograft nephropathy was also significantly lower in the belatacept-treated patients [109, 110]. Based on this data, belatacept was recently approved by the FDA for kidney transplantation. The additional long-term benefits that accrue to patients on belatacept relating to improvements in metabolic parameters have been fully assessed.

Belatacept was next investigated in a phase 2b multicenter prospective partially blind clinical trial in LTX [111]. Five treatment groups were utilized: Group 1, anti-IL2RA + belatacept more intensive (MI) + MMF; Group 2, belatacept (MI) + MMF; Group 3, belatacept less intensive (LI) + MMF; Group 4, tacrolimus + MMF; and Group 5, tacrolimus. The primary objective was to evaluate the effects of belatacept relative to TAC on the triple composite endpoint of the incidence of acute rejection (AR), death, and graft loss by 6 months after receiving a deceased donor transplant. An imbalance in deaths in the belatacept treatment arms relative to the tacrolimus + MMF arm was noted. The frequencies of death were noted as 12, 21, and 22 % in the anti-IL2RA + belatacept MI + MMF, belatacept MI + MMF, and belatacept LI + MMF arms, respectively, in comparison to 6 % in the TAC + MMF arm and 14 % in the TAC arm. Of note was the marked difference in GFR in the belatacept groups compared to the control groups. There were two reports (one fatal) of PTLD and one report of fatal progressive multifocal leukoencephalopathy (PML). In addition there was an increase in viral and fungal infections in the combined belatacept groups versus the tacrolimus groups, potentially due to the degree of immunosuppression.

Future trials with belatacept may possibly include a short period of CNI exposure in the perioperative period, and these considerations are ongoing at this time.

Efalizumab

Efalizumab is a humanized IgG1 mAb directed against the leukocyte function-associated antigen-1 (LFA-1, CD11a). CD11a plays an important role in adhesion of leukocytes to endothelial cells and also serves as a costimulatory molecule. Approved for treatment of moderate to severe psoriasis, a pilot study was performed in 38 primary kidney transplant recipients [11]. Patients were randomized to receive efalizumab 0.5 or 2 mg/kg weekly subcutaneously for 12 weeks. Patients were maintained on full-dose CsA, MMF, and steroids ($n = 10$ 0.5 mg/kg efalizumab,

$n=10$ 2.0 mg/kg efalizumab) or half-dose CsA, sirolimus, and prednisone ($n=9$ 0.5 mg/kg efalizumab, $n=9$ 2.0 mg/kg efalizumab). At 6 months following transplant, patient survival was 97 % and graft survival was 95 %. Clinical biopsy-proven acute rejection in the first 6 months after transplantation was confirmed in one of each of the immunosuppressive combination (e.g., 4/38, 11 %). Three patients (8 %) developed PTLD, all in the highest dose efalizumab with full-dose CsA [112]. Although this drug appeared promising, subsequent reports of the development of PML in patients treated for extended periods with efalizumab for psoriasis resulted in withdrawal of this agent from the market [113]. Nevertheless, interference of this pathway is seemingly a novel approach for future trials.

Alefacept

Alefacept is a lymphocyte function-associated molecule 3/immunoglobulin G (LFA3-IgG1) fusion receptor protein, which functions by interfering with the CD2 receptor on T cells, causing apoptosis of effector memory T cells. By blocking LFA-3/CD2 interactions, alefacept can inhibit T-cell activation and proliferation. It has been approved for moderate to severe chronic plaque psoriasis and has no known nephrotoxicity. In addition, it has been used for the treatment of graft-versus-host disease in bone marrow transplantation [114]. In transplantation, a phase 2, multicenter, randomized, double-blind, placebo-controlled study in primary adult kidney transplant patients comparing alefacept, tacrolimus, and MMF to placebo, tacrolimus, and MMF was conducted. The primary endpoint was an incidence of biopsy-proven acute rejection at 6 months. No statistical differences between treatment arms were observed for the primary endpoint, patient or graft survival, as well as renal function. Alefacept was associated with statistically significant reduction in T memory lymphocyte subsets. Given that alefacept appears to react with a different population of T cells, i.e., effector memory T cells, rather than naïve T cells, it would seem that the results of this study are not surprising. There was an increased rate of malignancy in the alefacept group [115]. Alefacept may be better in models where memory T cells have a pathophysiologic role, e.g., sensitized or retransplant patients, GVHD after LTX. In fact, alefacept has been used successfully in such a case [116].

1.3.1.11 Signal 3 Inhibition

Tasocitinib

Tasocitinib (CP-690,550, tofacitinib) is an orally active immunosuppressant currently being tested for a variety of immune-mediated disorders, including prevention of transplant rejection. Tasocitinib specifically inhibits Janus-activated kinase 3 (JAK3), which is a hematopoietic cell-restricted tyrosine kinase involved in cytokine signal transduction associated with lymphocyte proliferation, specifically interfering with IL-2-mediated STAT5 activation in CD4+ T cells. A randomized pilot study compared two dosages of tasocitinib (15 mg BID and 30 mg BID, $n=20$ each) with tacrolimus ($n=21$) in de novo kidney allograft recipients [117]. Patients received anti-IL-2RA, MMF, and corticosteroids. The 6-month biopsy-proven acute

rejection rates were 5, 20, and 4.8 % for low- and high-dose tasocitinib and tacrolimus groups, respectively. The infectious complications were most frequent in the high-dose tasocitinib/MMF group with BK virus infection developing in 20 % and cytomegalovirus infection in 20 % of patients. Other side effects of tasocitinib included hyperlipidemia, anemia, and neutropenia. A larger study of tasocitinib in primary kidney transplantation utilized 15 mg BID for either 3 or 6 months followed by 10 mg BID thereafter and compared to a CsA control group – all groups were induced with anti-IL-2RA and given maintenance MPA and corticosteroids [118]. Of the 109 CsA patients, the ACR rate at 12 months was 18.8 % compared to longer high-dose tasocitinib ($n=106$) at 17.4 % and shorter high-dose tasocitinib ($n=107$) at 15.4 %. The finding that tasocitinib preserves regulatory T-cell function may be particularly important in LTX, as this may help to explain the immunologically privilege of LTX related to lack of chronic rejection and possible tolerance.

Anti-CD40 Ligand or Anti-CD40 Antibody

The CD40 molecule is expressed on antigen-presenting cells and serves as a costimulatory molecule through its interaction with CD154. Activation of CD40/CD154 has been shown to promote T-cell activation, B-cell proliferation and class switching, macrophage function, and a variety of other immunological processes. Page et al. have demonstrated that CD40- or CD40L-specific mAb could prevent and even reverse acute allograft rejection leading to prolongation of MHC-mismatched renal allografts in primates without the need of chronic maintenance immunosuppression [119]. Early studies with humanized anti-CD154 mAb were hampered by unexpected thromboembolic complications [120]. Further studies suggested that this was a function of the effects on integrin-binding sites on CD154 which are believed to aid in arterial plaque stabilization [121]. More recently, fully human anti-CD40 monoclonal antibodies, 4D11/ASKP1240, have been tested in a primate model with marked suppression of T-cell responses and prolongation of kidney allograft survival [122]. ASKP 1240 has recently undergone phase 1 evaluation, and currently phase 2a study is underway to assess the utility of ASKP1240 in MMF and CNI avoidant regiments (Basilixumab induction+ASKP1240+steroids+MMF vs Basilixumab+steroids+MMF+tacrolimus vs Basilixumab+ASKP1240+steroids +Tacrolimus) [123].

1.3.1.12 Chimerism

Tolerance

"Immunological tolerance," the state whereby the immune system fails to respond to a stimulus that would normally elicit an immunological response, is one of the "Holy Grails" of clinical transplantation. The ability to induce tolerance would obviate the need for maintenance immunosuppression and its long-term risks and associated complications as well as mitigate allograft rejection. The field of transplant tolerance was born in 1953 with the landmark report of Billingham, Brent, and Medawar where exposure during the fetal life of mice and chickens to homologous antigens leads to immunological tolerance. This was manifested by a lack of

response to skin grafting from the organism that was used in the inoculation process [124]. Subsequently, this neonatal tolerance has been demonstrated to be mediated through negative selection via mechanism(s) of thymic deletion of reactive T cells, also referred to as "central tolerance" [125]. However, similar approaches in adult recipients could not reproduce acceptance of donor tissues, unless the recipient had been cytoablated, in this early period, accomplished through lethal irradiation and reconstitution with donor bone marrow (radiation-induced chimerism) [126]. Chimerism refers to the development of an immune constitution that is comprised of cells of both donor and recipient lineages. In contrast to neonatally tolerant animals, the mechanism(s) of tolerance in these adult recipients involves not only clonal deletion but also active suppression [127].

Over the next 50 years, attempts were made in various animal models to induce tolerance with varying degrees of success, including early attempts of whole body and total lymphoid irradiation, shown to be necessary for the induction of immunological tolerance in bone marrow transplant patients. However, the development of potent immunosuppressive agents became the pathway to successful clinical solid organ transplantation. Because neither bone marrow nor any other kind of donor hematolymphopoietic cells were given adjunctively in solid organ transplantation, the enigmatic mechanisms of organ engraftment were assumed to be independent of leukocyte chimerism. However, there were clues that organ engraftment was a state of variable tolerance that in some cases became immunosuppression independent. Tolerance was inferred from a rapidly declining need for maintenance immunosuppression following the successful treatment of rejection. In addition, Starzl and coworkers demonstrated that long surviving allografts could be weaned from immunosuppression in a significant proportion of kidney and liver transplant recipients [128]. The finding that low-level multilineage donor leukocyte chimerism (microchimerism) was found in all tolerant patients and in one or more locations that included the skin, lymph nodes, heart, lungs, spleen, intestine, kidneys, bone marrow, and thymus emphasizes the importance of antigen migration and tolerance, as advocated by Starzl and Zinkernagel [129]. At any given site, the donor leukocytes were present in larger numbers in liver recipients than in kidney recipients studied at comparable posttransplant times. With the persistence of donor cells for as long as 30 years, it was inferred that the passenger leukocyte population of organ grafts was critical in establishing clinically operational tolerance. The migration of donor antigens, either as living cells or by shed alloantigens from the allograft, initiates a recipient immune response via direct or indirect antigen presentation pathways, respectively. Effector mechanisms include generation of cytotoxic T cells (CD8+) as well as downstream alloantibody as a result of CD4+ cytokines. As noted before, T-cell activation is essential for subsequent tolerance generation [130], and the use of potent immunosuppression is likely to delay or prevent the deletion of CD8+ effector T cells and the regulation of CD4+ helper T cells, likely mediated through apoptotic inducing clonal exhaustion as well as other peripheral tolerogenic pathways mediated through active suppressive regulation, such as T-regulatory cells (Treg) or myeloid-derived suppressor cells (MDSC). The liver allograft is naturally endowed with high levels of hepatic

stellate cells, Tregs, and MDSCs, which may facilitate the evolution to clinically operational tolerance [131].

The mechanism(s) by which tolerance is induced and maintained in clinical transplant recipients has been an area of ongoing investigation. A European consortium of liver transplant centers presented preliminary results of a prospective trial on 102 adult LTX recipients who were enrolled in a prospective immunosuppressive drug weaning study. A total of 41 recipients (40 %) were successfully weaned off immunosuppression a median to 11 years after LTX [132]. Similarly, in a pediatric recipient cohort receiving living donor liver allografts, a median of 7 years prior to enrollment in a prospective weaning protocol, 60 % of patients were successfully weaned [133], validating the earlier reports from the Kyoto University group of a 40 % success rate of weaning pediatric recipients of partial livers from parental donors from a steroid-sparing tacrolimus-based regimen [20]. While a variety of biomarkers have been associated with the development of clinical operational tolerance following LTX, including increased expression of hepatic iron homeostatic genes [21]; increased circulating CD4+, CD25+, CD127-, and FoxP3+ T-cell subsets [21]; and alterations in γδ1 T cells with an increased γδ1/γδ2 ratio [22], these preliminary results suggest that although the mechanism(s) associated with liver allograft tolerance are still being elucidated, obtaining success in clinically operational tolerance in liver transplantation is strictly related to the careful selection of the candidates for long-term weaning and follow-up [133–135].

The following are preliminary results of current tolerance studies, primarily being conducted in kidney transplants because of the requirement for preconditioning that is inherent with the requirement for donor-specific activation and deletion. Unfortunately, extrapolation of findings in the living donor scenario to deceased donors may prove to be a considerable barrier. To this point all successful attempts at tolerance have been accomplished by co-induction of hematopoietic chimerism. Induction of persistent mixed chimerism has been difficult to achieve in humans. Despite this, several studies have suggested that persistence of chimerism may not be necessary for the development of allograft tolerance. Scandling et al. published their cohort of 16 patients who underwent kidney transplantation with an induction protocol including ten doses of 80 cGy TBI each and five doses of rabbit ATG to human recipients of combined HLA-matched G-CSF "mobilized" blood mononuclear cell and kidney transplants from HLA-matched sibling donors. The hematopoietic grafts in the latter protocol were selected CD34+ cells with 1×10^6 CD3+ T cells/kg added back to the hematopoietic cells. Four patients developed persistent mixed chimerism, and eight developed transient chimerism [136]. All those with persistent mixed chimerism, and several of those with transient chimerism, were weaned from their maintenance immunosuppression. With these proofs of concept studies, the next challenge has been to attempt tolerance in HLA-mismatched transplant pairs. Kawai et al. followed up their initial study of myeloma patients with ten patients who received HLA-mismatched kidney transplants with an induction regiment consisting of thymic radiation, anti-CD2 mAb, and cyclophosphamide +/− rituximab followed by ~9 months of calcineurin inhibitors. Seventy percent of the patients were weaned

from all maintenance immunosuppression up to 5 years posttransplant, even though donor hematopoietic chimerism was transient [137].

Another approach to allograft tolerance has been the induction of full donor chimerism, whereby the recipients' immune constitution is replaced by that of the donor. Studies in the bone marrow transplant literature where full donor chimerism was induced have been plagued by high rates of GVHD and engraftment syndrome. Recently, attempts have been made with some success, to induce full donor chimerism in renal allograft recipients. In one of the largest groups to date, Leventhal et al. reported on 15 patients who underwent HLA-mismatched kidney transplantation after an induction regiment of pretreatment with fludarabine, 200-cGy TBI, and cyclophosphamide followed by infusion of tolerance-promoting CD8+, TCR-facilitating cell (FC)-based hematopoietic stem cell (HSC) graft infusion, and posttransplant immunosuppression with tacrolimus and mycophenolate mofetil. Postoperative monitoring for donor chimerism was used to establish decision for immunosuppressive drug weaning. Ten of these 15 patients have achieved durable full or mixed hematopoietic chimerism without GVHD or engraftment syndrome. Eight of these ten achieved durable, high-level (>90 %) hematopoietic chimerism. Six of the eight have successfully completed immunosuppression withdrawn without allograft rejection or graft loss (range of between 10 and 22 months off IS). The two remaining patients with high-level chimerism are currently undergoing immunosuppression withdrawal. Two subjects achieved sustained, mixed chimerism, while three participants achieved transient chimerism [138]. The key to this experience relies on the coadministration of the proprietary "facilitating cell" first identified by Ildstad and coworkers [139].

Todo and coworkers recently reported on their prospective liver tolerance study – they utilized a protocol of T-regulatory cell (Treg) expansion ex vivo to determine whether Treg-based cell therapy affords COT in living donor LT (LDLT). The group from Hokkaido University treated ten consecutive LDLT adult patients with Tregs created from peripheral blood mononuclear cells collected from both donors and recipients by leukapheresis and expanded ex vivo with a 2-week culture of recipient PBMNs with irradiated donor PBMNs under the presence of anti-CD80/anti-CD86 mAbs. These cells were infused into the recipient on postoperative day (POD) 13 along with cyclophosphamide given on POD 5. Steroids and MMF were stopped within 1 month, while the patients were left on tacrolimus monotherapy. At 6 months after LDLT, when graft function and histology were normal, immunosuppression was gradually tapered by spaced doses until it was discontinued 12 months later. Thus far, of the ten recipients, seven are free from immunosuppression [140] (update provided by Todo S. personal communication, April 2014). Protocols for deceased donor liver transplantation are planned.

Graft-Versus-Host Disease (GVHD)

The potential downside of facilitating increasing levels of chimerism is the prospect of developing GVHD, which is a rare but serious complication after liver transplantation. It occurs when immunocompetent donor lymphocytes transferred through the liver allograft become activated and are able to carry out an

immune response against recipient tissues. Acute GVHD is more commonly seen after hematopoietic stem cell transplantation and uncommonly after solid organ transplantation. The true incidence of GVHD after LT is not clear, but according to the more recent reports, it is estimated to be around 1–2 % [141]. GVHD usually presents with fever, skin rash, diarrhea, and pancytopenia 2–10 weeks after liver transplantation. The diagnosis is confirmed by demonstration of substantial number of donor chimerism in the patient's peripheral blood. Studies have shown that these donor chimeric cells are usually CD8+ T cells [142]; however, multilineage donor hematopoietic chimerism has also been described [143–145]. Despite a variety of protocols for treatment of GVHD after LT, with different strategies to decrease or increase immunosuppression [141, 142, 145–147], response rate remains poor with 85 % mortality rate in affected patients. Mortality is usually as a result of multiorgan failure and especially bone marrow failure and infection. To date, only two forms of therapy have been successful, reprogramming the recipient's immune system with infusion of pre-transplant recipient bone marrow [148] and the promising use of alefacept, a fusion protein comprising the extracellular CD2-binding portion of the human leukocyte function antigen-3 (LFA-3) linked to the Fc portion of human-IgG1, and selectively targets memory T cells [149].

1.4 Conclusions

The field of transplant immunology and its applications in clinical transplant has undergone remarkable changes in the last 50 years. With the development and refinement in our understanding of the process underlying clinical transplantation, the ability to prolong graft survival has vastly improved. Despite these advances, long-term graft failure remains a significant problem. Additionally, many of our current immunosuppressants continue to have significant side effect profiles. Optimizing efficacy and decreasing toxicity of regiments continue to drive the efforts toward more efficacious and less toxic regiments. Coupled with new advances in immunological understanding, the field of transplantation continues its quest toward immunosuppressant optimization and even elimination, to improve the lives of the increasing number of transplant recipients.

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