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Moduler la costimulation des lymphocytes T pour la prévention du rejet de l'allogreffe rénale chez les primates non humains / Modulating T Cell Costimulation to Prevent Renal Allograft Rejection in Nonhuman Primates

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Moduler la costimulation des lymphocytes T pour la prévention du rejet de l'allogreffe rénale chez les primates non humains / Modulating T Cell Costimulation to Prevent Renal Allograft Rejection in Nonhuman Primates

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Résumé

La transplantation d'organes est souvent la meilleure approche thérapeutique pour l'insuffisance organique au stade terminal. Le rejet de greffe est le principal obstacle pour une transplantation réussie, car cette dernière est le plus fréquemment réalisée entre individus génétiquement distincts. Les tissus ou organes transplantés sont généralement reconnus par le système immunitaire comme corps étrangers et sont rapidement détruits. Une série d'approches a été réalisée en clinique pour augmenter l'acceptation de la transplantation d'organes. Les immunosuppresseurs ont un rôle clé dans le combat contre le rejet de greffe. Actuellement, les résultats à court terme de la transplantation d'organes ont été considérablement améliorés avec l'émergence des inhibiteurs de la calcineurine (ICN), mais les résultats à long terme sont encore insatisfaisants. L'une des principales raisons est que les médicaments immunosuppresseurs actuels manquent de spécificité. Ces agents, en particulier ICN, sont considérés comme les facteurs de risque pour la perte tardive du greffon et les décès avec un greffon fonctionnel en raison de la toxicité des médicaments, de la sur-immunosuppression et d'autres effets secondaires. Ainsi, il y a un besoin urgent de rechercher de nouvelles thérapies idéales.

Les lymphocytes T jouent un rôle central dans l'initiation du rejet des allogreffes. La pleine activation des cellules T nécessite au moins deux signaux combinés, mais distincts. En plus du signal généré par l'interaction entre le récepteur des cellules T (TCR) et les complexes CMH-peptides, le second signal, appelé signal de costimulation qui est délivré par

l'engagement du récepteur de costimulation avec son ligand, est essentiel. L'engagement du TCR en l'absence de signaux de costimulation peut donner lieu à l'anergie des cellules T, un état d'absence de réponse immunitaire. Les molécules costimulatrices acquièrent ainsi l'attention en tant que cibles thérapeutiques potentielles. Du fait que ces molécules soient largement limitées à des cellules T et/ou des cellules présentatrices d'antigène, cibler la voie de signalisation de costimulation offre la possibilité de moduler le système immunitaire d'une manière plus sélective par rapport à des agents immunosuppresseurs conventionnels.

À ce jour, de nombreuses molécules costimulatrices ont été identifiées et certaines ont été testées en tant que cibles thérapeutiques dans des modèles de transplantation d'organes. Les axes CD28–CD80/86 et CD40–CD40L sont importants et les deux voies de signalisation de costimulation les mieux connues. Bélatacept est un variant de l'antigène 4 des lymphocytes T cytotoxiques-immunoglobuline G (CTLA4-Ig) qui bloque la voie de signalisation CD28–CD80/86. C'est le seul bloqueur de la costimulation à être approuvé pour utilisation clinique en transplantation d'organes. Par rapport à la thérapie basée sur les ICN pour les receveurs de transplantation rénale, les thérapies à base de bélatacept montrent un taux similaire de survie, une fonction supérieure du greffon et l'amélioration du profil de risque cardiovasculaire. Cependant, bélatacept est également associée à des taux plus élevés de rejet aigu et de syndrome lymphoprolifératif post-greffe (SLPG).

Dans notre étude, l'efficacité d'ASKP1240, un nouvel AcM anti-CD40 complètement humain qui bloque la voie de costimulation CD40–CD40L, a été évaluée sur la prévention du rejet d'allogreffe avec le modèle de transplantation rénale chez le singe *cynomolgus*. Quand

ASKP1240 a été administré seul, il a réduit l'incidence du rejet aigu et a prolongé la survie de l'allogreffe rénale dépendamment de la dose administrée. L'acceptation de l'allogreffe rénale a été encore améliorée chez des singes qui ont reçus des traitements d'ASKP1240 combiné avec le tacrolimus (dose sub-thérapeutique) ou le mycophénolate mofétil (MMF). Le rejet aigu d'allogreffe a été totalement éliminé chez ces animaux et la médiane de survie du greffon rénal de ces groupes était significativement plus longue que ceux des groupes avec un traitement monothérapie. ASKP1240 a été bien toléré pour un traitement allant jusqu'à 180 jours. Il n'y avait pas d'effets secondaires évidents, y compris les complications thromboémboliques liées au médicament. L'administration d'ASKP1240 n'a pas induite de libération de cytokines.

Ensuite, nous avons étudié les effets d'ASP2409 sur le rejet de l'allogreffe rénale et la survie chez des singes cynomolgus. ASP2409 est une nouvelle CD86-sélective variante de CTLA4-Ig, qui possède une affinité de liaison au CD86 14 fois plus élevée que le bélatacept *in vitro* et une amélioration de la puissance immunosuppressive. Une haute dose d'ASP2409 en monothérapie a montré des résultats supérieurs dans la réduction de rejet aigu et la prolongation de la survie de l'allogreffe rénale en comparaison avec une faible dose d'ASP2409 en monothérapie. Une faible dose d'ASP2409 en combinaison avec tacrolimus (dose sub-thérapeutique) inhibe complètement le rejet aigu d'allogreffe et prolonge significativement la survie de l'allogreffe rénale par rapport à une monothérapie avec une faible dose d'ASP2409 ou une dose sub-thérapeutique de tacrolimus. La médiane de survie de l'allogreffe des animaux recevant un traitement à base d'une dose élevée d'ASP2409, bélatacept, ou une dose thérapeutique de tacrolimus étaient identiques (> 91 jours). Les

traitements à base d'une dose élevée d'ASP2409 présentaient de meilleurs résultats histopathologiques que le traitement à base de bélatacept. En outre, la fréquence des cellules $FoxP3^{+}$ dans les allogreffes rénales a été observée plus haute dans les traitements à base d'ASP2409 et de bélatacept comparés aux traitements à base de tacrolimus. L'étude a également montré que ASP2409 est sans danger pour les animaux pour les doses qui ont été testées. Nous n'avons pas trouvé de graves effets secondaires liés à ASP2409 au cours des 91 jours d'étude.

Collectivement, ces résultats suggèrent que la modulation sélective de la costimulation des cellules T avec des bloqueurs de la costimulation est une stratégie réalisable pour la prévention et le traitement du rejet d'allogreffe. ASKP1240 et ASP2409 sont tous deux des agents immunosuppresseurs prometteurs pour les régimes d'évitement ou de réduction des inhibiteurs de la calcineurine.

Mots-clés: blocage de la costimulation, transplantation rénale, primate non humain, rejet aigu

Abstract

Organ transplantation is often the best therapeutic approach for end-stage organ failure. Graft rejection is the major obstacle to successful transplantation because transplantation is most frequently carried out between genetically distinct individuals. Transplanted tissues or organs are usually recognized by the immune system as foreign and are rapidly destructed without immune interventions. A series of approaches have thus been applied in clinic to inhibit the allogenic immune responses and in turn increase organ transplant acceptance. Immunosuppressive drugs are the key players in the "war" against immune cell-mediated rejection of allogenic transplants. Currently, the use of calcineurin inhibitors (CNIs) has dramatically decreased the risk of acute transplant rejection and improved the short-term outcomes of organ transplantation, but the long-term outcomes are still unsatisfied. One of the main reasons causing unsatisfied long-term outcomes is that current immunosuppressive drugs do not specifically target immune cells that cause transplant rejection. These immunosuppressive agents, especially CNIs, are the risk factors for late graft loss and death with functioning graft (DWFG) due to drug toxicity, over-immunosuppression, and other side-effects. Thus there is an urgent need for seeking novel therapies that can selectively eliminate the alloreactive immune responses while preserving the integrity of the remainder of the host immune system.

It has been known that T cells play a central role in initiating allograft rejection. Full activation of T cells requires at least two collaborative but distinct signals. The first signal is

generated by the interaction between T cell receptor (TCR) and MHC-peptide complex. The second signal, termed costimulatory signal, is delivered through the engagement of costimulatory receptors by their ligands. Importantly, TCR engagement in the absence of costimulatory signals can result in T cell anergy, a state of T cell unresponsiveness. Costimulatory molecules thus gain attention as potential therapeutic targets. Because these molecules are primary expressed on T cells and/or antigen-presenting cells, targeting costimulatory signaling pathway offers the oppertumities to modulate immune system in a more selective way relative to conventional immunosuppressive agents.

To date, numerous costimulatory molecules have been identified and some have been tested as therapeutic targets in organ transplant models. CD28–CD80/86 and CD40–CD40L axis are two important and the most well known costimulatory signaling pathways. Belatacept, a variant of cytotoxic T lymphocyte antigen 4-immunoglobulin G (CTLA4-Ig) that blocks CD28–CD80/86 signaling pathway, is the only costimulation blocker to be approved for clinical use in organ transplantation. Compared to CNI-based regimen for kidney transplant recipients, belatacept-based regimens show similar patient and graft survival rate, superior graft function, and improved cardiovascular risk profile. However, belatacept is also associated with higher rates of acute rejection and posttransplant lymphoproliferative disorder (PTLD). Disruption of CD40–CD40L interaction with anti-CD40L mAbs has also been demonstrated to be a reliable approach for preventing acute rejection and for prolonging allograft survival. Unfortunately, unexpected thromboembolic complications in preclinical studies and clinical trials discontinued the development of anti-CD40L mAbs. The main objective of this thesis is to identify the optimal T cell costimulation blockers that can show

improved safety and non-inferior efficacy in promoting allograft acceptance relative to current costimulatory blocking agents.

Anti-CD40 mAbs are an alternative approach to block CD40–CD40L costimulatory pathway. CD40 is not involved in the stability of platelet thrombi and anti-CD40 mAbs are expected to not cause thromboembolic events. ASKP1240 is a novel fully human anti-CD40 mAb. In this study, the efficacy of ASKP1240 in the prevention of renal allograft rejection was evaluated in *cynomolgus* monkey kidney transplantation model. When ASKP1240 was administered alone, it dose-dependently reduced the incidence of acute rejection and prolonged renal allograft survival. Renal allograft acceptance was further improved in the monkey which received regiments using ASKP1240 combined with conventional immunosuppressive drugs tacrolimus (sub-therapeutic dose) or mycophenolate mofetil (MMF). Acute allograft rejection was totally eliminated in these animals and the kidney allograft median survival times (MST) of these groups were significantly longer than those of monotherapy groups. ASKP1240 administration did not induce cytokine release. This agent was well tolerated in monkey kidney transplant recipients during the 180-day treatment period. There were no obvious side effects including drug-related thromboembolic complications.

Next, we tested the hypothesis that a CD86-selective variant of CTLA4-Ig might show non-inferior efficacy on the prevention of allograft rejection and prolongation of graft survival in comparison with belatacept. CD86 is the dominating ligand between the two natural ligands for CD28 in alloimmune response. Improvements in CD86 binding affinity of CTLA4-Igs confer increased immunosuppressive potency. A CD86-selective CTLA4-Ig variant may

reach therapeutic levels of CD86 occupancy while occupies substantially less CD80 ligand than non-CD86-selective CTLA4-Igs. Preservation of CD80 signaling may be favoring to retain protective immunity and to improve safety of CTLA4-Ig therapy. This thesis investigated the effects of a novel CD86-selective CTLA4-Ig variant, ASP2409, on renal allograft rejection and survival in *cynomolgus* monkeys. ASP2409 possesses 14-fold higher *in vitro* CD86 binding affinity than belatacept and improved immunosuppressive potency. Compared to no-treatment control, low-dose (0.3 mg/kg) ASP2409 monotherapy significantly prolonged renal allograft survival (MST = 5 and 26 days, respectively) but did not decrease the incidence of acute rejection (8/8). The rate of acute renal allograft rejection was reduced in the group of high-dose ASP2409 monotherapy (2/7) and, correspondingly, a much longer MST (>91 days) was shown in this group. Combination therapy of low-dose ASP2409 and tacrolimus (sub-therapeutic dose) completely eliminated acute allograft rejection and notably prolonged renal allograft survival (MST >91 days). The MSTs of renal allografts in the animals receiving high-dose ASP2409-, belatacept-, and therapeutic dose tacrolimus-based immunosuppressive regimens were same (>91 days). High-dose ASP2409-based regimens exhibited better histopathological results than belatacept-based regimen. Furthermore, higher frequencies of $FoxP3^+$ Tregs in renal allografts were observed in ASP2409- and belatacept-based regimens compared to tacrolimus-based regimen. ASP2409 was also demonstrated to be safe for animals with the doses to be tested. There were no serious side effects related to ASP2409 to be found in term of 91-day study.

This study demonstrates that ASKP1240 is as effective as anti-CD40L mAbs on inhibition of acute rejection and prolongation of renal allograft survival, and do not cause thromboembolic

complications. Previous studies indicated that CNIs could diminish the effects of anti-CD40L mAbs. In our study, ASKP1240 in combination with tacrolimus or MMF shows improved efficiency. Furthermore, the effects of ASP2409 on promoting renal allograft acceptance are not inferior to belatacept. These results collectively suggest that ASKP1240 and ASP2409 both are the promising immunosuppressive agents for solid organ transplantation.

Keywords: costimulation blockade, kidney transplantation, nonhuman primate, acute rejection

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List of Abbreviations

ACR: Acute cellular rejection

ADCC: Antibody-dependent cell-mediated cytotoxicity

AHR: Acute humoral rejection

ALG: Antilymphocyte globulin

AP-1: Activation protein-1

APCs: Antigen-presenting cells

ATG: Antithymocyte globulin

AZA: Azathioprine

BCR: B cell receptor

BUN: Blood urea nitrogen

CAMs: Cell adhesion molecules

CAN: Chronic allograft nephropathy

CD: Cluster of differentiation

CDC: Complement dependent cytotoxicity

CNIs: Calcineurin inhibitors

CsA: Cyclosporine

CTL: Cytotoxic T-lymphocyte

CTLA4: Cytotoxic T-lymphocyte-associated protein 4

CTLA4-Ig: Cytotoxic T lymphocyte antigen 4-immunoglobulin G

CVF: Cobra venom factor

DAG: Diacylglycerol

DC: Dendritic cell

DD: Death domain

DSAs: Donor-specific antibodies

DST: Donor-specific transfusion

DTH: Delayed-type hypersensitivity

DWFG: Death with functioning graft

ECs: Endothelial cells

ECL: Electrochemiluminescent

ECM: Extracellular matrix

FasL: Fas ligand

FcγRs: Fc gamma receptors

FDA: Food and Drug Administration

FoxP3: Forkhead box protein 3

GITR: Glucocorticoid-induced TNFR family-related protein

Grb2: Growth factor receptor-bound protein 2

GVHD: Graft-versus-host disease

HCT: Hematocrit

HGB: Hemoglobin

HLA: Human Leukocyte Antigens

HVEM: Herpes virus entry mediator

ICOS: Inducible T cell costimulator

IDO: Indoleamine 2, 3-dioxygenase

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IgSF: Immunoglobulin superfamily

IKK: IκB kinase

ILA: Induced by lymphocyte activation

IP_3: Inositol trisphosphate

ITAM: Immunoreceptor tyrosine-based activation motif

ITIM: Immunoreceptor tyrosine-based inhibition motif

iTregs: Induced regulatory T cells

JAK-3: Janus kinase-3

JNK: C-Jun N-terminal kinase

LAT: Linker for activation of T cells

LFA-1: Leukocyte function-associated antigen 1

liCTLA4: Ligand-independent CTLA4

LIGHT: Homologous to **l**ymphotoxin, exhibits **i**nducible expression and competes with HSV **g**lycoprotein D for binding to **h**erpesvirus entry mediator, a receptor expressed on **T** lymphocytes

mAbs: Monoclonal antibodies

MALT: Mucosa-associated lymphoid tissue

MAPK: Mitogen-activated protein kinase

MFI: Mean fluorescence intensity

MHC: Major histocompatibility complex

miH: Minor histocompatibility antigen

MMF: Mycophenolate mofetil

MPA: Mycophenolic acid

MST: Median survival time

mTOR: Mammalian target of rapamycin

NCAM: Neural cell adhesion molecule

NFAT: Nuclear factor of activated T cells

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NHP: Nonhuman primate

NK: Natural killer

nTregs: Naturally occurring Tregs

PAG: Phosphoprotein associated with glycosphingolipid-enriched microdomains

PAS: Periodic Acid-Schiff

PBMCs: Peripheral blood mononuclear cells

PD: Pharmacodynamics

PD-1: Programmed death-1

PI_3K: Phosphatidylinositol 3-kinase

PIP_2: Phosphatidylinositol 4,5-bisphosphate

PK: Pharmacokinetic

PKC: Protein kinase C

PLCγ1: Phospholipase Cγ1

PLT: Platelets

PODs: Post-transplantation days

PtdSer: Phosphatidylserine

PTLD: Post-transplant lymphoproliferative disorder

RasGRP: Ras guanyl nucleotide-releasing protein

RBC: Red blood cell

S1P: Sphingosine-1-phosphate

SCr: Serum creatinine

Sema4A: Semaphorin 4A

SIT: Src homology 2 domain-containing protein tyrosine phosphatase-interacting TRAP

SLP-76: SH2 domain-containing leukocyte phosphoprotein of 76 kDa

SNBL: Shin Nippon Biomedical

TBI: Total body irradiation

TCR: T cell receptor

TFH cell: T follicular helper cell

Th cell: Helper T cell

TIM: T cell Ig domain and mucin domain

TNF: Tumor necrosis factor

TNFR: Tumor necrosis factor receptor

TNFRSF9: Tumor necrosis factor receptor superfamily member 9

Tr1: Type 1 regulatory T cells

TRAF: TNF receptor associated factor

TRAP: Transmembrane adapter protein

Tregs: Regulatory T cells

TRIM: T cell receptor interacting molecule

TTx: Anti-tetanus toxoid

VCAM-1: Vascular cell adhesion molecule 1

VLA-4: Very late antigen 4

WBC: White blood cell

ZAP-70: Zeta-chain-associated protein kinase 70

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Chapter 1

Introduction

In 1954, organ transplantation from an idea in ancient legend successfully became a realistic therapeutic approach in clinical practice (Merrill 1956). Since then, transplantation of organs including kidney, heart, lung, liver, pancreas *etc.* gradually become a routine procedure to save the life of the patient with end-stage organ failure. Every year tens of thousands organ transplantations are preformed in the world. To date, organ transplantation is still the only cure for some end-stage organ failure, such as heart and liver failure.

1.1 Rejection – the major cause of organ transplant failure

Organ transplantations are mostly carried out between genetically distinct individuals because the donor who is genetically identical to the recipient is very rare. Although the outcome of transplantation could be affected by many factors including surgical techniques, organ quality, age and race of recipient, post-transplant complications *etc.*, allograft rejection is the most common cause of transplant failure.

The word "rejection" come from the French word "réjection", or directly come from the Latin word "reiectionem". It means "act of throwing back". In the 1940s, through serial studies of skin transplantation, Medawar and his colleagues demonstrated that transplant rejection was an immunologic event (Gibson 1943; Medawar 1944). During and after transplantation, the recipient's immune system recognizes certain components of transplanted cells, tissues, or organs as foreign, and then initiates a cascade of reactions aimed towards the allografts. Transplant injury caused by donor disease, organ harvesting, organ preservation, implantation, and reperfusion triggers a non-specific inflammatory response. Numerous proinflammatory

cytokines, chemokines and adhesion molecules are released during this process (Kim 2008). They promote various inflammatory cells including dendritic cell (DC), monocyte, and lymphocyte to migrate in and out the allograft and lead to antigen specific immune response to eliminate the transplant. Allograft rejection is usually an integrated result that many factors and multiple mechanisms involve in. Both innate and adaptive immunity play a role in this event even though one component of the immune system may dominate a certain type of rejection (Moreau 2013; Farrar 2013).

The antigenic disparity between donors and recipients is the main factor to determine the potency of the alloimmune response (Sánchez-Fueyo 2011). Studies have demonstrated that incompatibility of the surface glycoproteins named major histocompatibility complex (MHC) is the most important cause of rapid graft rejection. MHC is found in all jawed vertebrates. These molecules are encoded by a set of genes and are involved in a lot of physiological processes including immunological and non-immunological activities (Gruen 1997; Kelley 2005). In humans, the MHC is also known as Human Leukocyte Antigens (HLA). The HLA gene group is located on the short arm of chromosome 6 and consists of more than 200 genes (Shiina 2009). Based on gene locations, product functions and structures, these genes are categorized into class I–III three subgroups. HLA Class I molecules are found on most nucleated cells. On contrary, expression of HLA class II molecules are seen on DCs, B cells, and macrophages/monocytes. Molecules of these two groups serve as both stimulus and target in alloimmune response. Genes of HLA class III subgroup encode complements, cytokines, heat shock proteins, and the molecules not related to immune function (Shankarkumar 2004). Besides MHC, minor histocompatibility antigens (miH) that are MHC-bound peptides derived

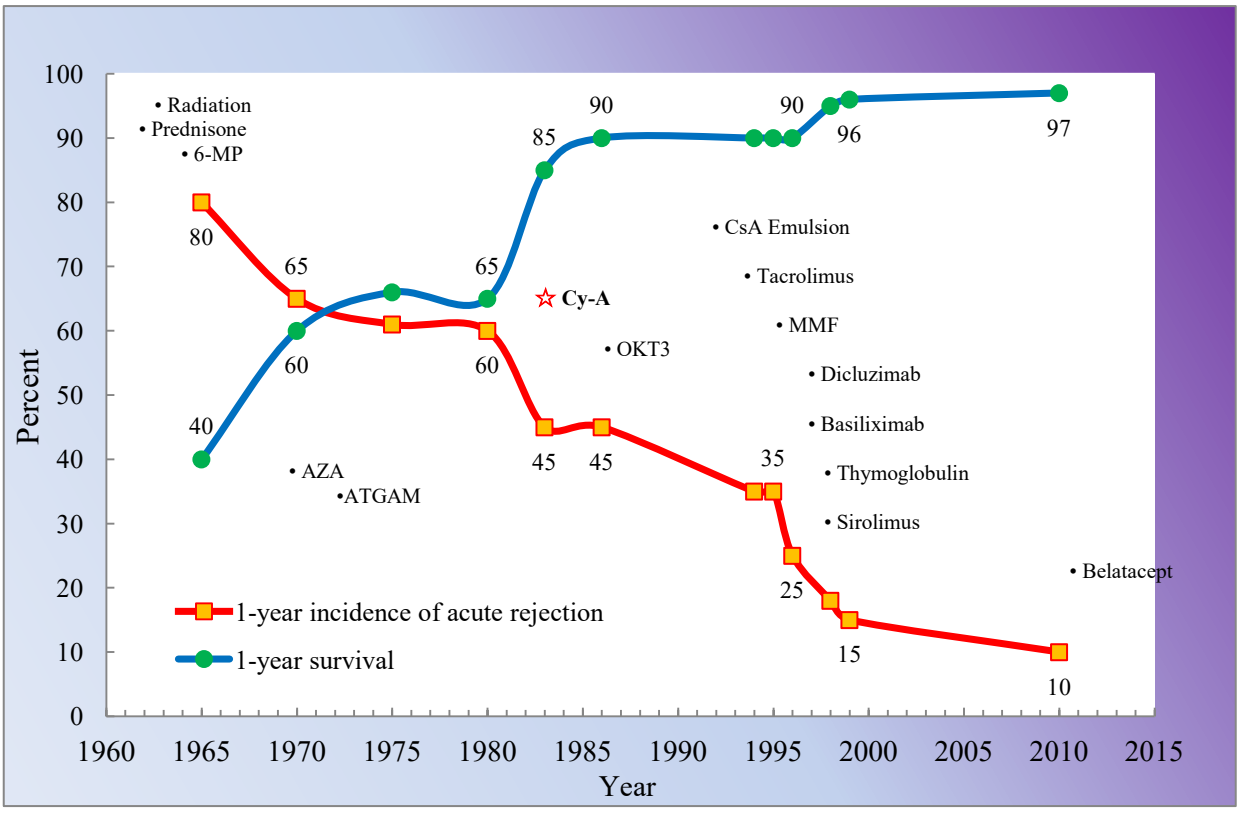
from various endogenous proteins, can also act as alloantigens. They elicit cytotoxic T cell mediated immune responses and cause allograft rejection in MHC-matched individuals (Dzierzak-Mietla 2012; Dierselhuis 2009).

Rejection occurs in almost all allografts if there are no immunomodulatory therapies to be applied to suppress the immune response against the transplant. Based on its clinical features, allograft rejection is classified into three types: hyperacute rejection, acute rejection, and chronic rejection.

The most common pattern of allograft rejection is acute rejection. According to the literatures of the 1980s and 1990s, acute rejection occurred in 50% to 75% of kidney transplants (Gulanikar 1992), 64% of liver transplants (Wiesner 1998), and 70% to 85% heart transplants (Hunt 1983). The incidence of acute rejection dramatically declined with the advent of calcineurin inhibitors and other new immunosuppressive drugs (Figure 1.1). Organ Procurement and Transplantation Network data showed that the acute rejection rate within one year in kidney transplant recipients was on average approximately 15% in the period from 2001 to 2003 (Djamali 2006).

Acute rejection is a rapid reaction against allograft resulting in the injury of transplant parenchyma and blood vessels. This event can be mediated by T cells (acute cellular rejection, ACR) or antibodies (acute humoral rejection, AHR), or both together (Nankivell 2010). Acute rejection is most likely to happen within the first several posttransplant weeks, but may occur at any time, even many years after transplantation. In the process of acute rejection, the

allograft encounters inflammation, cell injury, and upregulation of MHC class I and II expression. The release of various inflammatory cytokines and infiltration of different cells lead to destruction of parenchymal and endothelial cells (ECs), edema and blood flow decline in tissues. Partial or whole transplanted tissue infarction may occur in the case of destruction of blood vessels (Moreau 2013; Nankivell 2010). Although nowadays the incidence of acute rejection has been significantly decreased, it still contributes to about 12% renal graft loss due to functional failure during the first six months after transplantation (El-Zoghby 2009).



Adapted from Stewart F. Organ Transplantation. 1999

Figure 1.1 Rejection rate and graft survival in kidney transplantation.

The principal mechanisms of cell-mediated acute rejection are to evoke cluster of differentiation (CD) 4^+ T helper (Th) cell-mediated delayed-type hypersensitivity (DTH) reactions and $CD8^+$ cytotoxic T-lymphocyte (CTL)-mediated cytotoxicity (Rocha 2003; Wood 2012). To date various $CD4^+$ T cell subsets including Th1, Th2, Th9, Th17, Th22, T follicular helper (TFH) cells, and regulatory T cells (Tregs) have been identified. Each subset exhibits distinct cytokine production patterns and effector functions. Traditionally Th1 cells such as interferon gamma (IFN-γ)- and tumor necrosis factor (TNF)-secreting Th1 cells are the common $CD4^+$ Th cell subsets that cause DTH. They secrete proinflammatory cytokines such as IFN-γ, TNF-α, and IL-1. These proinflammatory cytokines help to recruit and activate non-antigen-specific cells including monocytes and macrophages. These activated cells release proteolytic enzymes, nitric oxide, and other soluble factors that cause nonspecific local inflammation, and affect vascular tone and permeability, as well as enhance chemotaxis to result in cellular infiltrate, edema, and tissue damage. In turn, these non-antigen-specific activities promote the further activation and differentiation of antigen-specific T cells. Recently $CD4^+$ Th17 cells are found to promote macrophage and endothelial activation, and to cause inflammatory tissue damage and graft rejection (Yuan 2008; Heidt 2010). Suppression of DTH leading to long term allograft acceptance from another angle demonstrates that DTH plays important role in allograft rejection (VanBuskirk 1998).

$CD8^+$ CTLs are the major effector cells in the cell-mediated cytotoxicity. Activation and differentiation of a naïve $CD8^+$ T cell (CTL precursor) into a functional CTL occur within secondary lymphoid tissue. CTL precursors are activated by the interaction of the TCR and $CD8^+$ molecules with MHC class I molecules expressed on donor APCs. $CD4^+$ Th cells

provide help during the process via cell-to-cell contact or secretion of IL-2/IFN-γ cytokines (Ridge 1998). Activated CTL precursors differentiate into functional CTLs with up-regulating expression of IL-2 receptor, and forming lytic granules which contain perforins, granzymes, serglycin, calreticulin, and granulysin *etc*. CTLs then migrate into the allograft and identify target cells by specific interactions between MHC class I on the target cell and TCR as well as CD8 on the CTL. The contents within granules are released into the immunological synapse after cell-cell contact (Rocha 2003). Target cell apoptosis is induced via perforin/granzyme cell death pathway, or via the Fas/FasL pathway.

AHR is less common than ACR. The incidence of AHR in kidney transplant recipients is about 5–7% (Takemoto 2008). This type of rejection is mediated by donor-specific antibodies (DSAs) that may either be pre-existed or *de novo* generated after transplantation. The prognosis of AHR is poorer than ACR because conventional immunosuppressive therapy is typically not effective enough for AHR. The vasculature of graft is the primary target in this type of rejection. The binding of DSAs to alloantigens (mainly MHC molecules) on the graft vascular ECs triggers activation of both the classical complement and coagulation cascades, and results in endothelial injury, intravascular thrombosis, necrosis of smaller vessels, edema and haemorrhage, and eventually destruction of the transplanted organ (Puttarajappa 2012; Lucas 2011). DSAs may also cause cell injury via antibody-dependent cell-mediated cytotoxicity (ADCC).

Hyperacute rejection is the most rapid and aggressive form of allograft rejection, and fortunately it is extremely rare nowadays due to pretransplantation cross-matching is routinely

applied as a prophylaxis. This type of rejection is mediated by high levels of preformed alloantibodies against donor antigens, particularly MHC class I or ABO blood group antigens. The vascular ECs are generally the main targets. Preexisting alloantibodies bind to endothelial antigens in transplanted organs, and lead to activation of complement and coagulation cascades. Both of them promote extensive intravascular thrombosis and irreversible tissue injury in the graft. Transplanted organ failure may occur within hours or even minutes (Colvin 2006).

Chronic rejection is a slow and progressive process over months to years after transplantation. It usually develops insidiously and is the major cause of later graft loss. The pathologic features of chronic rejection in vascularized grafts include intimal thickening (caused by edema and cell infiltration), smooth muscle cell proliferation in the medial layer, and disruption of elastic lamina *etc*. These changes result in vascular occlusion and interstitial fibrosis in kidney and heart allograft (Gloor 2006; Tan 2007), bronchiolitis obliterans in lung allograft (Belperio 2009), and vanishing bile duct syndrome in liver allograft (Inomata 2001). The pathogenesis of chronic rejection is not fully understood. Multiple factors including DSAs, the degree of HLA mismatch, previous acute rejection episodes, immunosuppressive drug toxicity, ischaemia/reperfusion injury, infection, and hyperlipidaemia are associated with the development of chronic rejection. It implies that the pathogenesis of chronic rejection may involve both immune and nonimmune factors (Costello 2013). For this reason, in kidney transplant recipients, chronic allograft rejection was replaced by the term chronic allograft nephropathy (CAN). Recently the more descriptive phrase interstitial fibrosis and tubular atrophy (IFTA) took the place of CAN to define the level and severity of chronic renal

allograft damage. IFTA can be found in recipients with good allograft function and is closely associated with late renal transplant failure (Fletcher 2009; Shimizu 2016).

1.2 T cells play a central role in allograft rejection

T cells are essential in the event of allograft rejection. This notion was demonstrated by the experimental studies that fully mismatched transplants were not rejected in the animals lacking T cells, and adoptive transfer of normal T cells could restore allograft rejection in those animals (Pennycuik 1971; Manning 1973; Whitby 1990). Naïve T cells recognize foreign histocompatibility antigens particularly MHC is a key step to initiate allorejection. There are three different ways for immune recognition known as direct, indirect, and semi-direct allorecognition (Afzali 2008).

As soon as the recirculation is established in the transplanted organ, recipient APCs enter the allograft where they encounter foreign antigen derived from donor and become activated. The donor-derived DCs in the transplant may also be activated as a result of cell injury incurred during the transplant procedure. Those activated APCs (either donor-derived or recipient-derived) then migrate to the recipient's draining lymph nodes. Meanwhile, the soluble MHC proteins that come from transplant may be bound, processed, and presented by B cells. Naïve T cells circulating in the blood enter the lymph node via the afferent lymphatic vessels. They encounter and interact with APCs at the T cell zones in the lymph node paracortex, where allorecognition initially takes place. In the case of direct allorecognition, recipient T cells recognize intact foreign class I and class II MHC molecules expressed on donor-derived

APCs via interaction between TCR and MHC-peptide complexes. Donor-derived DCs are irreplaceable in this type of antigen recognition (Lechler 1982). This process induces a vigorous polyclonal T cell response; as a result, both $CD4^+$ and $CD8^+$ T cells that target donor antigens are generated. Direct allorecognition plays a significant role in acute allograft rejection (van Besouw 2005).

Indirect allorecognition is similar to the way of T cell activation in response to pathogens. The allogeneic antigens from donor (*e.g.* cell membrane fragments, soluble MHC and miH molecules) are captured by recipient APCs (DCs and B cells) and are degraded into peptides within cells. These peptides bind to newly synthesized recipient MHC class II molecules, and then are transported to the cell surface. They integrate into the cell membrane of recipient APCs in the form of MHC-peptide complexes. $CD4^+$ T cells recognize those peptide fragments via interactions between TCRs and MHC-peptide complexes, and the process of cell activation is then initiated. Beside of $CD4^+$ T cells, $CD8^+$ T cells can also be activated by indirect antigen recognition pathway (Carbone 1990; Zinkernagel 2002). Some studies have indicated that indirect allorecognition is involved in the induction and progression of chronic transplant rejection (Liu 1996; Vella 1997; Womer 2001).

The third mechanism of antigen recognition is semi-direct allorecognition. This type of allorecognition pathway is proposed based on a well-recognized biological process that immunological cells such as DCs have the capacity to transfer membrane components between them via cell-to-cell contact or the exosomal route (Smyth 2008). Semi-direct allorecognition occurs when entire MHC peptide complexes released from donor DCs and

ECs are captured by recipient APCs (Bedford 1999; Herrera 2004). This kind of recipient APCs can then present donor peptide antigens either to $CD4^+$ T cells in the form of self-MHC II peptide complexes or to $CD8^+$ T cells via captured donor MHC I molecule. They possess the ability that same APC activates both $CD4^+$ T help cell via indirect antigen recognition pathway and $CD8^+$ cytotoxic T cell via direct antigen recognition pathway, even simultaneously (Afzali 2008).

Allorecognition triggers further molecular interactions between T cells and APCs to extend the contact time. Naïve T cells that fail to make prolonged contact with specific antigen then leave the lymph node and return to the circulation. T cells that encounter antigen-specific interaction with APCs remain in the lymph node for 3–4 days. With the help of costimulatory molecules and other molecules, they become fully activated and undergo proliferation and differentiation.

In adaptive immune response mediated allograft rejection, DTH, CTL-mediated cytotoxicity, and antibody-mediated responses are three principal mechanisms that cause destruction of transplanted tissues and organs. The $CD4^+$ T cells orchestrate all three processes. Upon activation, with stimulation of cytokines secreted activated T cells, naïve $CD4^+$ T cells differentiate into various Th cell subsets. Th cells secrete a range of cytokines and chemokines including IL-2, IL-4, IL-5, IFN-γ, and TNF-α *etc.* These cytokines and chemokines attract and activate corresponding effector cells, and lead to various immune responses. Th 1 and Th 17 involve in DTH (Cher 1987; Ishii 2010). The proinflammatory cytokines produced by them cause inflammatory damage of allografts.

In CTL-mediated cytotoxicity, although the effector cell is MHC class I restricted $CD8^+$ T cell, $CD4^+$ T cell is required for this immune response process. The differentiation and expansion of $CD8^+$ T cells are $CD4^+$ T cell dependent (Behrens 2004). $CD8^+$ T cell and $CD4^+$ T cell need to be activated by same APC (Bennett 1997). APC is firstly licensed via interaction with $CD4^+$ T cell, with the help of IL-2 produced by $CD4^+$ T cell, the same APC engages with $CD8^+$ T cell and trigger cell activation and proliferation. Another model to generate functional effector CTLs is that $CD4^+$ T cell, $CD8^+$ T cell and APC form a three-cell cluster. Activated CTLs leave the secondary lymphoid organs and migrate to the graft where they attack donor cells and destruct transplanted organs. In addition, $CD4^+$ T cells promote $CD8^+$ T cell priming by amplifying DC production of IL-15 via CD40–CD40L interactions (Greyer 2016).

Antibody-mediated responses are also T cell dependent. T cells involve in various steps of B cell immune response including activation, differentiation, and isotype switching. Naïve B cells in the circulation can only survive for a limited time unless they encounter their antigen. The engagement of B cell receptor (BCR) with antigen can trigger the activation of B cells. But, in the case of T cell absence, their interaction induces only low-level B cell proliferation. As a result of that, few antibodies are produced, or even there are no specific antibody productions in some time. The participation of $CD4^+$ Th cells can remarkably enhance B cell functions (Reinitz 1990; Noelle 1991). When T cells and B cells encounter each other, $CD4^+$ Th cells recognize the processed donor MHC peptides through the interaction between TCRs on the $CD4^+$ cell membranes and MHC class II molecules on B cells. With the help of costimulatory molecules, coreceptors and adhesion molecules, $CD4^+$ Th cells become

activated. Activated $CD4^+$ Th cells release various types of cytokines. Those cytokines promote B cell proliferation, differentiation into plasma cells, and early isotype switching. Plasma cells produce specific alloantibodies primarily targeted ECs. The interaction between alloantibodies and ECs lining the donor vasculature activates both complement and coagulation cascades, and induces ADCC resulting in cell and organ damage (Wasowska 2010).

1.3 T cell receptor signaling in T cell activation

In the context of transplantation, the binding of the TCR variable regions to the MHC-peptide complex is an essential for generating signals to initiate T cell activation. The TCR is a heterodimer composed of two (either α/β or γ/δ) transmembrane polypeptide chains. The cytoplasmic domains of the TCR α and β chain are very short and there are no any specific protein-binding motifs. To possess the capability of transmembrane signal transduction, therefore, the TCR assembles with CD3 molecules to form TCR-CD3 complex (Weiss 1994). The CD3 is a multimeric protein complex and is composed of four (γ, δ, ε and ζ) different chains. The intracellular domains of all CD3 chains contain a characteristic sequence motif for tyrosine phosphorylation, known as immunoreceptor tyrosine-based activation motif (ITAM). The ITAM is required to transduce receptor-mediated antigen recognition signals into the cytoplasm of T cells and to induce a series of intracellular signaling cascades (Flaswinkel 1995; Guy 2009). In addition to the CD3 complex, coreceptor CD4 or CD8 is also involved in the antigen recognition process. CD4 and CD8 bind to nonpolymorphic

regions of MHC class II and class I molecules, respectively, to stabilize the T cell–APC immunological synapse and to promote effective signal transduction (Barber 1989).

Following TCR engagement, activation signals that are transmitted into the cell by CD3 trigger a phosphorylation cascade. PCR signaling is initiated by the activations of two Src-related protein tyrosine kinases (PTKs), Fyn that associates with the cytoplasmic tails of the CD3 complex and lymphocyte protein tyrosine kinase (Lck) that noncovalently associates with the cytoplasmic domains of the CD4 and CD8 coreceptors (Veillette 1988). Inactivated Lck has a closed configuration. Upon activation, Lck changes its conformation from the closed shape to an open structure by CD45-mediated dephosphorylation, and possesses active kinase function. CD3 is one of the substrates of Lck. Lck is recruited to CD3 complex to induce the tyrosine phosphorylation of the ITAMs (Barber 1989). This creates a docking site to facilitate another tyrosine kinase protein zeta-chain associated protein kinase 70 (ZAP-70) bind to phosphorylated CD3ζ-chain ITAMs and being activated. The activation of ZAP-70 further amplifies the response by phosphorylating tyrosine residues on various adapter molecules including linker for activation of T cells (LAT), SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76), phospholipase Cγ1 (PLCγ1), T cell receptor interacting molecule (TRIM) *etc* (Williams 1999; Zhang 1998; Bruyns 1998). These activated adapter molecules serve as docking sites for a number of cellular enzymes and lead to the activation of multiple downstream signaling pathways. PLCγ1 hydrolyzes the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces the calcium release from calcium pool within the endoplasmic reticulum and the activation of plasma membrane calcium channel. The

serine/threonine phosphatase calcineurin is then activated along with the increase of intracellular calcium level. Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT). NFAT is then able to translocate to the nucleus and execute its function as transcription factor. DAG is an activator of various signaling molecules including protein kinase C (PKC), Ras guanyl nucleotide-releasing protein (RasGRP). The activations of PKC and RasGRP initiate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) cascades. These lead to the generation of transcription factors including activation protein-1 (AP-1), NF-κB, and NFAT *etc*. These transcription factors enter the nucleus to modulate transcription of various genes that are involved in cell cycle initiation, proliferation, and differentiation (Smith-Garvin 2009; Zhong 2008).

Various factors are involved in the regulation of TCR signaling. The costimulatory molecule CD28 engagement by CD80/CD86 provides an initial adhesion capacity to allow T cell approaching APC that is an essential for T cell activation. With CD28 signals, naïve T cells become more sensitive to the stimulation of alloantigen because the TCR number required for activation is decreased. The activation of ZAP-70 is also affected by CD28 signals. Furthermore, CD28 signals allow T cell to enter cell cycle G 1 phase to achieve clonal expansion, and to activate transcription of the IL-2 gene by the induction of the NFAT (Appleman 2002; Boomer 2010). CD45 is also a critical regulator of TCR signaling because it induces the dephosphorylation of the tyrosine kinases including Lck and Fyn (Saunders 2010). Other factors that modulate TCR signaling include actin cytoskeleton (Beemiller 2013).

TCR signaling is also controlled by negative regulators to prevent the hyperactivation of T cells. Activated T cells may upregulate the expression of a costimulatory molecule CTLA4. CTLA4 shares same ligands with CD28 but possesses 10–20 fold higher affinity. Hence, the ligands (CD80/CD86) preferentially bind to CTLA4 and CD28 signals are antagonized. CTLA4 also execute it negative regulation via inhibition of the phosphorylation of TCR (Gough 2005). A transmembrane adapter protein (TRAP), Src homology 2 domain-containing protein tyrosine phosphatase-interacting TRAP (SIT), is a critical negative regulator of TCR signaling (Simeoni 2005). And another TRAP, phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), negatively regulates Ras activation by altering Src kinase activity (Brdicka 2000; Simeoni 2008).

1.4 Costimulatory signals are required for the full activation of T cells

T cells are the crucial mediators and controllers in the alloimmune response induced by foreign histoincompatible alloantigens. Full activation of T cell is a result of cooperation of multiple signals (Durrbach 2010). In 1987, Helen Quill and Ron Schwartz described a phenomenon termed T cell anergy. When functional APCs were absent, purified MHC/peptide complexes that were incorporated into artificial planar lipid membranes could induce high-affinity interaction between TCR and MHC/peptide complexes, but the T cells presented a state of proliferative nonresponsiveness rather than activation (Quill 1987). It was hypothesized that, besides the signal provided by the TCR engagement with MHC/peptide complexes, accessory signals that emanated from the interaction between functional APCs

and T cells were required for T cell to be fully activated. Results from subsequent studies provided concrete evidences to support this notion (Turka 1990; Koulova 1991; Gimmi 1993). The two-signal model of T cell activation was then recognized (Bernard 2002). In the process of T cell activation, TCR engagement with MHC/peptide complexes generates Signal 1 that is antigen-specific. This signal is transmitted into the cell via CD3 complex and triggers various early activation events including the activation calcium-dependent calcineurin protein, tyrosine phosphorylation, and inositol metabolism. The binding of TCR with MHC/peptide complexes is quite brief (Lanzavecchia 2000). The intrinsic affinity between them is very low and some coreceptors and adhesion molecules which stabilize the engagement between TCR and MHC/peptide complexes may take part in the process to enhance the activity. Signal 1 alone is insufficient to lead to T cell being efficiently activated. An additional signal termed costimulatory signal (Signal 2) is essential for the full activation of T cells. Costimulatory signal is induced by the interaction between T cell costimulatory receptors and their corresponding ligands that are expressed by the functional APCs. This signal is non-antigen-specific. In the absence of costimulatory signal, T cells cannot acquire the capacity of cell proliferation and become effector cells (Jain 1995; Serfling 1995). Costimulatory signals play an important role in the enhancement of tyrosine kinase activity. If there is no costimulatory signal, ZAP-70 is unable to achieve the critical threshold to activate downstream molecules. As a result of that, the entire intracellular phosphorylation cascade will be terminated. Consequently, the fates of T cells are either anergy or apoptosis (Figure 1.2). The exact molecular mechanisms of T cell anergy are still unclear. Studies indicated that both immune signal transduction pathways and the ubiquitin proteasome system are associated with clonal anergy (Appleman 2003; Safford 2005; Zheng 2008; Fathman 2007).

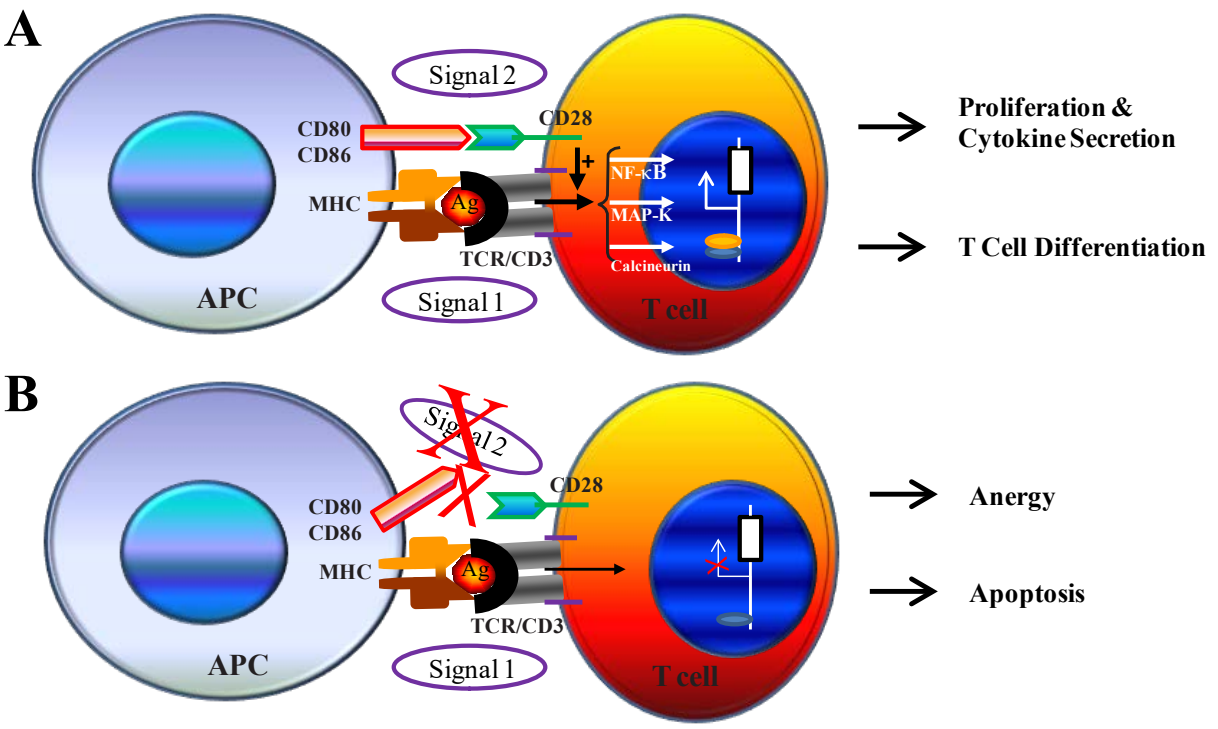


Figure 1.2 Costimulatory signals are essential for full T cell activation. The combined actions of Signal 1 and Signal 2 result in T cells to become fully activated (A). In the absence of Signal 2, T cells exhibit a state of proliferative unresponsiveness rather than activation (B).

1.5 Costimulatory molecules

CD40 and CD28 are the two best characterized costimulatory molecules. They were identified in 1985 and 1986, respectively. Since then, numerous molecules that are involved in costimulatory signal transduction have been discovered. These molecules form a large group known as second signal family. Among them, some molecules provide enhancement signals to promote T cell activation, survival, and/or differentiation. These molecules are named positive costimulatory receptors and ligands. In contrast to them, some other molecules produce signals to antagonize TCR signaling resulting in termination of T cell activation.

They are referred as negative costimulatory receptors and ligands. Negative costimulatory signaling pathways play important roles in the maintenance of peripheral T cell tolerance and reducing inflammation after infection. However, the activations of naïve T cells will not be affected because naïve T cells do not express negative costimulatory molecules. The negative costimulatory molecules are expressed by effector T cells, and the expression of these molecules will be up-regulated at the end of an immune response. Apart from basing on the functional properties of costimulatory receptors mentioned above, costimulatory molecules can also be sorted according to their structural properties. Basing on their molecular structures, costimulatory molecules are classified into four distinct groups *i.e.* immunoglobulin (Ig) superfamily, tumor necrosis factor receptor (TNF-R) family, cell adhesion molecules, and T cell immunoglobulin and mucin domain (TIM) family.

1.5.1 The immunoglobulin superfamily

Costimulatory molecules in the Ig superfamily all possess a characteristic domain referred to Ig domain in their extracellular region. Ig domain contains a sandwich-like structure known as Ig fold which is formed by two sheets of antiparallel beta strands (Chattopadhyay 2009). Members in this superfamily play a central role in both immune activation and immune regulation. Some members of this superfamily promote (costimulatory) T cell activation and differentiation, whereas other members exhibit opposing effects (coinhibitory) on the activation of T cells. Generally the transductions of stimulatory and inhibitory signals are mediated by two loosely conserved motifs in their cytoplasmic region termed ITAM and immunoreceptor tyrosine-based inhibition motifs (ITIM), respectively (Sharpe 2002).

However, in some circumstances, ITAM may propagate inhibitory signals and ITIM may transmit activation signals (Blank 2009; Waterman 2010; Barrow 2006). In addition, some molecules of Ig superfamily such as CD28 also play an important role in the homeostasis and function of a population of Tregs (Sansom 2006).

1.5.1.1 CD28/CTLA4–CD80/CD86 pathway

The CD28 molecule is a 44 kDa homodimeric transmembrane glycoprotein which consists of 202 amino acids. The human CD28 gene is localized on chromosome 2q33, and the mouse CD28 gene maps to chromosome 1 at band C (Lafage-Pochitaloff 1990; Howard 1991). The extracellular region (134 amino acids in length) of CD28 receptor comprises a single disulphide-linked V-like domain. A hexapeptide motif (MYPPPY) is essential for the interaction between CD28 and its ligands (Peach 1994; Boćko 2002). Forty one amino acids constitute the intracellular region of the CD28 receptor. This region contains four tyrosine residues that can be inducibly phosphorylated. They provide the binding sites for phosphatidylinositol 3-kinase (PI_3K) and growth factor receptor-bound protein 2 (Grb2), and involve the regulation of downstream signaling cascade (Zhou 1993; Schneider 1995; Truitt 1994; Cai 1995).

In humans, CD28 is constitutively expressed on the surface of all naïve T cells, 80–95% $CD4^+$ T cells, and approximately 50% of $CD8^+$ T cells. In contrast, CD28 is expressed by all $CD4^+$ and $CD8^+$ mouse T cells (June 1994; Hutchcroft 1994; Paterson 2009). CD28 expression has also been found on γδ T cells (Testi 1989), some human plasmablasts and plasma cells (Pellat-Deceunynck 1994; Kozbor 1987), and human fetal peripheral blood

natural killer (NK) cells (Nagler 1989). The expression of CD28 in humans is down-regulated with age and in chronic disease states, but it is not the case in mice.

CD80 (B7-1) and CD86 (B7-2) are two known ligands for CD28. CD80 is a 60 kDa type I transmembrane monomeric glycoprotein that is constituted by 262 amino acids. CD86 is also a glycoprotein with molecular weight of 70 kDa. It shares structural homology to CD80 (Freeman 1989; Schwartz 1992). The genes encoding CD80 and CD86 are localized to the same region in human chromosome 3q13.3-3q21 and 3q13-3q23 respectively (Freeman 1992; Fernández-Ruiz 1995). Both CD80 and CD86 molecules contain a single IgV-like domain and a single IgC2-like domain within their extracellular region. The amino-acid sequence analysis indicates that the SQDXXXELY motif in the immunoglobulin C-like domain is a putative CD28-binding sequence (Freeman 1991; Engel 1994; Fargeas 1995).

CD80 is expressed on activated APCs including B cells, macrophages and DCs. Activated T cells and FoxP3^{+} Tregs also express CD80. In contrast, CD86 is constitutively expressed on APCs, and the level of CD86 expression will be upregulated upon stimulation by inflammatory cytokines. In addition, CD86 is also expressed on activated T cells (Azuma 1993). Some studies indicated that CD86 probably acted as the major initial ligand for CD28, and CD80 was the preferential ligand for CD28 because CD80 possessed approximately 10-fold higher binding affinity compared to CD86 and appeared to be a more potent costimulus in terms of T cell activation (Fields 1998; Olsson 1998). However, another *in vitro* study indicated that CD86 was the dominated ligand to induce naïve T cells to become IL-4 producers (Freeman 1995).

When the TCR is properly engaged, CD28 costimulatory signal produced by the interaction between CD28 and its B7 ligands can result in a dramatic augmentation of the expression of genes induced by TCR signaling alone. The cooperative effects between TCR and CD28 signals will lead to full activation of T cells (Figure 1.2). CD28 signaling pathway involves numerous activities. It participates in the regulation of glucose metabolism (Frauwirth 2002), promotes the production of IL-2 (June 1989), mediates entry of T cells into the cell cycle (Appleman 2000), increases the generation of cytokines and chemokines (Thompson 1989), controls Treg cell homeostasis (Salomon 2000), and reinforces resistance to apoptosis (Sperling 1996). CD28 costimulation can lower the threshold of activation by decreasing the number of TCRs required for T cell activation (Viola 1996). Together with CD28 signal can also promote cytoskeleton reorganization to the TCR contact site (Viola 1999), these might partially explain how CD28 exerts its costimulatory effects.

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4, CD152) is a T cell surface molecule with high sequence and structural homology to CD28. It is a glycoprotein constituted of 223 amino acids. The genes for CTLA4 and CD28 are located on the same chromosomal region and share the same overall intron/exon organization. The human CTLA4 gene contains four exons. The exon 1 consists of the leader peptide sequence, and exon 2 to 4 encodes the ligand binding site, the transmembrane region, and the cytoplasmic tail respectively (Ling 1999). Between CTLA4 and CD28, there is about 20% identity at the gene structure level and about 30% identity at the amino acid level (Howard 1991, Greenwald 2005). Similar to CD28, the extracellular domain of CTLA4 also contains a hexapeptide motif MYPPPY which is required for ligand binding.

CTLA4 is mainly expressed on T cells (Brunet 1987). The expression of CTLA4 on the surface of B cells (Kuiper 1995) and thymocytes (Wagner 1996) has also been reported. Full-length CTLA4 is only detected on the surface of activated T cells, but not on resting T cells (Linsley 1992). Approximately 90% of CTLA4 exists as intracellular vesicles due to the rapid and constitutive endocytosis of surface CTLA4 (Alegre 1996). However, Treg is an exception. CTLA4 is constitutively expressed on the surface of Tregs. Studies also indicated that Tregs contained higher levels of CTLA4 than conventional T cells (Sakaguchi 2004; Tai 2012). A splice variant of CTLA4 known as ligand-independent CTLA4 (liCTLA4) that contains exons 1, 3, and 4, unlike full-length CTLA4, is expressed on murine resting T cells. However, the level of liCTLA4 expression is downregulated along with T cell activation (Vijayakrishnan 2004; Araki 2009).

Same as CD28, CD80 and CD86 are two main ligands for CTLA4. Although it is highly homologous to CD28, CTLA4 appears as a negative regulator on T cell function by switching off T cell activation, proliferation and IL-2 production (Krummel 1996; Walunas 1996). How CTLA4 exerts its inhibiting effects on T cells still remains uncertain. It may down-regulate T cell immunity via various ways. CTLA4 is thought to directly affect CD28–CD80/CD86 engagement by ligand competition because it binds to CD80 and CD86 with much higher affinity compared with CD28 (Linsley 1994; Greene 1996). CTLA4 can raise the threshold of TCR signaling for full activation of T cells (Chambers 2001). Moreover, CTLA4 coligation induces the activation of a cytosolic phosphatase and then blocks TCR-transmitted signal including to inhibit the formation of ZAP-70 containing microclusters (Harper 1991; Schneider 2007; Lee 1998). The CTLA4–CD80/CD86 interaction can induce the release of

indoleamine 2,3-dioxygenase (IDO). IDO can result in tryptophan catabolism and then suppress T cell proliferation (Boasso 2005; Fallarino 2003). CTLA4 on the surface of Treg cells can physically remove CD80 and CD86 from the surface of APCs through trans-endocytosis (Oderup 2006; Qureshi 2011).

1.5.1.2 ICOS –B7h pathway

The inducible T cell costimulator (ICOS, CD278) is a member of Ig superfamily. This T cell surface receptor is a CD28 homolog. The cDNA of ICOS shares approximate 30–40% sequence similarity with CD28 and CTLA4 (Hutloff 1999). Induced expression of ICOS is detected on $CD4^+$ and $CD8^+$ T cells when these cells are activated. In contrast, resting $CD4^+$ and $CD8^+$ T cells do not express ICOS (Coyle 2000). B7h (also known as ICOS-L, B7h-2, B7RP-1, and GL-50) is the ligand for ICOS. It is a transmembrane glycoprotein. Althoug B7h shares the homologous structure of CD80 and CD86 costimulatory molecules, B7h does not contain the unique SQDXXXELY motif (Chattopadhyay 2006). The expression of B7h is found on B cells, monocytes and DCs (Yoshinaga 1999). Some non-lymphoid tissues including lung and heart also express B7h (Swallow 1999). ICOS–B7h engagement can provide additional costimulatory signals for T cell activation, differentiation and cytokine production. These signals show synergistic effects with CD28 signaling on T cell activation. Unlike CD28 signaling which mainly promotes initial T cell activation, ICOS signaling plays an important role in the activation of antigen-experienced T cells (Yoshinaga 1999). The interactions between ICOS and its ligand B7h can promote B cells, Tregs, Th17, Th1 and follicular B helper T cells responses (Kornete 2012; Herman 2004; Smith 2009). ICOS is also involved T–B cell interactions, immunoglobulin class switching (Dong 2001) and splenic

germinal centre formation (Tafuri 2001). In addition, ICOS signaling can induce T cell expansion in a CD28-independent manner (Swallow 1999).

1.5.1.3 PD-1–PD-L1/PD-L2 pathway

Another member of the Ig superfamily is programmed death-1 (PD-1, CD279). PD-1 is a 50–55 kDa type I transmembrane protein. Unlike CD28 and ICOS as mentioned above, PD-1 exerts coinhibitory effects on T cell activation. Structurally, PD-1 shares 23% similarity with CTLA4 but does not contain the amino acid sequence MYPPPY (Freeman 2000). The expression of most members of the Ig family are restricted to T cells, whereas PD-1 is expressed on activated $CD4^+$ and $CD8^+$ T cells, Treg cells, NK cells, B cells, DCs, macrophages but not resting T cells (Agata 1996; Chemnitz 2004; Keir 2008). PD-1 has two known ligands termed PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) (Latchman 2001). PD-L1 is constitutively expressed on APCs, T and B lymphocytes, mast cells, mesenchymal stem cells and certain parenchymal tissues including heart, lung, kidney, pancreas, and placenta, ECs (Yamazaki 2002; Sharpe 2007; Liang 2003). The expression of PD-L1 is upregulated upon cell activation. Compared to PD-L1, the expression of PD-L2 is much more restricted. It is detected on DCs, macrophages, bone marrow-derived mast cells (Loke 2003). Recent study indicated that PD-L2 was also expressed on 50–70% resting peritoneal B1 cells (Zhong 2007).

PD-1 signaling is critical for the regulation of autoimmunity, tumor immunity, transplantation immunity, and infectious immunity. Similar to CTLA4, engagement of PD-1 by its ligands generates the potent inhibitory signals that suppress T cell activation, proliferation and

cytokine production by arresting the cell cycle in the G0/G1 phase (Carter 2002; Chemnitz 2004). Various studies (Parekh 2009; Francisco 2009) have demonstrated that PD-1 signaling is critical for the induction of anergy and the development of induced regulatory T cells (iTregs).

1.5.2 The TNF/TNF-R family

Numerous TNFs and TNF-Rs constitute another costimulatory molecule superfamily that is known as TNF/TNF-R family (Croft 2003). CD40L and CD40 are the first ligand-receptor pair of TNF/TNF-R family to be identified. Thereafter, the inventory of this family has been expanding rapidly. At present, 18 different genes that encode TNF have been identified in humans (Hymowitz 2007; Zhang 2004; Pradet-Balade 2002). The TNFs are important cytokines. All members of the family are type II transmembrane proteins, and they are homologous and share about 30% similarities (Bodmer 2002). According to their structures, TNFs are classified into three groups: conventional ligands (L-THD), EF-disulfide ligands (S-THD) and the divergent ligands (V-THD) (Compaan 2006; Won 2010). In humans, the TNF-Rs superfamily contains 29 reported members. Among them, 22 molecules belong to type I transmembrane receptors and the remaining 7 molecules are either type III transmembrane receptors or soluble receptors (Zhang 2004). TNF-Rs exhibit functional difference based on their structural diversities. TNF-Rs which contain a death domain (DD) motif with the length of approximately 80 amino acids can activate caspase cascades and result in apoptosis (Leist 1996). In contrast, some TNF-Rs possess a TNF receptor associated factor (TRAF)-binding sequence that recruits TRAF proteins. TRAF is involved in cell activation, differentiation, and

survival. Therefore signals from these TNF-Rs participate in the regulation of T cell activation instead of the induction of T cell apoptosis (Borst 1989).

Most TNF-Rs such as OX40, 4-1BB, and CD30 are expressed predominantly by activated T cells (de Jong 1991; Ellis 1993; Gramaglia 2000). Their ligands OX40L, 4-1BBL, and CD30L are expressed by activated APCs and can be detected within one to several days after activation (Croft 2003). These facts suggest that these costimulatory molecules are mainly involved in the effector and memory phases rather than the initial phase of T cell immune response. On the other hand, some costimulatory molecules such as the herpes virus entry mediator (HVEM) and its ligand LIGHT (homologous to **l**ymphotoxin, exhibits **i**nducible expression and competes with HSV **g**lycoprotein D for binding to **h**erpesvirus entry mediator, a receptor expressed on **T** lymphocytes, CD258) are constitutively expressed by naïve T cells and APCs, respectively (Morel 2000). It suggests that they play a role in the early activation of T cells and APCs.

1.5.2.1 CD40–CD40L pathway

CD40 is the first member of TNF-R superfamily. This cell surface receptor is a 48-kDa type I transmembrane glycoprotein (Stamenkovic 1989). The CD40 genes are located on chromosome 20 in humans and chromosome 2 in mice (Grimaldi 1992). CD40 is constitutively expressed on APCs and the expression level of CD40 could be dramatically upregulated upon activation of the cells. The expression of CD40 is also seen on some parenchymal cells such as ECs, fibroblasts, and smooth muscle cells (van Kooten 2000; Schönbeck 2001; Yellin 1995). The CD40 ligand (CD40L, CD154) belongs to the TNF

superfamily. It is a 32 to 33-kDa type II transmembrane protein (Armitage 1992). In both humans and mice, the genes that encode CD40L are located on the X chromosome (Villa 1994). CD40L is expressed on activated T cells ($CD4^+$ T lymphocyte lineage and a subset of $CD8^+$ T cells), NK cells, B cells, mast cells, eosinophils, basophils, DCs and platelets (Grewal 1998; Mach 1997). Studies have indicated that CD40L expressed on platelets is involved in the formation and stabilization of thrombi (André 2002). The CD40–CD40L signaling pathway is involved in both cellular and humoral immune responses (Figure 1.3). The receptor ligand interaction generates important signals to promote B-cell activation, proliferation, Ig production, Ig isotype switching, the generation of B-cell memory, and T cell activation (Foy 1996; Durie 1994; Kawabe 1994). CD40–CD40L engagement is a key step in APC activation. CD40-mediated activation of DCs is one of the mechanisms by which CD40 signaling promotes immune response. Upon CD40 ligation by CD40L, DCs mature and become activated. Activated DCs produce high levels of proinflammatory cytokines and chemokines, and upregulate the expression of MHC class II and other costimulatory molecules including CD80, CD86 (Yang 1996; van Kooten 1997; Ma 2009). All these factors contribute to the augmentation of immune response.

1.5.2.2 CD27–CD70 pathway

CD27 is a 120-kDa type I transmembrane homodimeric glycoprotein. As a member of the TNF-R family, this molecule was identified in human cells in 1987. Low level constitutive expression of CD27 is seen on naïve T cells, B cells, and a subset of NK cells. CD27 exists on resting T cells in the form of disulfide-linked homodimer with subunits of 50–55 kDa (p55). A soluble form of CD27 (sCD27) can be generated from the p55 form by proteolytic cleavage

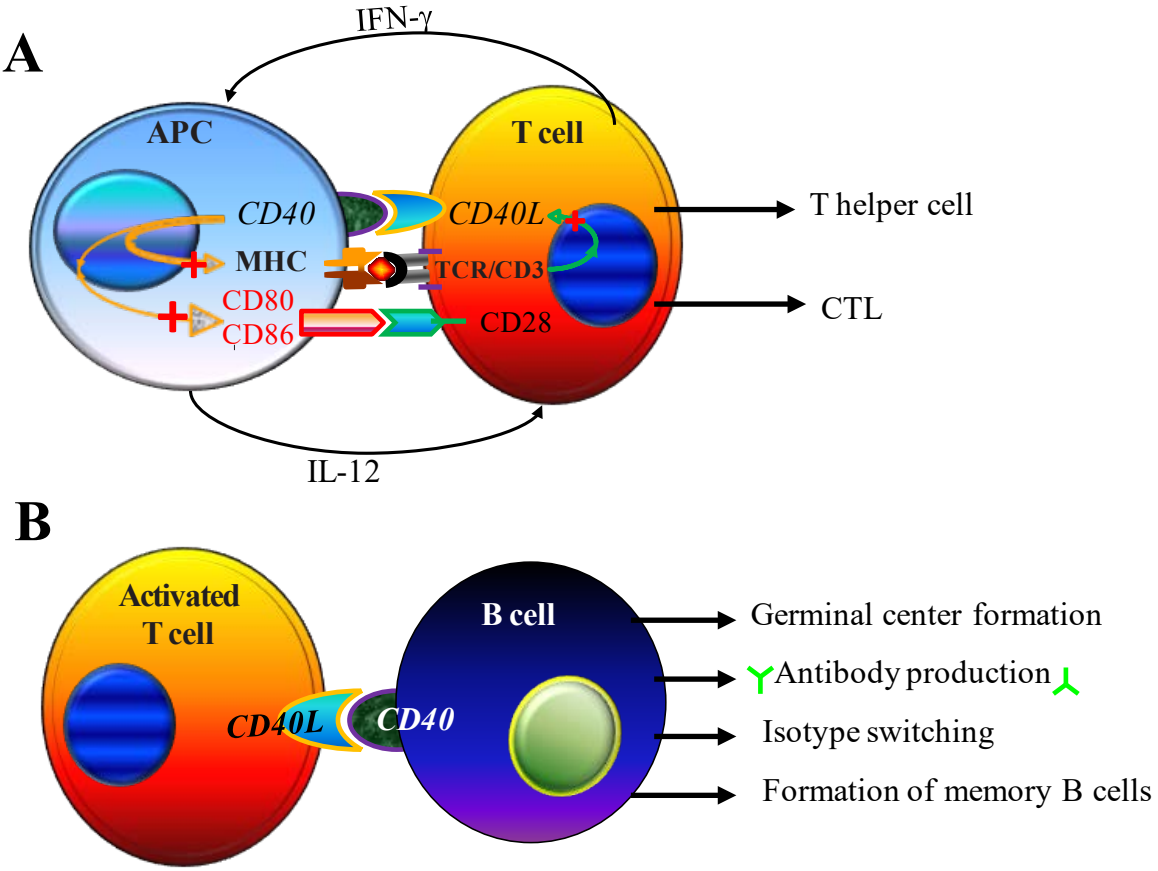


Figure 1.3 CD40–CD40L pathway plays a role in both cellular and humoral immune responses. The engagement of the CD40 and CD40L is critical for full activation of the T cell and T cell mediated effector functions. Upon CD40 ligation by CD40L, DCs become activated and the expressions of CD80, CD86 and MHC class II are upregulated. Activated DCs produce high levels of proinflammatory cytokines and chemokines. They jointly promote T cell activation and differentiation (A). CD40 signaling in B cells is involved in B cell proliferation, differentiation, immunoglobulin class switching, cytokine production, protection from apoptosis, development of GC B cells, and formation of long-lived plasma cells and memory B cells (B).

after T cell activation (van Lier 1987; Loenen 1992). Upon activation, the expression of CD27 is transiently upregulated on naïve and memory T cells (Hendriks 2000; Hintzen 1995). CD70, a unique ligand for CD27 belongs to TNF family. Initially it was found as a marker of Reed-

Sternberg cells in Hodgkin's disease and non-Hodgkin's lymphomas. CD70 is a homotrimeric type II transmembrane glycoprotein. Expression of CD70 is found on APCs, activated T cells and B cells. Epithelial cells in the thymic medulla constitutively express CD70 (Tesselaar 1997; Oshima 1998; Hintzen 1994). CD27–CD70 interactions promote T cell activation, proliferation and cytokine production. The costimulatory signals provided to T cells by CD27 engagement are CD28-independent. CD27–CD70 signaling pathway plays an important role in T cell–B cell interactions and T cell–DC communication. This pathway is involved in regulating B cell activation and T cell-dependent antibody production, and also participates in the NK-mediated innate immunity (Yamada 2005; Jacquot 2000; Taraban 2004). In addition, CD27 can trigger apoptotic cell death (Prasad 1997) by binding to a proapoptotic protein – CD27-binding protein (Siva).

1.5.2.3 OX40–OX40L pathway

OX40 is a membrane-bound member of TNF-R superfamily. This molecule was initially characterized as a T cell activation marker. It is a 50-kDa protein and is also known as CD134. OX40 is abundantly expressed on activated T cells rather than on naïve T cells. Generally OX40 is induced on T cells 12 to 24 hours after activation instead of immediately following the TCR ligation (Gramaglia 1998). The expression of OX40 is preferentially found on activated $CD4^+$ T cells rather than on $CD8^+$ T cells. Within few days, the level of expression of OX40 on activated T cells will be downregulated (Sugamura 2004). Th2 cell subset is the predominant group to express OX40, but Th1 cells can also express OX40 (Kim 2003; Roos 1998). In addition, OX40 is expressed on activated $CD4^+CD25^+$ Tregs. Ligation of CD28 by its ligand can promote the expression of OX40 although CD28 is not required for OX40

expression (Rogers 2001; Walker 1999). OX40L (CD252) is the ligand for OX40, and is a member of TNF superfamily. Structurally, human OX40L shares approximately 40% sequence identity with murine OX40L (Compaan 2006). OX40L is expressed on activated DCs, B cells, T cells, macrophages and inflamed vascular ECs (Gramaglia 1998; Lepisto 2007).

OX40–OX40L signaling pathway exhibits an extensive impact on the T cell immunity. OX40 signals play an important role in the survival and homeostasis of effector T cells, and in promoting the generation of memory T cells (especially memory $CD4^+$ T cells) (Gramaglia 1998; Rogers 2001). It is able to upregulate the expression of antiapoptotic proteins such as Bcl-xL and Bcl-2, and then inhibits apoptosis. OX40-deficient naïve $CD4^+$ T cells are capable of IL-2 production, cell division, and expansion. However, the colony expansion is at low level and the cells die through apoptosis after activation (Rogers 2001). OX40 engagement delivers signal to promote IL-17 production (Nakae 2003). OX40 and OX40L expression can be induced in the $CD8^+$ T cells which come from intraepithelial lymphocytes of the gut. This is considered to be related to cytotoxic effector function (Wang 2001). In addition, in activated $CD4^+CD25^+$ Tregs, OX40 stimulation can profoundly inhibit the expression of FoxP3 gene and therefore downregulate the expansion of Tregs (Vu 2007). OX40–OX40L interaction also enhances T cell-dependent B cell proliferation and differentiation, as well as the immunoglobulin production (Stüber 1995).

1.5.2.4 4-1BB–4-1BBL pathway

4-1BB (CD137) is a type I transmembrane glycoprotein. This costimulatory molecule is also known as tumor necrosis factor receptor superfamily member 9 (TNFRSF9) or "induced by lymphocyte activation" (ILA) in humans. The human 4-1BB consists of 255 amino acids with an N-terminal signal sequence (17 amino acids), an extracellular domain (169 amino acids), a transmembrane domain (27 amino acids), and a cytoplasmic domain (42 amino acids). The murine 4-1BB is composed of 256 amino acids and shares approximately 60% amino acid sequence identity with its human counterpart. From mouse 4-1BB intracellular domain to human 4-1BB intracellular domain, there are 5 conserved amino acid sequences (Zhou 1995; Kwon 1989). 4-1BB is mainly expressed on the surface of activated $CD8^+$ and $CD4^+$ T cells as a 30 kDa monomer and a 55 kDa dimer. The soluble forms of 4-1BB have also been reported. 4-1BB is also constitutively expressed on human monocytes, DCs, blood vessel ECs, B cells, $CD4^+CD25^+$ Tregs, and NK cells (Cannons 2001; Kienzle 2000; Broll 2001).

4-1BBL (CD137L), a member of TNF superfamily, is the ligand for 4-1BB. In mice, the 4-1BBL encoding gene is located on chromosome 17, and contains three exons and two introns. 4-1BBL is a 34-kDa type II transmembrane glycoprotein with a C-terminal extracellular domain (Goodwin 1993). It is primarily expressed on activated APCs including B cells, macrophages and DCs. T cells, hematopoieitic stem cells, myeloid progenitors, and tumor cells also express 4-1BBL (Lee 2008; DeBenedette 1995).

4-1BB receptor ligation by 4-1BBL generates a costimulatory signal for T cell activation, proliferation and cytokine production (Vinay 1998; DeBenedette 1997). 4-1BB costimulatory

signal could exert its effect in a CD28 independent manner and replace the role of CD28 signal on inducing T cell proliferation during early T cell expansion (Saoulli 1998). Studies have indicated that 4-1BB signal play an important role in the generation of cytotoxic T cells and in the maintenance of $CD8^+$ T cell memory (Shuford 1997; Sabbagh 2007). 4-1BB signal promotes cell survival by suppressing activation-induced cell death via the upregulation of anti-apoptotic protein c-$FLIP_{short}$ and Bcl-xL (Stärck 2005). On DC, 4-1BB signaling upregulates the expression of CD80 and CD86, and enhances IL-6 and IL-12 secretion (Futagawa 2002). 4-1BB promotes IL-8 and TNF-α production, but down-regulates the expression of IL-10 in monocytes (Kienzle 2000). It has also been obsevered that 4-1BB is involved in B cell survival and proliferation, as well as the production of TNF-α and -β (Zhang 2010). 4-1BB–4-1BBL engagement can also provide a reverse signaling to APCs. 4-1BBL signaling is important in monocyte activation, migration, survival and proliferation (Drenkard 2007; Langstein 2000). When human monocytes are cultured with a recombinant 4-1BB protein, 4-1BBL signaling induces monocyte to DC differentiation (Kwajah 2010). In DCs, 4-1BBL mediates the maturation of immature DC via autocrine, and promotes the expression of CD80, CD86, MHC class II and IL-12. 4-1BBL signal also plays a key role in the regulation of DC migration by enhancing expression of the CCR7 chemokine receptor. In addition, this signaling increases the capacity of DC in inducing antigen-specific T cell differentiation (Kim 2002; Lippert 2008).

1.5.3 The T cell immunoglobulin and mucin domain family

The TIM family refers to a group of emerging cell surface costimulatory molecules. Its alternative name is transmembrane immunoglobulin and mucin domain family. All members in TIM family are type I transmembrane glycoproteins containing an extracellular region, a transmembrane region, and an intracellular tail. TIM molecules possess a unique structural feature to distinguish them with other costimulatory molecules *i.e.*, in their extracellular domain, there are a heavily glycosylated mucin motif and an N-terminal Cys-rich immunoglobulin V motif (Freeman 2010). To date, in mice, the TIM gene family includes eight members named TIM-1 to TIM-8, and these genes are located on chromosome 11B1.1. In humans, three members (*i.e.,* TIM-1, TIM-3, and TIM-4) have joined the TIM gene family. They are found to be clustered in human chromosome 5q33.2 which is a region to be linked with multiple autoimmune and allergic diseases (McIntire 2004; Marsh 1994). The members of TIM protein family that have been identified include mouse TIM-1–4 and human TIM-1, TIM-3, and TIM-4.

TIM molecules are widely expressed on hematopoietic and nonhematopoietic cells including T cells, B cells, DCs, macrophages, mast cells, renal epithelial cells, and hepatocytes. They are involved in many immune responses such as promoting T cell activation, proliferation, cytokine production, and APCs activation (Umetsu 2005; Meyers 2005).

TIM-1 is expressed by activated T cells, mast cells, and a B cell subpopulation. Upon differentiation, Th2 cells are the predominant cells to express TIM-1 (Nakae 2007; Sizing 2007). Following the kidney ischemia/reperfusion injury, the expression of TIM-1 on tubular

epithelial cells is highly upregulated (Ichimura 1998). TIM-4, one of the ligands for TIM-1, is constitutively expressed on APCs (Meyers 2005). TIM-1–TIM-4 interaction can generate potent costimulatory signals to promote T cell proliferation, survival, and cytokine production (Umetsu 2005). In Tregs, TIM-1 signaling reduces the expression of forkhead box protein 3 (FoxP3) and thereby hinders Treg cell development (Degauque 2008). TIM-1 is also involved in modulating the phagocytosis of apoptotic cells (Ichimura 2008). TIM-3 is expressed on the surface of differentiated Th1 cells, some $CD8^+$ T cells, Th17 cells, mast cells, macrophages, DCs, microglias, NK and NKT cells (Hastings 2009; Khademi 2004; Anderson 2007). The ligands for TIM-3 are galectin-9 and PtdSer. Galectin-9, an S-type lectin, is expressed by mast cells, Tregs, T cells, B cells, macrophages, ECs, and fibroblasts (Wada 1997). Ligation of TIM-3 by galectin-9 leads to downregulation of Th1 responses by induction of cell death (Zhu 2005), inhibition of Th17 development, but promoting Treg generation (Seki 2008). TIM-3–PtdSer interaction plays an important role in the clearance of apoptotic cells (Nakayama 2009). In DCs and microglias, TIM-3 signaling enhances the expression of costimulatory molecules and cytokines (Anderson 2007). TIM-2 has not been found in the human genome. Mouse TIM-2 is a homologue of mouse TIM-1, and shares high sequence similarity with mouse TIM-1. TIM-2 is expressed on activated T cells, B cells, bile duct epithelial cells, and renal tubule cells (Kumanogoh 2002; Chen 2005). H-ferritin is a ligand for TIM-2. TIM-2–H-ferritin interaction is involved in extracellular H-ferritin endocytosis and iron transfer (Chen 2005). Another possible ligand for TIM-2 is class IV semaphorin 4A (Sema4A) which is a type I transmembrane protein found on the surfaces of DCs and B cells (Kumanogoh 2002). The TIM-2 protein function in the immune systems remains to be determined.

1.5.4 Cell adhesion molecules

Cell adhesion molecules (CAMs) are a group of cell surface proteins which modulate cell adhesion. Cell adhesion is the interaction process between cells and the surrounding extracellular matrix (ECM). It is essential for multiple cellular processes including cell migration, proliferation, morphogenesis, cell trafficking, gene expression, cell division and survival. CAMs are typically transmembrane proteins which consist of three domains: an extracellular domain, a transmembrane domain and a cytoplasmic tail. Their extracellular domains contain ligand binding sites that allow CAMs to engage with their ligands on other cells or within ECM, while their cytoplasmic tails are usually connected to the cytoskeletal proteins to obtain the intracellular anchors. Based on the molecular structure of these proteins, most CAMs can be classified into four main families: integrins, immunoglobulin superfamily (IgSF), selectins and cadherins. Another classification system is to divide CAMs into calcium dependent and calcium independent two different groups.

Integrins, one of the major groups of CAMs, firstly appeared in the article in 1986 although they are evolutionary old (Tamkun 1986). These molecules are heterodimeric transmembrane glycoproteins that contain two subunits: an α-chain and a β-chain. In mammalians, 18 α and 8 β subunits have been identified, and the noncovalently associated α subunit and β subunit combinations generate at least 24 different integrin heterodimers. The expression manners of integrins base on the cell type and environmental context. Most of them are widely expressed by nucleated cells; however, some integrins such as αIIbβ3 and α6β4 are restricted to be expressed in certain cell types (Hynes 2002). Integrin receptors bind to various ligands including ECM ligands, cell surface ligands, and soluble ligands. The divalent cations such as

Ca^{2+}, Mg^{2+}, and Mn^{2+} are important for the interactions between integrin receptors and their ligands. Integrins are critical to a number of biological processes such as cell migration, cell–ECM interactions, actin cytoskeleton organization, and embryonic development. The integrin–ligand interaction can provide both inside-out signaling and outside-in signaling, and the generated intracellular signals activate multiple signaling cascades to modulate cell proliferation, differentiation, motility, adhesion, and apoptosis (Schwartz 1995; Dedhar 1999). Integrins are also involved in T cell recirculation, antigen recognition, providing costimulatory signals, and stabilizing T cell–APC interactions during immune response (Dustin 1989; Shimizu 1990). Targeting integrins such as leukocyte function-associated antigen 1 (LFA-1) and very late antigen-4 (VLA-4) has been demonstrated to prolong allograft survival in the experimental transplantation models (Nicolls 2000; Paul 1993; Yang 1995).

IgSF is a very large family that includes numerous members. In humans, 765 genes that encode Ig domain have been identified via genome analysis (Lander 2001). Members of this superfamily all possess characteristic Ig-like domains consisting of 70–110 amino acids. These globular, loop structures are insensitive to proteases. According to the similarity to the variable and constant regions of antibodies, Ig-like domains are classified as 4 types *i.e.*, IgC1, IgC2, IgV, and IgI. Structurally, IgSF molecules mostly are type I transmembrane proteins. IgSF CAMs possess small transmembrane domains, whereas the lengths of their intracellular domains vary from case to case because many of them contain signaling motifs to interact with cytoskeletal elements (Barclay 2003). The ligands for IgSF CAMs are integrins or different IgSF CAMs, and they exhibit either homophilic or heterophilic receptor–ligand

interactions. IgSF CAMs are mainly involved in modulating cell–cell interactions. Some of them, like neural cell adhesion molecule (NCAM, CD56), vascular cell adhesion molecule 1 (VCAM-1, CD106), and T cell surface antigen T11/Leu-5 (CD2, LFA-2), play a role in immune response. In humans, LFA-2 is expressed on T cells, NK cells and most thymocytes. LFA-2 modulates adhesion between T cells and their target cells. In addition, it also transduces costimulatory signals when ligated by its ligand, LFA-3 (CD58) on APCs (Moingeon 1989). The costimulatory function of LFA-2 is particularly important for the memory T cell responses (Majeau 1994; Lo 2011). Alefacept, a fusion protein targeting LFA-2 signaling pathway, was shown synergistic effects with CTLA4-Ig on the prolongation of renal allograft survival in NHP transplantation model (Weaver 2009).

The cadherins are Ca^{2+} dependent CAMs. To date, there are more than 350 known members in this superfamily (Hulpiau 2009). Most cadherins are transmembrane glycoproteins that contain multiple cadherin domains in their extracellular regions. Each cadherin domain consists of about 110 amino acid residues (Takeichi 1995). Based on the sequence similarity of extracellular region, members of the cadherin superfamily are classified into different subfamilies: classical (type I) cadherins, atypical (type II) cadherins, flamingo cadherins, desmosomal cadherins, protocadherins, and solitary members (Nollet 2000). Almost all vertebrate tissues express cadherins, while the expression pattern of cadherin types is tissue-specific (Takeichi 1988). The cadherins mediate cell–cell adhesion via homophilic binding interactions. They are essential for tissue organization and morphogenesis, cell–cell recognition and cell sorting, cell migration, desmosome formation, and maintenance of normal tissue architecture (Ivanov 2001).

The selectins are a family of transmembrane carbohydrate-binding proteins containing C-type lectin-like domains in their N-terminal sections. They interact with glycoproteins and/or glycolipids via heterophilic binding activities. The selectins paly an important role in lymphocyte homing and circulating leukocytes to migrate to sites of inflammation (Geng 1990; Bevilacqua 1993). There are three known members in the selectin family: E-selectin, L-selectin, and P-selectin that are primarily expressed on activated ECs, leukocytes, and platelets, respectively.

1.6 Immunosuppressive strategies in organ transplantation

To avoid destruction of transplanted organs or tissues by host immune system, the clinically applied approaches include to reduce the antigenic differences between donor and recipient and to inhibit immune response against the grafted organs and tissues. The pre-transplantation tests including ABO blood typing, tissue typing and cross-matching *etc.* aim to minimize immunogenicity of allograft to the greatest extent. Peritransplantation and posttransplantation immunosuppression, usually to be achieved by administration of several different types of immunosuppressive drugs, is the key to promote allograft acceptance.

Medawar and his colleagues (Gibson 1943; Medawar 1944) indicated that allograft rejection was an immunologic event in the 1940s. Their research results inspired people in this field to explore the approach by suppression of host immunity to promote allograft survival. One of the means was to physically destroy the immune tissues and organs. Sublethal total body irradiation (TBI) was tested in a skin transplantation model in the 1950s (Dempster 1950).

Previous studies had demonstrated that TBI was able to suppress antibody production to bacteria. The rationale for this approach was that the immune response agaist the allograft could be minimized by generating generalized immunosuppression via ablation of the bone marrow. Cortisone, as another early attempt, was studied in a rabbit skin transplant model almost at the same time (Billingham 1951). Unfortunately, the effects of both two techniques were limited; the survival time of skin graft was prolonged for only a few days. Other therapeutic approaches targeting lymphocyte depletion such as splenectomy, thymectomy and thoracic duct drainage were also attempted (Starzl 1979). TBI was subsequently replaced by anti-cancer drugs including azathioaprine (AZA), methotrexate, actinomycin C and cyclophosphamide in the 1960s (Murray 1962; Goodwin 1963; Martin 1965). The mechanism of action of these myelotoxic agents was similar to TBI with the idea of inhibiting proliferation of donor-reactive cells. As with TBI, these agents also caused severe bone marrow depression and increased the risk of serious infections. The benefit of the myelotoxic agents was that, unlike TBI, the effects of these drugs could be reversible with drug withdrawal. The regimen of antimetabolites coupled with corticosteroids then became popular in clinical practice (Martin 1965; Starzl 1964). Corticosteroids were the main component of maintenance therapy, and in high dose, were the effective agents to reverse rejection crises (Goodwin 1963; Starzl 1963). The major drawback of corticosteroids was their multiple side effects including gastrointestinal bleeding, hypertension, infection, hyperglycemia, fluid retention, osteoporosis, hirsutism, weight gain, cataracts *etc.* Shortly afterward, heterologous antilymphocyte globulin (ALG) as a new immunosuppressive agent was introduced into transplantation realm (Starzl 1967a; Starzl 1967b). Compared to azathioprine and prednisone treated group, patients receiving treatment of ALG combined with azathioprine and

prednisone showed better graft function, less acute rejection episodes, improved graft survival, and reduction of the prednisone dose (Doak 1969; Taylor 1976). However, ALG was a heterologous antibody that had potential antigenic property, and this agent could non-specifically bind to various hematopoietic cells leading to different types of side effects including fever, hypotension, thrombocytopenia, anemia, neutropenia, and anaphylaxis *etc* (Rosenberg 1975; Henricsson 1977; Grant 1995). Polyclonal ALG was typically made in rabbits or horses. Batch-to-batch variation in potency was another problem because the method of preparation of ALG was not standardized. These drawbacks of ALG limited its application in clinical practice.

In 1976, a novel antilymphocytic agent termed cyclosporine that was extracted from the soil fungi *Cylindrocarpon lucidum* and *Trichoderma polysporum* (Dreyfuss 1976), was found to be able to prolong skin allograft survival and to delay the onset of graft-versus-host disease (GVHD) (Borel 1976). The anti-rejection effects of cyclosporine A (CsA) were subsequently demonstrated in various organ transplantation studies (Calne 1978; Calne 1979). CsA can inhibit the expression of several genes that are critical in T cell activation, and thus prevent T cell responses to alloantigen.

Comparison of the effects of corticosteroids and AZA on immune system, CsA is a relatively selective immunosuppressive agent. CsA exhibited potent immunosuppressive effect even in low dose, and its risk of bone marrow suppression was less than previous immunosuppressive drugs. The results of organ transplantation were dramatically improved from the wide use of this agent in clinical practice (Starzl 1980; Starzl 1981; Canafax 1983). The regimens based

on CsA became the standard clinical therapeutics for the prevention of acute rejection in solid organ transplantation in the 1980s. In 1989, tacrolimus, an extract of the soil fungus *Streptomyces tsukabaensis* that displayed much more potent immunosuppressive ability (Kino 1987), was introduced into clinical practice as rescue therapy for the liver transplant recipients who had failed with conventional immunosuppression (Starzl 1989). Tacrolimus, similar to CsA, was found to inhibit early events in T cell activation by suppressing calcineurin. Therefore both of them were called calcineurin inhibitors (CNIs). This novel agent showed better clinical efficacy than CsA (Busuttil 2004; Haddad 2006), and has gradually replaced CsA in many transplant programs. Also in the late 1980s, a mTOR inhibitor termed sirolimus joined the family of anti-rejection medications (Saunders 2001; Geissler 2010; Klintmalm 2014). Sirolimus inhibits a protein called mammalian target of rapamycin (mTOR) and subsequently blocks cell cycle progression from the late G1 to the S phase (Terada 1993; Crespo 2002). As a result, the growth and proliferation of T cells are suppressed. At almost the same period, some novel agents such as the anti-CD3 monoclonal antibody (mAb) Orthoclone OKT3 (muromonab-CD3) (Thistlethwaite 1984; Thistlethwaite 1987) which aims at all T cells (naïve or activated), and the antimetabolites mycophenolate mofetil (MMF) which targets all lymphocytes (selective inhibition of T and B lymphocytes purine nucleotides synthesis) (Sollinger 1992; Sollinger 1995), were added to the growing list of immunosuppressive drugs.

The use of CNIs has tremendously improved the short-term outcomes of solid organ transplantation due to the incidence of acute rejection was substantially decreased. However, the improvement of long-term survival rates in solid organ transplantation has been

disappointing in the past decades (Meier-Kriesche 2004). Although the long-term outcome of organ transplantation can be influenced by a lot of factors, evidence does suggest that the side effects of immunosuppressive drugs (especially CNIs) play a role in late graft loss (Nankivell 2003; Stratta 2005; Alam 2007). Long-term use of CNIs is associated with higher risk of hypertension, post-transplant diabetes, nephrotoxicity and neurotoxicity *etc*. These adverse effects of CNIs have been found to cause graft loss or death with functioning graft. To minimize the negative impact of CNIs on the long-term outcome of organ transplantation, various regimens to reduce CNIs exposure such as CNI minimization, avoidance, and early withdrawal have been tested. Combined use of a CNI and other immunosuppressive drugs that are synergistic is one of the most effective ways to reduce CNI exposure. The synergistic effect refers to a combination of two or more drugs produces the overall effect that is greater than the sum of their individual effects. Low-dose tacrolimus in combination with MMF and steroids is one example of CNI minimization protocols (Ekberg 2009). In CNI avoidance regimens, MMF and mTOR inhibitors are mostly chosen to replace the role of CNIs in maintenance therapy. However, the reported results from CNIs sparing trials are mixed and there are no concrete evidence to support the elimination of CNIs from this field (Larson 2006; Mathis 2014; Goralczyk 2012). To date, CNIs remain the cornerstone in posttransplantation immunosuppresion in clinical practice. In addition, all conventional immunosuppressive drugs are antigen nonspecific agents. They suppress the function of all lymphocytes or all T cells instead of selectively abolishing allogeneic immune responses. The non-specific immune suppression of these agents increases the risk of infection and malignancy that are also the major causes of death with graft function (Kahwaji 2011). Hence there is a need to seek novel

immunosuppressive strategies which specifically inhibit the alloimmune response initiated by donor antigens while preserving the integrity of the remainder of the host immune system.

1.7 Costimulatory signaling pathways as novel targets for immunosuppression

The notion that costimulatory signals are critical for the activation of T cells initiates the attempt by blocking costimulatory pathway to prevent allograft rejection (Lenschow 1992; Turka 1992). The rationale for this therapeutic approach is based on, in the absence of effective costimulation, interaction between TCR and MHC-bound peptides could induce T cells into a state of unresponsiveness to antigenic stimulation instead of activation (Schwartz 1990). Thus the allorejection is interrupted because of the missing key component of the event. The actions of costimulation blockers are mainly focused on the specific signaling pathways and the T cells activated by TCR stimulation. Compared to the conventional immunosuppressive drugs, they modulate alloimmune response in a more selective and precise way. Targeting costimulatory pathways offers the potential to inhibit pathologic T cells while to retain protective T cell responses as much as possible. CD28-deficient mice showed impaired immune responses to viral antigens or alloantigens (Shahinian 1993; Kawai 1996). In contrast, their responses to exogenous antigens were normal (Wu 1998). Blockade of CD28 signal with anti-CD28 mAb JJ319 in rats could successfully inhibit allogeneic T cell responses, whereas responses to exogenous antigens were unaffected (Haspot 2002). Costimulation blockers mostly are large molecule biologics. Their effects are mainly limited

to T cells and/or APCs. These features will be helpful for minimizing their side-effects on other organs and systems.

In 1986, the costimulatory molecule CD28 was identified. Since then, a lot of novel costimulatory molecules have been discovered and various costimulatory pathways have been tested as targets to inhibit alloimmune responses (Table 1.1). CD28–CD80/86 and CD40–CD40L are two well-characterized costimulatory signaling pathways. Numerous agents targeting these two pathways emerged over the past several decades. Some of them such as anti-CD40L mAbs and cytotoxic T lymphocyte antigen 4-immunoglobulin G (CTLA4-Ig) that competitively blocks CD28–CD80/86 interactions showed promising results in the prolongation of allograft survival in experimental animal models of transplantation. Development of anti-CD40L mAbs was discontinued because those agents were associated with thromboembolic complications. On the other hand, belatacept, a high-affinity variant of CTLA4-Ig, was demonstrated to promote allograft acceptance in further studies and clinical trials (Larsen 2005; Durrbach 2010). In 2011, belatacept was approved by the U.S. Food and Drug Administration (FDA) for use as an immunosuppressant to prevent acute rejection in adult patients who undergo renal transplantation.

The agents targeting costimulatory molecules other than CD28–CD80/86 and CD40–CD40L pathways have also shown promise in prolonging allograft survival. It has been reported that TS-1/22, an anti-LFA-1 antibody, dramatically prolongs islet allograft survival when it is combined with basiliximab and sirolimus. Moreover, the combined use of TS-1/22 and belatacept exhibits synergistic effect on promoting islet allograft acceptance in nonhuman

primates (Badell 2010). OX40–OX40L costimulatory pathway have been found to play a critical role in CD28- and CD40-independent allograft rejection. Blocking OX40–OX40L pathway with anti-OX40L mAb (RM134L) extended skin allograft survival in CD28/CD40L double knockout mice. RM134L in combination with murine CTLA4-Ig (m CTLA4-Ig) and anti-CD40L mAb (MR1) showed much longer skin allograft survival time (>100 days) than m CTLA4-Ig and MR1 combination therapy as well as RM134L monotherapy (<15 days) in wild type C57BL/6 mice (Demirci 2004). Further studies demonstrated that RM134L was able to induce islet allograft tolerance in CD154 deficient mice. In addition, combined use of RM134L and MR1 in wild type C57BL/6 mice also yielded long-term islet allograft survival (Chen 2008).

1.8 Hypothesis and objectives of this thesis

The studies of costimulatory blockade in transplantation have been carried out more than two decades. Various costimulatory blockers that target different costimulatory signaling pathway have been tested. Belatacept is the only authorized agent targeting costimulatory molecules for clinical use in organ transplantation up to now. This biologic agent shows its advantage with similar graft survival rate and superior graft function relative to CNI-based regimen. However, higher rates of acute rejection in belatacept-based regimens implicate that there is still a need for improvement.

Since the studies of anti-CD40L treatment were halted, anti-CD40 mAbs as an alternative approach have been investigated for many years. Anti-CD40 mAbs are expected not to cause

thromboembolic complications because CD40 do not involve platelet activation and the stability of platelet thrombi as CD40L do. To date, various anti-CD40 agents have been tested, and the efforts to seek an optimal anti-CD40 mAb for the prevention of transplant rejection are still ongoing. ASKP1240 is a novel fully human anti-CD40 mAb. Previous studies have demonstrated that ASKP1240 monotherapy could successfully prolong allograft survival, and the results of those studies also suggested that ASKP1240 need to be combined with additional immunosuppressive agents. Some studies have indicated that CNIs antagonize the effects of anti-CD40L mAbs, it raises the question of whether anti-CD40 mAbs are applicable for CNI minimization protocols. A main aim of this thesis is to test the hypothesis that ASKP1240 in combination with conventional immunosuppressive drugs including CNI can produce synergistic effects on promoting renal allograft survival, and does not cause thrombolytic complication. Meanwhile, the pharmacokinetics (PK) of ASKP1240 in monkey kidney transplant recipients are also observed.

Current CTLA4-Igs including belatacept display high binding affinity for CD80. Between the two natural ligands for CD28, compared to CD80, CD86 exhibits higher binding affinity for CD28 and is the dominating ligand to mediate costimulatory signals in alloimmune response. Improvements in CD86 binding affinity confer increased immunosuppressive potency of CTLA4-Igs. Therefore, we hypothesize that ASP2409, a novel CTLA4-Ig variant with more specific binding to a CD86 costimulatory molecule, has improved efficacy in preventing kidney allograft rejection. To this end, we thus decided to investigate the effects of ASP2409 on acute renal allograft rejection and graft survival in a NHP kidney transplantation model. At the same time, the PK and pharmacodynamic (PD) profiles of ASP2409, as well as the

influence of ASP2409 in intragraft Tregs are determined to provide valuable information for clinical trials.

Table 1.1 Costimulation blockers in organ transplantation

Agent	Target	Model	Regimen	Graft Survival (days)	Ref.
Anti-CD28-PV1-IgG3	CD28	Heart/Rat	Anti-CD28 (1.2 mg/kg)	14, 22, 23, 26, 36	Jang 2008
			Anti-CD28 (2.5 mg/kg)	26, 27, 31, 31, 39	
			Anti-CD28 (5.0 mg/kg)	26, 60, 61, >100, >100	
			Anti-CD28 (1.2 mg/kg) + tacrolimus	24, 24, 32, 46, >100	
			Tacrolimus	9, 10, 12, 14, 14	
			Untreated	6, 6, 7, 7, 7	
FR104	CD28	Kidney/Baboon	FR104	14, 16, 21, 28	Poirier 2015
			FR104 + MMF + Cs	21, 49, 158, 291	
			FR104 + tacrolimus	77, 77, 84, 112	
			FR104 + rapamycin	84, 91, 123	
			Untreated	6, 6, 6, 7	
B7–24 1G10	CD80 CD86	Kidney/Rhesus	B7-24 + 1G10	21, 22, 34, 35	Ossevoort 1999
			B7-24 + 1G10 + CsA	18, 53, 59, 225	
			CsA	25, 69, 74, 266, 312	
h1F1 h3D1	CD80 CD86	Kidney/Cynomolgus	h1F1 + h3D1	9, 48, >119, >119	Hausen 2001
			h1F1 + h3D1 + CsA	96, >119, >119, >119	
			h1F1 + h3D1 + CsA + prednisone	50, 57, 59, 59	
			CsA	22, 25, 38, 71	

(continued)

Table 1.1 Costimulation blockers in organ transplantation (continued)

Agent	Target	Model	Regimen	Graft Survival (days)	Ref.
CTLA4-Ig	CD80 & CD86	Kidney/Rhesus	CTLA4-Ig	8, 8, 8, 58	Larsen 2005
			LEA29Y	38, 39, 45, 99, 134	
LEA29Y (belatacept)	CD80 & CD86		LEA29Y + MMF + methylprednisolone	39, 45, 155, 221, 375	
			MMF + methylprednisolone	8, 25, 36, 50	
			LEA29Y + basiliximab	28, 116, 120, 129, 130, 145	
hu5C8	CD40L	Kidney/Rhesus	hu5C8	>73, >85, >136, >143, 173, >206, >506, >519, >527	Kirk 1999
			hu5C8 + MMF + steroids	36, 39, >272, >283, >295	
			hu5C8 + tacrolimus	73, 148, >199	
			hu5C8 + dacluzimab	74, 298, 428, 479	Xu 2003
ABI793	CD40L	Kidney/Cynomolgus	ABI793 (7 mg/kg)	8, 8, 9, 9	Schuler 2004
			ABI793 (20 mg/kg)	23, 30, 32, 70, 108, 148, 206, 266, 328	
		Kidney/Rhesus	ABI793 (20 mg/kg)	13*, 44, 139, 149, 154, 158, 221	Kanmaz 2004

(continued)

Table 1.1 Costimulation blockers in organ transplantation (continued)

Agent	Target	Model	Regimen	Graft Survival (days)	Ref.
IDEC-131	CD40L	Skin/Rhesus	IDEC-131	10, 16	Xu 2003
			IDEC-131 + sirolimus	84, 246	
			IDEC-131 + sirolimus + DST	33, 202, 246	
		Kidney/Rhesus	IDEC-131	3, 7, 21, 44, 352	Preston 2005
			IDEC-131 + sirolimus	9, 9, 42, 108, 178	
			IDEC-131 + sirolimus + DST	168, 269, >559, >993, >1012	
			Sirolimus	10, 21, 23	
		Kidney/Cynomolgus	IDEC-131 + sirolimus + DST	108, 153, 177	Pearl 2007
H106	CD40L	Kidney/Rhesus	Chi220	30*, 41, 56, 70	Pearson 2002
			CTLA4-Ig	8, 8, 8, 58	
Chi220	CD40		Chi220 + CTLA4-Ig	28, 35, 50, 84, 91	
			H106 + CTLA4-Ig	35, 36*, 42*, 129, 290	
Chi220	CD40	Islet/Pig-to-rhesus	Chi220+ basiliximab + sirolimus + belatacept	56, >59, >203	Thompson 2011
H106	CD40L		H106 + basiliximab + sirolimus + belatacept	30, 76, >86, >147, >169, 194, 344	
			Basiliximab + sirolimus + belatacept	26, 33, 71	
			Untreated	15, 15	
AH.F5	CD40L	Small bowel/Rat	AH.F5	14, 15, 16, 18, 19	Fishbein 2002
			AH.F5 + DST	41, 50, 88, 116, 119	
			Untreated	8, 9, 11, 12, 12	

(continued)

Table 1.1 Costimulation blockers in organ transplantation (continued)

Agent	Target	Model	Regimen	Graft Survival (days)	Ref.
MR1	CD40L	Heart/Mouse	MR1	30, 48, >75, >75, >75, >75, >75, >75, >75, >75	Larsen 1996
			Untreated	12, 12, 12, 12, 13, 16, 18	
			MR1 + DST	37, >60, >60, >60, >60, >60, >120, >120, >120	Valujskikh 2002
			MR1 + DST + ATT (B10.A)	18, 23, 23, 26	
			ATT (B10.A)	10, 12, 12, 14	
			MR1 + DST + ATT (SJL)	>70, >80, >80	
			MR1 + DST + CD4 ATT (B10.A)	20, 25, 28	
			MR1 + DST + CD8 ATT (B10.A)	23, 29, 30	
			Untreated	11, 13, 14, 15, 15, 16	
ch5D12	CD40	Kidney/Rhesus	ch5D12 (low)	8, 30	Haanstra 2003
			ch5D12 (high)	12, 42, 91, 135, 217	
			ch5D12 (low) + chFun-1	7, 71	
chFun-1	CD86		ch5D12 (high) + chFun-1	61, 75, 78, 116	
			CsA	25, 69, 74, 266, 312	
			Untreated	5, 6, 6, 7	

(continued)

Table 1.1 Costimulation blockers in organ transplantation (continued)

Agent	Target	Model	Regimen	Graft Survival (days)	Ref.
3A8	CD40	Islet/Rhesus	3A8 + basiliximab + sirolimus	155, 158, 202, 208, 312	Badell 2012
			3A8	7, 9	
2C10R4	CD40	Islet/Rhesus	2C10R4 + basiliximab + sirolimus	163, 265, 296, 304	Lowe 2012
			Basiliximab + sirolimus	8, 8, 10	
		Heart/Pig-to-baboon	3A8 + anti-CD20 + ATG + CVF + MMF + Cs	21, 21, 28	Mohiuddin 2013
			2C10R4 + anti-CD20 + ATG + CVF + MMF + Cs	>30, >40, 60, 107, 146, 149	
			2C10R4 + anti-CD20 + ATG + CVF + MMF + methylprednisolone	146*, 159, 298, 616, 945	Mohiuddin 2016
CFZ533	CD40	Kidney/Cynomolgus	CFZ533	76, 98, 100, 100, 100	Cordoba 2015
ICOS-Ig	ICOS	Kidney/Rhesus	ICOS-Ig	6, 6, 6	Lo 2015
			ICOS-Ig + belatacept	36, 44, 45	
			Belatacept	44, 71, 385	
			Untreated	5, 6, 7, 7, 8	
JTT.1	ICOS	Liver/Rat	JTT.1	10, 10, 11, 11, 12, 13	Guo 2004
			JTT.1 + tacrolimus	35, 38, 41, 45, 47, 49, 56, 58, >100, >100, >100	
			Tacrolimus	19, 22, 24, 26, 30, 42, 45	
			Untreated	10, 11, 11, 11, 12, 12	

(continued)

Table 1.1 Costimulation blockers in organ transplantation (continued)

Agent	Target	Model	Regimen	Graft Survival (days)	Ref.
4D11 (ASKP1240)	CD40	Kidney/Cynomolgus	4D11 (1 mg/kg, induction)	31, 64, 69	Aoyagi 2009
			4D11 (5 mg/kg, induction)	75, 79, 317	
			4D11 (10 mg/kg, induction)	92, 107, 320	
			4D11 (20 mg/kg, induction)	90, 105, 169	
			4D11 (1 mg/kg, maintenance)	10, 79, 80	
			4D11 (5 mg/kg, maintenance)	43, 98, 220	
			4D11 (10 mg/kg, maintenance)	27, 254, 373	
			4D11 (20 mg/kg, maintenance)	153, 169, 216	
			Untreated	5, 6, 7	
		Liver/Cynomolgus	ASKP1240 (10 mg/kg, induction)	11, 90, 188, 209	Oura 2012
			ASKP1240 (10 mg/kg, maintenance)	19, 31, 98, 168, 272, 278, 1035	
			Untreated	4, 6, 7	
		Liver/Cynomolgus	ASKP1240 (10 mg/kg, induction)	>15, >23, 210, 250, >608	Watanabe 2013
			ASKP1240 (10 mg/kg, maintenance)	>96, >115, 523, >607	
			Untreated	7, 8, 9	
OX86	OX40	Islet/Mouse	OX86 + CD154 KO	17, 18, 25, 25, 34	Chen 2008
			RM134L + CD154 KO	>150, >150, >150, >150, >150, >150, >150, >150	
RM134L	OX40L		MR1 + RM134L	>150, >150, >150, >150, >150	
			CD154 KO	13, 17, 17, 37, 82, 104	
			Untreated	12, 15, 15, 17, 22	

(continued)

Table 1.1 Costimulation blockers in organ transplantation (continued)

Agent	Target	Model	Regimen	Graft Survival (days)	Ref.
OX40-Ig	OX40	Heart/Mouse (BALB/c to CBA/Ca)	OX40-Ig, 150μg	8, 8, 9, 9, 10, 10	Curry 2004
			OX40-Ig, 400μg	8, 8, 8, 9, 9	
			OX40-Ig + CTLA4-Ig	10, 11, 11, 12	
			OX40-Ig + sIL-15Rα	9, 10, 12, 12, 12	
			Mouse IgG	7, 7, 8, 8, 9, 9	
		Heart/Mouse (B10.BR to CBA/Ca)	OX40-Ig, days 0, 2, 4, 6, 8, 10	81, 87, 88, 88, 88, 94, 100, 100, 100, 100, 100	
			OX40-Ig, days 0–15	71, 96, 96, >100, >100, >100, >100, >100, >100, >100, >100	
			Mouse IgG	13, 13, 13, 14, 14, 14, 15	
RM134L	OX40L	Skin/Mouse (CD4KO)	RM134L	10, 12, 18, 20	Vu 2004
			RM134L + CTLA4-Ig + MR1	82, 98, 98, >100, >100	
			CTLA4-Ig + MR1	11, 15, 19, 22, 24	
			Control	11, 11, 11, 12, 12, 13, 14, 15	
		Skin/Mouse (CD8KO)	RM134L	8, 8, 10, 12, 12	
			RM134L + CTLA4-Ig + MR1	70, 78, 78, >100, >100, >100, >100, >100	
			CTLA4-Ig + MR1	12, 18, 22, 24	
			Control	7, 7, 7, 8, 8, 8, 10	

(continued)

Table 1.1 Costimulation blockers in organ transplantation (continued)

Agent	Target	Model	Regimen	Graft Survival (days)	Ref.
M-17.5.2	LFA-1	Islet/Mouse	MR1 + CTLA4-Ig	4, 4, 6, 7, 8, 8, 16	Diab 2010
			M-17.5.2 + MR1 + CTLA4-Ig	4, 5, 6, 7, 7, 8, 13, 14, 17, 26	
			Isotype control	0, 0, 4, 5, 8, 9, 13, 14	
			Naïve	7, 14, 14, 15, 29	
TS-1/22	LFA-1	Islet/Rhesus	TS-1/22 + belatacept	73, 223, 338, >367, >373	Badell 2010
			TS-1/22	10, 12	
			Belatacept	8, 58, 60	
			TS-1/22 +basiliximab + sirolimus	>17, 67, 154, >371, >375	
			Basiliximab + sirolimus	8, 8, 10	
4-1BBL mAB	4-1BBL	Rat/Liver	4-1BBL mAB	9, 12, 16, 18, 19, 20, 20, 24	Qin 2010
			Isotype mAB	7, 8, 10, 11, 11, 12, 14, 14	
3H3	4-1BB	Corneal/Mouse	3H3 (200 mg)	6, 7, 8, 8, 8, 8, 8	Asai 2007
			3H3 (100 mg)	8, 9, 9, 10, 11, 12	
TKS-1	4-1BBL		TKS-1 (200 mg)	10, 11, 11, 12, 12, 13	
			TKS-1 (100 mg)	8, 8, 11, 12, 12, 15	
			Rat IgG (200 mg)	9, 10, 10, 10, 12, 14, 15	

ATT, adoptive transfer of T cells from skin-grafted-primed mice;

Cs, corticosteroids;

CVF, cobra venom factor;

DST, donor-specific transfusion;

*, died of complications

Chapter 2

Effects of ASKP1240 Combined with Tacrolimus or Mycophenolate Mofetil on Renal Allograft Survival in *Cynomolgus* Monkeys

Effects of ASKP1240 Combined with Tacrolimus or Mycophenolate Mofetil on Renal Allograft Survival in *Cynomolgus* Monkeys

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Summary: In this paper, we evaluated the efficacies of ASKP1240, monotherapy or in combination with conventional immunosuppressive drugs, on the prevention of acute rejection and the prolongation of graft survival in a nonhuman primate renal transplantation model. The PK and PD profiles of ASKP1240 in transplanted monkey and the side effects of this agent were observed simultaneously.

Author Contributions: Lijun Song performed experiments, processed and analyzed data, drafted manuscript; Huifang Chen directed the experiment design and participated in experiments; Anlun Ma performed experiments, processed and analyzed FACS data; Fumitaka Kinugasa, Yuji Sudo, Yasuhiro Miyao, Kazumichi Okimura, Toru Miura participated in experiment design; Hao Dun, Yanxin Hu, Lin Zeng, Jieying Bai, Guangzhou Zhang participated in the performance of the research; Pierre Daloze provided critical revision of the article for important intellectual content.

Effects of ASKP1240 Combined with Tacrolimus or Mycophenolate Mofetil on Renal Allograft Survival in *Cynomolgus* Monkeys

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Keywords: ASKP1240, costimulation blockade, kidney transplantation, nonhuman primate

Abstract

Background. Blocking the CD40–CD40L signal pathway has previously shown promise as a strategy to prevent allograft rejection. In this study, the efficacy of a novel fully human anti-CD40 mAb — ASKP1240, administered as a mono- or combination-therapy (sub-therapeutic dose of tacrolimus or mycophenolate mofetil [MMF]), on the prevention of renal allograft rejection was evaluated in *Cynomolgus* monkeys.

Methods. Heterotopic kidney transplants were performed in ABO compatible, stimulation index ≥ 2.5 in the two-way mixed lymphocyte reaction (MLR) monkey pairs. Animals were divided into twelve groups and observed for maximum of 180 days. Histopathological, hematology and biochemistry analyses were conducted in all groups. Cytokine release (IL-2, 4, 5, 6, TNF and IFN-γ) was investigated in several groups.

Results. ASKP1240 prolonged renal allograft survival in a dose-dependent manner in monotherapy. Low (2 mg/kg) or high (5 mg/kg) dose ASKP1240 in combination with MMF (15 mg/kg) or tacrolimus (1 mg/kg), showed a significantly longer allograft survival time compared with monotherapy groups. No obvious side effects including drug-related thromboembolic complications were found. Cytokine release was not induced by ASKP1240 administration.

Conclusions. The present study indicates that ASKP1240, either alone or in combination with other immunosuppressive drugs, could be a promising anti-rejection agent in organ transplantation.

Introduction

Costimulatory signals play crucial roles in fully activating T cells that are involved in allograft rejection (Jenkins 1987; Jenkins 1988; Frauwirth 2002). The CD40–CD40L costimulatory pathway, has previously been shown to be an important interaction that mediates both the humoral and cellular immune responses (Eliopoulos 2000; Pype 2000; Li 2008; Elgueta 2009). It has been demonstrated that blocking CD40–CD40L signaling pathway is a successful strategy to reduce allograft rejection (Larsen 1996; Parker 1995; Kirk 1999). CD40L was initially chosen as primary therapeutic target, but the further studies encountered setbacks due to the unexpected thromboembolic complications associated with the administration of anti-CD40L antibodies (Kawai 2000; Schuler 2004; Koyama 2004; Kirk 2001). Targeting CD40 as an alternate therapeutic strategy was developed when it was recognized that these thrombotic complications were CD40-independent (André 2002; Crow 2003). To date, several anti-CD40 antibodies that are engineered as IgG1 or IgG4 chimeric isotypes, or fully human IgG4 have been shown to prevent allograft rejection in experimental studies (Haanstra 2003; Pearson 2002; Imai 2007; Badell 2012; Page 2012).

ASKP1240 is an anti-CD40 mAb consisting of fully human IgG4. It interrupts the CD40–CD40L pathway by preventing the interaction between CD40 and CD40L. This masking antibody was generated from trans-chromosome mice. As a fully human mAb, ASKP1240 is expected to have lower immunological risk than mouse or chimeric antibodies. In addition, ASKP1240 does not cause antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) (Imai 2007; Okimura 2014; van der Pol 1998). ASKP1240 monotherapy has been demonstrated to delay renal, islet and hepatic allograft

rejection in nonhuman primate (NHP) models (Imai 2007; Aoyagi 2009; Watanabe 2011; Oura 2012).

This study evaluated the effects of ASKP1240 when administered as a monotherapy or in combination with a sub-therapeutic dose of tacrolimus or mycophenolate mofetil (MMF), on renal allograft survival in NHPs. We also assessed the pharmacokinetic (PK) profile of ASKP1240 and its effect on cytokine release in transplanted monkeys. We found that ASKP1240 significantly prolonged allograft survival either as a monotherapy or in combination with tacrolimus or MMF without causing obvious side effects.

Results

Renal allograft survival

Renal allograft survival day was recorded according to the following definitions: 1) The final day that the serum creatinine (sCr) level did not exceed a value of 10 mg/dL; 2) The day when significant asthenia had been observed; 3) A day before the finding of death; 4) Animal that survived to the end of study was described as >180 days. As shown in Table 2.1, the median survival times (MSTs) were 7 days for the control group, 10 days for the low-dose (0.5 mg/kg) tacrolimus monotherapy group, 52 days for the low-dose ASKP1240 monotherapy group, 100.5 days for the high-dose ASKP1240 monotherapy group, 53 days for the low-dose ASKP1240 and low-dose tacrolimus combination group, 57 days for the high-dose ASKP1240 and low-dose tacrolimus combination group, 29 days for the MMF plus steroid treatment group, 121 days for the low-dose ASKP1240, MMF and steroid combination group, >180 days for the high-dose ASKP1240, MMF and steroid combination group, 28.5

days for the sub-therapeutic dose (1.0 mg/kg) tacrolimus monotherapy group, >180 days for the low-dose ASKP1240 and sub-therapeutic dose tacrolimus combination group, and >180 days for the high-dose ASKP1240 and sub-therapeutic dose tacrolimus combination group. Compared with control group, the MSTs of all treatment groups except low-dose tacrolimus monotherapy group were significantly prolonged (Group 3 to 12, p = 0.005, 0.012, 0.005, 0.001, 0.002, 0.005, 0.005, 0.002, 0.001, 0.001, respectively). ASKP1240 monotherapy improved renal allograft survival in a dose-dependent manner (MSTs of low-dose group and high-dose group were 52 days and 100.5 days, respectively). ASKP1240 combined with low-dose of tacrolimus did not show a prolongation in the MST compared with ASKP1240 monotherapy (Figure 2.1A & 2.1B). However, ASKP1240, either in low-dose or high-dose, combined with a sub-therapeutic dose of tacrolimus showed significantly longer MST compared with either tacrolimus monotherapy groups (Group 11 and 12 *vs.* Group 10, p = 0.006 and 0.025, respectively) or the ASKP1240 monotherapy group (Group 11 *vs.* Group 3, p = 0.011; Group 12 *vs.* Group 4, p = 0.329) (Figure 2.1C & 2.1D). ASKP1240 combined with MMF and steroid showed much longer MSTs than either that of MMF plus steroid treatment group (Group 8 and 9 *vs.* Group 7, p = 0.001 and 0.017, respectively) or those of ASKP1240 monotherapy groups (Figure 2.1E & 2.1F). In all 12 groups, 19 animals survived to the end of study. Except of one animal was in the sub-therapeutic dose tacrolimus monotherapy group, the remaining animals were treated with low- or high-dose of ASKP1240, either as a monotherapy or in combination with tacrolimus or MMF.

Table 2.1 Renal Allograft Survival and Histopathological Evaluation

Group	Therapeutics	Number	MST	Graft Survival Time (days) / Histolopathogical Diagnoses of Renal Allografts							
1	Naïve	3	7	7 AR III (v3/i1)	7 AR IIB (v2/i3)	10 AR III (t2/v3/i3)					
2	Tac 0.5mg/kg	6	10	6 AR III (v3/i2)	6 AR IIA (t2/v1/i3)	10 AR III (v3/i3)	10 AR IIA (t2/v1/i3)	14 AR IIB (v2/i3)	35 AR III (v3/i3)		
3	ASKP 2mg/kg	5	52	50 AR III (v3/i3/ct2/cv1/ci1)	51 AR III (v3/i3/ct1/ci1)	52 CAN IB (t1/i3/ct1/ci1/cg1)	86 AR IIA (v1/i3/ct2/ci1/mm1)	>180 i2			
4	ASKP 5mg/kg	6	100.5	10 AR III (v3/i3)	19 BC (t1/i2)	74 CAN IIIA (i3/ct3 /ci3)	127 CAN IIB (i3/ct3/cv1/ci2)	180 CAN IIB (i3/ct2/cv2/ci3)	>180 CAN IIB (i3/ct2/cv3/ci2)		
5	ASKP 2mg/kg + Tac 0.5mg/kg	5	53	27 AR IIA (t1/v1/i3)	35 AR III (v3/i3)	53 AR III (v3/i3/ct1/cv3/ci1)	70 CAN IB (ct1/cv1/ci1)	>180 CAN IB (i1/ct1/cv3/ci1)			
6	ASKP 5mg/kg + Tac 0.5mg/kg	7	57	25 AR IIA (v1/i2)	46 AR III (v3/i3)	48 i1	57 AR IIA (t1/v1/i2)	70 AR IIA (v1/i3)	94 CAN IIIB (i2/ct3/cv3/ci3)	>180 NR	
7	MMF + Steroid	6	29	27 AR IIA (t2/v1/i3)	28 AR IIB (t1/v2/i1)	29 AR IIB (t1/v2/i3)	29 AR III (v3/i3)	32 AR IIA (t1/v1/i3)	43 CAN IB (i3/ct1/cv2/ci1)		
8	ASKP 2mg/kg + MMF + Steroid	5	121	48 BC (t1/i1/ct1)	70 i1	121 i1	122 CAN IA (i1/ct1/mm2/ci1)	175 CAN IB (i1/ct1/cv1/ci1)			
9	ASKP 5mg/kg + MMF + Steroid	5	>180	28 i1	116 CAN IIB (i2/ct2/cv2/ci2)	>180 i1/cv2	>180 CAN IB (i3/ct1/cv3/ci1)	>180 CAN IB (i1/ct2/cv2/ci1/cg1/mm2)			
10	Tac 1.0mg/kg	6	28.5	17 AR IIA (v1/i1)	21 AR IIA (v1/t1/i3)	24 CAN IA (i2/ct1/cv1/ci1/mm1)	33 CAN IB (i2/ct1/cv1/ci2)	33 CAN IA (i1/ct1/ci1/mm1)	>180 CAN IB (i1/ct1/cv2/ci1/mm1)		
11	ASKP 2mg/kg + Tac 1.0mg/kg	8	>180	105 CAN IB (i1/ct1/cv2/ci2/mm1)	125 CAN IIB (i2/ct2/cv2/ci3)	>180 CAN IA (i2/ct1/ci1/cv1)	>180 i1/ct0/ci0/cv3	>180 CAN IB (i1/ct1/cv1/ci2)	>180 CAN IA (i2/ct1/cv1/ci1)	>180 i1	>180 CAN IIIB (i2/ct3/cv3/ci3)
12	ASKP 5mg/kg + Tac 1.0mg/kg	7	>180	44 CAN IIB (i1/ct3/cv1/ci2)	56 CAN IA (i1/ct2/ci1)	145 CAN IIIB (i2/ct3/ci3/mm1)	>180 CAN IIB (i3/ct2/cv3/ci2)	>180 i1/ci1	>180 CAN IB (i1/ct1/cv1/ci1/mm1)	>180 ct1	

AR, acute rejection; BC, borderline change; CAN, chronic allograft nephropathy; cg, glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; cv, fibrous intimal thickening; i, interstitial inflammation; mm, mesangial matrix increase; NR, no rejection; t, tubulitis; v, intimal arteritis; ▭ anti-ASKP1240 antibody was detected during the maintenance treatment period; Tac, Tacrolimus; ASKP, ASKP1240; MMF, mycophenolate mofetil.

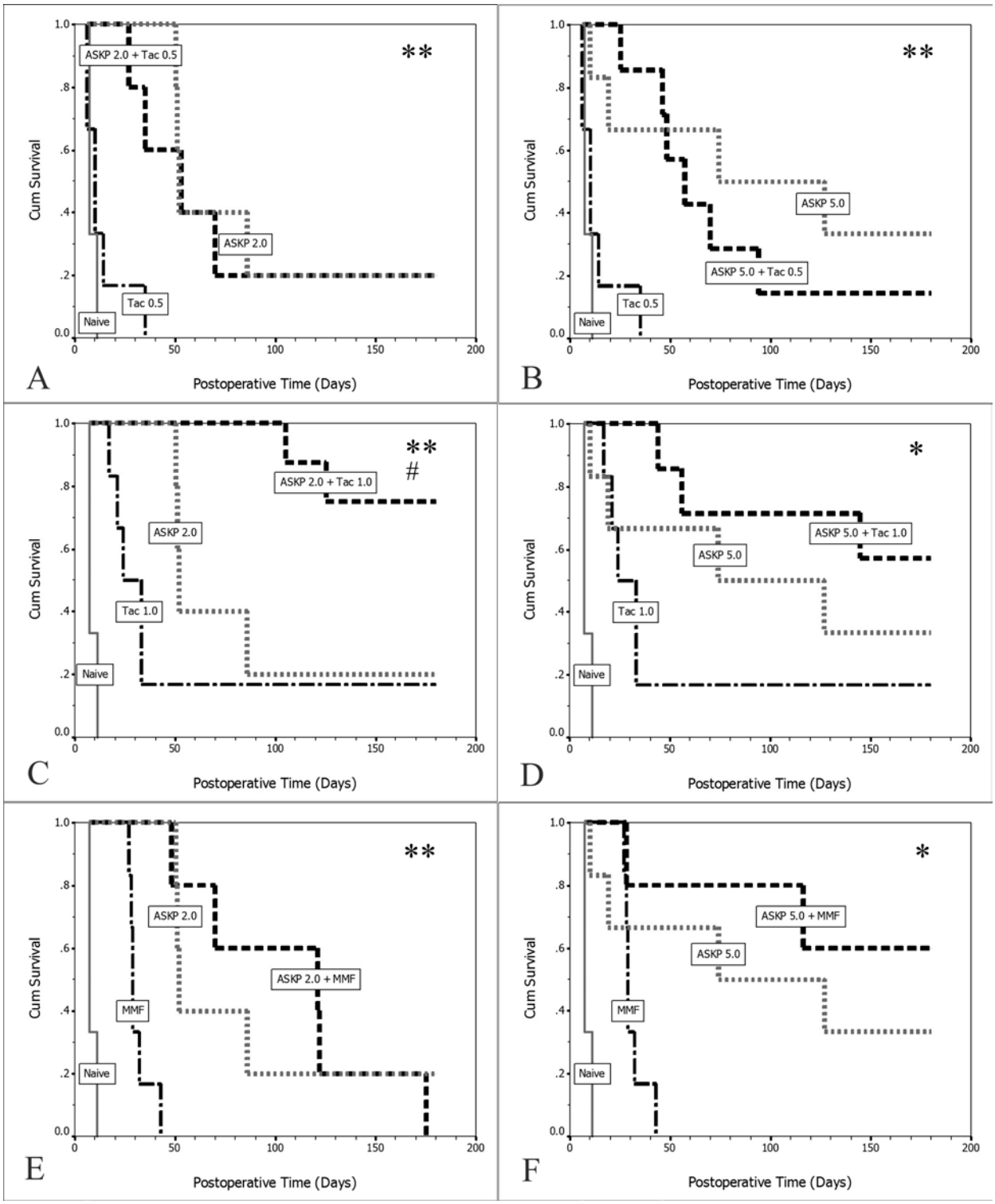

Figure 2.1 Renal allograft survival in each group. For comparison, each ASKP1240 combination therapy group and the relevant control groups were replicated in panels A to F. The curves represent renal graft survival

in naïve control (gray solid line), tacrolimus or MMF monotherapy (black dot dash line), ASKP1240 monotherapy (gray dotted line), and combination therapy (black dashed line) group. (Tac 0.5 = tacrolimus 0.5 mg/kg, Tac 1.0 = tacrolimus 1.0 mg/kg, ASKP 2.0 = ASKP1240 2.0 mg/kg, ASKP 5.0 = ASKP1240 5.0 mg/kg, MMF = mycophenolate mofetil 15 mg/kg; *: $p < 0.05$ ASKP1240 combination group *vs*. Tacrolimus or MMF monotherapy group, **: $p < 0.01$ ASKP1240 combination group *vs.* Tacrolimus or MMF monotherapy group, #: $p < 0.05$ ASKP1240 combination group *vs.* ASKP1240 monotherapy group).

Renal graft function

In general, when acute allograft rejection occurred, the sCr and blood urea nitrogen (BUN) levels of recipient monkeys were rapidly elevated. The renal function parameters were relatively stable in long-term survival monkeys (Figure 2.2A and 2.2B).

Biochemistry

Serum liver enzymes, creatinine kinase, electrolyte levels and all other biochemical parameters were unaffected by the administration of ASKP1240 (SDC, Table S2.1–S2.8).

Hematological determinations

The red blood cell (RBC), hemoglobin (HGB), and hematocrit (HCT) decreased on week 1 in Group 1, 11 and 12. These parameters were stable in other groups. This phenomenon can be explained based on the blood loss that may occur during the transplantation surgery. There were no consistent changes in white blood cell (WBC) or platelets (PLT) counts. The number of $CD3^+$ T cells or $CD20^+$ B cells in peripheral blood mononuclear cells (PBMCs) fluctuated

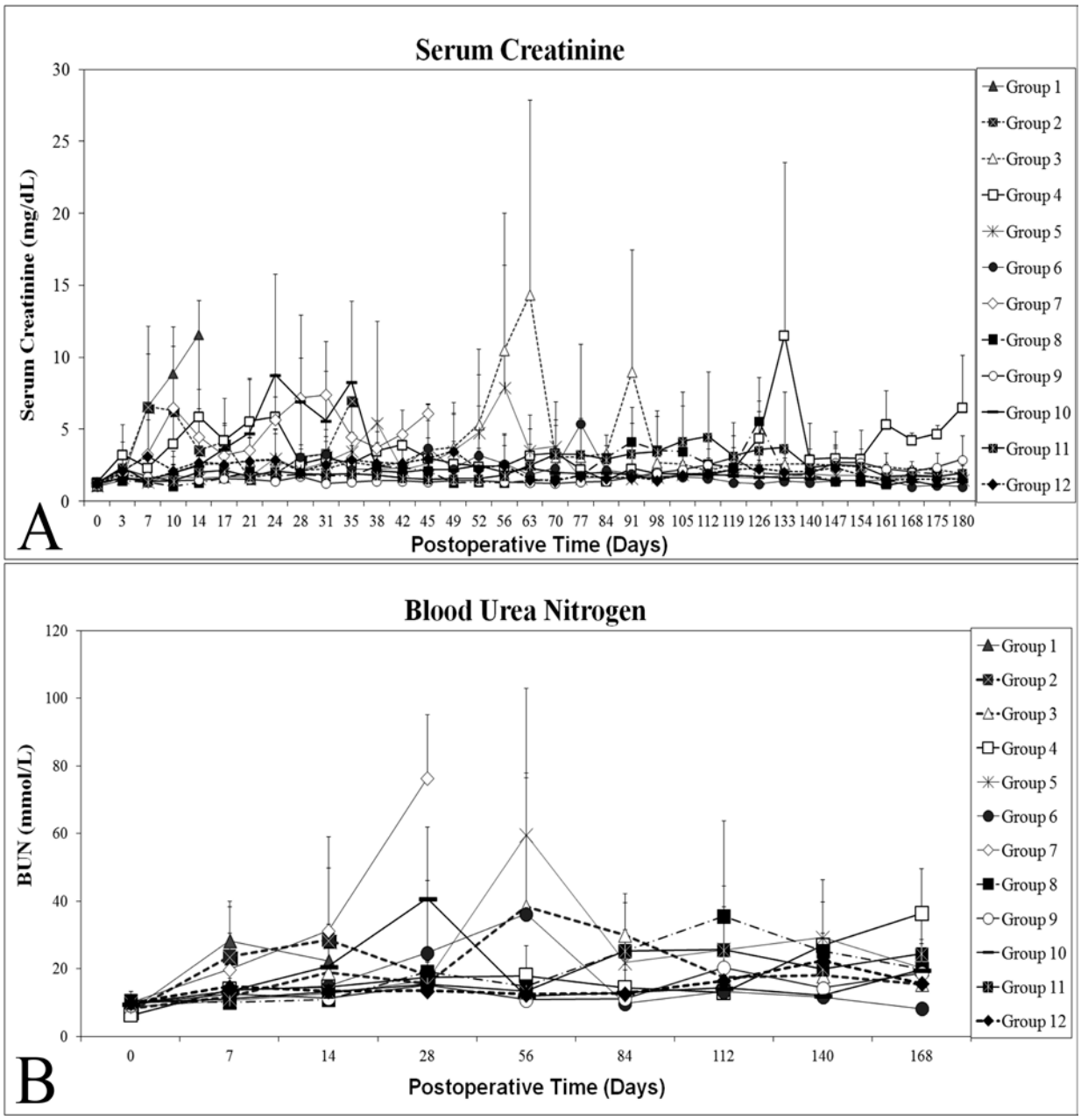

Figure 2.2 Renal graft function. A. Mean (± SD) serum creatinine level in each group. The elevation of sCr level was consistent with the occurrence of acute allograft rejection. B. Mean (± SD) blood urea nitrogen level in all groups. Changes of mean BUN levels were similar to sCr.

during the study period in all groups. However, no clear relationship between $CD3^+$ and $CD20^+$ cell counts and the administration of ASKP1240 was observed.

Body weight and clinic symptoms

Most of recipient monkey weights remained stable during the study. After recovery from surgery, all recipient monkeys were very active with good appetite. However, when acute rejection occurred, animal became lethargic, lost appetite and weight, and developed oliguria which was accompanied by an increase of sCr.

Pharmacokinetic evaluation

Serum ASKP1240 concentrations: The ASKP1240 concentration-time data are depicted in Figure 2.3A. In general, serum concentrations of ASKP1240 increased in a dose-dependent manner. The highest mean values of ASKP1240 trough level in most group appeared on Day 14. Thereafter, these values decreased to relative lower levels that were consistent with dose reduction in maintenance phase. From Day 56, serum ASKP1240 trough level in most animals dropped to a very low level. That was different with what was observed in normal monkeys in another study (Ma 2014).

Blood concentration of tacrolimus: The profiles of mean blood trough level of tacrolimus are illustrated in Figure 2.3B. The blood trough level of tacrolimus ranged from 0.31 to 85.47 ng/mL. Blood tacrolimus trough level increased in a dose-related manner.

Plasma concentration of Mycophenolic acid (MPA): The mean plasma trough levels of MPA are shown in Figure 2.3C. MPA was detected in all submitted samples with the minimum value 0.126 μg/mL and the maximum value 10.3 μg/mL from Day 7 to Day 168. The range of mean trough level of MPA in each group was 1.45 to 3.70 μg/mL for Group 7, 0.437 to 3.22 μg/mL for Group 8, and 0.411 to 1.47 μg/mL for Group 9.

Anti-ASKP1240 antibody assay

Anti-ASKP1240 antibodies in serum were detected in 15 of 48 animals during the maintenance treatment period (Table 2.1). They were mainly found in ASKP1240 2 mg/kg monotherapy or combination therapy groups (14/23), and only one was found in ASKP1240 5 mg/kg treated groups (1/25).

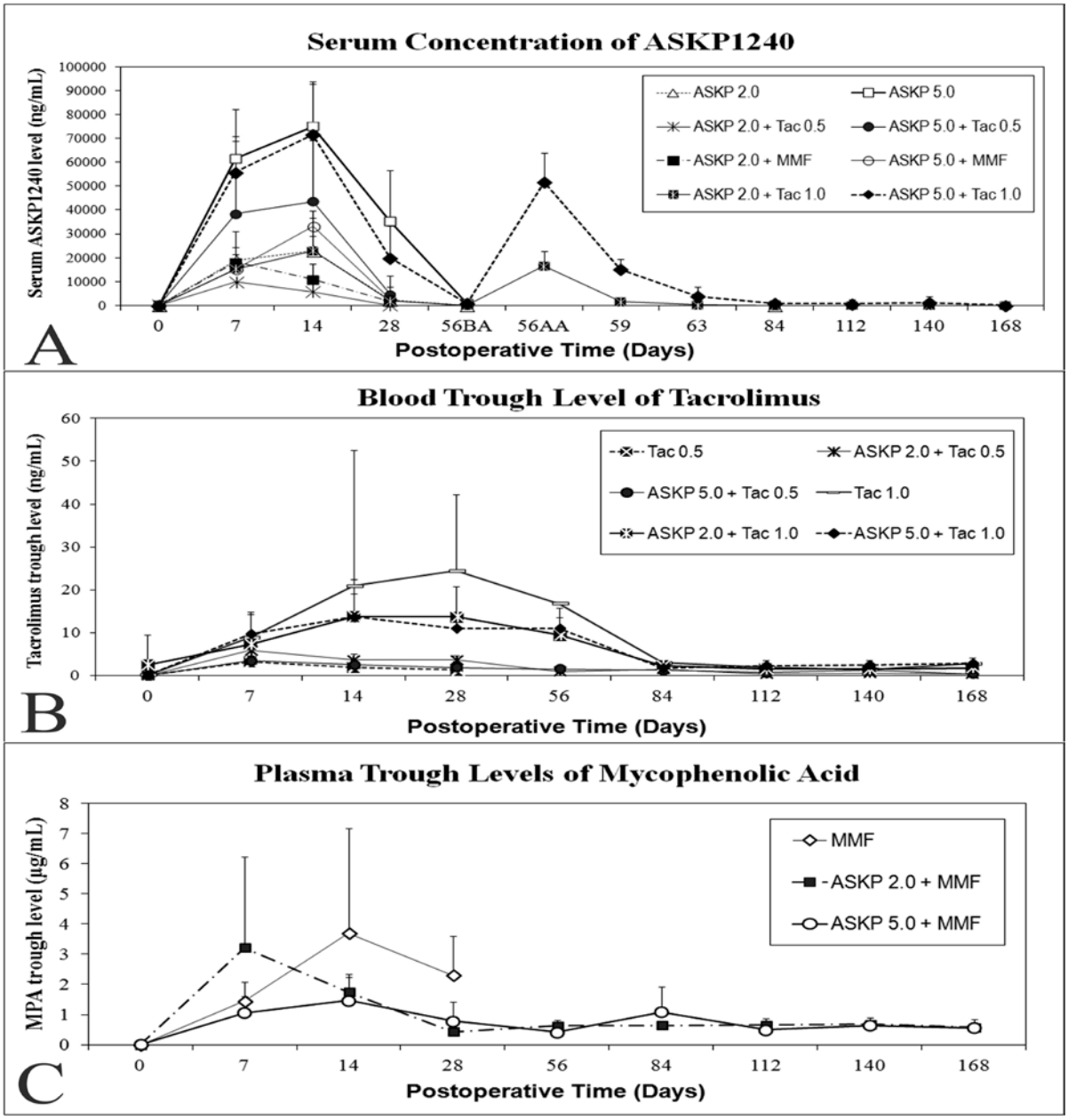

Figure 2.3 Pharmacokinetic evaluation. A. The mean (± SD) serum trough levels of ASKP1240 in Group 3–6, 8 and 9, as well as mean (± SD) serum concentration transitions of ASKP1240 in Group 11 and 12. Serum concentrations of ASKP1240 increased in a dose-dependent manner, and decreased to relative lower levels two weeks after drug administration in maintenance phases. (56BA = before ASKP1240 administration on Day 56, 56AA = one hour after ASKP1240 administration on Day 56, Day 59 = 3 days after ASKP1240 administration, Day 63 = 7 days after ASKP1240 administration). B. The mean (± SD) whole blood tacrolimus trough levels increased in a dose-related manner. C. Mean (± SD) plasma trough levels of Mycophenolic acid. In general, in

the induction phrase, plasma trough levels of Mycophenolic acid revealed relatively higher level that was associated with the frequency of MMF administration. The plasma trough levels of Mycophenolic acid were stable in maintenance phases. (Tac 0.5, Tacrolimus 0.5 mg/kg, Tac 1.0, Tacrolimus 1.0 mg/kg, ASKP 2.0, ASKP1240 2.0 mg/kg, ASKP 5.0, ASKP1240 5.0 mg/kg, MMF, mycophenolate mofetil 15 mg/kg).

Cytokine Assay

Serum interleukin (IL)-2, 4, 5, 6, tumor necrosis factor (TNF), and interferon-gamma (IFN-γ) were measured in Group 10, 11 and 12. IL-2, 4, 5, TNF, and IFN-γ were not detected in any animals from Day 0 to Day 168. IL-6 was found in three samples (one of each group) on Day 0 before surgery and dosing. Apparent elevated levels of IL-6 were detected in all Day 0 (after surgery and dosing) samples and most Day 1 (before and after dosing) samples. From Day 3 to Day 168, IL-6 was sporadically detected in some animals. No clear difference was noted between the ASKP1240 and tacrolimus combination groups and the tacrolimus mono group. It was concluded that changes of serum IL-6 level were not related ASKP1240 administration under the condition of this study.

Histopathology

The details of histological features of transplanted kidneys were summarized in Table 2.1. The percentage proportions of histological types in each group are depicted in Figure 4. Acute rejections were found in all renal grafts of Group 1 and Group 2. The incidence of acute renal rejection, in ASKP1240 monotherapy groups, dropped to 3/5 of Group 3 (ASKP1240 2 mg/kg) and 1/6 of Group 4 (ASKP1240 5 mg/kg). ASKP1240 combined with tacrolimus 0.5 mg/kg (Group 5 and 6) did not show benefits in reducing acute allograft rejection compared with

ASKP1240 monotherapy groups. When ASKP1240 combined with tacrolimus 1 mg/kg (Group 11 and 12) or MMF plus steroids (Group 8 and 9), no acute rejections were found in these groups. Varying degrees of chronic allograft nephropathy were the common pathological changes observed in long-term survival animals, except for one animal in Group 6 that displayed no rejection. The histopathological results of 19 animals that survived to the end of study were following: No rejection = 1 (5.3%); Nonspecific changes = 6 (31.6%); Grade I chronic nephropathy = 8 (42.1%); Grade II chronic nephropathy = 3 (15.8%); Grade III chronic nephropathy = 1 (5.3%).

Renal artery thrombosis, a common technical complication in the kidney transplantation model (Song 2010), was found in some animals. The incidence of renal artery thrombosis between ASKP1240-treated (5.00%) and non-ASKP1240-treated (4.35%) groups was not significantly different (p = 1.000; cases with technical complications were excluded from the analysis of survival and other parameters). No thromboembolism was found in other organs including the brain, lung, heart, liver, pancreas, and spleen in all animals at the time of necropsy, and at the histological assessments in obtained tissue specimens.

Hepatic abscess was developed in one monkey in Group 9 and lymphoma was observed in another monkey in Group 12 in kidney, mesentery, mediastinum, left lung and heart.

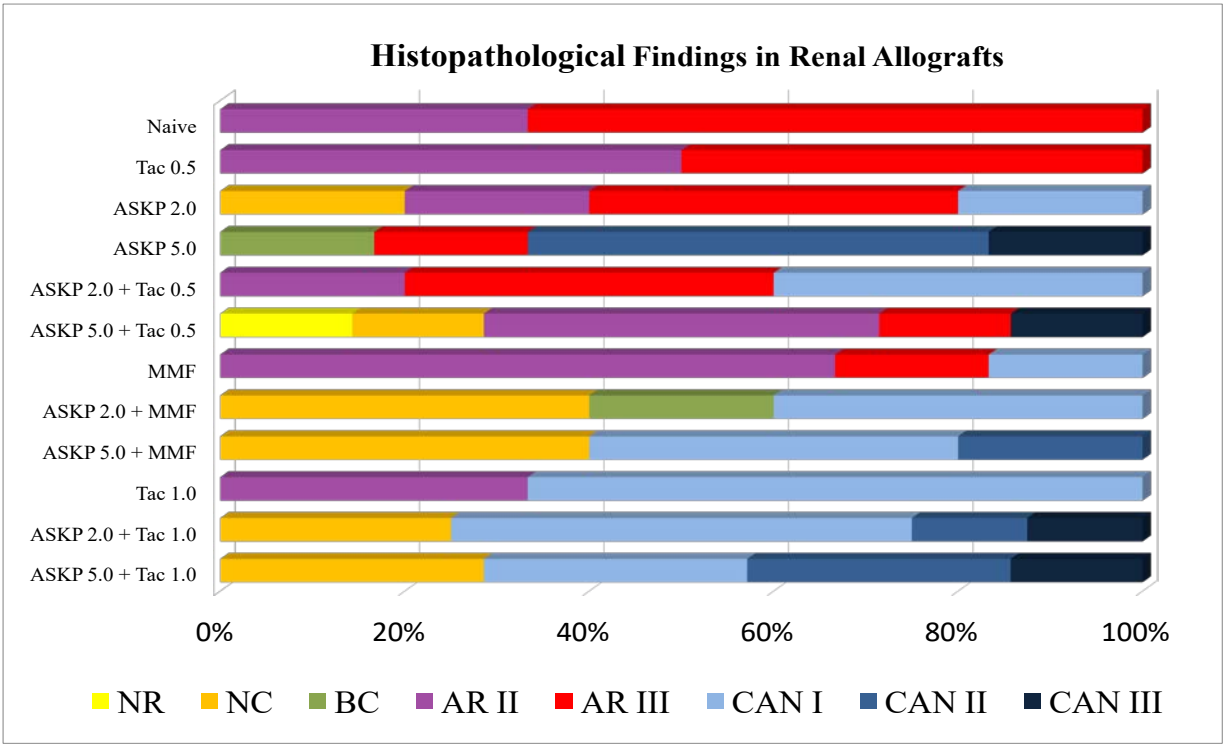

Figure 2.4 Proportions of histological types in each group. Acute rejection (red and pink bar) was the only histological type in Group 1 and Group 2, and was also the primary histological type in Group 3, 5, 6 and 7. Varying degree of chronic allograft nephropathy (bars with baby blue, cobalt blue or navy blue) constituted the main parts of pathological findings in Group 4, 9–12. (NR = no rejection, NC = nonspecific changes, BC = borderline changes, AR = acute rejection, CAN = chronic allograft nephropathy, Tac 0.5 = tacrolimus 0.5 mg/kg, Tac 1.0 = tacrolimus 1.0 mg/kg, ASKP 2.0 = ASKP1240 2.0 mg/kg, ASKP 5.0 = ASKP1240 5.0 mg/kg, MMF = mycophenolate mofetil 15 mg/kg).

Discussion

Since the introduction of cyclosporine A (CsA) into clinical practice in the early 1980s, calcineurin inhibitors (CNIs) have dramatically improved short-term outcomes of renal transplantation. However, the extensive adverse effects of CNIs including nephrotoxicity,

neurotoxicity, posttransplantation diabetes, hypertension, and hyperlipidemia impact the long-term success rates of renal transplants. It has been known that most toxicities of CNIs are dose-dependent. To minimize the side effects associated with CNI exposure, strategies applied in clinic include CNI reduction, avoidance, and withdrawal (Flechner 2008; Barbari 2007). In the *Cynomolgus* monkey kidney transplant model, tacrolimus 2 mg/kg was the therapeutic dose, while 1 mg/kg was considered to be a sub-therapeutic dose (Kinugasa 2008). Here we show the notable additive effects when ASKP1240 either in high-dose (5 mg/kg) or in low-dose (2 mg/kg) combines with sub-therapeutic dose of tacrolimus. MSTs of renal allograft were also significantly prolonged in groups of ASKP1240 in combination with MMF and steroid. This implies that ASKP1240 can work in both CNI sparing and avoiding regimens. Previous report indicates the effect of anti-CD40L mAb can be abrogated when it is paired with CNIs (Blaha 2003). Our results demonstrate that it is not the same case with ASKP1240. Further studies to explore the effects of these regimens on immune components at the cellular and molecular level will be helpful to elucidate the exact mechanisms.

Histopathologic results of this study revealed that ASKP1240 monotherapy could successfully inhibit acute renal allograft rejection in a dose-dependent manner. When ASKP1240 combined with MMF or a sub-therapeutic dose of tacrolimus, acute allograft rejections were abolished. Previous studies have shown that high-dose ASKP1240 can suppress the generation of donor specific antibodies (DSA) (Aoyagi 2009; Oura 2012) which are strongly associated with chronic allograft rejection (Mao 2007). The effect of ASKP1240 on inhibiting acute rejection has been verified by this and other studies. To clarify the role of ASKP1240 in chronic allograft nephropathy will be an attractive topic for future.

It is notable during maintenance phase, with two weeks dosing interval, serum concentration of ASKP1240 in most animals decreased to a very low level. Correspondingly, ASKP1240 trough levels in normal monkeys, especially in the high dose group, were apparently high. It also found that ASKP1240 half-life of the high dose group during maintenance phase was markedly shorter than that of normal monkeys (Ma 2014). These results imply that transplanted monkey may possess a different ASKP1240 metabolic profile compared with that of normal monkey. One of the possibilities of these differences is due to CD40 overexpression in transplanted monkey (Ma 2014). These factors should be taken into consideration during designing optimal dosing regimens.

Previous studies showed that interference with the CD40–CD40L pathway by the administration of anti-CD40L mAbs was associated with thromboembolic events. The possible correlation between anti-CD40 mAbs and thromboembolism has been of concern although it is generally thought that these adverse effects are only associated with anti-CD40L mAbs (Crow 2003; Larsen 2006). To date, there have been no preclinical reports indicating that ASKP1240 or any other anti-CD40 mAbs are involved in the thromboembolic complications. In this study, except for renal artery thromboses which are common surgical complications and did not correlate to ASKP1240 treatment, there have been no thromboembolic findings in other organs.

We also measured serum IL-2, 4, 5, 6, TNF, and IFN-γ level in renal transplanted monkeys. The results indicate that ASKP1240 does not induced cytokine release. Anti-ASKP1240 antibodies were detected in some animals (mainly in low-dose groups). The partial reason for

the production of these antibodies is due to ASKP1240 is a human antibody and a foreign protein for monkey. On the other hand, anti-ASKP1240 antibody is rarely found in high-dose group. One of possible explanation is that higher dose ASKP1240 may suppress antibody production.

ASKP1240 was shown to be a potent immunosuppressive agent in this NHP kidney transplantation model. Treatment of these animals for up to 180 days was not associated with a deterioration in the general clinical observational signs (activity or appetite) nor was it associated with weight loss. A recent phase I clinical trial supports and extends these animal findings by demonstrating that ASKP1240 is well tolerated in healthy subjects and is not associated with either drug induced cytokine release or with thromboembolic events (Goldwater 2013).

In conclusion, ASKP1240, a fully human anti-CD40 mAb, prolongs renal allograft survival in a dose-dependent manner when administered as a monotherapy to renal allograft transplanted *cynomolgus* monkeys. ASKP1240, when combined with sub-therapeutic dose tacrolimus or MMF plus steroid shows the additive effect on prolonging renal graft survival. The combination of ASKP1240 and sub-therapeutic dose tacrolimus, relatively, is more effective than ASKP1240 in combination with MMF plus steroid. With this treatment protocol, even though the low-dose ASKP1240, can successfully eliminate acute rejection and dramatically improve renal allograft survival. In view of the efficiency and safety, the combined use of low-dose ASKP1240 and sub-therapeutic dose tacrolimus is recommended. Collectively,

ASKP1240 appears to be a promising anti-rejection agent in solid organ transplantation. The present results provide concrete support for further clinical studies.

Materials and Methods

Animals

Sixty-nine bred male *Cynomolgus* monkeys, with body weights ranging from 3.1–6.0 kg, hepatitis B virus (HBV), hepatitis C virus (HCV), simian immunodeficiency virus (SIV) and Herpes B virus free, were obtained from Laboratory Animals Center of the Academy of Military Medical Sciences (AMMS), Beijing, China. All experimental procedures were approved by the Ethical Committee for Animal Experimentation at Laboratory Animals Center of the AMMS, and were performed in accordance with the standards described in the Guide for the Care and Use of Laboratory Animals, National Institutes of Health Office of Animal Care and Use. Each animal was identified by number and randomly assigned to a dose group. All animals were screened for general health and quarantined for two weeks before study entry. They were housed in individual cages and were allowed free access to water, fruits and monkey chow.

Life supporting kidney transplantation

Renal transplantation was performed in ABO compatible, stimulation index ≥ 2.5 in the one-way MLR monkey pairs. Each animal in this study acted as both donor and recipient. Left renal transplantations were performed as previously described (Song 2010; Qi 2000; Chen 2000). Briefly, all donor and recipient monkeys were anesthetized by intramuscular injection of ketamine and xylazine. The left kidneys were exchanged in paired monkeys by

transplanting into the upper part of abdomen with end-to-side anastomoses of renal artery to aorta and renal vein to vena cava, and with end-to-end anastomosis of donor and recipient ureters. The right native kidney was removed after the transplanted kidney was reperfused.

Experimental group and treatment

Animals were divided into twelve groups as shown in Table 1: *i.e.* Naïve control, Tacrolimus monotherapy, ASKP1240 monotherapy, MMF monotherapy, ASKP1240 and Tacrolimus combination, ASKP1240 and MMF combination. ASKP1240 (2 mg/kg or 5 mg/kg) was given intravenously on Day 0 (before and after surgery), 3, 7, 11, 14, and then from Day 28 to Day 168 half dose (1 mg/kg or 2.5 mg/kg) bi-weekly. Tacrolimus (0.5 mg/kg or 1.0 mg/kg, non-therapeutic dose) (Kinugasa 2008) was orally administered immediately after kidney transplantation, then once daily, until Day 179. MMF (15 mg/kg) was given subcutaneously twice daily from Day 0 to Day 14, and then once daily till Day 179. Methylprednisolone (steroid) was administered subcutaneously once a day in a tapering manner. Recipients were observed for maximum of 180 days.

Biochemical and hematological determinations

Serum creatinine (sCr) was monitored at least twice a week for the first two postoperative months then weekly. Blood urea nitrogen, total protein, albumin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, total bilirubin, creatinine kinase and electrolyte levels (K^+, Na^+, Cl^-) were measured using the auto analyzer.

Hematological parameters including RBC, WBC, HGB, HCT, PLT and lymphocyte phenotypes (CD3, CD20) were examined in PBMCs using the automated hematology analyzer.

Pharmacokinetic evaluation

Serum ASKP1240 trough levels were measured using enzyme-linked immunosorbent assay (ELISA) as described previously (Ma 2014) in Group 3–6, 8 and 9 on Day 0, 7, 14, 28, 56, 84, 112, 140, and 168. The whole blood trough levels of tacrolimus in Group 2, 5, 6, 10–12 and plasma trough levels of mycophenolic acid (MPA) in Group 7–9 were determined using a validated LC-Tandem Mass Spectrometry (API 5000 LC/MS/MS System) assay on Day 0, 7, 14, 28, 56, 84, 112, 140, and 168. In group 11 and 12, the serum concentration transition of ASKP1240 was monitored on Day 0, 7, 14, 28, 42, 56 (before and one hour after administration), 59, 63, 84, 112, 140 and 168.

Immunological assays

Anti-ASKP1240 antibody: Monkey anti-ASKP1240 antibodies were detected using an ELISA screening assay and further confirmed using immunodepletion assays at Shin Nippon Biomedical Laboratories (SNBL) (Ma 2014).

Cytokine Assay: The serum concentrations of IL-2, 4, 5, 6, TNF, and IFN-γ were analyzed with CBA (Cytometoric Beads Array) Nonhuman Primate Th1/Th2 Cytokine Kit (BD Biosciences) using flow cytometer (FACSCalibur, BD Biosciences).

Histopathological determinations

All recipient monkeys were subjected to complete gross necropsies. Routine hematoxylin and eosin staining were performed on all paraffin-embedded samples sections including graft kidney, liver, pancreas, spleen, heart, lung, stomach, jejunum, thoracic aorta, and mesentery lymph nodes. Graft kidney tissues were stained with Masson's Trichrome and Periodic acid–Schiff (PAS) as well. The Banff 97 classification of kidney pathology was used for scoring the presence and degree of renal rejection (Racusen 1999).

Statistical analysis

All results of body weight, biochemistry analyses, and drug concentrations were presented as mean ± SD. The statistical differences among groups were analyzed using one-way analysis of variance. Survival of renal allograft was presented as MST, with comparisons among groups performed by log rank test. A p value < 0.05 was considered statistically significant.

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Conflict of Interest Statement:

All authors declare that there are no conflicts of interest.

Supplemental Digital Content (SDC)

Table S2.1 Mean (SD) serum alanine aminotransferase (U/L) in each group

Group	Postoperative Time (Days)								
	0	**7**	**14**	**28**	**56**	**84**	**112**	**140**	**168**
Group 1	20.0 (9.6)	84.0 (123.1)	15.0						
Group 2	32.5 (14.1)	53.8 (59.2)	20.0 (1.4)	22.0					
Group 3	52.6 (29.2)	32.8 (16.6)	33.8 (14.5)	30.0 (10.6)	34.3 (5.7)	45.5 (36.1)	53.0	33.0	29.0
Group 4	32.3 (10.9)	15.0 (4.2)	19.2 (3.4)	22.8 (15.8)	31.0 (12.5)	30.7 (11.7)	26.3 (3.5)	61.5 (48.8)	28.5 (0.7)
Group 5	54.8 (37.0)	62.4 (66.6)	35.4 (18.0)	27.3 (5.6)	41.7 (20.0)	60.0	38.0	35.0	31.0
Group 6	54.3 (18.5)	36.9 (13.5)	42.0 (12.2)	41.0 (11.0)	37.0 (11.0)	37.5 (7.6)	67.0	56.0	54.0
Group 7	70.7 (11.8)	36.5 (12.1)	46.8 (13.5)	48.8 (39.6)					
Group 8	60.6 (7.9)	54.6 (19.1)	68.6 (22.3)	52.8 (23.5)	57.0 (31.0)	34.7 (7.4)	32.3 (9.6)	31.0	34.0
Group 9	93.6 (42.8)	43.6 (18.6)	44.6 (17.5)	37.6 (10.2)	43.8 (17.5)	55.5 (11.0)	50.8 (17.1)	51.0 (13.7)	43.3 (13.8)
Group 10	64.5 (47.6)	33.3 (11.0)	26.8 (3.8)	20.8 (9.7)	39.0	41.0	49.0	77.0	128.0
Group 11	44.4 (13.9)	32.0 (9.5)	33.5 (17.4)	26.8 (9.1)	28.6 (7.9)	27.0 (8.7)	29.1 (9.1)	33.8 (8.8)	48.8 (22.8)
Group 12	57.7 (63.7)	36.4 (15.5)	30.1 (12.3)	45.4 (45.4)	30.8 (6.9)	46.2 (41.6)	31.2 (11.8)	34.0 (9.5)	46.3 (11.7)

Table S2.2 Mean (SD) serum aspartate aminotransferase (U/L) in each group

Group	Postoperative Time (Days)								
	0	**7**	**14**	**28**	**56**	**84**	**112**	**140**	**168**
Group 1	41.7 (4.5)	24.0 (20.9)	25.0						
Group 2	40.0 (6.2)	72.0 (70.6)	36.0 (0.0)	38.0					
Group 3	51.8 (20.7)	37.8 (9.1)	30.4 (10.0)	33.0 (12.0)	34.8 (5.1)	28.0 (5.7)	37.0	29.0	53.0
Group 4	48.0 (16.9)	46.7 (29.6)	32.0 (4.6)	37.3 (8.1)	46.0 (5.6)	47.7 (5.5)	34.0 (5.3)	96.0 (69.3)	42.0 (1.4)
Group 5	67.2 (27.4)	102.0 (117.0)	41.8 (17.7)	34.3 (1.7)	40.7 (10.7)	54.0	33.0	49.0	71.0
Group 6	82.7 (47.4)	62.0 (37.4)	48.9 (16.1)	42.6 (6.4)	47.5 (20.3)	59.0 (53.7)	33.0	32.0	36.0
Group 7	124.2 (61.4)	65.8 (31.4)	63.0 (19.8)	79.4 (72.7)					
Group 8	114.6 (11.7)	70.0 (10.7)	122.2 (55.1)	56.0 (24.8)	77.8 (47.6)	40.3 (7.0)	41.0 (12.5)	63.0	61.0
Group 9	112.8 (39.2)	59.0 (11.7)	66.8 (28.6)	38.4 (7.8)	50.8 (17.2)	48.8 (23.5)	44.3 (11.2)	52.0 (7.9)	40.0 (11.8)
Group 10	79.8 (46.6)	36.8 (3.8)	30.5 (3.6)	29.0 (6.6)	36.0	35.0	42.0	65.0	106.0
Group 11	45.1 (6.8)	30.3 (4.5)	32.4 (11.0)	30.9 (8.4)	30.4 (4.1)	32.9 (4.7)	30.0 (6.7)	44.7 (6.9)	54.3 (9.8)
Group 12	50.9 (19.9)	43.3 (12.4)	38.4 (17.7)	55.1 (31.5)	35.6 (8.1)	35.8 (10.8)	39.2 (12.2)	44.8 (5.3)	55.3 (13.7)

Table S2.3 Mean (SD) serum albumin (g/L) in each group

Group	Postoperative Time (Days)								
	0	**7**	**14**	**28**	**56**	**84**	**112**	**140**	**168**
Group 1	28.7 (1.2)	21.3 (3.8)	28.0						
Group 2	35.2 (3.7)	25.7 (4.3)	26.5 (10.6)	28.0					
Group 3	35.4 (3.1)	27.4 (2.6)	31.2 (4.6)	31.2 (3.9)	25.5 (4.8)	23.0 (4.1)	30.0	31.0	37.0
Group 4	32.2 (4.2)	24.3 (4.7)	27.8 (5.0)	29.0 (7.4)	27.0 (7.6)	30.3 (8.6)	29.3 (7.2)	20.0 (7.1)	32.0 (5.7)
Group 5	33.4 (3.0)	26.8 (6.1)	29.0 (4.8)	30.8 (4.1)	25.7 (6.8)	28.0	25.0	18.0	11.0
Group 6	31.7 (3.0)	26.4 (2.4)	28.3 (4.5)	27.9 (4.0)	28.3 (5.7)	28.5 (10.6)	40.0	33.0	28.0
Group 7	35.0 (3.7)	23.0 (3.0)	24.5 (3.3)	19.8 (2.7)					
Group 8	32.6 (0.9)	22.8 (2.6)	23.2 (2.8)	24.6 (2.5)	24.8 (4.5)	25.3 (0.6)	21.0 (2.6)	15.0	11.0
Group 9	33.0 (1.0)	22.4 (3.8)	17.8 (5.0)	21.6 (7.4)	17.5 (5.4)	21.0 (3.2)	19.3 (1.9)	21.7 (2.1)	20.3 (2.9)
Group 10	35.5 (5.2)	22.3 (5.2)	22.8 (4.3)	21.5 (4.5)	27.0	29.0	26.0	27.0	27.0
Group 11	37.4 (7.1)	24.1 (3.8)	26.4 (3.5)	31.5 (6.7)	28.4 (2.6)	28.9 (2.3)	28.3 (4.3)	30.0 (5.3)	25.3 (5.8)
Group 12	37.4 (4.0)	24.0 (3.1)	25.3 (3.1)	29.4 (2.5)	24.4 (3.8)	25.6 (4.3)	25.2 (2.2)	24.0 (2.3)	25.5 (3.9)

Table S2.4 Mean (SD) serum creatine kinase (U/L) in each group

Group	Postoperative Time (Days)								
	0	**7**	**14**	**28**	**56**	**84**	**112**	**140**	**168**
Group 1	106.7 (75.7)	197.3 (274.3)	45.0						
Group 2	293.5 (292.3)	73.2 (60.5)	48.5 (13.4)	90.0					
Group 3	194.8 (146.8)	53.6 (11.8)	72.4 (18.0)	97.4 (12.4)	143.3 (36.2)	90.5 (17.7)	289.0	110.0	553.0
Group 4	354.0 (640.0)	408.2 (915.1)	81.0 (27.0)	100.8 (90.4)	113.0 (29.3)	90.0 (14.2)	93.0 (24.3)	692.0 (790.5)	126.0 (14.1)
Group 5	144.6 (66.7)	49.8 (14.2)	82.2 (32.2)	146.8 (104.9)	87.0 (23.5)	111.0	85.0	98.0	153.0
Group 6	669.0 (599.3)	68.6 (29.4)	92.7 (28.1)	93.9 (37.2)	352.0 (381.1)	196.0 (166.9)	150.0	117.0	109.0
Group 7	1326.7 (1114.3)	305.3 (270.2)	351.5 (302.4)	199.6 (82.4)					
Group 8	843.6 (492.3)	332.4 (393.4)	571.6 (392.9)	365.4 (303.3)	337.3 (154.0)	135.3 (116.7)	101.0 (35.1)	400.0	1205.0
Group 9	650.4 (426.5)	210.2 (159.7)	268.4 (146.6)	123.2 (30.2)	197.0 (205.8)	140.8 (63.5)	525.0 (529.3)	85.3 (12.7)	132.7 (88.7)
Group 10	481.5 (800.4)	56.5 (23.0)	70.0 (32.2)	79.3 (29.8)	110.0	68.0	81.0	63.0	68.0
Group 11	158.8 (64.1)	75.5 (55.9)	73.6 (41.3)	75.4 (36.3)	123.3 (100.5)	93.8 (41.2)	118.1 (96.8)	98.8 (53.8)	106.5 (32.2)
Group 12	390.4 (676.9)	82.4 (53.7)	207.0 (367.8)	125.7 (69.0)	103.8 (76.8)	94.4 (49.8)	130.8 (117.1)	86.0 (40.7)	106.8 (52.3)

Table S2.5 Mean (SD) serum potassium (mmol/L) in each group

Group	Postoperative Time (Days)								
	0	**7**	**14**	**28**	**56**	**84**	**112**	**140**	**168**
Group 1	5.2 (0.9)	6.4 (2.3)	5.6						
Group 2	5.3 (0.8)	6.7 (1.0)	5.8 (1.1)	5.5					
Group 3	5.6 (1.2)	5.6 (1.0)	5.4 (0.7)	5.2 (0.8)	6.0 (2.3)	5.1 (0.6)	5.6	5.6	7.6
Group 4	5.6 (0.6)	5.8 (0.4)	6.0 (1.1)	5.5 (1.2)	6.2 (1.7)	6.3 (1.2)	6.4 (1.0)	6.7 (0.8)	7.4 (0.2)
Group 5	5.3 (1.0)	5.8 (0.8)	5.5 (1.7)	5.4 (0.8)	7.9 (4.8)	5.6	4.2	6.6	7.4
Group 6	4.9 (0.8)	6.1 (0.5)	6.4 (1.3)	6.1 (1.3)	5.6 (0.9)	8.1 (2.5)	5.6	5.4	6.8
Group 7	6.0 (0.8)	6.1 (0.5)	5.5 (0.6)	7.5 (1.0)					
Group 8	4.8 (0.6)	5.5 (0.4)	6.0 (0.8)	5.6 (1.2)	5.3 (0.7)	5.8 (0.6)	6.3 (1.3)	6.3	5.8
Group 9	5.3 (1.0)	5.5 (1.0)	5.6 (0.6)	4.7 (0.5)	5.0 (0.5)	6.0 (0.9)	5.9 (1.2)	6.5 (0.8)	6.5 (2.1)
Group 10	7.9 (1.3)	6.3 (1.5)	6.0 (0.6)	6.7 (0.8)	5.9	5.9	5.2	6.7	12.1
Group 11	6.8 (1.2)	5.1 (0.6)	5.3 (0.8)	5.3 (0.5)	5.5 (0.4)	5.5 (0.8)	5.4 (0.9)	5.9 (0.9)	6.1 (1.6)
Group 12	7.5 (0.8)	9.6 (3.4)	5.5 (1.4)	6.1 (0.9)	5.7 (0.6)	5.6 (1.1)	5.5 (1.0)	5.5 (1.1)	5.8 (1.1)

Table S2.6 Mean (SD) serum sodium (mmol/L) in each group

Group	Postoperative Time (Days)								
	0	**7**	**14**	**28**	**56**	**84**	**112**	**140**	**168**
Group 1	153.9 (16.5)	135.3 (8.9)	140.2						
Group 2	148.1 (1.9)	143.3 (6.3)	141.6 (8.7)	143.6					
Group 3	151.0 (8.9)	145.9 (1.9)	149.1 (3.0)	147.7 (2.8)	144.0 (4.2)	139.8 (3.8)	143.1	144.2	146.7
Group 4	146.7 (4.9)	150.5 (5.9)	155.5 (14.7)	147.3 (6.5)	157.7 (6.4)	147.0 (2.9)	151.3 (6.3)	138.9 (3.2)	147.7 (1.9)
Group 5	167.7 (4.1)	147.7 (4.1)	146.2 (3.7)	145.0 (2.6)	142.2 (5.0)	146.5	149.5	149.3	145.5
Group 6	158.6 (10.8)	146.3 (3.4)	148.6 (6.1)	145.8 (8.7)	144.6 (7.3)	138.2 (10.7)	147.2	149.4	144.7
Group 7	163.3 (11.0)	145.4 (2.9)	146.5 (3.4)	136.0 (6.3)					
Group 8	166.6 (2.3)	152.1 (1.7)	149.3 (4.6)	147.5 (1.4)	146.0 (2.0)	149.3 (0.8)	147.1 (6.1)	139.7	142.5
Group 9	168.9 (6.8)	148.1 (3.2)	146.9 (3.9)	145.8 (3.6)	144.8 (3.9)	145.1 (3.2)	145.7 (4.0)	147.2 (4.3)	142.3 (4.5)
Group 10	174.2 (17.2)	150.5 (3.1)	152.7 (4.8)	143.0 (9.6)	151.9	144.0	154.4	151.1	150.1
Group 11	169.1 (18.9)	151.1 (7.0)	146.4 (6.7)	146.4 (3.0)	151.2 (2.8)	143.9 (3.1)	146.2 (4.2)	145.4 (4.3)	147.3 (2.8)
Group 12	175.0 (15.0)	165.8 (23.2)	142.5 (24.0)	147.8 (3.2)	147.0 (2.0)	144.2 (6.9)	146.0 (6.1)	139.8 (13.8)	152.6 (3.0)

Table S2.7 Mean (SD) serum chloridion (mmol/L) in each group

Group	Postoperative Time (Days)								
	0	**7**	**14**	**28**	**56**	**84**	**112**	**140**	**168**
Group 1	109.4 (2.3)	99.0 (10.9)	100.9						
Group 2	110.9 (3.5)	100.3 (8.9)	102.8 (13.4)	111.8					
Group 3	110.1 (3.1)	104.3 (2.6)	108.5 (3.6)	108.1 (4.8)	104.9 (7.6)	106.5 (1.9)	101.1	103.7	103.7
Group 4	108.4 (2.5)	105.8 (2.6)	109.7 (12.1)	106.3 (5.7)	114.5 (4.6)	109.2 (5.3)	109.3 (6.3)	97.0 (2.0)	100.0 (3.0)
Group 5	111.5 (3.9)	105.0 (1.1)	104.3 (2.3)	105.3 (2.2)	101.6 (4.4)	109.3	108.7	107.3	106.3
Group 6	112.5 (4.9)	107.3 (4.1)	107.2 (4.8)	103.4 (13.5)	101.4 (11.1)	104.6 (7.8)	105.0	109.0	107.8
Group 7	117.8 (3.1)	103.3 (2.9)	103.2 (4.0)	96.4 (6.5)					
Group 8	114.5 (2.0)	106.4 (0.7)	106.2 (1.5)	105.4 (1.2)	106.0 (1.4)	107.6 (2.7)	106.9 (3.3)	102.8	112.1
Group 9	115.7 (3.0)	105.1 (3.1)	105.3 (4.4)	104.5 (5.5)	104.1 (2.2)	106.4 (4.8)	108.6 (2.4)	111.3 (0.5)	109.7 (2.9)
Group 10	129.2 (14.1)	105.3 (5.6)	109.2 (2.6)	105.7 (11.1)	106.4	104.3	109.8	105.4	106.2
Group 11	122.7 (13.7)	107.7 (6.9)	108.9 (7.4)	108.1 (2.0)	110.0 (2.5)	110.6 (4.4)	108.2 (4.1)	104.6 (3.6)	105.4 (2.6)
Group 12	128.3 (11.0)	121.6 (17.9)	103.1 (12.1)	110.0 (4.3)	111.0 (2.9)	111.2 (2.5)	108.4 (2.6)	100.7 (11.6)	108.7 (1.4)

Table S2.8 Mean (SD) serum alkaline phosphatase (U/L) in each group

Group	Postoperative Time (Days)								
	0	**7**	**14**	**28**	**56**	**84**	**112**	**140**	**168**
Group 1	217.7 (60.5)	194.3 (30.2)	230.0						
Group 2	239.7 (79.2)	214.5 (67.2)	316.5 (164.8)	345.0					
Group 3	229.0 (37.2)	191.8 (34.5)	239.6 (48.0)	280.6 (138.3)	202.3 (48.0)	149.0 (67.9)	203.0	248.0	209.0
Group 4	257.8 (107.2)	203.0 (59.2)	231.6 (48.4)	191.5 (66.4)	234.5 (62.6)	216.0 (34.4)	163.3 (43.8)	394.5 (320.3)	177.0 (45.3)
Group 5	277.2 (88.2)	284.4 (156.4)	286.2 (133.2)	421.5 (145.3)	256.0 (41.6)	212.0	172.0	132.0	162.0
Group 6	225.0 (64.6)	192.9 (41.3)	247.6 (115.1)	281.4 (108.6)	164.5 (39.5)	217.5 (16.3)	238.0	153.0	205.0
Group 7	207.8 (68.7)	195.7 (52.2)	184.5 (32.9)	267.0 (135.9)					
Group 8	235.8 (65.5)	191.0 (51.9)	216.0 (74.3)	296.6 (94.0)	190.3 (33.2)	153.3 (32.9)	126.3 (4.5)	89.0	194.0
Group 9	308.2 (105.4)	254.8 (82.7)	320.6 (89.1)	369.8 (138.1)	382.8 (199.5)	251.5 (92.2)	184.8 (42.9)	134.3 (54.6)	208.7 (86.2)
Group 10	208.8 (90.8)	246.8 (96.0)	263.3 (116.7)	215.5 (101.5)	77.0	65.0	74.0	86.0	85.0
Group 11	353.0 (163.4)	284.6 (124.8)	295.5 (165.9)	343.8 (182.5)	306.0 (300.9)	203.9 (129.5)	327.7 (141.1)	274.8 (53.7)	198.8 (59.7)
Group 12	302.9 (99.3)	278.4 (53.7)	288.9 (86.1)	305.3 (92.6)	167.8 (38.8)	122.2 (31.5)	229.6 (94.5)	212.0 (98.0)	204.8 (52.4)

Chapter 3

ASP2409, a Next-Generation CTLA4-Ig, Versus Belatacept in Renal Allograft Survival in *Cynomolgus* Monkeys

ASP2409, a Next-Generation CTLA4-Ig, Versus Belatacept in Renal Allograft Survival in *Cynomolgus* Monkeys

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Summary: This paper is mainly focused on exploring the effects of ASP2409, the first CD-86 selective variant of CTLA4-Ig, on preventing acute kidney transplant rejection and promoting renal allograft acceptance in nonhuman primates. At same time, the influence of ASP2409 in the frequency of $FoxP3^+$ Tregs in renal allografts, the PK and PD of ASP2409 in transplanted monkey, and the side effects of ASP2409 were studied.

Author Contributions: Lijun Song participated in studies, data collection, data analysis and interpretation, and preparation of the manuscript; Huifang Chen directed the study design and participated in studies; Anlun Ma processed and analyzed PD and IHC data; Yanxin Hu participated in studies, processed and analyzed pathological data; Hao Dun participated in studies; Yasutomo Fujii, Fumitaka Kinugasa, Shinsuke Oshima, and Yasuyuki Higashi participated in study design; Pierre Daloze reviewed the manuscript and provided critical revision.

ASP2409, a Next-Generation CTLA4-Ig, Versus Belatacept in Renal Allograft Survival in *Cynomolgus* Monkeys

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Running title: ASP2409 in Renal Allograft Survival in Primates

Abbreviations: +, positive; A0.3, low-dose ASP2409 monotherapy; A0.3MP, low-dose ASP2409 combined with mycophenolate mofetil and pridol (methylprednisolone); A0.3T1.0, low-dose ASP2409 combined with subtherapeutic-dose tacrolimus; A1.0, high-dose ASP2409 monotherapy; A1.0MP, high-dose ASP2409 combined with mycophenolate mofetil and pridol; A1.0T0.5, high-dose ASP2409 combined with very low-dose tacrolimus; A1.0T1.0, high-dose ASP2409 combined with subtherapeutic-dose tacrolimus; AMMS, Academy of Military Medical Sciences; APC, antigen-presenting cell; AR, acute rejection; BLOQ, below the lower limit of quantification; BMP, belatacept combined with mycophenolate mofetil and pridol; BUN, blood urea nitrogen; CAN, chronic allograft nephropathy; ci, interstitial fibrosis; CNI, calcineurin inhibitor; CTLA4-Ig, cytotoxic T-lymphocyte associated protein 4-immunoglobulin; ct, tubular atrophy; cum, cumulative; cv, fibrous intimal thickening; DAPI, 40,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; i, interstitial inflammation; MFI, mean fluorescence intensity; MMF, mycophenolate mofetil; MPA, mycophenolic acid; MST, median survival time; NR, no rejection; PE, phycoerythrin; PK, pharmacokinetic; POD, posttransplant day; PTLD, posttransplant lymphoproliferative disorder; sCr, serum creatinine; T0.5, very low-dose tacrolimus monotherapy; T1.0, subtherapeutic-dose tacrolimus monotherapy; T2.0MP, therapeuticdose tacrolimus combined with mycophenolate mofetil and pridol; Treg, regulatory T cell

Abstract

Belatacept is the first costimulatory blockade agent approved for maintenance immunosuppression in kidney transplant recipients. Clinical results have indicated that belatacept is associated with superior renal function and improved metabolic profile. However, higher incidence of acute rejection and PTLD are the shortcomings of this agent. In this study, ASP2409, a new CTLA4-Ig possessing 14-fold higher *in vitro* CD86 binding affinity than belatacept, was tested for renal allograft survival in *cynomolgus* monkeys. ASP2409 monotherapy dose-dependently prolonged renal allograft survival. Low-dose ASP2409 in combination with a sub-therapeutic dose of tacrolimus showed much longer MST than monotherapy. Similar allograft survival results were observed in high-dose ASP2409-, belatacept- and therapeutic dose tacrolimus-based regimens. The results of renal allograft histopathology of high-dose ASP2409-based regimens were not inferior to the belatacept-based regimen. Moreover, higher frequencies of $FoxP3^{+}$ Tregs in renal allografts were observed in ASP2409- and belatacept-based regimens compared to tacrolimus-based regimens. There were no serious side effects related to ASP2409 administration to be found during the study. The present data suggest that ASP2409 is a promising candidate for CNI sparing or avoidance regimens.

Introduction

Organ transplantation opened a new era in the history of treatment of end-stage organ failure. Currently, rejection is still the major obstacle to success because most organ transplantations are carried out between genetically distinct individuals. T cells are the crucial mediators and controllers in graft rejection induced by histoincompatible alloantigens (Ingulli 2010). It is known that full T cell activation requires several collaborative but distinct signals. In addition to the triggered signal generated by the interaction between T cell receptor (TCR) and major histocompatibility complex (MHC)-bound peptide, costimulatory signals are also essential for T cells to be efficiently activated. In absence of these signals, T cells will enter a state of proliferative nonresponsiveness and undergo apoptosis (Jenkins 1987; Jenkins 1988; Frauwirth 2002).

Currently, numerous costimulatory molecules have been identified. Unlike the targets of conventional immunosuppressants, these molecules are largely limited to T cells and/or antigen-presenting cells. Targeting costimulatory pathways offers the potential for selective inhibition of alloimmune response. CD28–CD80 (B7-1)/CD86 (B7-2) is one of the well characterized costimulatory receptor/ligand pairs. CD28 signals amplify TCR-induced signaling events, promote IL-2 production (June 1989), mediate T cells to enter the cell cycle (Appleman 2000), increase the generation of cytokines and chemokines (Thompson 1989), control regulatory T cell (Treg) homeostasis (Salomon 2000) and reinforce resistance to apoptosis (Sperling 1996).

Blocking the CD28–CD80/CD86 pathway with CTLA4-Ig has been demonstrated to successfully prevent allograft rejection. Belatacept, a high affinity variant of CTLA4-Ig, has been approved for clinical use in renal transplantation recipients. Compared to cyclosporine-based regimen, belatacept-based regimens exhibited similar patient/graft survival, superior renal function, and improved metabolic profile (Vincenti 2005). However, belatacept was found to be associated with higher rates and grades of acute rejection. In addition, post-transplant lymphoproliferative disorder (PTLD) was observed more frequently in belatacept-treated patients (Vincenti 2010; Vincenti 2012).

ASP2409 is a fusion protein recombining the modified extracellular portion of human CTLA4 with the wild-type fragment crystallizable (Fc) region of human IgG2. This compound is created through directed evolution using DNA shuffling techniques. Compared with wild-type CTLA4-Ig, there are total 10 amino acid mutations (A24E, A50M, G55E, I65S, L104E, N56D, S64P, S70F, T30N, V32I) within the protein structure of ASP2409. Thus this novel CTLA4-Ig variant has binding affinity to human CD80 and CD86 with K_D values of 64.2 pM and 30.0 pM, respectively. That means ASP2409 possesses approximately 14-fold higher CD86 binding affinity compared to belatacept, whereas the CD80 binding affinity of belatacept is 1.8-fold higher than that of ASP2409. Of note, previous CTLA4-Igs including belatacept are all selective for CD80 binding. ASP2409 is a CD86-selective CTLA4-Ig with 2.1-fold higher binding affinity for CD86 than that for CD80 (Oshima 2016a).

This study evaluated the effects of ASP2409 on renal allograft survival in nonhuman primates. We found that ASP2409, monotherapy and in combination with sub-therapeutic dose

tacrolimus or mycophenolate mofetil (MMF), significantly prolonged allograft survival. High-dose ASP2409-based regimens showed similar allograft survival results compared with the belatacept-based regimen and the therapeutic dose tacrolimus-based regimen, and non-inferior histopathological results compared to the belatacept-based regimen.

Materials and Methods

Animals

This study utilized specific pathogen (hepatitis B virus, hepatitis C virus, simian immunodeficiency virus, and Herpes B virus) free, 3.3 to 6.3 kg male *cynomolgus* monkeys, obtained from Laboratory Animals Center of the Academy of Military Medical Sciences (AMMS), Beijing, China. All experimental procedures were approved by the Ethical Committee for Animal Experimentation at Laboratory Animals Center of the AMMS, and were performed in accordance with the Guide for the Care and Use of Laboratory Animals. The recipient and donor pairs chosen were ABO antigen compatible with a stimulation index of 2.5 or higher in a one-way mixed lymphocyte reaction, as previously described (Ma 2014).

Life supporting kidney transplantation

Heterotopic renal transplantations were performed as described previously (Song 2010; Chen 2000). Briefly, each animal in this study acted as both donor and recipient. Monkeys were anesthetized by intramuscular injection of ketamine and xylazine and a midline incision was made to open the abdominal cavity. The left kidney and ureter were removed *en bloc* and exchanged between paired monkeys. The allograft then was implanted 2–4 cm distal on the left native kidney by end-to-side anastomoses of renal artery to aorta and renal vein to vena

cava, and end-to-end anastomosis of donor and recipient ureters. The right native kidney was removed after the transplanted kidney was reperfused.

Experimental group and treatment

Animals were divided into twelve groups as shown in Table 3.1: *i.e.*, no-treatment control, low-dose ASP2409 monotherapy (A0.3), high-dose ASP2409 monotherapy (A1.0), very-low-dose tacrolimus monotherapy (T0.5), sub-therapeutic dose tacrolimus monotherapy (T1.0), low-dose ASP2409 combined with sub-therapeutic dose tacrolimus (A0.3T1.0), high-dose ASP2409 combined with very-low-dose tacrolimus (A1.0T0.5), high-dose ASP2409 combined with sub-therapeutic dose tacrolimus (A1.0T1.0), low-dose ASP2409 combined with MMF and pridol (methylprednisolone) (A0.3MP), high-dose ASP2409 combined with MMF and pridol (A1.0MP), belatacept combined with MMF and pridol (BMP), therapeutic dose tacrolimus combined with MMF and pridol (T2.0MP). ASP2409 was provided by Astellas. All procedures for generation and production of ASP2409 were performed under the control of Perseid Therapeutics (Redwood city, CA). ASP2409 was administered subcutaneously at doses of 0.3 mg/kg or 1 mg/kg (0.2 mL/kg) on post-transplantation days (PODs) 0, 4, 7, then weekly to day 84. Tacrolimus (Astellas) was orally administered immediately after kidney transplantation, then once daily, until day 90. MMF (Hoffmann-La Roche Ltd.) 15 mg/kg was given subcutaneously twice daily from PODs 0 to 14, and then once daily until day 90. Pridol (Alfresa Pharma Corporation) was administered subcutaneously once a day in a tapering manner from PODs 0 to 90. Belatacept (Bristol-Myers Squibb) was administered subcutaneously 5 mg/kg on PODs 0, 4, 7, and then once weekly to day 84. Study period was determined as 91 days.

Biochemical and hematological determinations

Serum creatinine (sCr) and blood urea nitrogen (BUN) were monitored to determine renal function. Other biochemical parameters including total protein, glucose, amylase, cholesterol, triglyceride, lactate dehydrogenase, albumin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, total bilirubin, and creatine kinase were measured using the auto analyzer (Beckman Synchron CX5 Pro, Beckman Coulter Inc.).

Hematological parameters, red blood cell, white blood cell, lymphocyte counts and differential, hemoglobin, hematocrit, and platelet counts were examined in peripheral blood samples using the automated hematology analyzer.

Pharmacokinetic (PK) evaluation

Serial serum samples were collected before drug administration on days 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, and on days 16, 30, 58, 86, 91 to measure ASP2409 and belatacept levels. To measure tacrolimus levels, whole blood samples were taken before drug administration on days 0, 7, 14, 28, 56, 84, and 1 hr after drug administration on days 14, 28, 56, 84. The plasma mycophenolic acid (MPA) levels were determined on days 0, 7, 14, 28, 56, and 84 before drug administration, and on days 14, 28, 56, and 84 at 1 h after drug administration.

Anti-ASP2409 antibody

Monkey anti-ASP2409 antibodies were detected with electrochemiluminescent (ECL) immunoassay at PPD (VA, USA) under the control of Shin Nippon Biomedical (SNBL: Kagoshima, Japan). The sampling schedule was same as PK evaluation for ASP2409.

Pharmacodynamics (PD) evaluation

The following monoclonal antibodies (mAbs) were used in PD evaluation: FITC mouse anti-human CD80 Clone: L307.4 (BD 557226), PE mouse anti-human CD86 Clone: 2331 (FUN-1) (BD 557344), APC mouse anti-human HLA-DR Clone: L243 (G46-6) (BD 559866), APC anti-CD14 antibody Clone: TÜK4 (MACS 130-091-243), FITC mouse IgG1, κ Isotype control Clone: MOPC-21 (BD 555748), PE mouse IgG1, κ Isotype control Clone: MOPC-21 (BD 556650).

A 1 mL of heparin anticoagulation blood was drawn from each monkey with same sampling schedule as PK evaluation for ASP2409. The CD80 receptor occupancy on MHC-DR positive cells and CD86 receptor occupancy on CD14 positive cells were evaluated using binding competition assay with commoditized CD80/CD86 mAbs to detect the mean fluorescence intensity (MFI) of the CD80/86-bound antibody with flow cytometry. The occupancy rate was calculated using the following formula: Occupancy rate (%) = ([ΔMFI before treatment] - [ΔMFI at each time point] / [ΔMFI before treatment]) × 100, (ΔMFI = MFI - MFI control).

Detection of regulatory T cells by immunohistochemical staining assay in paraffin embedded sections of renal allografts

The infiltration of $CD3^+FoxP3^+$ and $CD3^+$ T cells were examined by immunohistochemistry assay on kidney allograft paraffin sections in monkeys that survived till the end of study, and on normal kidneys that had no any therapy and transplantation.

FoxP3 has been reported to be transiently expressed by activated nonregulatory T cells (Wang 2007). High infiltration of $FoxP3^+$ cells was observed in samples from kidney allograft with acute cellular rejection (Veronese 2007). Besides of Tregs, these intragraft $FoxP3^+$ cells can also be activated effector T cells. To minimize the influence of this factor, the staining assay in this study was conducted in renal grafts that pathological diagnoses were not acute rejection.

Histopathological determinations

All recipient monkeys were subjected to complete gross necropsies. Routine hematoxylin and eosin staining were performed on all paraffin-embedded sample sections including kidney graft, liver, pancreas, spleen, heart, lung, stomach, jejunum, thoracic aorta, and mesentery lymph nodes. Kidney graft sections were stained with Masson's Trichrome and Periodic Acid-Schiff (PAS) as well. The Banff 97 classification was used for scoring the presence and degree of renal rejection (Racusen 1999).

Statistical analysis

In this study, all analyses were conducted using SPSS 13. All laboratory assay data including the percentage of $CD3^+FoxP3^+$ T cells in renal allografts were analyzed by running post hoc tests. Renal allograft survival was presented as median survival time (MST), with

comparisons among groups performed by log rank test. A p value less than 0.05 was considered statistically significant.

Results

Renal allograft survival

Renal allograft survival day was recorded according to the following definitions: 1) The final day that the sCr level did not exceed a value of 10 mg/dL; 2) The day when significant asthenia had been observed; 3) A day before the finding of death; 4) Animal that survived to the end of study period was described as >91 days. As shown in Table 3.1 and Figure 3.1C, the MSTs were 5 days for the no-treatment control group, 26 days for the low-dose ASP2409 monotherapy group, >91 days for the high-dose ASP2409 monotherapy group, 7 days for the very-low-dose tacrolimus monotherapy group, 38.5 days for the sub-therapeutic dose tacrolimus monotherapy group, 47 days for the high-dose ASP2409 combined with very-low-dose tacrolimus group, >91 days for the low-dose ASP2409 combined with sub-therapeutic dose tacrolimus group, >91 days for the high-dose ASP2409 combined with sub-therapeutic dose tacrolimus group, 42 days for the low-dose ASP2409 combined with MMF and pridol group, >91 days for the high-dose ASP2409 combined with MMF and pridol group, >91 days for the belatacept combined with MMF and pridol group, and >91 days for the therapeutic dose tacrolimus combined with MMF and pridol group. Compared with no-treatment control group, the MSTs of all treatment groups except T0.5 group were significantly prolonged (p = 0.0082, 0.0003, 0.0566, 0.0013, 0.0006, 0.0003, 0.0001, 0.0001, 0.0001, 0.0003, 0.0001, respectively). Increasing ASP2409 dosage from 0.3 mg/kg to 1 mg/kg, the allograft survival times in ASP2409 monotherapy groups were dramatically increased (MST, 26 days *vs.* >91

Table 3.1 Renal Allograft Survival and Histopathological Evaluation

Group	Therapeutics	Number	MST	Individual Graft Survival Time (days)							
				Renal Allograft Pathology at Necropsy (Banff 1997)							
Control	No Treatment	6	5	5 AR III	5 AR IIB	5 AR III	5 AR IB	7 AR IIA	7 AR III		
A0.3	ASP2409 0.3 mg/kg	8	26*	5 AR IIA	5 AR IIA	10 AR IIA	17 AR III	35 AR IA	36 AR IIA	44 AR IIA/CAN Ib	49 AR IIA/CAN Ib
A1.0	ASP2409 1 mg/kg	7	>91*	17 AR IA	35 AR IIA/CAN Ib	46 CAN Ia	>91 CAN IIIa	>91 CAN Ib	>91 CAN Ia	>91 CAN IIa	
T0.5	Tacrolimus 0.5 mg/kg	8	7	5 AR III	5 AR IIA	7 AR III	7 AR IIA	7 AR III	16 CAN Ia	17 AR IA	42 AR IIA/CAN Ib
T1.0	Tacrolimus 1 mg/kg	8	38.5*	7 AR IIA	7 AR III	24 AR III	32 CAN Ia	45 CAN Ia	45 CAN Ia	>91 CAN Ib	>91 NR
A1.0T0.5	ASP2409 1 mg/kg + Tacrolimus 0.5 mg/kg	6	47*	29 CAN IIb	39 CAN IIa	42 CAN Ib	52 CAN Ia	>91 CAN Ib	>91 NR		
A0.3T1.0	ASP2409 0.3 mg/kg + Tacrolimus 1 mg/kg	7	>91*#&	24 CAN Ia	>91 NR	>91 NR	>91 NR	>91 CAN IIb	>91 CAN IIb	>91 CAN IIb	
A1.0T1.0	ASP2409 1 mg/kg + Tacrolimus 1 mg/kg	8	>91*&	68 CAN Ia	85 CAN Ia	>91 NR (ci1)	>91 NR	>91 NR	>91 CAN IIa	>91 NR (cv1)	>91 CAN Ia
A0.3MP	ASP2409 0.3 mg/kg + MMF 15 mg/kg + Pridol	8	42*	28 AR IIA	35 AR IIA	40 AR IIA	42 CAN Ib	42 AR III	45 AR IA	58 AR IIA/CAN Ia	72 AR IIA
A1.0MP	ASP2409 1 mg/kg + MMF 15 mg/kg + Pridol	8	>91*	34 NR (i1)	64 CAN Ia	70 AR IA/CAN Ib	>91 CAN Ib	>91 CAN Ia	>91 CAN Ia	>91 NR (i1/ci0/ct1)	>91 CAN Ia
BMP	Belatacept 5 mg/kg + MMF 15 mg/kg + Pridol	7	>91*	30 AR IIA	49 CAN Ib	53 CAN Ia	>91 CAN Ia	>91 CAN Ib	>91 CAN Ia	>91 CAN IIa	
T2.0MP	Tacrolimus 2 mg/kg + MMF 15 mg/kg + Pridol	8	>91*	51 CAN Ib	>91 NR (i1)	>91 CAN Ia	>91 CAN Ia	>91 NR	>91 NR	>91 NR (ct1)	>91 NR

AR = acute rejection; CAN = chronic allograft nephropathy; ci = interstitial fibrosis; ct = tubular atrophy; cv = fibrous intimal thickening; i = interstitial inflammation; MST = median survival time; NR = no rejection.

* $p < 0.01$ *vs.* no-treatment group, # $p < 0.01$ *vs.* A0.3 group, & $p < 0.05$ *vs.* T1.0 group. anti-ASP2409 antibody was detected during the maintenance treatment period.

days, $p = 0.0123$). In terms of the 91-day observation period, ASP2409 (either in low-dose or high-dose) combined with sub-therapeutic tacrolimus, and high-dose ASP2409 in combination with MMF showed similar graft survival results with the belatacept-based regimen and the conventional therapeutic dose of tacrolimus-based regimen.

Renal graft function

Generally, rapid elevation of sCr and BUN levels implied the occurrence of acute graft rejection. The renal function parameters were relatively stable in long-term surviving monkeys (Figure 3.1A and 3.1B).

Biochemistry

There was no evidence that administration of ASP2409 affected serum liver enzymes, creatinine kinase, and other biochemical parameters.

Hematological determinations

Red blood cell counts, hemoglobin, and haematocrit decreased shortly after surgery in most groups. These parameters recovered and kept stable two weeks after surgery.

Body weight and clinical symptoms

Most of the monkey weights remained stable during the study. After recovery from surgery, all monkeys were very active with good appetites. However, when acute rejection occurred, animals became lethargic, lost appetite and weight, and developed oliguria which was accompanied by an increase of sCr.

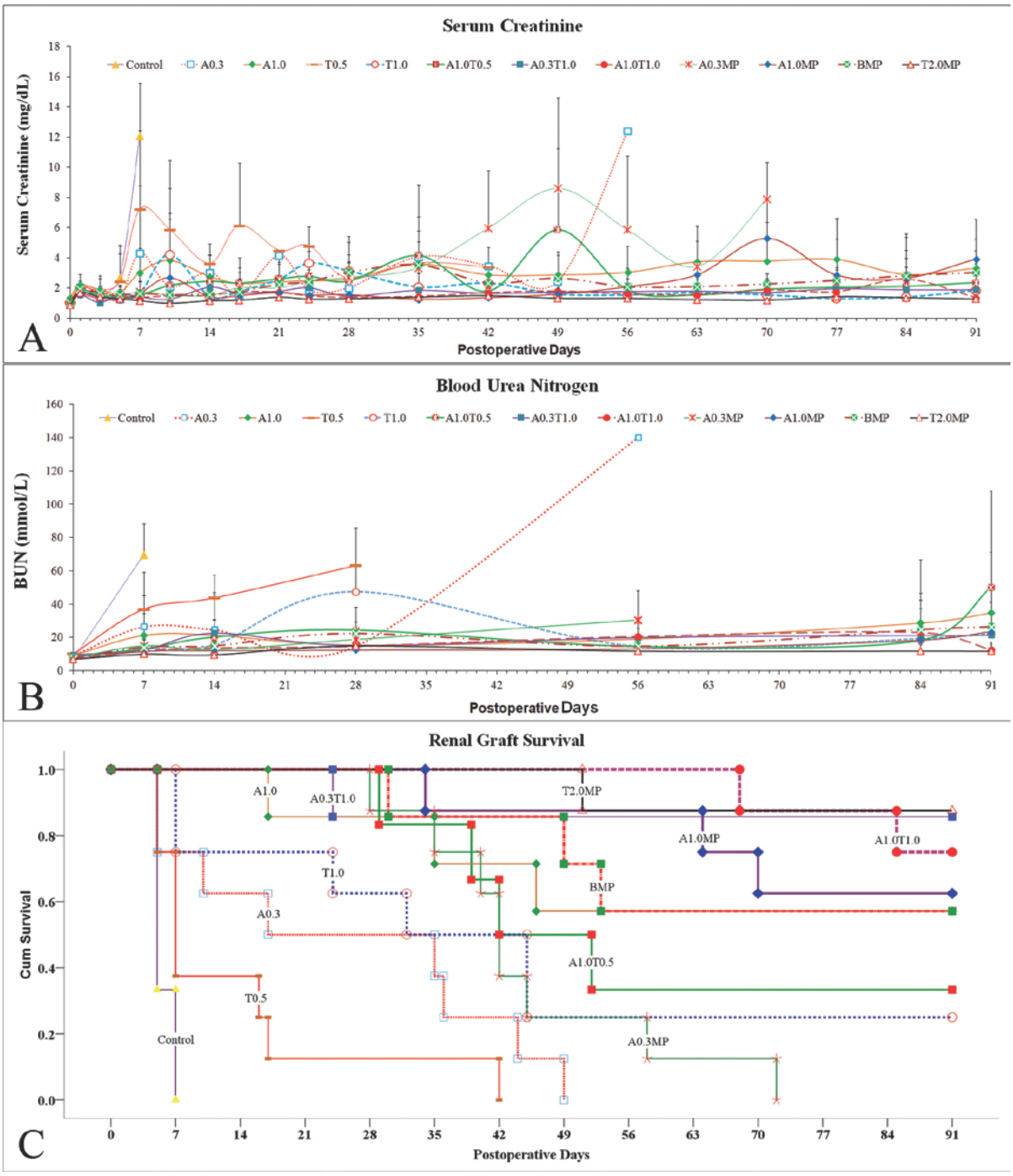


Figure 3.1 Renal graft function and survival. A. Mean serum creatinine level in each group. The elevation of sCr level was consistent with the occurrence of acute allograft rejection. B. Mean blood urea nitrogen level in all groups. Changes of mean BUN levels were similar to sCr. C. Renal allograft survival in each group. (T0.5 =

tacrolimus 0.5 mg/kg, T1.0 = tacrolimus 1.0 mg/kg, T2.0 = tacrolimus 2.0 mg/kg, A0.3 = ASP2409 0.3 mg/kg, A1.0 = ASP2409 1.0 mg/kg, B = belatacept 5 mg/kg, M = mycophenolate mofetil 15 mg/kg, *P* = pridol).

Pharmacokinetic evaluation

Serum ASP2409 concentration: In general, serum concentrations of ASP2409 increased in a dose-dependent manner. During maintenance phase, the mean ASP2409 C_{trough} values in low-dose ASP2409 treated groups were from below the lower limit of quantification (BLOQ, <0.04 μg/mL) to 0.23 μg/mL, and the mean ASP2409 C_{2days} values ranged from BLOQ to 1.10 μg/mL. In contrast, the range of the mean ASP2409 C_{trough} and ASP2409 C_{2days} values in high-dose ASP2409 treated groups were 0.67–4.46 μg/mL and 3.48–10.80 μg/mL, respectively (Figure 3.2A).

Serum belatacept concentration: The mean belatacept C_{trough} values during maintenance phase ranged from 10.7 to 16.8 μg/mL. The mean belatacept C_{2days} values on days 16, 30, 58, and 86 of dosing were 31.7, 29.3, 35.0, and 30.6 μg/mL, respectively (Figure 3.2B).

Blood concentration of tacrolimus: The profiles of mean concentrations of tacrolimus are illustrated in Figure 3.2C. The whole blood tacrolimus concentrations increased in a dose-related manner. The mean tacrolimus C_{trough} values in 0.5 mg/kg, 1 mg/kg, and 2 mg/kg tacrolimus-treated groups were 2.87–13.09 ng/mL, 7.36–15.63 ng/mL, and 10.72–17.03 ng/mL, respectively. The mean tacrolimus C_{1h} values in 0.5 mg/kg, 1 mg/kg, and 2 mg/kg tacrolimus treated groups were 27.06–49.28 ng/mL, 36.64–51.05 ng/mL, and 41.55–82.99 ng/mL, respectively.

Plasma concentration of mycophenolic acid: The mean plasma MPA levels are shown in Figure 3.2D. Generally the mean MPA C_{trough} and C_{1h} values in each group were similar. The mean MPA trough level fluctuated at the range of 0.54–2.60 μg/mL, and the mean MPA C_{1h} values ranged from 4.13 to 10.98 μg/mL.

Anti-ASP2409 antibody assay

Monkey anti-ASP2409 antibodies were detected in 17 of 52 ASP2409 treated animals during the maintenance treatment period (Table 3.1). There were four cases in high-dose ASP2409 treated groups (Two of each in groups A1.0T0.5 and A1.0MP). The remaining cases were all found in low-dose ASP2409 treated monkeys.

Pharmacodynamics evaluation

The CD80 and CD86 receptor occupancy by ASP2409 increased in a dose-dependent manner. The CD86 receptor occupancy rate reached an almost saturated state in all high-dose ASP2409 treated groups on POD 7, and remained stable in the maintenance phase. The low-dose ASP2409 treated groups showed slightly lower CD86 receptor occupancy rate than high-dose ASP2409 treated groups on POD 7, but they dramatically decreased during the maintenance phase. The CD80 receptor occupancy by ASP2409 was relatively lower compared to CD86. The means of CD80 receptor occupancy in high-dose ASP2409 treated groups were higher than those of low-dose ASP2409 treated groups, especially during the maintenance phase. Compared to ASP2409, belatacept showed higher CD80 receptor occupancy and lower CD86 receptor occupancy (Figure 3.3A and 3.3B).

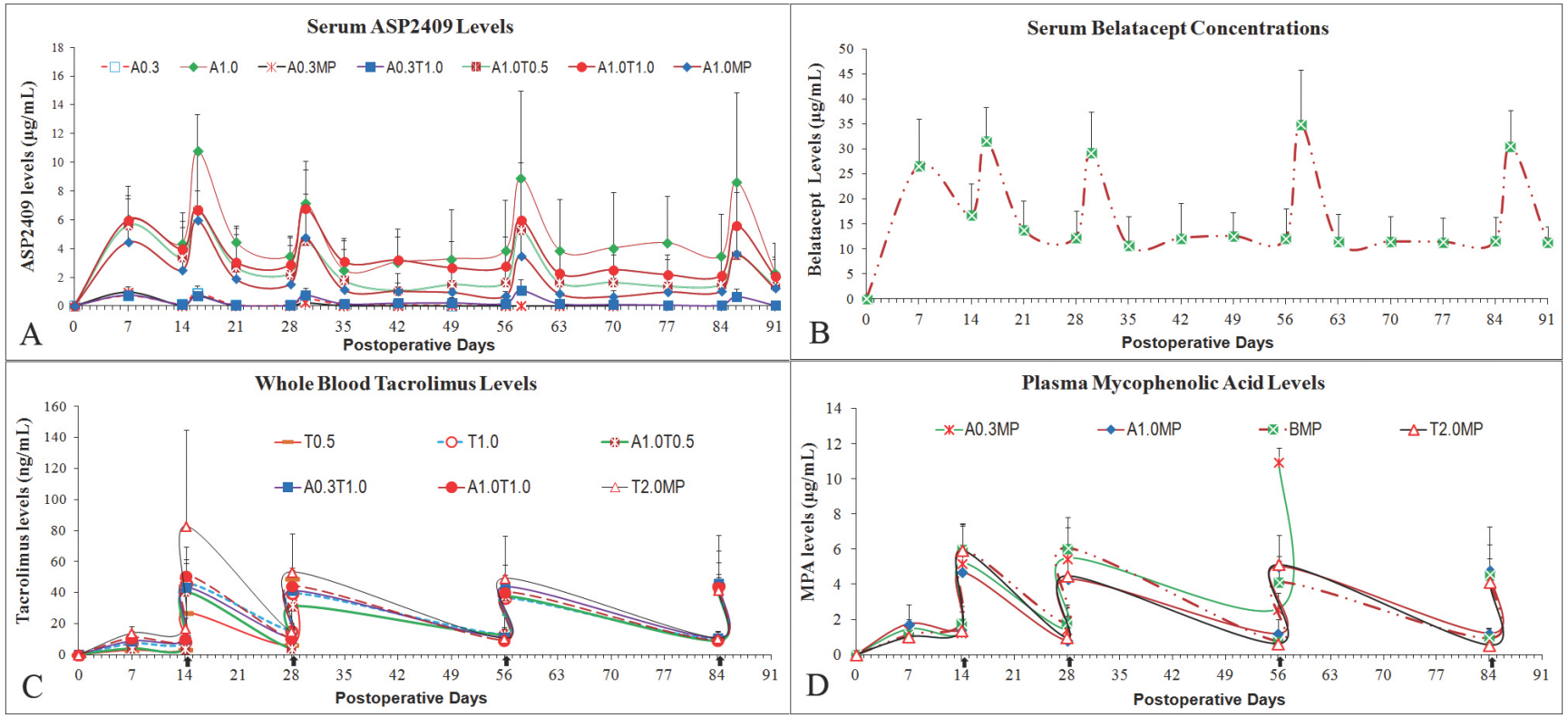


Figure 3.2 Pharmacokinetic evaluation. A. The mean serum concentration transitions of ASP2409 in each group. Serum concentrations of ASP2409 increased in a dose-dependent manner. B. The mean serum concentration of belatacept. C. The mean whole blood tacrolimus trough levels and C1h increased in a dose-related manner. D. Mean plasma concentrations of mycophenolic acid. In general, plasma trough levels and C1h of MPA were similar in each group. (T0.5 = tacrolimus 0.5 mg/kg, T1.0 = tacrolimus 1.0 mg/kg, T2.0 = tacrolimus 2.0 mg/kg, A0.3 = ASP2409 0.3 mg/kg, A1.0 = ASP2409 1.0 mg/kg, B = belatacept 5 mg/kg, M = mycophenolate mofetil 15 mg/kg, *P* = pridol, ↑ = one hour after drug administration).

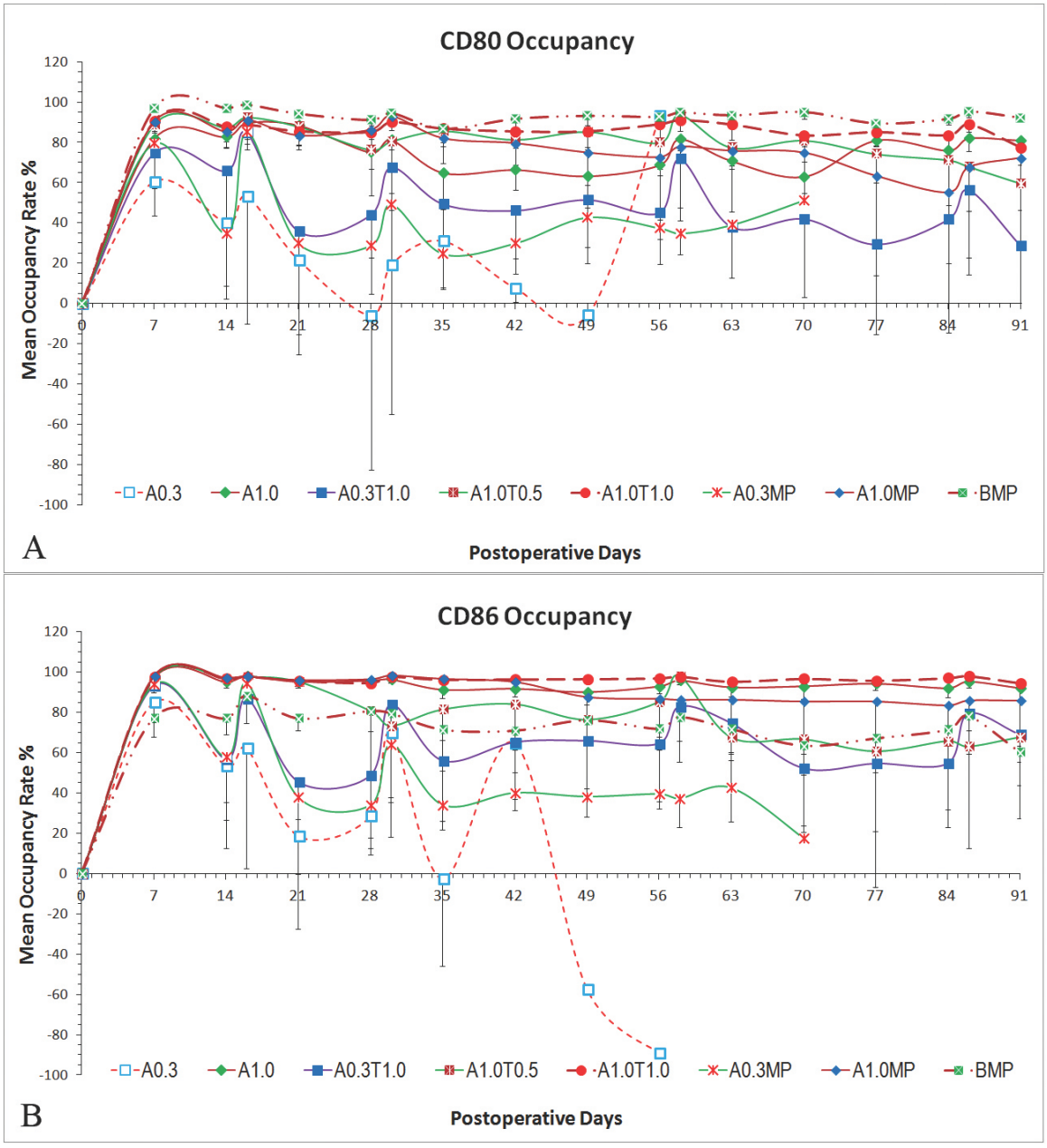


Figure 3.3 Pharmacodynamics evaluation. A. The mean level of CD80 receptor occupancy in each group. The rate of CD80 receptor occupancy by ASP2409 increased in a dose-dependent manner. Belatacept exhibited higher CD80 receptor occupancy than ASP2409, and it is consistent with belatacept possessing higher CD80 binding affinity. B. The mean level of CD86 receptor occupancy in each group. The rate of CD86 receptor occupancy by ASP2409 dose-dependently increased. In the maintenance phase, high-dose ASP2409 treated groups exhibited high level of CD86 receptor occupancy, whereas CD86 receptor occupancy in low-dose

ASP2409 treated groups dramatically decreased. ASP2409 showed higher percentage of CD86 receptor occupancy than belatacept. It is consistent with ASP2409 to be designed possessing higher CD86 binding affinity than belatacept.

$CD3^+FoxP3^+$ Tregs in renal allografts

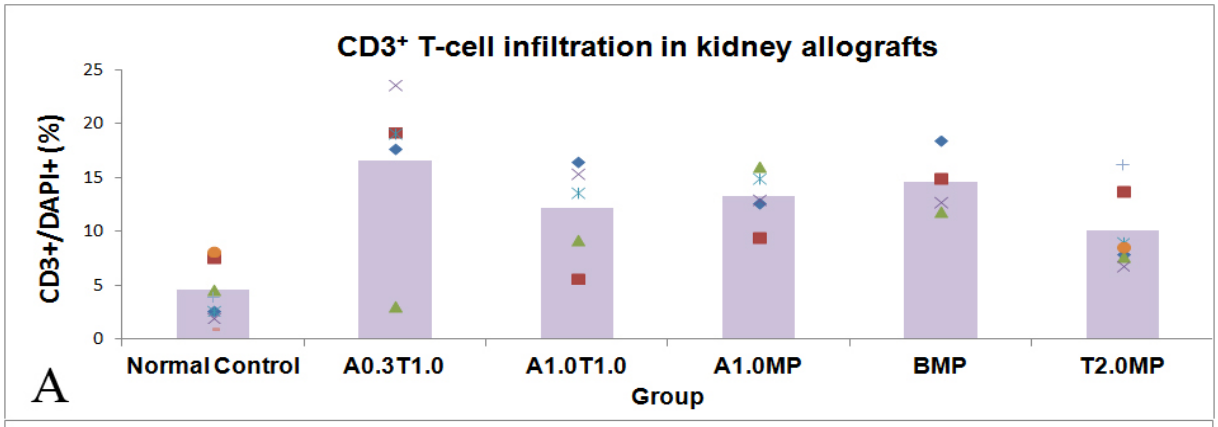
To evaluate whether blockade of CD28–CD80/CD86 costimulatory pathway affects Treg cells in kidney transplant, we examined the frequencies of $CD3^+FoxP3^+$ and $CD3^+$ T cells in renal allograft by immunohistochemistry assay.

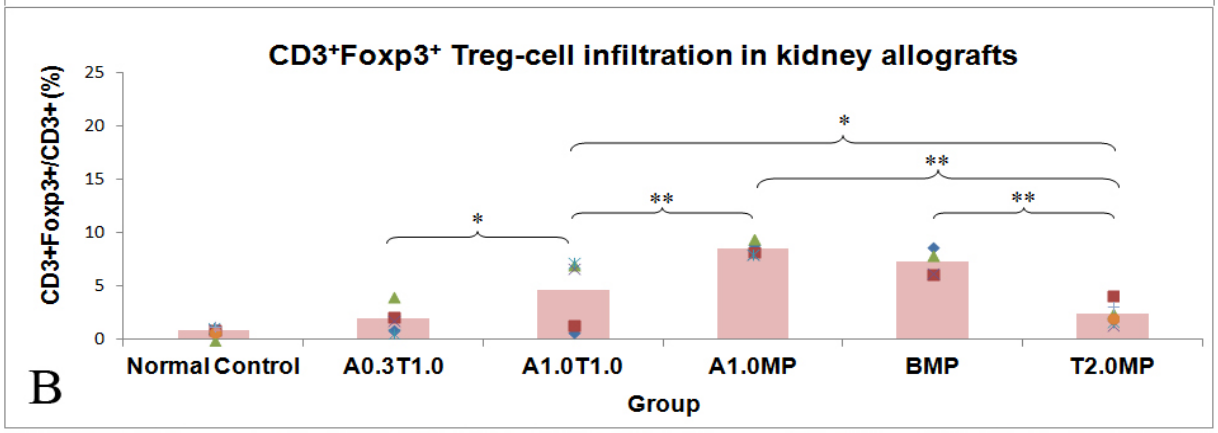
As shown in Figure 3.4, the percentage of $CD3^+FoxP3^+$ Treg cells among total $CD3^+$ T cells was very low in normal monkey kidneys (0.89%). In two combinations of ASP2409 and sub-therapeutic dose tacrolimus, high-dose ASP2409 group exhibited higher proportion of $CD3^+FoxP3^+$ cells than low-dose ASP2409 group (4.60% *vs.* 1.92% $p < 0.05$). Substantially higher percentages of $CD3^+FoxP3^+$ cells were seen in renal allografts in groups high-dose ASP2409 combined with MMF (8.51%) and belatacept combined with MMF (7.28%) relative to the combination of therapeutic dose tacrolimus and MMF (2.41%).

Histopathology

The details of histological features of transplanted kidneys are summarized in Table 3.1. All renal allografts in groups no-treatment control and low-dose ASP2409 monotherapy exhibited acute rejection. Acute rejection was also found in most transplanted kidneys in groups very-low-dose tacrolimus monotherapy (7/8) and low-dose ASP2409 combined with MMF (7/8).

The incidence of acute rejection was significantly decreased in groups sub-therapeutic dose tacrolimus monotherapy (3/8), high-dose ASP2409 monotherapy (2/7), high-dose ASP2409 combined with MMF (1/8), and belatacept combined with MMF (1/7). No acute rejection was discovered in groups high-dose ASP2409 combined with very-low-dose tacrolimus, low-dose ASP2409 combined with sub-therapeutic dose tacrolimus, high-dose ASP2409 combined with sub-therapeutic dose tacrolimus, and therapeutic dose tacrolimus combined with MMF. Varying degrees of chronic allograft nephropathy (CAN) were the common pathological changes observed in long-term survival animals. Some animals in various cohorts displayed no rejection.


CD3⁺ T-cell infiltration in kidney allografts
CD3+/DAPI+ (%)
25
20
15
10
5
0
Normal Control
A0.3T1.0
A1.0T1.0
A1.0MP
BMP
T2.0MP
Group
A


CD3⁺Foxp3⁺ Treg-cell infiltration in kidney allografts
CD3+Foxp3+/CD3+ (%)
25
20
15
10
5
0
*
**
**
*
**
Normal Control
A0.3T1.0
A1.0T1.0
A1.0MP
BMP
T2.0MP
Group
B

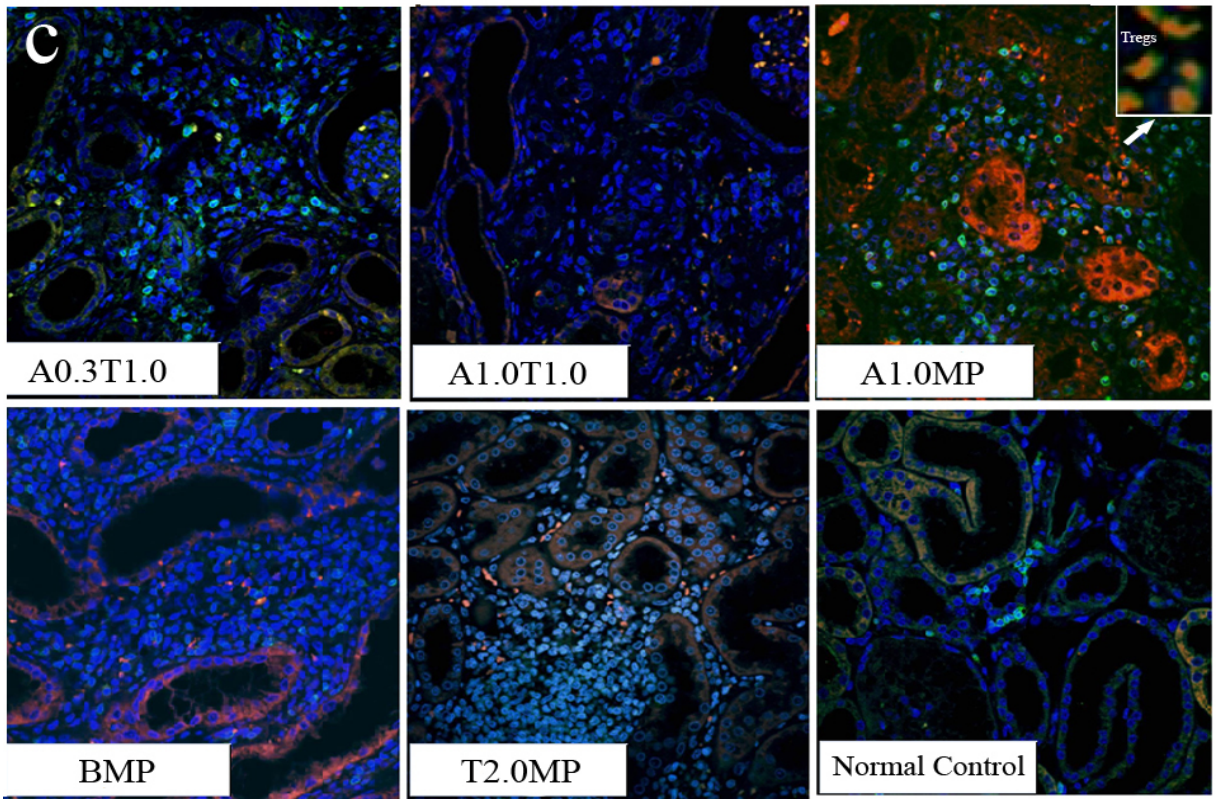

C
Tregs
A0.3T1.0
A1.0T1.0
A1.0MP
BMP
T2.0MP
Normal Control

Figure 3.4 The infiltration of CD3/FoxP3 double-positive Tregs in renal allografts. A. The percentage of $CD3^+$ cells in $DAPI^+$ cells in renal allografts. DAPI, 40,6-diamidino-2-phenylindole. B. The percentage of $CD3^+FoxP3^+$ cells in $CD3^+$ cells in renal allografts (* $p < 0.05$, ** $p < 0.01$). C. The fluorescent detection of $CD3^+$ (green membrane staining) $FoxP3^+$ (red intracellular staining) Tregs (inset shows enlarged image of Tregs) on paraffin sections of renal allografts. DNA was stained with DAPI (blue). Original magnifications: ×400.

Discussion

Currently calcineurin inhibitors (CNIs) are still the cornerstone for maintenance therapy in organ transplantation. However, the extensive adverse effects of CNIs that may cause graft dysfunction, loss and/or patient death impact the long-term outcomes of organ transplants. Several therapeutic regimens such as CNI reduction, avoidance, and early withdrawal have been tested to minimize the consequences associated with CNI exposure (Flechner 2008; Barbari 2007). The reported results of these immunosuppressive regimens have shown mixed results. Some of them increased the risk for acute and chronic rejection (Salvadori 2013; Grinyó 2009). The belatacept-based regimens shed light on CNI avoidance protocols. Through a series of renal transplantation clinical trials, belatacept-based regimens were demonstrated to confer similar patient and graft survival rates with cyclosporine-based regimens, and superior renal function, as well as an improved metabolic profile (Vincenti 2005; Vincenti 2010; Vincenti 2012, Rostaing 2013). Unfortunately, higher incidence of acute rejection and PTLD is the potential “Achilles’ heel” of belatacept to slow the progress of this novel agent on other organ transplant clinical application (Vincenti 2010, Knechtle 2014).

CD28 signaling plays a pivotal role in T cell activation, especially in the early post-transplant stage. Between the two natural ligands for CD28, CD86 exhibits higher affinity than CD80. In contrast, CD80 has a higher binding affinity for CTLA4 (Collins 2002; Linsley 1994). For the purpose to durably suppress allograft rejection, complete and equal blockade of the costimulatory pathway is required (Greene 1996). To develop new CTLA4-Ig proteins which possess more potent immunosuppressive properties, it is reasonable to engineer a molecule with higher binding affinity for its B7 ligands, especially CD86. ASP2409 is designed to reach this goal. *In vitro* pharmacology studies indicated that this new generation CTLA4-Ig had 220-fold and 14-fold improvement in human CD86 binding affinity compared to abatacept and belatacept, respectively (Oshima 2016a). The *in vivo* tests in *cynomolgus* monkeys showed that ASP2409 dose-dependently inhibited anti-tetanus toxoid (TTx) antibody production and delayed-type hypersensitivity (DTH) reactions. When the dosage reached 1 mg/kg, ASP2409 showed almost complete inhibitory effect on both TTx antibody production and DTH reactions, while belatacept with dose 10 mg/kg showed just partial suppression (Oshima 2016b).

ASP2409 monotherapy dose-dependently prolonged the MST of allografts. Low-dose (0.3 mg/kg) ASP2409 monotherapy showed significantly longer MST compared to no-treatment control. However, acute rejections were only delayed but not eliminated in this group. This implies that ASP2409 0.3 mg/kg is not a therapeutic dose for allograft rejection. Interestingly, when this dosage of ASP2409 was combined with sub-therapeutic dose tacrolimus (Kinugasa 2008), the MST was dramatically increased and acute rejection was totally abolished. When

the dosage of ASP2409 was increased to 1 mg/kg, more than half animals in this monotherapy group survived to the end of the study and the incidence of acute rejection was significantly reduced. This dosage of ASP2409 in combination with either sub-therapeutic dose tacrolimus or MMF, revealed comparable MST to belatacept-based and therapeutic dose tacrolimus-based regimens. Histopathological findings of kidney grafts in high-dose ASP2409 combined with sub-therapeutic dose tacrolimus group were similar to therapeutic dose tacrolimus and MMF combination group. These collectively imply that ASP2409 may work for both CNI sparing and avoidance regimens.

Serum concentrations of ASP2409 increase in a dose-related manner. Compared to low-dose groups, ASP2409 C_{trough} and C_{day2} values in high-dose groups present relatively low variability. It has been known that CD86 receptor saturation correlates with inhibition of T cell alloresponses (Latek 2009). In the induction phase, in either high-dose or low-dose, ASP2409 exhibited higher CD86 receptor occupancy than belatacept. The high level of CD86 receptor occupancy was kept in ASP2409 high-dose groups in the maintenance phase. It is consistent with ASP2409 to be designed to possess higher CD86 binding affinity. However, with the low ASP2409 C_{trough} during the maintenance phase, the levels of CD86 receptor occupancy in ASP2409 low-dose groups dramatically decreased. The development of anti-ASP2409 antibodies was confirmed in 17 of 52 animals (≈33%) following administration of ASP2409 during the maintenance period. The partial reason for the high incidence of anti-ASP2409 antibodies in this study could attribute to ASP2409, as a human protein, is a foreign antigen for monkeys. On the other hand, the incidence of anti-ASP2409 antibodies in high-dose treated animals (≈14%) is much lower than that of low-dose treated animals (≈57%). It

implies that higher dose ASP2409 may involve in inhibition of the production of anti-ASP2409 antibodies. High incidence of anti-ASP2409 antibodies in low-dose treated animals corresponds to low ASP2409 C_{trough} values and decreased CD80 and CD86 receptor occupancies. One possible explanation for this phenomenon is that anti-ASP2409 antibodies neutralize the biological activity of ASP2409 and/or lower its half life in the circulatory system (Shankar 2010; Wadhwa 2010).

Tregs are important regulators of immune tolerance. CD28 signals play a key role in the development of Tregs in the thymus. CD28 costimulation is also critical for the survival of periphery Tregs (Tang 2003; Sharpe 2006). It raises a concern that targeting the CD28–CD80/CD86 pathway may interfere with Treg development and peripheral expansion. CTLA4-Ig has been reported to have a deleterious effect on Treg development and function in animal models (Riella 2012) and inhibits the *in vitro* human Treg generation (Levitsky 2013). This agent is also found to restore skin allograft rejection in Treg-mediated tolerance model by disabling IL-2-expanded Tregs (Charbonnier 2012). On the other hand, there is also the report to indicate that CTLA4-Ig induced the *in vitro* swine Treg generation via an IL-10-dependent mechanism (Lee 2001). In renal transplant patients who received belatacept-based regimens, belatacept administrations did not affect the number or function of circulating Tregs (Bluestone 2008). The effects of CTLA4-Ig on Treg generation and function may be complicated.

It has been known that CD28 costimulation is unnecessary for the generation or function of induced Tregs, whereas the development and maintenance of natural Tregs are strongly

dependent on CD28–CD80/CD86 signals (Pletinckx 2011). In addition, the effects of CTLA4-Ig on CTLA4 signaling pathways are still unclear. Further investigation is helpful to explore the overall profile of the effects of CTLA4-Ig on Tregs. In this study, we did not measure the number of circulating Tregs. Interestingly, we found that renal allografts treated with ASP2409- or belatacept-based regimens exhibited significantly higher proportion of $FoxP3^{+}/CD3^{+}$ T cells than normal kidney and renal allografts treated with CNI-based regimens. The situation is similar to what is found in graft biopsies of the patients who are treated with belatacept-based regimens (Bluestone 2008). These findings suggest that both ASP2409 and belatacept may have no significant deleterious effect on the frequency of Tregs in renal allografts.

In this study, ASP2409 as an immunosuppressive agent was administered to *cynomolgus* monkey kidney transplant recipients for up to 90 days. Treatment with ASP2409 did not cause deterioration in the general clinical observational signs including daily activity and appetite. There was also no weight loss and other side-effects associated with ASP2409 administration during the study period. It was demonstrated that ASP2409 was safe and well tolerated for the monkey at the doses tested.

We conclude that ASP2409 exhibits potent immunosuppressive effects on the monkey renal transplantation model without causing serious side effects. When administered alone, this agent dose-dependently prolongs renal allograft survival. ASP2409 in combination with sub-therapeutic dose tacrolimus or MMF shows similar MST to the belatacept-based regimen and tacrolimus-based regimens in terms of 91-day observation period. Histopathological findings

of kidney grafts in ASP2409-based regimen are non-inferior to the belatacept-based regimen. Chronic administration of ASP2409 does not negatively affect Treg frequency in renal allografts. These results imply that ASP2409 is a promising candidate for CNI sparing or avoidance regimens. They provide solid evidence for conducting further research on this compound including clinical trials.

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Disclosure

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Chapter 4

Discussion

The postgrafting immunosuppressive therapy, especially CNIs-based regimens, has greatly reduced the incidence of acute allograft rejection and has tremendously improved the short-term outcomes in organ transplantation. In the USA, 1-year allograft survival rate has exceeded 90% among adult kidney transplant recipients (Knoll 2008; Hart 2016). However, the improvement in long-term results is not satisfied over the past several decades. Despite significant progresses have been made in transplantation techniques, organ preservation, recipient selection and the development of new immunosuppressive drugs, no major changes in 5–10-year death-censored graft attrition rate in kidney transplant recipients were observed during a 10-year period from 1989 to 1999 (Lamb 2011). Multiply factors, either antigen-dependent or antigen-independent, are associated with late graft loss. Among them, the side-effects of current "standard" immunosuppressive drugs are unignorable. These agents such as CNIs may directly cause graft injury, or lead to cardiovascular disease and diabetes mellitus, as well as infection and malignancy due to over immunosuppression. As a result of that, transplanted organs are eventually lost due to graft function failure or death with a functioning graft (Cosio 2008; Morales 2012). The disadvantages of conventional immunosuppression urge the need to find novel immunosuppressive methods that specifically target alloimmune responses. Costimulation blockade is one option among them. The research and clinical data suggest that blocking T cell costimulatory signals can successfully promote allograft acceptance, but challenges remain.

4.1 Blockade of the CD40–CD40L costimulatory pathway in organ transplantation

CD40–CD40L signaling pathway is critical for the allogeneic antigen-induced immune responses. CD40 was first identified on B cells. Nowadays, it has been known that this molecule is broadly expressed on B cells, monocytes, ECs, platelets, epithelial cells, mesenchymal cells, activated DCs, and activated T cells (van Kooten 2000; Li 2008). The signals transmitted through CD40–CD40L pathway are involved in both cellular and humoral adaptive immunity. Ligation of the CD40–CD40L axis on APCs leads to upregulated expression of CD80, CD86, MHC class II, adhesion molecule, and cytokine (Caux 1994; Kawabe 2011). Interaction of CD40–CD40L plays pivotal roles in promoting effective T cell activation. It is also intimately involved in the activation of DCs, ECs, fibroblasts, and platelets (Ma, 2009; Inwald 2003). CD40 signaling in B cells activates B cell proliferation, antibody production, isotype switching, and germinal center formation (Durie 1994; van Kooten 2000). In terms of organ transplantation, the CD40–CD40L signaling pathway is closely associated with acute and chronic rejection.

Anti-CD40L mAbs were demonstrated to successfully prolong allograft survival in experimental organ transplantation in rodents and in NHP (Larsen 1996; Kirk 1999). These facts suggest that bocking CD40–CD40L signaling pathway was a feasible strategy to promote allograft acceptance. Unfortunately, further studies were discontinued because of the thromboembolic complications of anti-CD40L mAbs in NHP model and clinical trials (Kawai 2000). Therefore, CD40 was chosen as an alternative therapeutic target. Some anti-CD40 mAbs such as ch5D12 (Haanstra 2003) and Chi220 (Pearson 2002) has been shown the effects to extend allograft survival in NHP model. However, the potencies of those agents were less than that of anti-CD40L mAbs.

This thesis (Chapter 2) investigates the efficacy of ASKP1240, a novel fully human antagonistic anti-CD40 mAb, on renal allograft survival in NHP model. Compared to control group, low-dose ASKP1240 monotherapy significantly prolonged survival time of renal transplant. The MST of allograft was further extended in high-dose monotherapy group. ASKP1240 monotherapy also decreased the incidence of acute transplant rejection in a dose-related manner. However, acute rejection has not been totally abolished, even in high-dose group. These observations are consistent with other author's results (Imai 2007; Aoyagi 2009).

When ASKP1240, either in low-dose or in high-dose, was combined with conventional immunosuppressant MMF or tacrolimus (sub-therapeutic dose), a notable improvement in graft survival was observed. In term of 180-day study period, except group low-dose ASKP1240 in combination with MMF, the MSTs of allograft in remaining three groups were 180 days. The graft MST of low-dose ASKP1240 and MMF combination group was 121 days that was approximately two times and four times those of monotherapy groups of low-dose ASKP1240 and MMF, respectively. Results of the histopathological examination indicated that no acute rejection was found in the combination groups mentioned above. Previous studies showed CNIs could abrogate donor-specific tolerance induced by anti-CD40L mAb (Blaha 2003). In this study, we found that ASKP1240 in combinantion with tacrolimus (sub-therapeutic dose) exhibited excellent results in both graft survival and prevention of acute rejection. Our results imply that ASKP1240 might work for both CNI-sparing and CNI-free regimens.

The thromboembolic events associated with anti-CD40L mAb raise the question of whether anti-CD40 mAb causes smilar complications. To date, how anti-CD40L mAbs involving in thromboembolism is not fully understood. Most anti-CD40L mAbs (*e.g.* hu5C8 and IDEC-131) are recombinant IgG1 antibodies. One speculated mechanism is these molecules can cross-link with platelet Fc gamma receptors (FcγRs) leading to platelet activation and aggregation (Robles-Carrillo 2010; Prasad 2003). ASKP1240 was designed as a fully human IgG4 mAb. Compared to IgG1, IgG4 has an extremely lower binding affinity for FcγRIIa/b and FcγRIIIa, and has no detectable binding for FcγRIIIb (Bruhns 2009). Moreover, the stability of platelet thrombi is related to CD40L. Studies have indicated that CD40L-deficient mice formed unstable thrombi. These thrombi easily detach from vascular wall and circulate blood vessels as emboli. However, the stability of platelet thrombi was not affected in CD40-deficient mice (André 2002). Thus, ASKP1240 is expected to not cause unwanted thromboembolic events. Okimura et al (2014) investigated the influence of ASKP1240 and anti-CD40L antibody (mu5C8) in human platelet thrombus formation. ASKP1240 did not activate platelet and ECs. Under physiological high shear stress condition, normal platelet thrombi were observed in ASKP1240-treated blood sample, in contrast, only smaller thrombi were seen in mu5C8-treated blood sample. These results suggested that ASKP1240 did not affect the stability of thrombi but mu5C8 did. To date several anti-CD40 mAbs had been tested in preclinical studies. There were no reports that anti-CD40 mAbs were associated with thromboembolic events. In this study, renal artery thrombosis, a common kidney transplant complication, was obseverved in some animals. However, the incidence of renal artery thrombosis was not significant different between ASKP1240-treated groups and non-ASKP1240-treated groups. Futhermore, there were no thrombosis-related events to be found

in other organs. Together with the observations in previous studies about ASKP1240 (Aoyagi 2009; Oura 2012), it could be concluded that ASKP1240 may be less likely to cause thromboembolism.

In previous studies, the IgG1 anti-CD40 mAbs such as chi220 exhibited high cytotoxicity and led to circulating B cell depletion that increased the chance of cytomegalovirus infection (Pearson 2002). The constant region of ASKP1240 is designed to be IgG4, thus the probability of ASKP1240 to deplete CD40 positive cells (*e.g.* B cell, platelet) via ADCC and CDC activities is extremely reduced. Even though ASKP1240 was weekly administered at doses up to 100 mg/kg, the number of peripheral B cells did not decline significantly (Okimura 2014). The administration of ASKP1240 does not induce cytokine release. In this study, IL-2, IL-4, IL-5, TNFα, and IFN-F and IFN-γ were not detected in any samples to be tested. The safety of ASKP1240 is further confirmed in healthy volunteers. A double-blind randomized phase I clinical trial demonstrated that ASKP1240 could be well tolerated in subjects at doses up to 10 mg/kg. ASKP1240 did not cause serious side effects including drug-induced cytokine release and thromboembolic events (Goldwater 2013).

4.2 Blockade of CD28-mediated T cell costimulation in organ transplantation

CD28 is the primary positive costimulatory receptor that constitutively expressed on naïve T cells. It interacts with its legends CD80 and CD86 to generate the most critical costimulatory signals for T cell activation. Thus, blocking the CD28–CD80/CD86 pathway to prevent T

cell-mediated allograft rejection is a rational choice. The attempts that directly targeting CD28 with mAb met some diffculty (Suntharalingam 2006), whereas CTLA4-Ig, an agent targeting both CD80 and CD86, showed promise in the rodent transplantation models (Lin 1993; Baliga 1994). Beside of preventing CD28–CD80/CD86 engagement, CTLA4-Ig also induces IDO production by DCs. IDO causes tryptophan degradation and subsequently suppresses T cell proliferation (Boasso 2005; Hwu 2000). However, CTLA4-Ig did not show the effects on the prevention of allorejection in further studies in NHP models (Kirk 1997). This molecule was then modified by mutating the binding domain to increase its binding affinity for both CD80 and CD86 (Larsen 2005). The novel CTLA4-Ig molecule is known as belatacept. Compared to its parent compound, belatacept showed much higher binding affinity to its ligands especially to CD86, and exhibited enhanced immunosuppressive activity in either *in vitro* or *in vivo* studies.

Preclinical studies in NHPs indicated that belatacept, either in monotherapy or in combination with conventional immunosuppressive agents, significantly prolonged renal allograft survial (Larsen 2005). Subsequent phase II and III clinical trials in kidney transplantation also showed encouraging results. Compared to cyclosporine arm, belatacept-based regimens presented similar patient and graft survival rate, but superior renal graft function, improved cardiovascular risk profile, and a trend of less chronic allograft nephropathy. However, this agent was found to be associated with higher incidence of acute rejection than conventional CNI-based immunosuppression. Moreover, PTLD, a post-transplant malignancy, was more common in the recipients who received belatacept-based immunosuppression, although the overall incidence is rather rare (Vincenti 2005; Grinyo 2012; Vincenti 2010). Belatacept-

based immunosuppression was also tested to determine if this approch would benefit the recipients besides renal transplantation. Unfortunately, the results of a recent phase II clinical trial in liver transplantation were disappointed. The incidence of acute rejection, graft loss, and death in the belatacept groups was higher than the tacrolimus groups (Klintmalm 2014).

The effect of ASP2409, a novel CD86-selective variant of CTLA4-Ig, on renal allograft survival in NHPs was investigated in this thesis (Chapter 3). ASP2409 is a recombinant fusion protein created using DNA shuffling directed evolution methods. This new compound is comprised of the modified extracellular domain of human CTLA4 and the wild-type Fc region of human IgG2. Similar to belatacept, ASP2409 is also generated by mutating the B7 binding region of CTLA4-Ig. Structurally, there are 10 amino acid substitutions in ASP2409 molecule instead of two in belatacept. These differences confer ASP2409 possessing 14-fold higher human CD86 binding affinity than belatacept, and a lower CD80 binding affinity relative to belatacept (Oshima 2016).

Although both CD80 and CD86 bind to their common receptors CD28 and CTLA4, they may not play equal roles in CD28/CTLA4–CD80/CD86 signaling pathway. CD86 exhibits higher binding affinity for CD28 relative to CD80. Thus CD86 is considered to be the dominant costimulatory ligand for signaling through CD28. In contrast, CD80 possesses a higher binding affinity for CTLA4 relative to CD86, and is the main ligand for CTLA4 (Linsley 1994; Pentcheva-Hoang 2004). CD28 costimulatory signal is a key element for T cell activation. Improvements in CD86 binding affinity and selectivity would provide increased immunosuppressive efficacy of CTLA4-Ig. It is the theoretical basis for the development of

belatacept. Although the CD86 binding affinity of belatacept has been significantly improved compared to CTLA4-Ig, this compound is still a CD80-selective rather than CD86-selective CTLA4-Ig variant because its *in vitro* binding affinity for CD80 is 12-fold higher than CD86. In contrast, ASP2409 is a real CD86-selective CTLA4-Ig variant with 2.1-fold higher binding affinity for CD86 relative to CD80 (Oshima 2016). In our *in vivo* study, ASP2409, even in low-dose, exhibited higher CD86 receptor occupancy than belatacept in the induction phase. CD86 receptor occupancy was kept at nearly saturated level in high-dose ASP2409 treated animals during the whole maintenance phase. While the CD80 receptor occupancy in these animals mostly fluctuated in the range of 70% to 90%, and the CD80 occupancy rate in low-dose ASP2409 treated animals was further lowered. On the other hand, belatacept treated animals showed high CD80 occupancy rate (almost saturated) and relative low CD86 occupancy rate (ranged from 88% to 60%). It is consistent with the observation from *in vitro* studies. While CD86 ligands are almost fully occupied by ASP2409, CD80 ligands are partially unoccupied. CD80 plays an important role in antiviral immune responses (Lumsden 2000), provides negative signal for the proliferation B cell lymphomas (Suvas 2002). Preservation of CD80 signaling may be helpful to reduce the complications related to current CTLA4-Igs such as increased susceptibility of infection and tumorigenesis, thus improves the safety of CTLA4-Ig therapy. However, the question is if an agent that completely blocks CD86 but partially blocks CD80 can effectively prevent allograft rejection.

The results of present study in a *cynomolgus* monkey renal transplantation model give an affirmative answer. Low-dose ASP2409 monotherapy significantly prolonged renal allograft survival time compared to control animals. However, acute rejections were only delayed but

not eliminated in these amimals. The MST of renal allograft was further prolonged along with increasing of the dosage of ASP2409, and the incidence of acute rejection was also significantly reduced. ASP2409, either in low-dose or high-dose, in combination with sub-therapeutic dose of tacrolimus showed similar renal allograft MST with therapeutic-dose tacrolimus-based immunosuppressive regimen, and acute rejection was totally eliminated in these groups. The regimen of 1 mg/kg ASP2409 in combination with MMF and methylprednisolone obtained equal MST and superior histopathological findings in renal allograft relative to the regimen of 5 mg/kg belatacept in combination with MMF and methylprednisolone. These results collectively demonstrate that ASP2409 can successfully promote renal allograft acceptance by inhibiting acute allograft rejection. These findings further support the hypothesis that CD86 is the dominating ligand to mediate costimulatory signals in alloimmune response, and increased CD86 receptor occupancy by CTLA4-Ig correlates with improved immunosuppressive potency (Latek 2009).

Improvements in CD86 binding affinity and selectivity may also confer clinically-meaningful advantages in drug safety. With improved CD86 binding affinity, the novel CTLA4-Ig would be able to provide therapeutic levels of CD86 occupancy at substantially lower serum drug concentrations than current CTLA4-Igs. To achieve similar *in vitro* inhibition of primary human T cell proliferation, required protein concentration of ASP2409 is 18-fold lower relative to belatacept (Oshima 2016). This makes it possible to reach similar or better therapeutic efficacy as belatacept with lower dose level of ASP2409 by virtue of providing more complete saturation of the dominant costimulatory ligand CD86 throughout the dosing cycle and therefore may reduce the incidence of dose related side effects of CTLA4-Igs.

ASP2409 was demonstrated to be safe and well tolerated for the monkey at the doses tested in this study. Our *cynomolgus* monkey renal allograft recipients were treated with ASP2409 for up to 90 days. ASP2409 administration did not cause deterioration in the general condition of monkey including daily activity, appetite, and body weight during the study period. A dose escalation phase I clinical trial was recently performed in stable rheumatoid arthritis patients. ASP2409 was administered intravenously with a single dose ranging from 0.01 to 3.0 mg/kg. The results indicated that ASP2409 was well tolerated at all doses, and there were no clinically relevant safety issues (Zhang 2016).

4.3 Costimulation blockade and regulatory T cells

The Treg cells are a subgroup of T cells that are specifically dedicated to the suppression of immune responses. They play an important role in the maintenance of tolerance to self-antigens, regulation of the magnitude of immune responses, and the prevention of autoimmune disease (Sakaguchi 1985; Wang 2016). Therefore, increasing the number and/or function of Treg might lead to tumour development; on the contrary, dysfunction of Treg cells contributes autoimmunity. In terms of organ transplantation, Tregs are involved in the inhibition of acute rejection and are the key players in the induction and maintenance of transplant tolerance. Tregs suppress alloimmune responses in an antigen-specific manner, and these cells can also be isolated and expanded *in vitro*. Treg therapy has been chosen as the novel therapeutic strategies against allorejection and is now on the road from bench to bed (Kingsley 2002; Kendal 2011).

To date two main classes of Tregs have been identified, *i.e.*, $CD4^+$ and $CD8^+$ Tregs. The majority of Treg cells are $CD4^+$ T lymphocytes. $CD8^+$ Treg cells constitute a very small proportion of the Treg population (Ligocki 2015; Churlaud 2015). $CD4^+$ Treg cells consist of two varieties: naturally occurring Tregs (nTregs) and induced (adaptive) Tregs (iTregs). nTregs develop in the thymus and constitutively express CD25 and FoxP3. iTregs are generated extrathymically at peripheral sites such as the mucosa-associated lymphoid tissue (MALT), inflammatory tissues, and transplanted tissues. They convert from conventional $CD4^+$ T cells under the proper physiological conditions and some subsets like Type 1 regulatory T cells (Tr1) may not express FoxP3 and CD25 (Pot 2011). nTregs constitute approximately 5–10% of peripheral $CD4^+$ T cell population and are the critical regulators of immune homeostasis. They exert their suppressive effects via cell–cell contact, granzyme B-dependent, and to produce TGF-β. While iTregs, unlike nTregs, mainly manifest their suppressive functions through the secretion of cytokines such as IL-10 and TGF-β. These molecules may induce effector T cell apoptosis or cell-cycle arrest, and inhibit DCs maturation (Jonuleit 2003; Workman 2009). iTregs are essential for the induction and maintenance of mucosal immune tolerance, and they also play an important role in the control of allergic inflammation (Curotto de Lafaille 2008; Huang 2013).

Costimulatory signals are required for the generation of Treg cells. CD28 is the earliest identified costimulatory molecule which is critical for the development of nTreg cells in the thymus. The absolute number of the $CD4^+CD25^+$ T cells were reduced 5 to 6-fold in both CD28-deficient and CD80/CD86-deficient NOD mice (Salomon 2000). CD28 knockout mice exhibited significantly low frequencies of $FoxP3^+$ T cells in thymus and peripheral lymph

nodes in comparison with those of wildtype mice (Sansom 2006). In contrast, in CTLA4-deficient mice that revealed excessive CD28 stimulation, both frequency and number of $CD4^+FoxP3^+$ T cells increased enormously although these Tregs showed functional defects (Wing 2008). Moreover, CD28 costimulation promotes nTreg survival via induction of Treg-extrinsic survival factor, and enhances their self-renewal. It is a key player to control peripheral homeostasis of nTregs (Tang 2003). iTreg cells are generated in the periphery under more varied conditions. CD28 is not necessary for the development of iTregs. Conventional $CD4^+$ T cells are able to be converted into iTregs in the absence of CD28 stimulation (Curotto de Lafaille1 2009; Taylor 2002). In fact, stimulation through high-level CD28 was shown to hinder TGF-β-induced Treg cell differentiation *in vitro* (Benson 2007). In contrast, CTLA4 is required for generation of iTreg cells. TGF-β can induce $CD4^+CD25^-$ cells FoxP3 expression and conversion to $FoxP3^+$ iTregs. However, neither normal level expression of FoxP3 nor suppressor activity was induced in $CD4^+CD25^-$ cells from CTLA4-deficient mice under the stimulation of TGF-β. Moreover, blockade of CTLA4 abolished TGF-β to induce activated wild-type $CD4^+CD25^-$ cells to become $CD4^+CD25^+FoxP3^+$ suppressor cells (Zheng 2006). Other stimulatory molecules such as OX40, glucocorticoid-induced TNFR family-related protein (GITR), 4-1BB, were also found to affect the development and function of Tregs, and the details still needed to be clarified by further studies (Croft 2014).

CTLA4-Ig and its variants block CD28–CD80/CD86 signaling by competitive inhibition of CD28 intreraction with CD80 and CD86. Recently, concerns that use of CTLA4-Igs may interfere with the development and function of Treg cells have been raised. Levitsky and

colleagues (2013) demonstrated that belatacept alone or in combination with other immunosuppressants inhibited the human Treg generation *in vitro*. Administration of CTLA4-Ig significantly reduced the number of mouse Treg cells *in vivo* (Riella 2012). Skin allograft rejection and Th1 alloreactivity were restored by CTLA4-Ig administration in a mouse transplantation model that Treg-dependent long-term allograft acceptance had been achieved by short-term administration of IL-2/anti-IL-2 complex. CTLA4-Ig suppressed IL-2-induced *in vivo* Treg expansion and their immunosuppressive capacity (Charbonnier 2012). In contrast, some authors reported that CTLA4-Ig could induce the generation of porcine Treg cells *in vitro* using IL-10-dependent mechanisms (Lee 2001). Studies of mouse thymus-derived Treg cells indicated that CTLA4-Ig had no negative effect on Treg frequencies and function *in vitro* (Ahmadi 2014). The number and function of circulating Tregs were unaffected by belatacept administrations in kidney transplant recipients who received belatacept-based treatment (Bluestone 2008). Mixed results indicate the intricate effects of CTLA4-Igs on Tregs and the need to further delineate their relationship.

Although FoxP3 is transiently expressed by activated nonregulatory T cells and some Treg cells do not express FoxP3, this nuclear transcription factor remains the best and the most lineage-specific marker of Treg cells to date. In this thesis, the percentage of $FoxP3^+$ T cells among total $CD3^+$ T cells was measured in paraffin embedded sections of renal allograft by immunohistochemical staining to evaluate the effects of ASP2409 on Tregs. The frequency of $FoxP3^+$ T cells is very low in normal kidney that does not encounter surgical injuries and inflammatory stimulation. All kidney allografts including those of CNI-treated animals exhibit higher frequencies of $FoxP3^+$ T cells compared to normal kidneys. Previous studies

have indicated that CNIs have a negative impact on Treg development and survival (Baan 2005). Our reuslts also support this view. The frequencies of $FoxP3^+$ T cells of ASP2409 combined with CNI treatment groups are much lower than that of ASP2409 combined with MMF treatment group. In the regimens of ASP2409 combined with CNI, high-dose ASP2409 group shows higher ratio of $FoxP3^+/CD3^+$ T cells compared to low-dose ASP2409 group. When either ASP2409 or belatacept in combination with non-CNI immunosuppressive agents, significantly higher proportion $FoxP3^+$ T cells infiltration in renal allografts are observed relative to CNI-based regimens. Similarly greater amounts of $FoxP3^+$ T cells were found in renal graft biopsy samples in patients who had received belatacept-based regimens (Bluestone 2008). These results indicate that ASP2409 may promote Treg infiltration in renal allografts, at least ASP2409 do not show the significantly negative effects on the frequencies of Tregs in renal allografts.

4.4 T cell costimulation blockers in combination with other immunosuppressive drugs

Combination therapy is a commonly used therapeutic strategy in clinic. The purpose of this strategy is, by taken more than one medication or therapy to treat a single disease, to maximize overall effectiveness while the adverse effects and toxic effects of each drug or modality are minimized. Immunosuppressive therapy after organ transplantation is critical for the long-term allograft survival. Clinical immunosuppressive regimens may vary due to a multitude of factors such as drug compliance, side effects, intercurrent disease and cost *etc* (Popoola 2014). Conventionally, they are a combination of 2–3 immunosuppressive drugs

that target various immune components especially different steps in T cell activation. The combinations of agents with different mechanisms of action provide the chance to promote overall effectiveness of immunosuppressive regimens because a subgroup of immune cells which are resistant to one agent may be inhibited by another agent. Meanwhile, several agents act on various steps of cell activity such as T cell activation offers the opportunity to lower doses of each individual immunosuppressant. Thus dose-related side effects and toxicity of each immunosuppressant could be minimized. Although the monotherapies of T cell costimulation blockers have shown promising results in the prolongation of allograft survival, they still need to be combined with additional immunosuppressive drugs to achieve a better antirejection effect and to lower drug-related side effects (Kirk 2009).

In our study, two costimulation blockers, either ASKP1240 that targets the CD40–CD40L pathway or ASP2409 that blocks the CD28–CD80/CD86 signaling pathway, were demonstrated to inhibit acute renal allograft rejection and to prolong kidney transplant survival time in a dose-dependent manner. However, costimulation blocker monotherapy did not eliminate acute rejection in kidney transplant. Even in high-dose costimulation blocker treated animals, there was a certain percentage of acute renal allograft rejection to be found. In contrast, the rates of acute rejection were dramatically reduced in groups either ASKP1240 or ASP2409 in combination with tacrolimus or MMF plus methylprednisolone. Histological analysis revealed that acute renal allograft rejection was completely abolished in most combination therapy groups (Figure 4.1). These groups correspondingly showed better renal allograft survival results. A typical example is the combination of low-dose ASP2409 and sub-therapeutic dose of tacrolimus. All aminals of the low-dose ASP2409 monotherapy group

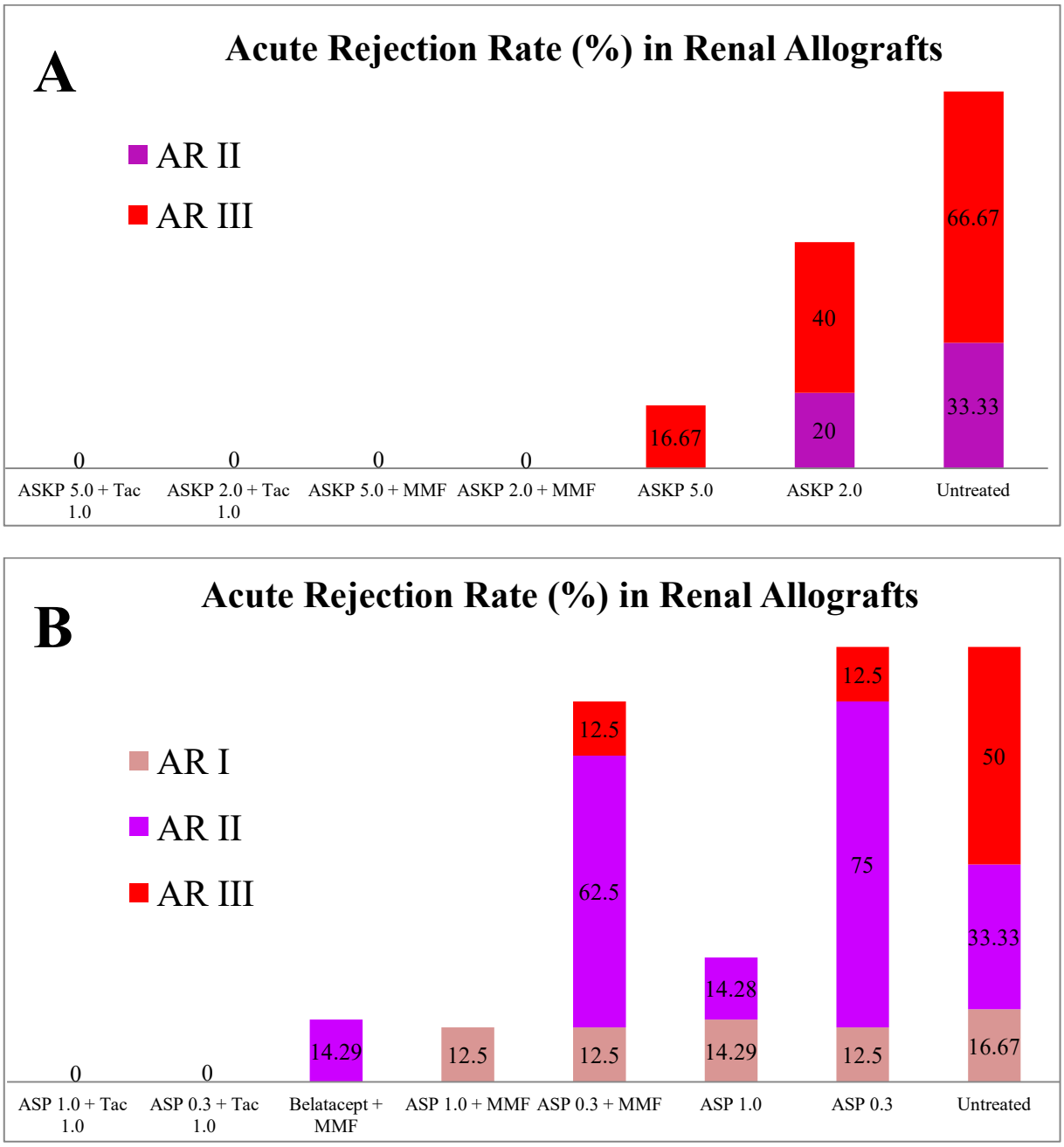

Figure 4.1 The rate of acute renal allograft rejection in ASKP1240-based (A) or ASP2409-based (B) treatment groups. AR, acute rejection; Tac 0.5, tacrolimus 0.5 mg/kg; Tac 1.0, tacrolimus 1.0 mg/kg; ASKP 2.0, ASKP1240 2.0 mg/kg; ASKP 5.0, ASKP1240 5.0 mg/kg; ASP 0.3, ASP249 0.3 mg/kg; ASP 1.0, ASP2409 1.0 mg/kg; MMF, mycophenolate mofetil 15 mg/kg.

were euthanized due to acute rejection although they showed a better survival profile and a low incidence of high-grade acute rejection compared to untreated control group. Surprisingly, when low-dose ASP2409 was combined with sub-therapeutic dose of tacrolimus, none of acute renal allograft rejection was found in this combination group. All animals except one (1/7) receiving combination therapy survived to the end of the study. These results (Figure 4.2) were also much better than sub-therapeutic dose tacrolimus monotherapy group in either the rate of acute rejection (none *vs.* 37.5%) or graft survival profile (MST >91 *vs.* 38.5 days). A proper combination of costimulation blocker and conventional immunosuppressive drug produces synergistic effect.

Clinical experience in kidney transplantation indicates that belatacept is associated with a high incidence of cell-mediated acute rejection (Grinyo 2012; Vincenti 2010). The exact mechanisms involved in the increase of allograft rejection in costimulation blockade-based immunosuppressive regimens have not yet been fully understood. Experimental data demonstrate that alloreactive memory T cells are insusceptible to costimulatory blockers and they likely play an important role in costimulatory blockade-resistant rejection (Zhai 2002; Valujskikh 2006). Memory cells are the products of immune responses and are critical elements in protective immunity against invading pathogens. Alloreactive memory T cells may be generated through previous antigen exposure (*e.g.* previous transplantation, blood transfusion, and pregnancy), or through the process of heterologous immunity (Amir 2010). They are able to persist in the host in an antigen-independent manner and have the capacity for self-renewal *in vivo*. Compared to naïve T cells, memory T cells exhibit a much lower threshold for activation. Their proliferation can occur in the environment of low concentration

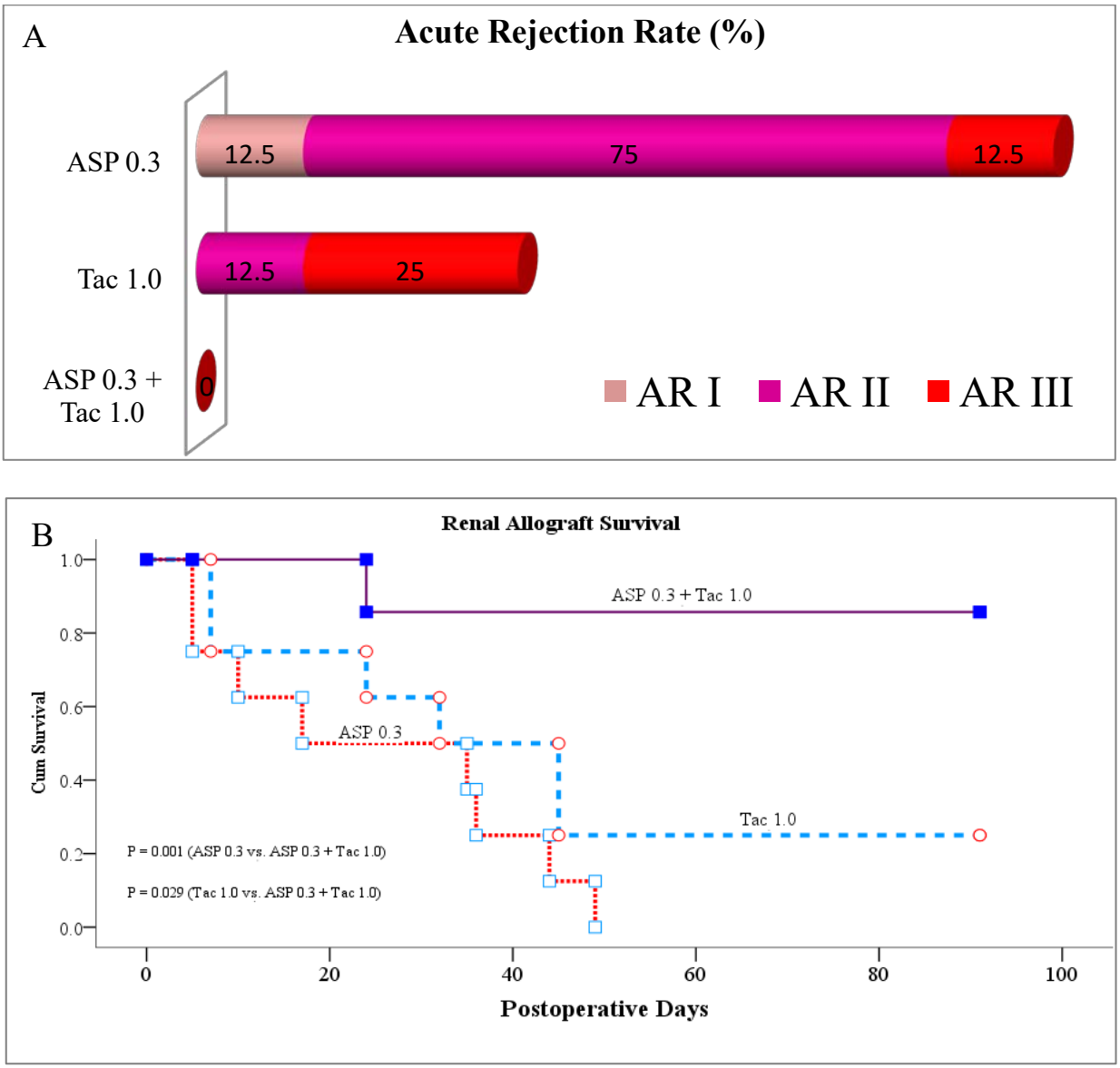

Figure 4.2 The acute renal allograft rejection rates (A) and graft survival (B) in low-dose ASP2409 monotherapy, sub-therapeutic dose tacrolimus monotherapy, and low-dose ASP2409 and sub-therapeutic dose tacrolimus combination therapy groups. Combination therapy totally eliminates acute rejection and shows much better graft survival results relative to the two monotherapies. AR, acute rejection; Tac 1.0, tacrolimus 1.0 mg/kg; ASP 0.3, ASP249 0.3 mg/kg.

of antigen. CD28–CD80/CD86 and CD40–CD40L costimulatory signaling pathways are not necessary during the activation process of memory T cells. The alternative costimulatory signals such as that are generated by OX40–OX40L or 4-1BB–4-1BBL pathways may play a role in memory T cell activation (London 2000; Vu 2006). In addition, memory T cells can also be activated in the places other than secondary lymphoid organs in which the activation of naïve T cells takes place (Chalasani 2002). That makes memory T cells to have more chance to encounter the alloantigen and facilitates them to arouse a vigorous immune response. In contrast to naïve T cells, memory T cells are more resistant to regulation by Tregs (Yang 2007) and they are able to quickly expand following T cell depletion therapies (Pearl 2005; Neujahr 2006). Taken together, all these features of memory T cells make them become a large obstacle to successful transplantation especially in costimulation blockade-based immunosuppression. The levels of donor-reactive memory T cells are considered to be related to posttransplant acute rejection episodes, and long-term allograft function (Heeger 1999; Augustine 2005).

Thus, the immunosuppressive regimens for organ transplantation should include the memory T cell-directed intervention therapies, especially considering the increasing application of the costimulation blockade-based protocols in clinical practice. Current strategies to control alloreactive memory T cells include T cell depletion, suppressing their activation and proliferation, and blocking their infiltration into the graft. As mention above, memory T cells exhibit resistance to T cell depleting agents such as polyclonal antithymocyte globulin (ATG). Lymphocytopenia caused by ATG induction can induce compensatory clonal expansion of memory T cells throgh homeostatic proliferation (Moxham 2008). On the other hand, some

studies have demonstrated that ATG promotes Treg survival and expansion; consequently, this agent shows overall immunosuppressive capabilities (Valdez-Ortiz 2015; Wittenbecher 2013). In contrast to peri-transplant lymphoablation, targeting pre-existing memory T cells by pre-transplant administration of ATG is more efficient in controlling donor-reactive memory T cell, reducing their recovery speed from T cell depletion, and prolonging allograft survival (Ayasoufi 2013). Some studies indicate that the alternative costimulatory pathways such as 4-1BB and OX40 involve in the activation and effector functions of memory T cells. These alternative costimulatory pathways are thus considered to be the novel therapeutic targets. Blocking OX40–OX40L pathway with anti-OX40L mAb has been demonstrated to prevent CD28 and CD154-independent rejection and to dramatically prolong skin allograft survival in a CD28/CD154 double-knockout mouse model (Demirci 2004). Targeting 4-1BB–4-1BBL interactions also shows efficacy in prolonging allograft survival (Cho 2004). Janus kinase-3 (JAK-3) plays important roles in cytokine-driven proliferation of memory T cells. Conventional immunosuppressive drugs and JAK-3 inhibitors such as PNU156804 and CP-690550 (tofacitinib) act synergistically to extend allograft survial (Stepkowski 2002; Borie 2005). FTY720, a synthetic analogue of sphingosine, is able to retain lymphocytes within lymph nodes through modulating sphingosine-1-phosphate (S1P) signaling, which regulates T cell egress from thymus and secondary lymphoid organs. This agent is demonstrated to prevent infiltration of T cells including donor-specific memory T cells into allografts and appears to delay cardiac transplant rejection (Zhang 2006). Additionally, agents targeting adhesion molecules such as LFA-1 and VLA-4 have shown efficacy in inhibiting the infiltration of memory T cells into allografts. They can suppress T cell trafficking into grafts or/and donor-reactive memory recall responses, and reveal synergistic effects with

conventional costimulation blockers on the prolongation of allograft survival (Setoguchi 2011; Kitchens 2012; Badell 2010). LFA-3-Ig (alefacept), a fusion protein disrupting CD2–LFA-3 interaction, is able to selectively eliminate memory T cells through multiple mechanisms including limiting Th cell adhesion to APCs and complement-mediated lysis (Weaver 2009). There is an increasing amount of evidence to suggest that conventional immunosuppression drugs have only a limited effect on memory T cells. Agents mentioned above should be considered when design the costimulation blockade-based anti-rejection regimens. Currently, some agents are not yet clinically available, further investigations about the efficacies and safeties of these agents are highly recommended. On the other hand, memory T cells are an important component in protective immunity. To suppress memory T cells while minimizing the influence on their protective effects is a big challenge for memory T cell-specific therapeutics.

Concluding remarks

Belatacept to be approved for use in the clinic presaged the arrival of the era of costimulation blockers as novel immunosuppressive agents in organ transplantation. Post-transplant immunosuppression based on this compound is associated with improvement of renal allograft function and reduction of CNI-related cardiovascular and metabolic side effects, while the patient and graft survival rates are similar to CNI-based regimens. These results indicate that costimulation blockade is a feasible strategy for allograft acceptance. However, relatively high incidences of acute rejection and PTLD imply that there is still room for improvement. Along with the increase of knowledge about costimulatory signaling in alloimmune response, more and more agents targeting costimulatory pathway have emerged over the past decade. In this thesis, we investigated the effects of two new costimulation blockers, ASKP1240 and ASP2409, as a monotherapy or in combination with conventional immunosuppressive drugs on preventing acute allograft rejection and promoting transplant acceptance.

Our results demonstrated that interrupting the CD40–CD40L interaction with anti-CD40 mAb ASKP1240 could successfully inhibit acute kidney transplant rejection and prolong renal allograft survival time. Meanwhile, taken together with the results of other NHP studies, we found that ASKP1240 as a monotherapy, even in high dose, was insufficient to abrogate all acute renal allograft rejection. However, acute rejection was totally eliminated when ASKP1240 was combined with tacrolimus or MMF, and the animals in these combination groups survived much longer than animals receiving single-drug therapy. ASKP1240 showed

comparable efficacy to anti-CD40L mAbs but did not cause thromboembolic complications that anti-CD40L mAbs did.

We also firstly demonstrated that ASP2409, a CD86-selective CTLA4-Ig variant that could almost completely occupy CD86 but partially occupy CD80, dose-dependently reduced the incidence of acute rejection and prolonged MST of renal allograft when it was administered alone. ASP2409 in combination with tacrolimus or MMF further minimized acute renal allograft rejection episode and improved graft survival. Interestingly, similar graft survival results were seen in ASP2409 (1 mg/kg)/MMF combination and belatacept (5 mg/kg)/MMF combination. The results further confirm the hypothesis that CD86 is the dominant costimulatory ligand for CD28, and plays the main role in alloimmune response against transplated organs. Furthermore, our data indicated that both ASP2409 and belatacept did not exhibit adverse effects on Treg frequency in renal allografts although CD28 signals were critical for Treg development and survival.

The encouraging results suggest that ASKP1240 and ASP2409 both are promising candidates for CNI-sparing or complete CNI avoidance immunosuppressive regimens. The studies also provide concrete evidence to support the application of costimulation-based approaches in organ transplantation. However, there are still lots of questions need to be addressed by further studies. Will ASKP1240 or ASP2409 based therapies improve the long-term outcomes of organ transplantation? Can these two agents reduce chronic allograft rejection? If memory T cell-directed intervention therapies are included in immunosuppressive regimens, will the overall results be improved as expected? An agent fully blocking CD86 and partially blocking

CD80 has shown promising results in inhibition of acute rejection and prolongation of graft survival. Will leaving CD80 partially unoccupied preserve the protective immunity, minimize side-effects, and achieve the goal of ASP2409 to be designed? We now have to wait for the answer from the further facts although theoretically T cell costimulatory blockade represents a more specific and selective immunosuppressive approach, and is expected to be a promising therapeutic strategy with reliable effect and minimizing adverse effects.

Chapter 5

Bibliography

Afzali B, Lombardi G, Lechler RI. Pathways of major histocompatibility complex allorecognition. Curr Opin Organ Transplant 2008; 13:438–444.

Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int Immunol 1996; 8:765–772.

Ahmadi SM, Hölzl MA, Mayer E, Wekerle T, Heitger A. CTLA4-Ig preserves thymus-derived T regulatory cells. Transplantation 2014; 98:1158–1164.

Alam A, Badovinac K, Ivis F, Trpeski L, Cantarovich M. The outcome of heart transplant recipients following the development of end-stage renal disease: analysis of the Canadian Organ Replacement Register (CORR). Am J Transplant 2007; 7:461–465.

Alegre ML, Noel PJ, Eisfelder BJ, Chuang E, Clark MR, Reiner SL, Thompson CB. Regulation of surface and intracellular expression of CTLA4 on mouse T cells. J Immunol 1996; 157:4762–4770.

Amir AL, D'Orsogna LJ, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, van der Hoorn MA, Kester MG, Doxiadis II, Falkenburg JH, Claas FH, Heemskerk MH. Allo-HLA reactivity of virus-specific memory T cells is common. Blood 2010; 115:3146–3157.

Anderson AC, Anderson DE, Bregoli L, Hastings WD, Kassam N, Lei C, Chandwaskar R, Karman J, Su EW, Hirashima M, Bruce JN, Kane LP, Kuchroo VK, Hafler DA. Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. Science 2007; 318:1141–1143.

André P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, Phillips DR, Wagner DD. CD40L stabilizes arterial thrombi by a beta3 integrin: dependent mechanism. Nat Med 2002; 8:247–252.

Aoyagi T, Yamashita K, Suzuki T, Uno M, Goto R, Taniguchi M, Shimamura T, Takahashi N, Miura T, Okimura K, Itoh T, Shimizu A, Furukawa H, Todo S. A human anti-CD40 monoclonal antibody, 4D11, for kidney transplantation in cynomolgus monkeys: Induction and maintenance therapy. Am J Transplant 2009; 9:1732–1741.

Appleman LJ, Berezovskaya A, Grass I, Boussiotis VA. CD28 costimulation mediates T cell expansion via IL-2-independent and IL-2-dependent regulation of cell cycle progression. J Immunol 2000; 164:144–151.

Appleman LJ, Boussiotis VA. T cell anergy and costimulation. Immunol Rev 2003; 192:161–180.

Araki M, Chung D, Liu S, Rainbow DB, Chamberlain G, Garner V, Hunter KM, Vijayakrishnan L, Peterson LB, Oukka M, Sharpe AH, Sobel R, Kuchroo VK, Wicker LS. Genetic evidence that the differential expression of the ligand-independent isoform of CTLA-4 is the molecular basis of the Idd5.1 type 1 diabetes region in nonobese diabetic mice. J Immunol 2009; 183:5146–5157.

Armitage RJ, Fanslow WC, Strockbine L, Sato TA, Clifford KN, Macduff BM, Anderson DM, Gimpel SD, Davis-Smith T, Maliszewski CR. Molecular and biological characterization of a murine ligand for CD40. Nature 1992; 357:80–82.

Asai T, Choi BK, Kwon PM, Kim WY, Kim JD, Vinay DS, Gebhardt BM, Kwon BS. Blockade of the 4-1BB (CD137)/4-1BBL and/or CD28/CD80/CD86 costimulatory pathways promotes corneal allograft survival in mice. Immunology 2007; 121:349–358.

Augustine JJ, Siu DS, Clemente MJ, Schulak JA, Heeger PS, Hricik DE. Pre-transplant IFN-gamma ELISPOTs are associated with post-transplant renal function in African American renal transplant recipients. Am J Transplant 2005; 5:1971–1975.

Ayasoufi K, Yu H, Fan R, Wang X, Williams J, Valujskikh A. Pretransplant antithymocyte globulin has increased efficacy in controlling donor-reactive memory T cells in mice. Am J Transplant 2013; 13:589–599.

Azuma M, Ito D, Yagita H, Okumura K, Phillips JH, Lanier LL, Somoza C. B70 antigen is a second ligand for CTLA-4 and CD28. Nature 1993; 366:76–79.

Baan CC, van der Mast BJ, Klepper M, Mol WM, Peeters AM, Korevaar SS, Balk AH, Weimar W. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. Transplantation 2005; 80:110–117.

Badell IR, Russell MC, Thompson PW, Turner AP, Weaver TA, Robertson JM, Avila JG, Cano JA, Johnson BE, Song M, Leopardi FV, Swygert S, Strobert EA, Ford ML, Kirk AD, Larsen CP. LFA-1-specific therapy prolongs allograft survival in rhesus macaques. J Clin Invest 2010; 120:4520–4531.

Badell IR, Thompson PW, Turner AP, Russell MC, Avila JG, Cano JA, Robertson JM, Leopardi FV, Strobert EA, Iwakoshi NN, Reimann KA, Ford ML, Kirk AD, Larsen CP. Nondepleting anti-CD40-based therapy prolongs allograft survival in nonhuman primates. Am J Transplant 2012; 12:126–135.

Baliga P, Chavin KD, Qin L, Woodward J, Lin J, Linsley PS, Bromberg JS. CTLA4Ig prolongs allograft survival while suppressing cell-mediated immunity. Transplantation 1994; 58:1082–1090.

Barbari AG, Stephan AG, Masri MA. Calcineurin inhibitor-free protocols: risks and benefits. Saudi J Kidney Dis Transpl 2007; 18:1–23.

Barbari AG, Stephan AG, Masri MA. Calcineurin inhibitor-free protocols: risks and benefits. Saudi J Kidney Dis Transpl 2007; 18:1–23.

Barber EK, Dasgupta JD, Schlossman SF, Trevillyan JM, Rudd CE. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. Proc Natl Acad Sci USA 1989; 86:3277–3281.

Barclay AN. Membrane proteins with immunoglobulin-like domains--a master superfamily of interaction molecules. Semin Immunol 2003; 15:215–223.

Barrow AD, Trowsdale J. You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling. Eur J Immunol 2006; 36:1646–1653.

Bedford P, Garner K, Knight SC. MHC class II molecules transferred between allogeneic DCs stimulate primary mixed leukocyte reactions. Int Immunol 1999; 11:1739–1744.

Beemiller P, Krummel MF. Regulation of T-cell receptor signaling by the actin cytoskeleton and poroelastic cytoplasm. Immunol Rev 2013; 256:148–159.

Behrens G, Li M, Smith CM, Belz GT, Mintern J, Carbone FR, Heath WR. Helper T cells, DCs and CTL Immunity. Immunol Cell Biol 2004; 82:84–90.

Belperio JA, Weigt SS, Fishbein MC, Lynch JP 3rd. Chronic lung allograft rejection: mechanisms and therapy. Proc Am Thorac Soc 2009; 6:108–121.

Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J Exp Med 1997; 186:65–70.

Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced Treg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J Exp Med 2007; 204:1765–1774.

Bernard A, Lamy L, Alberti I. The two-signal model of T-cell activation after 30 years. Transplantation 2002; 73(1 Suppl):S31–S35.

Bevilacqua MP, Nelson RM. Selectins. J Clin Invest 1993; 91:379–387.

Billingham RE, Krohn PL, Medawar PB. Effect of cortisone on survival of skin homografts in rabbits. BMJ 1951; 1:1157–1163.

Blaha P, Bigenzahn S, Koporc Z, Schmid M, Langer F, Selzer E, Bergmeister H, Wrba F, Kurtz J, Kiss C, Roth E, Muehlbacher F, Sykes M, Wekerle T. The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. Blood 2003; 101:2886–2893.

Blank U, Launay P, Benhamou M, Monteiro RC. Inhibitory ITAMs as novel regulators of immunity. Immunol Rev 2009; 232:59–71.

Bluestone JA, Liu W, Yabu JM, Laszik ZG, Putnam A, Belingheri M, Gross DM, Townsend RM, Vincenti F. The effect of costimulatory and interleukin 2 receptor blockade on regulatory T cells in renal transplantation. Am J Transplant 2008; 8:2086–2096.

Boasso A, Herbeuval JP, Hardy AW, Winkler C, Shearer GM. Regulation of indoleamine 2,3-dioxygenase and tryptophanyl-tRNA-synthetase by CTLA-4-Fc in human CD4+ T cells. Blood 2005; 105:1574–1581.

Boćko D, Kosmaczewska A, Ciszak L, Teodorowska R, Frydecka I. CD28 costimulatory molecule--expression, structure and function. Arch Immunol Ther Exp (Warsz) 2002; 50:169–177.

Bodmer JL, Schneider P, Tschopp J. The molecular architecture of the TNF superfamily. Trends Biochem Sci 2002; 27:19–26.

Boomer JS, Green JM. An enigmatic tail of CD28 signaling. Cold Spring Harb Perspect Biol 2010; 2:a002436.

Borel JF, Feurer C, Gubler HU, Stahelin H. Biological effects of cyclosporin A: a new antilymphocytic agent. Agents Actions 1976; 6:468–475.

Borie DC, Larson MJ, Flores MG, Campbell A, Rousvoal G, Zhang S, Higgins JP, Ball DJ, Kudlacz EM, Brissette WH, Elliott EA, Reitz BA, Changelian PS. Combined use of the JAK3 inhibitor CP-690,550 with mycophenolate mofetil to prevent kidney allograft rejection in nonhuman primates. Transplantation 2005; 80:1756–1764.

Borst J, Sluyser C, De Vries E, Klein H, Melief CJ, Van Lier RA. Alternative molecular form of human T cellspecific antigen CD27 expressed upon T cell activation. Eur J Immunol 1989; 19:357–364.

Brdicka T, Pavlistová D, Leo A, Bruyns E, Korínek V, Angelisová P, Scherer J, Shevchenko A, Hilgert I, Cerný J, Drbal K, Kuramitsu Y, Kornacker B, Horejsí V, Schraven B. Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. J Exp Med 2000; 191:1591–1604.

Broll K, Richter G, Pauly S, Hofstaedter F, Schwarz H. CD137 expression in tumor vessel walls. High correlation with malignant tumors. Am J Clin Pathol 2001; 115:543–549.

Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, Daëron M. Specificity and affinity of human Fcγ receptors and their polymorphic variants for human IgG subclasses. Blood 2009;113:3716–3725.

Brunet JF, Denizot F, Luciani MF, Roux-Dosseto M, Suzan M, Mattei MG, Golstein P. A new member of the immunoglobulin superfamily—CTLA-4. Nature 1987; 328:267–270.

Bruyns E, Marie-Cardine A, Kirchgessner H, Sagolla K, Shevchenko A, Mann M, Autschbach F, Bensussan A, Meuer S, Schraven B. T cell receptor (TCR) interacting molecule (TRIM), a novel disulfide-linked dimer associated with the TCR-CD3-zeta complex, recruits intracellular signaling proteins to the plasma membrane. J Exp Med 1998; 188:561–575.

Busuttil RW, Lake JR. Role of tacrolimus in the evolution of liver transplantation. Transplantation 2004; 77(9 Suppl):S44–51.

Cai YC, Cefai D, Schneider H, Raab M, Nabavi N, Rudd CE. Selective CD28pYMNM mutations implicate phosphatidylinositol 3-kinase in CD86-CD28-mediated costimulation. Immunity 1995; 3:417–426.

Calne RY, White DJG, Rolles K, Smith DP, Herbertson BM. Prolonged survival of pig orthotopic heart grafts treated with cyclosporin A. Lancet 1978; 1:1183–1185.

Calne RY. Pharmacological immunosuppression in clinical organ grafting. Observations on four agents: cyclosporin A, Asta 5122 (cytimun), lambda carrageenan and promethazine hydrochloride. Clin Exp Immunol 1979; 35:1–9.

Canafax DM, Ascher NL. Cyclosporine immunosuppression. Clin Pharm 1983; 2:515–524.

Cannons JL, Lau P, Ghumman B, DeBenedette MA, Yagita H, Okumura K, Watts TH. 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy. J Immunol 2001; 167:1313–1324.

Carbone FR, Bevan MJ. Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. J Exp Med 1990; 171:377–387.

Carter L, Fouser LA, Jussif J, Fitz L, Deng B, Wood CR, Collins M, Honjo T, Freeman GJ, Carreno BM. PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. Eur J Immunol 2002; 32:634–643.

Caux C, Massacrier C, Vanbervliet B, Dubois B, Van Kooten C, Durand I, Banchereau J. Activation of human DCs through CD40 cross-linking. J Exp Med 1994; 180:1263–1272.

Chalasani G, Dai Z, Konieczny BT, Baddoura FK, Lakkis FG. Recall and propagation of allospecific memory T cells independent of secondary lymphoid organs. Proc Natl Acad Sci U S A 2002; 99:6175–6180.

Chambers CA, Kuhns MS, Egen JG, Allison JP. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. Annu Rev Immunol 2001; 19:565–594.

Charbonnier LM, Vokaer B, Lemaître PH, Field KA, Leo O, Le Moine A. CTLA4-Ig restores rejection of MHC class-II mismatched allografts by disabling IL-2-expanded regulatory T cells. Am J Transplant 2012; 12:2313–2321.

Chattopadhyay K, Bhatia S, Fiser A, Almo SC, Nathenson SG. Structural basis of inducible costimulator ligand costimulatory function: determination of the cell surface oligomeric state and functional mapping of the receptor binding site of the protein. J Immunol 2006; 177:3920–3929.

Chattopadhyay K, Lazar-Molnar E, Yan Q, Rubinstein R, Zhan C, Vigdorovich V, Ramagopal UA, Bonanno J, Nathenson SG, Almo SC. Sequence, structure, function, immunity: structural genomics of costimulation. Immunol Rev 2009; 229:356–386.

Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human

T cell stimulation, but only receptor ligation prevents T cell activation. J Immunol 2004; 173:945–954.

Chen H, Peng J, Luo H, Loubeau M, Wan X, Xu D, Qi S, Vu MD, Daloze P, Fitzsimmons WE, Bekersky I, Peets J, Sehgal SN, Wu J. Compromised kidney graft rejection response in Vervet monkeys after withdrawal of immunosuppressants tacrolimus and sirolimus. Transplantation 2000; 69:1555–1561.

Chen M, Xiao X, Demirci G, Li XC. OX40 controls islet allograft tolerance in CD154 deficient mice by regulating FOXP3+ Tregs. Transplantation 2008; 85:1659–1662.

Chen TT, Li L, Chung DH, Allen CD, Torti SV, Torti FM, Cyster JG, Chen CY, Brodsky FM, Niemi EC, Nakamura MC, Seaman WE, Daws MR. TIM-2 is expressed on B cells and in liver and kidney and is a receptor for H-ferritin endocytosis. J Exp Med 2005; 202:955–965.

Cher DJ, Mosmann TR. Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. J Immunol 1987; 138:3688–3694.

Cho HR, Kwon B, Yagita H, La S, Lee EA, Kim JE, Akiba H, Kim J, Suh JH, Vinay DS, Ju SA, Kim BS, Mittler RS, Okumura K, Kwon BS. Blockade of 4-1BB (CD137)/4-1BB ligand interactions increases allograft survival. Transpl Int 2004; 17:351–361.

Churlaud G, Pitoiset F, Jebbawi F, Lorenzon R, Bellier B, Rosenzwajg M, Klatzmann D. Human and mouse $CD8^{+}CD25^{+}FOXP3^{+}$ regulatory T cells at steady state and during Interleukin-2 therapy. Front Immunol 2015; 6:171. doi: 10.3389/fimmu.2015.00171. eCollection 2015.

Collins AV, Brodie DW, Gilbert RJ, Iaboni A, Manso-Sancho R, Walse B, Stuart DI, van der Merwe PA, Davis SJ. The interaction properties of costimulatory molecules revisited. Immunity 2002; 17:201–210.

Colvin RB, Smith RN. Antibody-mediated organ-allograft rejection. Nat Rev Immunol 2005; 5:807–817.

Compaan DM, Hymowitz SG. The crystal structure of the costimulatory OX40–OX40L complex. Structure 2006; 14:1321–1330.

Cordoba F, Wieczorek G, Audet M, Roth L, Schneider MA, Kunkler A, Stuber N, Erard M, Ceci M, Baumgartner R, Apolloni R, Cattini A, Robert G, Ristig D, Munz J, Haeberli L, Grau R, Sickert D, Heusser C, Espie P, Bruns C, Patel D, Rush JS. A novel, blocking, Fc-silent anti-CD40 monoclonal antibody prolongs nonhuman primate renal allograft survival in the absence of B cell depletion. Am J Transplant 2015; 15:2825–2836.

Cosio FG, Hickson LJ, Griffin MD, Stegall MD, Kudva Y. Patient survival and cardiovascular risk after kidney transplantation: The challenge of diabetes. Am J Transplant 2008; 8:593–599.

Costello JP, Mohanakumar T, Nath DS. Mechanisms of chronic cardiac allograft rejection. Tex Heart Inst J 2013; 40:395–399.

Coyle AJ, Lehar S, Lloyd C, Tian J, Delaney T, Manning S, Nguyen T, Burwell T, Schneider H, Gonzalo JA, Gosselin M, Owen LR, Rudd CE, Gutierrez-Ramos JC. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. Immunity 2000; 13:95–105.

Crespo JL, Hall MN. Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae. Microbiol Mol Biol Rev 2002; 66:579–591.

Croft M. Costimulation of T cells by OX40, 4-1BB, and CD27. Cytokine Growth Factor Rev 2003; 14:265–273.

Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? Nat Rev Immunol 2003; 3:609–620.

Croft M. The TNF family in T cell differentiation and function--unanswered questions and future directions. Semin Immunol 2014; 26:183–190.

Crow AR, Leytin V, Starkey AF, Rand ML, Lazarus AH. CD154 (CD40 ligand)-deficient mice exhibit prolonged bleeding time and decreased shear-induced platelet aggregates. J Thromb Haemost 2003; 1:850.

Curotto de Lafaille MA, Kutchukhidze N, Shen S, Ding Y, Yee H, Lafaille JJ. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. Immunity 2008; 29:114–126.

Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? Immunity 2009; 30:626–635.

Curry AJ, Chikwe J, Smith XG, Cai M, Schwarz H, Bradley JA, Bolton EM. OX40 (CD134) blockade inhibits the co-stimulatory cascade and promotes heart allograft survival. Transplantation 2004; 78:807–814.

de Jong R, Loenen WA, Brouwer M, van Emmerik L, de Vries EF, Borst J, van Lier RA. Regulation of expression of CD27, a T cell-specific member of a novel family of membrane receptors. J Immunol 1991; 146:2488–2494.

DeBenedette MA, Chu NR, Pollok KE, Hurtado J, Wade WF, Kwon BS, Watts TH. Role of 4-1BB ligand in costimulation of T lymphocyte growth and its up-regulation on M12 B lymphoma by cAMP. J Exp Med 1995; 181:985–991.

DeBenedette MA, Shahinian A, Mak TW, Watts TH. Costimulation of CD28- T lymphocytes by 4-1BB ligand. J Immunol 1997; 158:551–559.

Dedhar S. Integrins and signal transduction. Curr Opin Hematol. 1999; 6:37–43.

Degauque N, Mariat C, Kenny J, Zhang D, Gao W, Vu MD, Alexopoulos S, Oukka M, Umetsu DT, DeKruyff RH, Kuchroo V, Zheng XX, Strom TB. Immunostimulatory Tim-1-specific antibody deprograms Tregs and prevents transplant tolerance in mice. J Clin Invest 2008; 118:735–741.

Demirci G, Amanullah F, Kewalaramani R, Yagita H, Strom TB, Sayegh MH, Li XC. Critical role of OX40 in CD28 and CD154-independent rejection. J Immunol 2004; 172:1691–1698.

Dempster WJ, Lennox B, Boag JW. Prolongation of survival of skin homotransplants in the rabbit by irradiation of the host. Br J Exp Pathol 1950; 31:670–679.

Diab R, Iwata T, Corbascio M, Tibell A, Ekberg H, Holgersson J, Kumagai-Braesch M. Effect of triple costimulation blockade on islet allograft survival in sensitized mice. Transplant Proc 2010; 42:2109–2111.

Dierselhuis M, Goulmy E. The relevance of minor histocompatibility antigens in solid organ transplantation. Curr Opin Organ Transplant 2009; 14:419–425.

Djamali A, Samaniego M, Muth B, Muehrer R, Hofmann RM, Pirsch J, Howard A, Mourad G, Becker BN. Medical care of kidney transplant recipients after the first posttransplant year. Clin J Am Soc Nephrol 2006; 1:623–640.

Doak PB, Dalton NT, Meredith J, Montgomerie JZ, North JD. Use of antilymphocyte globulin after cadaveric renal transplantation. Br Med J 1969; 4:522–525.

Dong C, Juedes AE, Temann UA, Shresta S, Allison JP, Ruddle NH, Flavell RA. ICOS co-stimulatory receptor is essential for T-cell activation and function. Nature 2001; 409:97–101.

Drenkard D, Becke FM, Langstein J, Spruss T, Kunz-Schughart LA, Tan TE, Lim YC, Schwarz H. CD137 is expressed on blood vessel walls at sites of inflammation and enhances monocyte migratory activity. FASEB J 2007; 21:456–463.

Dreyfuss M, Harri E, Hofmann H, Kobel H, Pache W, Tscherter H. Cyclosporin A and C: new metabolites from Trichoderma polysporum (Link ex Pers) Rifai. Eur J Appl Microbiol 1976; 3:125–133.

Durie FH, Foy TM, Masters SR, Laman JD, Noelle RJ. The role of CD40 in the regulation of humoral and cell-mediated immunity. Immunol Today 1994; 15:406–411.

Durrbach A, Francois H, Jacquet A, Beaudreuil S, Charpentier B. Co-signals in organ transplantation. Curr Opin Organ Transplant 2010; 15:474–480.

Durrbach A, Pestana JM, Pearson T, Vincenti F, Garcia VD, Campistol J, Rial Mdel C, Florman S, Block A, Di Russo G, Xing J, Garg P, Grinyó J. A phase III study of belatacept versus cyclosporine in kidney transplants from extended criteria donors (BENEFIT-EXT study). Am J Transplant 2010; 10:547–557.

Dustin ML, Springer TA. T cell receptor cross linking transiently stimulates adhesiveness through LFA-1. Nature 1989; 341:619–624.

Dzierzak-Mietla M, Markiewicz M, Siekiera U, Mizia S, Koclega A, Zielinska P, Sobczyk-Kruszelnicka M, Kyrcz-Krzemien S. Occurrence and impact of minor histocompatibility antigens' disparities on outcomes of hematopoietic stem cell transplantation from HLA-matched sibling donors. Bone Marrow Res 2012; 2012:257086. doi:10.1155/2012/257086.

Ekberg H, Bernasconi C, Tedesco-Silva H, Vítko S, Hugo C, Demirbas A, Acevedo RR, Grinyó J, Frei U, Vanrenterghem Y, Daloze P, Halloran P. Calcineurin inhibitor minimization in the Symphony study: observational results 3 years after transplantation. Am J Transplant 2009; 9:1876–1885.

Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunol Rev 2009; 229: 152–172.

Eliopoulos AG, Davies C, Knox PG, Gallagher NJ, Afford SC, Adams DH, Young LS. CD40 induces apoptosis in carcinoma cells through activation of cytotoxic ligands of the tumor necrosis factor superfamily. Mol Cell Biol 2000; 20:5503–5515.

Ellis TM, Simms PE, Slivnick DJ, Jäck HM, Fisher RI. CD30 is a signal-transducing molecule that defines a subset of human activated CD45RO+ T cells. J Immunol 1993; 151:2380–2389.

El-Zoghby ZM, Stegall MD, Lager DJ, Kremers WK, Amer H, Gloor JM, Cosio FG. Identifying specific causes of kidney allograft loss. Am J Transplant 2009; 9:527–535.

Engel P, Gribben JG, Freeman GJ, Zhou LJ, Nozawa Y, Abe M, Nadler LM, Wakasa H, Tedder TF. The B7-2 (B70) costimulatory molecule expressed by monocytes and activated B lymphocytes is the CD86 differentiation antigen. Blood 1994; 84:1402–1407.

Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, Belladonna ML, Fioretti MC, Alegre ML, Puccetti P. Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol 2003; 4:1206–1212.

Fargeas CA, Truneh A, Reddy M, Hurle M, Sweet R, Sékaly RP. Identification of residues in the V domain of CD80 (B7-1) implicated in functional interactions with CD28 and CTLA4. J Exp Med 1995; 182:667–675.

Farrar CA, Kupiec-Weglinski JW, Sacks SH. The innate immune system and transplantation. Cold Spring Harb Perspect Med 2013; 3:a015479. doi: 10.1101/cshperspect.a015479.

Fathman CG, Lineberry NB. Molecular mechanisms of CD4+ T-cell anergy. Nat Rev Immunol 2007; 7:599–609.

Fernández-Ruiz E, Somoza C, Sánchez-Madrid F, Lanier LL. CD28/CTLA-4 ligands: the gene encoding CD86 (B70/B7.2) maps to the same region as CD80 (B7/B7.1) gene in human chromosome 3q13-q23. Eur J Immunol 1995; 25:1453–1456.

Fields PE, Finch RJ, Gray GS, Zollner R, Thomas JL, Sturmhoefel K, Lee K, Wolf S, Gajewski TF, Fitch FW. B7.1 is a quantitatively stronger costimulus than B7.2 in the activation of naive CD8+ TCR-transgenic T cells. J Immunol 1998; 161:5268–5275.

Fishbein TM, Wang L, Benjamin C, Liu J, Tarcsafalvi A, Leytin A, Miller CM, Boros P. Successful tolerance induction under CD40 ligation in a rodent small bowel transplant model: first report of a study with the novel antibody AH.F5. Transplantation 2002; 73:1943–1948.

Flaswinkel H, Barner M, Reth M. The tyrosine activation motif as a target of protein tyrosine kinases and SH2 domains. Semin Immunol 1995; 7:21–27.

Flechner SM, Kobashigawa J, Klintmalm G. Calcineurin inhibitor-sparing regimens in solid organ transplantation: focus on improving renal function and nephrotoxicity. Clin Transplant 2008; 22:1–15.

Fletcher JT, Nankivell BJ, Alexander SI. Chronic allograft nephropathy. Pediatr Nephrol 2009; 24:1465–1471.

Foy TM, Aruffo A, Bajorath J, Buhlmann JE, Noelle RJ. Immune regulation by CD40 and its ligand GP39. Annu Rev Immunol 1996; 14:591–617.

Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, Sharpe AH. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J Exp Med 2009; 206:3015–3029.

Frauwirth KA, Riley JL, Harris MH, Parry RV, Rathmell JC, Plas DR, Elstrom RL, June CH, Thompson CB. The CD28 signaling pathway regulates glucose metabolism. Immunity 2002; 16:769–777.

Frauwirth KA, Thompson CB. Activation and inhibition of lymphocytes by costimulation. J Clin Invest 2002; 109:295–299.

Freeman GJ, Boussiotis VA, Anumanthan A, Bernstein GM, Ke XY, Rennert PD, Gray GS, Gribben JG, Nadler LM. B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. Immunity 1995; 2:523–532.

Freeman GJ, Casasnovas JM, Umetsu DT, DeKruyff RH. TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity. Immunol Rev 2010; 235:172–189.

Freeman GJ, Disteche CM, Gribben JG, Adler DA, Freedman AS, Dougery J, Nadler LM. The gene for B7, a costimulatory signal for T-cell activation, maps to chromosomal region 3q13.3-3q21. Blood 1992; 79:489–494.

Freeman GJ, Freedman AS, Segil JM, Lee G, Whitman JF, Nadler LM. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. J Immunol 1989; 143:2714–2722.

Freeman GJ, Gray GS, Gimmi CD, Lombard DB, Zhou LJ, White M, Fingeroth JD, Gribben JG, Nadler LM. Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. J Exp Med 1991; 174:625–631.

Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000; 192:1027–1034.

Futagawa T, Akiba H, Kodama T, Takeda K, Hosoda Y, Yagita H, Okumura K. Expression and function of 4-1BB and 4-1BB ligand on murine DCs. Int Immunol 2002; 14:275–286.

Geissler EK, Schlitt HJ. The potential benefits of rapamycin on renal function, tolerance, fibrosis, and malignancy following transplantation. Kidney Int 2010; 78:1075–1079.

Geng J, Bevilacqua MP, Moore KL, Mclntyre RM, Prescott SM, Kim JM, Bliss GA, Zimmerman GA, McEver RP. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. Nature 1990; 343:757–760.

Gibson T, Medawar PB. The fate of skin homografts in man. J Anat 1943; 77:299–310.

Gimmi CD, Freeman GJ, Gribben JG, Gray G, Nadler LM. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. Proc Natl Acad Sci U S A 1993; 90:6586–6590.

Gloor JM, Cosio FG, Rea DJ, Wadei HM, Winters JL, Moore SB, DeGoey SR, Lager DJ, Grande JP, Stegall MD. Histologic findings one year after positive crossmatch or ABO blood group incompatible living donor kidney transplantation. Am J Transplant 2006; 6:1841–1847.

Goldwater R, Keirns J, Blahunka P, First R, Sawamoto T, Zhang W, Kowalski D, Kaibara A, Holman J. A phase 1, randomized ascending single-dose study of antagonist anti-human CD40 ASKP1240 in healthy subjects. Am J Transplant 2013; 13:1040–1046.

Goodwin RG, Din WS, Davis-Smith T, Anderson DM, Gimpel SD, Sato TA, Maliszewski CR, Brannan CI, Copeland NG, Jenkins NA, Farrah T, Armitage RJ, Fanslow WC, Smith CA. Molecular cloning of a ligand for the inducible T cell gene 4-1BB: A member of an emerging family of cytokines with homology to tumor necrosis factor. Eur J Immunol 1993; 23:2631–2641.

Goodwin WE, Kaufmann JJ, Mims MM, Turner RD, Glassock R, Goldman R, Maxwell MM. Human renal transplantation. I Clinical experience with six cases of renal homotransplantation. J Urol 1963; 89:13–24.

Goralczyk AD, Bari N, Abu-Ajaj W, Lorf T, Ramadori G, Friede T, Obed A. Calcineurin inhibitor sparing with mycophenolate mofetil in liver transplantion: a systematic review of randomized controlled trials. Am J Transplant 2012; 12:2601–2607.

Gough SC, Walker LS, Sansom DM CTLA4 gene polymorphism and autoimmunity. Immunol Rev 2005; 204:102–115.

Gramaglia I, Jember A, Pippig SD, Weinberg AD, Killeen N, Croft M. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. J Immunol 2000; 165:3043–3050.

Gramaglia I, Weinberg AD, Lemon M, Croft M. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T-cell responses. J Immunol 1998; 161:6510−6517.

Grant SC, Lamb WR, Yonan N, Hutchinson IV, Brenchley PE. Antithymocyte globulin preparations after heart transplantation. Cytokine responses in vivo and in vitro. Transplantation 1995; 60:684–689.

Greene JL, Leytze GM, Emswiler J, Peach R, Bajorath J, Cosand W, Linsley PS. Covalent dimerization of CD28/CTLA-4 and oligomerization of CD80/CD86 regulate T cell costimulatory interactions. J Biol Chem 1996; 271:26762–26771.

Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol 2005; 23:515–548.

Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. Annu Rev Immunol 1998; 16:111–135.

Greyer M, Whitney PG, Stock AT, Davey GM, Tebartz C, Bachem A, Mintern JD, Strugnell RA, Turner SJ, Gebhardt T, O'Keeffe M, Heath WR, Bedoui S. T cell help amplifies innate signals in $CD8^+$ DCs for optimal $CD8^+$ T cell priming. Cell Rep 2016; 14:586–597.

Grimaldi JC, Torres R, Kozak CA, Chang R, Clark EA, Howard M, Cockayne DA. Genomic structure and chromosomal mapping of the murine CD40 gene. J Immunol 1992; 149:3921–3926.

Grinyo J, Alberu J, Contieri FL, Manfro RC, Mondragon G, Nainan G, Rial Mdel C, Steinberg S, Vincenti F, Dong Y, Thomas D, Kamar N. Improvement in renal function in kidney transplant recipients switched from cyclosporine or tacrolimus to belatacept: 2-year results from the long-term extension of a phase II study. Transpl Int 2012; 25:1059–1064.

Grinyó JM, Cruzado JM. Mycophenolate mofetil and calcineurin-inhibitor reduction: recent progress. Am J Transplant 2009; 9:2447–2452.

Gruen JR, Weissman SM. Evolving views of the major histocompatibility complex. Blood 1997; 90:4252–4265.

Gulanikar AC, MacDonald AS, Sungurtekin U, Belitsky P. The incidence and impact of early rejection episodes on graft outcome in recipients of first cadaver kidney transplants. Transplantation 1992; 53:323–328.

Guo L, Li XK, Enosawa S, Funeshima N, Suzuki S, Kimura H, Sugawara Y, Tezuka K, Makuuchi M. Significant enhancement by anti-ICOS antibody of suboptimal tacrolimus immunosuppression in rat liver transplantation. Liver Transpl 2004; 10:743–747.

Guy CS, Vignali DA. Organization of proximal signal initiation at the TCR:CD3 complex. Immunol Rev 2009; 232:7–21.

Haanstra KG, Ringers J, Sick EA, Ramdien-Murli S, Kuhn EM, Boon L, Jonker M. Prevention of kidney allograft rejection using anti-CD40 and anti-CD86 in primates. Transplantation 2003; 75:637–643.

Haddad EM, McAlister VC, Renouf E, Malthaner R, Kjaer MS, Gluud LL. Cyclosporin versus tacrolimus for liver transplanted patients. Cochrane Database Syst Rev 2006; (4):CD005161.

Harper K, Balzano C, Rouvier E, Mattéi MG, Luciani MF, Golstein P. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. J Immunol 1991; 147:1037–1044.

Hart A, Smith JM, Skeans MA, Gustafson SK, Stewart DE, Cherikh WS, Wainright JL, Boyle G, Snyder JJ, Kasiske BL, Israni AK. OPTN/SRTR 2014 Annual Data Report: kidney. Am J Transplant 2016; 16 Suppl 2:11–46.

Haspot F, Villemain F, Laflamme G, Coulon F, Olive D, Tiollier J, Soulillou JP, Vanhove B. Differential effect of CD28 versus B7 blockade on direct pathway of allorecognition and self-restricted responses. Blood 2002; 99:2228–2234.

Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, Kent SC, Zheng XX, Strom TB, Hafler DA, Kuchroo VK. TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines. Eur J Immunol 2009; 39:2492–2501.

Hausen B, Klupp J, Christians U, Higgins JP, Baumgartner RE, Hook LE, Friedrich S, Celnicker A, Morris RE. Coadministration of either cyclosporine or steroids with humanized monoclonal antibodies against CD80 and CD86 successfully prolong allograft survival after life supporting renal transplantation in cynomolgus monkeys. Transplantation 2001; 72:1128–1137.

Heeger PS, Greenspan NS, Kuhlenschmidt S, Dejelo C, Hricik DE, Schulak JA, Tary-Lehmann M. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. J Immunol 1999; 163:2267–2275.

Heidt S, Segundo DS, Chadha R, Wood KJ. The impact of Th17 cells on transplant rejection and the induction of tolerance. Curr Opin Organ Transplant 2010; 15:456–461.

Hendriks J, Gravestein LA, Tesselaar K, van Lier RA, Schumacher TN, Borst J. CD27 is required for generation and long-term maintenance of T cell immunity. Nat Immunol 2000; 1:433–440.

Henricsson A, Husberg B, Bergentz SE. The mechanism behind the effect of ALG on platelets in vivo. Clin Exp Immunol 1977; 29:515–522.

Herman AE, Freeman GJ, Mathis D, Benoist C. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. J Exp Med 2004; 199:1479–1489.

Herrera OB, Golshayan D, Tibbott R, Salcido Ochoa F, James MJ, Marelli-Berg FM, Lechler RI. A novel pathway of alloantigen presentation by DCs. J Immunol 2004; 173:4828–4837.

Hintzen RQ, Lens SM, Beckmann MP, Goodwin RG, Lynch D, van Lier RA. Characterization of the human CD27 ligand, a novel member of the TNF gene family. J Immunol 1994; 152:1762–1773.

Hintzen RQ, Lens SM, Lammers K, Kuiper H, Beckmann MP, van Lier RA. Engagement of CD27 with its ligand CD70 provides a second signal for T cell activation. J Immunol 1995; 154:2612–2623.

Howard TA, Rochelle JM, Seldin MF. Cd28 and Ctla-4, two related members of the Ig supergene family, are tightly linked on proximal mouse chromosome 1. Immunogenetics 1991; 33:74–76.

Huang H, Ma Y, Dawicki W, Zhang X, Gordon JR. Comparison of induced versus natural regulatory T cells of the same TCR specificity for induction of tolerance to an environmental antigen. J Immunol. 2013; 191:1136–1143.

Hulpiau P, van Roy F. Molecular evolution of the cadherin superfamily. Int J Biochem Cell Biol 2009; 41:349–369.

Hunt SA. Complications of heart transplantation. J Heart Transplant 1983; 3:70–74.

Hutchcroft JE, Bierer BE. Activation-dependent phosphorylation of the T-lymphocyte surface receptor CD28 and associated proteins. Proc Natl Acad Sci U S A 1994; 91:3260–3264.

Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczek RA. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 1999; 397:263–266.

Hwu P, Du MX, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2,3-dioxygenase production by human DCs results in the inhibition of T cell proliferation. J Immunol 2000; 164:3596–3599.

Hymowitz SG, Compaan DM, Yan M, Wallweber HJ, Dixit VM, Starovasnik MA, de Vos AM. The crystal structures of EDA-A1 and EDA-A2: splice variants with distinct receptor specificity. Structure 2003; 11:1513–1520.

Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002; 110:673–687.

Ichimura T, Asseldonk EJ, Humphreys BD, Gunaratnam L, Duffield JS, Bonventre JV. Kidney injury molecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. J Clin Invest 2008; 118:1657–1668.

Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, Sanicola M. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is upregulated in renal cells after injury. J Biol Chem 1998; 273:4135–4142.

Imai A, Suzuki T, Sugitani A, Itoh T, Ueki S, Aoyagi T, Yamashita K, Taniguchi M, Takahashi N, Miura T, Shimamura T, Furukawa H, Todo S. A novel fully human anti-CD40 monoclonal antibody, 4D11, for kidney transplantation in cynomolgus monkeys. Transplantation 2007; 84:1020–1028.

Ingulli E. Mechanism of cellular rejection in transplantation. Pediatr Nephrol 2010; 25:61–74.

Inomata Y, Tanaka K. Pathogenesis and treatment of bile duct loss after liver transplantation. J Hepatobiliary Pancreat Surg 2001; 8:316–322.

Inwald DP, McDowall A, Peters MJ, Callard RE, Klein NJ. CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. Circ Res 2003; 92:1041–1048.

Ishii A, Oboki K, Nambu A, Morita H, Ohno T, Kajiwara N, Arae K, Sudo H, Okumura K, Saito H, Nakae S. Development of IL-17-mediated delayed-type hypersensitivity is not affected by down-regulation of IL-25 expression. Allergol Int 2010; 59:399–408.

Ivanov DB, Philippova MP, Tkachuk VA. Structure and functions of classical cadherins. Biochemistry 2001; 66:1174–1186.

Jacquot S. CD27/CD70 interactions regulate T dependent B cell differentiation. Immunol Rev 2000; 21:23–30.

Jain J, Loh C, Rao A. Transcriptional regulation of the IL-2 gene. Curr Opin Immunol 1995; 7:333–342.

Jang MS, Pan F, Erickson LM, Fisniku O, Crews G, Wynn C, Hong IC, Tamura K, Kobayashi M, Jiang H. A blocking anti-CD28-specific antibody induces long-term heart allograft survival by suppression of the PKC theta-JNK signal pathway. Transplantation 2008; 85:1051–1055.

Jenkins MK, Ashwell JD, Schwartz RH. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. J Immunol 1988; 140:3324–3330.

Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J Exp Med 1987; 165:302–319.

Jonuleit H, Schmitt E. The regulatory T cell family: distinct subsets and their interrelations. J Immunol 2003; 171:6323–6327.

June CH, Bluestone JA, Nadler LM, Thompson CB. The B7 and CD28 receptor families. Immunol Today 1994; 15:321–331.

June CH, Ledbetter JA, Lindsten T, Thompson CB. Evidence for the involvement of three distinct signals in the induction of IL-2 gene expression in human T lymphocytes. J Immunol 1989; 143:153–161.

Kahwaji J, Bunnapradist S, Hsu JW, Idroos ML, Dudek R. Cause of death with graft function among renal transplant recipients in an integrated healthcare system. Transplantation 2011; 91:225–230.

Kanmaz T, Fechner JJ Jr, Torrealba J, Kim HT, Dong Y, Oberley TD, Schultz JM, Bloom DD, Katayama M, Dar W, Markovits J, Schuler W, Hu H, Hamawy MM, Knechtle SJ. Monotherapy with the novel human anti-CD154 monoclonal antibody ABI793 in rhesus monkey renal transplantation model. Transplantation 2004; 77:914–920.

Kawabe T, Matsushima M, Hashimoto N, Imaizumi K, Hasegawa Y. CD40/CD40 ligand interactions in immune responses and pulmonary immunity. Nagoya J Med Sci 2011; 73:69–78.

Kawabe T, Naka T, Yoshida K, Tanaka T, Fujiwara H, Suematsu S, Yoshida N, Kishimoto T, Kikutani H. The immune responses in CD40-deficient mice: Impaired immunoglobulin class switching and germinal center formation. Immunity 1994; 1:167–178.

Kawai K, Shahinian A, Mak TW, Ohashi PS. Skin allograft rejection in CD28-deficient mice. Transplantation 1996; 61:352–355.

Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. Nat Med 2000; 6:114.

Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 2008; 26:677–704.

Kelley J, Walter L, Trowsdale J. Comparative genomics of major histocompatibility complexes. Immunogenetics 2005; 56:683–695.

Kendal AR, Chen Y, Regateiro FS, Ma J, Adams E, Cobbold SP, Hori S, Waldmann H. Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance. J Exp Med. 2011; 208:2043–2053.

Khademi M, Illés Z, Gielen AW, Marta M, Takazawa N, Baecher-Allan C, Brundin L, Hannerz J, Martin C, Harris RA, Hafler DA, Kuchroo VK, Olsson T, Piehl F, Wallström E. T Cell Ig- and mucindomain-containing molecule-3 (TIM-3) and TIM-1 molecules are differentially expressed on human Th1 and Th2 cells and in cerebrospinal fluid-derived mononuclear cells in multiple sclerosis. J Immunol 2004; 172:7169–7176.

Kienzle G, von Kempis J. CD137 (ILA/4-1BB), expressed by primary human monocytes, induces monocyte activation and apoptosis of B lymphocytes. Int Immunol 2000; 12:73–82.

Kim IK, Bedi DS, Denecke C, Ge X, Tullius SG. Impact of innate and adaptive immunity on rejection and tolerance. Transplantation 2008; 86:889–894.

Kim MY, Gaspal FM, Wiggett HE, McConnell FM, Gulbranson-Judge A, Raykundalia C, Walker LS, Goodall MD, Lane PJ. $CD4^{+}CD3^{-}$ accessory cells costimulate primed CD4 T

cells through OX40 and CD30 at sites where T cells collaborate with B cells. Immunity 2003; 18:643–654.

Kim YJ, Li G, Broxmeyer HE. 4-1BB ligand stimulation enhances myeloid DC maturation from human umbilical cord blood CD34+ progenitor cells. J Hematother Stem Cell Res 2002; 11:895–903.

Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. J Immunol 2002; 168:1080–1086.

Kino T, Hatanaka H, Miyata S, Inamura N, Nishiyama M, Yajima T, Goto T, Okuhara M, Kohsaka M, Aoki H, Ochiai T. FK506, a novel immunousppressant isolated from Streptomyces. II Immunosuppressive effect of FK-506 in vitro. J Antibiot (Tokyo) 1987; 40:1256–1265.

Kinugasa F, Nagatomi I, Ishikawa H, Nakanishi T, Maeda M, Hirose J, Fukahori H, Ooshima S, Noto T, Higashi Y, Seki N, Mutoh S. Efficacy of oral treatment with tacrolimus in the renal transplant model in cynomolgus monkeys. J Pharmacol Sci 2008; 108:529–534.

Kirk AD, Burkly LC, Batty DS, Baumgartner RE, Berning JD, Buchanan K, Fechner JH Jr, Germond RL, Kampen RL, Patterson NB, Swanson SJ, Tadaki DK, TenHoor CN, White L, Knechtle SJ, Harlan DM. Treatmentwith humanizedmonoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. Nat Med 1999; 5:686–693.

Kirk AD, Harlan DM, Armstrong NN, Davis TA, Dong Y, Gray GS, Hong X, Thomas D, Fechner JH, Knechtle SJ. CTLA-4Ig and anti-CD40L prevent renal allograft rejection in primates. Proc Natl Acad Sci U S A 1997; 94:8789–8794.

Kirk AD, Knechtle SJ, Sollinger HW, Vincenti FG, Stecher S, Nadeau KC. Preliminary results of the use of humanized anti-CD154 in human renal allotransplantation [abstract]. Am J Transplant 2001; 1 Suppl 1:191.

Kirk AD. 4D11: The Second Mouse? Am J Transplant 2009; 9:1701–1702.

Kitchens WH, Haridas D, Wagener ME, Song M, Kirk AD, Larsen CP, Ford ML. Integrin antagonists prevent costimulatory blockade-resistant transplant rejection by $CD8^+$ memory T cells. Am J Transplant 2012; 12:69–80.

Klintmalm GB, Feng S, Lake JR, Vargas HE, Wekerle T, Agnes S, Brown KA, Nashan B, Rostaing L, Meadows-Shropshire S, Agarwal M, Harler MB, Garcia-Valdecasas JC. Belatacept-based immunosuppression in de novo liver transplant recipients: 1-year experience from a phase II randomized study. Am J Transplant 2014; 14:1817–1827.

Klintmalm GB, Nashan B. The Role of mTOR Inhibitors in Liver Transplantation: Reviewing the Evidence. J Transplant 2014; 2014:845438. doi: 10.1155/2014/845438.

Knechtle SJ, Adams AB. Belatacept: is there BENEFIT for liver transplantation too? Am J Transplant 2014; 14:1717–1718.

Knoll G. Trends in kidney transplantation over the past decade. Drugs 2008; 68 Suppl 1:3–10.

Kornete M, Sgouroudis E, Piccirillo CA. ICOS-dependent homeostasis and function of Foxp3+ regulatory T cells in islets of nonobese diabetic mice. J Immunol 2012; 188:1064–1067.

Koulova L, Clark EA, Shu G, Dupont B. The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4+ T cells. J Exp Med 1991; 173:759–762.

Koyama I, Kawai T, Andrews D, Boskovic S, Nadazdin O, Wee SL, Sogawa H, Wu DL, Smith RN, Colvin RB, Sachs DH, Cosimi AB. Thrombophilia associated with anti-CD154

monoclonal antibody treatment and its prophylaxis in nonhuman primates. Transplantation 2004; 77:460–462.

Kozbor D, Moretta A, Messner HA, Moretta L, Croce CM. Tp44 molecules involved in antigen-independent T cell activation are expressed on human plasma cells. J Immunol 1987; 138:4128–4132.

Krummel MF, Allison JP. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. J Exp Med 1996; 183:2533–2540.

Kuiper HM, Brouwer M, Linsley PS, van Lier RA. Activated T cells can induce high levels of CTLA-4 expression on B cells. J Immunol 1995; 155:1776–1783.

Kumanogoh A, Marukawa S, Suzuki K, Takegahara N, Watanabe C, Ch'ng E, Ishida I, Fujimura H, Sakoda S, Yoshida K, Kikutani H. Class IV semaphoring Sema4A enhances T-cell activation and interacts with Tim-2. Nature 2002; 419: 629–633.

Kwajah M M S, Schwarz H. CD137 ligand signaling induces human monocyte to DC differentiation. Eur J Immunol 2010; 40:1938–1949.

Kwon BS, Weissman SM. cDNA sequences of two inducible T-cell genes. Proc Natl Acad Sci U S A 1989; 86:1963–1967.

Lafage-Pochitaloff M, Costello R, Couez D, Simonetti J, Mannoni P, Mawas C, Olive D. Human CD28 and CTLA-4 Ig superfamily genes are located on chromosome 2 at bands q33-q34. Immunogenetics 1990; 31:198–201.

Lamb KE, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: a critical reappraisal. Am J Transplant 2011; 11:450–462.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L,

Lehoczky J, LeVine R, McEwan P, McKernan K, Meldrim J, Mesirov JP, Miranda C, Morris W, Naylor J, Raymond C, Rosetti M, Santos R, Sheridan A, Sougnez C, Stange-Thomann Y, Stojanovic N, Subramanian A, Wyman D, Rogers J, Sulston J, Ainscough R, Beck S, Bentley D, Burton J, Clee C, Carter N, Coulson A, Deadman R, Deloukas P, Dunham A, Dunham I, Durbin R, French L, Grafham D, Gregory S, Hubbard T, Humphray S, Hunt A, Jones M, Lloyd C, McMurray A, Matthews L, Mercer S, Milne S, Mullikin JC, Mungall A, Plumb R, Ross M, Shownkeen R, Sims S, Waterston RH, Wilson RK, Hillier LW, McPherson JD, Marra MA, Mardis ER, Fulton LA, Chinwalla AT, Pepin KH, Gish WR, Chissoe SL, Wendl MC, Delehaunty KD, Miner TL, Delehaunty A, Kramer JB, Cook LL, Fulton RS, Johnson DL, Minx PJ, Clifton SW, Hawkins T, Branscomb E, Predki P, Richardson P, Wenning S, Slezak T, Doggett N, Cheng JF, Olsen A, Lucas S, Elkin C, Uberbacher E, Frazier M, Gibbs RA, Muzny DM, Scherer SE, Bouck JB, Sodergren EJ, Worley KC, Rives CM, Gorrell JH, Metzker ML, Naylor SL, Kucherlapati RS, Nelson DL, Weinstock GM, Sakaki Y, Fujiyama A, Hattori M, Yada T, Toyoda A, Itoh T, Kawagoe C, Watanabe H, Totoki Y, Taylor T, Weissenbach J, Heilig R, Saurin W, Artiguenave F, Brottier P, Bruls T, Pelletier E, Robert C, Wincker P, Smith DR, Doucette-Stamm L, Rubenfield M, Weinstock K, Lee HM, Dubois J, Rosenthal A, Platzer M, Nyakatura G, Taudien S, Rump A, Yang H, Yu J, Wang J, Huang G, Gu J, Hood L, Rowen L, Madan A, Qin S, Davis RW, Federspiel NA, Abola AP, Proctor MJ, Myers RM, Schmutz J, Dickson M, Grimwood J, Cox DR, Olson MV, Kaul R, Raymond C, Shimizu N, Kawasaki K, Minoshima S, Evans GA, Athanasiou M, Schultz R, Roe BA, Chen F, Pan H, Ramser J, Lehrach H, Reinhardt R, McCombie WR, de la Bastide M, Dedhia N, Blöcker H, Hornischer K, Nordsiek G, Agarwala R, Aravind L, Bailey JA, Bateman A,

Batzoglou S, Birney E, Bork P, Brown DG, Burge CB, Cerutti L, Chen HC, Church D, Clamp M, Copley RR, Doerks T, Eddy SR, Eichler EE, Furey TS, Galagan J, Gilbert JG, Harmon C, Hayashizaki Y, Haussler D, Hermjakob H, Hokamp K, Jang W, Johnson LS, Jones TA, Kasif S, Kaspryzk A, Kennedy S, Kent WJ, Kitts P, Koonin EV, Korf I, Kulp D, Lancet D, Lowe TM, McLysaght A, Mikkelsen T, Moran JV, Mulder N, Pollara VJ, Ponting CP, Schuler G, Schultz J, Slater G, Smit AF, Stupka E, Szustakowki J, Thierry-Mieg D, Thierry-Mieg J, Wagner L, Wallis J, Wheeler R, Williams A, Wolf YI, Wolfe KH, Yang SP, Yeh RF, Collins F, Guyer MS, Peterson J, Felsenfeld A, Wetterstrand KA, Patrinos A, Morgan MJ, de Jong P, Catanese JJ, Osoegawa K, Shizuya H, Choi S, Chen YJ, Szustakowki J; International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. Nature 2001; 409:860–921.

Langstein J, Becke FM, Söllner L, Krause G, Brockhoff G, Kreutz M, Andreesen R, Schwarz H. Comparative analysis of CD137 and LPS effects on monocyte activation, survival, and proliferation. Biochem Biophys Res Commun 2000; 273:117–122.

Lanzavecchia A, Sallusto F. Dynamics of T lymphocyte response: intermediates, effectors, and memory cells. Science 2000; 290:92–97.

Larsen CP, Alexander DZ, Hollenbaugh D, Elwood ET, Ritchie SC, Aruffo A, Hendrix R, Pearson TC. CD40-gp39 interactions play a critical role during allograft rejection. Suppression of allograft rejection by blockade of the CD40-gp39 pathway. Transplantation 1996; 61:4–9.

Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, Cho HR, Aruffo A, Hollenbaugh D, Linsley PS, Winn KJ, Pearson TC. Long-term acceptance of

skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature 1996; 381:434–438.

Larsen CP, Knechtle SJ, Adams A, Pearson T, Kirk AD. A new look at blockade of T-cell costimulation: A therapeutic strategy for long-term maintenance immunosuppression. Am J Transplant 2006; 6:876–883.

Larsen CP, Pearson TC, Adams AB, Tso P, Shirasugi N, Strobert E, Anderson D, Cowan S, Price K, Naemura J, Emswiler J, Greene J, Turk LA, Bajorath J, Townsend R, Hagerty D, Linsley PS, Peach RJ. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. Am J Transplant 2005; 5:443–453.

Larson TS, Dean PG, Stegall MD, Griffin MD, Textor SC, Schwab TR, Gloor JM, Cosio FG, Lund WJ, Kremers WK, Nyberg SL, Ishitani MB, Prieto M, Velosa JA. Complete avoidance of calcineurin inhibitors in renal transplantation: a randomized trial comparing sirolimus and tacrolimus. Am J Transplant 2006; 6:514–522.

Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, Greenfield EA, Bourque K, Boussiotis VA, Carter LL, Carreno BM, Malenkovich N, Nishimura H, Okazaki T, Honjo T, Sharpe AH, Freeman GJ. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2001; 2:261–268.

Latek R, Fleener C, Lamian V, Kulbokas E 3rd, Davis PM, Suchard SJ, Curran M, Vincenti F, Townsend R. Assessment of belatacept mediated costimulation blockade through evaluation of CD80/86-receptor saturation. Transplantation 2009; 87:926–933.

Lechler RI, Batchelor JR. Restoration of immunogenicity to passenger celldepleted kidney allografts by the addition of donor strain DCs. J Exp Med 1982; 155:31–41.

Lee KM, Chuang E, Griffin M, Khattri R, Hong DK, Zhang W, Straus D, Samelson LE, Thompson CB, Bluestone JA. Molecular basis of T cell inactivation by CTLA-4. Science 1998; 282:2263–2266.

Lee RS, Rusche JR, Maloney ME, Sachs DH, Sayegh MH, Madsen JC. CTLA4Ig-induced linked regulation of allogeneic T cell responses. J Immunol 2001; 166:1572–1582.

Lee SW, Park Y, So T, Kwon BS, Cheroutre H, Mittler RS, Croft M. Identification of regulatory functions for 4-1BB and 4-1BBL in myelopoiesis and the development of DCs. Nat Immunol 2008; 9:917–926.

Leist M, Gantner F, Künstle G, Bohlinger I, Tiegs G, Bluethmann H, Wendel A. The 55-kD tumor necrosis factor receptor and CD95 independently signal murine hepatocyte apoptosis and subsequent liver failure. Mol Med 1996; 2:109–124.

Lenschow DJ, Zeng Y, Thistlethwaite JR, Montag A, Brady W, Gibson MG, Linsley PS, Bluestone JA. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. Science 1992; 257:789–792.

Lepisto AJ, Xu M, Yagita H, Weinberg AD, Hendricks RL. Expression and function of the OX40/OX40L costimulatory pair during herpes stromal keratitis. J Leukoc Biol 2007; 81:766–774.

Levitsky J, Miller J, Huang X, Chandrasekaran D, Chen L, Mathew JM. Inhibitory effects of belatacept on allospecific regulatory T-cell generation in humans. Transplantation 2013; 96:689–696.

Li XL, Ménoret S, Le Mauff B, Angin M, Anegon I. Promises and obstacles for the blockade of CD40-CD40L interactions in allotransplantation. Transplantation 2008; 86:10–15.

Liang SC, Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ, Sharpe AH. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. Eur J Immunol 2003; 33:2706–2716.

Ligocki AJ, Niederkorn JY. Advances on Non-CD4 + Foxp3+ T Regulatory Cells: CD8+, Type 1, and Double Negative T Regulatory Cells in Organ Transplantation. Transplantation. 2015; 99:1553–1559.

Lin H, Bolling SF, Linsley PS, Wei RQ, Gordon D, Thompson CB, Turka LA. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. J Exp Med 1993; 178:1801–1806.

Ling V, Wu PW, Finnerty HF, Sharpe AH, Gray GS, Collins M. Complete sequence determination of the mouse and human CTLA4 gene loci: cross-species DNA sequence similarity beyond exon borders. Genomics 1999; 60:341–355.

Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. Immunity 1994; 1:793–801.

Linsley PS, Greene JL, Tan P, Bradshaw J, Ledbetter JA, Anasetti C, Damle NK. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. J Exp Med 1992; 176:1595–1604.

Lippert U, Zachmann K, Ferrari DM, Schwarz H, Brunner E, Mahbub-Ul Latif AH, Neumann C, Soruri A. CD137 ligand reverse signaling has multiple functions in human DCs during an adaptive immune response. Eur J Immunol 2008; 38:1024–1032.

Liu Z, Colovai AI, Tugulea S, Reed EF, Fisher PE, Mancini D, Rose EA, Cortesini R, Michler RE, Suciu-Foca N. Indirect recognition of donor HLA-DR peptides in organ allograft rejection. J Clin Invest 1996; 98:1150–1157.

Lo DJ, Anderson DJ, Song M, Leopardi F, Farris AB, Strobert E, Chapin S, Devens B, Karrer E, Kirk AD. A pilot trial targeting the ICOS-ICOS-L pathway in nonhuman primate kidney transplantation. Am J Transplant 2015; 15:984–992.

Lo DJ, Weaver TA, Stempora L, Mehta AK, Ford ML, Larsen CP, Kirk AD. Selective targeting of human alloresponsive CD8+ effector memory T cells based on CD2 expression. Am J Transplant 2011; 11:22–33.

Loenen WA, De Vries E, Gravestein LA, Hintzen RQ, Van Lier RA, Borst J. The CD27 membrane receptor, a lymphocyte-specific member of the nerve growth factor receptor family, gives rise to a soluble form by protein processing that does not involve receptor endocytosis. Eur J Immunol 1992; 22:447–455.

Loke P, Allison JP. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. Proc Natl Acad Sci U S A 2003; 100:5336–5341.

London CA, Lodge MP, Abbas AK. Functional responses and costimulator dependence of memory $CD4^+$ T cells. J Immunol 2000; 164:265–272.

Lowe M, Badell IR, Thompson P, Martin B, Leopardi F, Strobert E, Price AA, Abdulkerim HS, Wang R, Iwakoshi NN, Adams AB, Kirk AD, Larsen CP, Reimann KA. A novel monoclonal antibody to CD40 prolongs islet allograft survival. Am J Transplant 2012; 12:2079–2087.

Lucas JG, Co JP, Nwaogwugwu UT, Dosani I, Sureshkumar KK. Antibody-mediated rejection in kidney transplantation: an update. Expert Opin Pharmacother 2011; 12:579–592.

Lumsden JM, Roberts JM, Harris NL, Peach RJ, Ronchese F. Differential requirement for CD80 and CD80/CD86-dependent costimulation in the lung immune response to an influenza virus infection. J Immunol 2000; 164:79–85.

Ma A, Dun H, Song L, Hu Y, Zeng L, Bai J, Zhang G, Kinugasa F, Miyao Y, Sakuma S, Okimura K, Kasai N, Daloze P, Chen H. Pharmacokinetics and Pharmacodynamics of ASKP1240, a Fully Human Anti-CD40 Antibody, in Normal and Renal Transplanted Cynomolgus Monkeys. Transplantation 2014; 97:397–404.

Ma DY, Clark EA. The role of CD40 and CD154/CD40L in DCs. Semin Immunol 2009; 21:265–272.

Mach F, Schönbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS, Libby P. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: Implications for CD40-CD40 ligand signaling in atherosclerosis. Proc Natl Acad Sci U S A 1997; 94:1931–1936.

Majeau GR, Meier W, Jimmo B, Kioussis D, Hochman PS. Mechanism of lymphocyte functionassociated molecule 3-Ig fusion proteins inhibition of T cell responses. Structure/function analysis in vitro and in human CD2 transgenic mice. J Immunol 1994; 152:2753–2767.

Manning DD, Reed ND, Shaffer CF. Maintenance of skin xenografts of widely divergent phylogenetic origin of congenitally athymic (nude) mice. J Exp Med 1973; 138:488–494.

Mao Q, Terasaki PI, Cai J, Briley K, Catrou P, Haisch C, Rebellato L. Extremely high association between appearance of HLA antibodies and failure of kidney grafts in a five-year longitudinal study. Am J Transplant 2007; 7:864–871.

Marsh DG, Neely JD, Breazeale DR, Ghosh B, Freidhoff LR, Ehrlich-Kautzky E, Schou C, Krishnaswamy G, Beaty TH. Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. Science 1994; 264:1152–1156.

Martin DC. Renal homotransplantation. Current status. Calif Med 1965; 103:427–432.

Mathis AS, Egloff G, Ghin HL. Calcineurin inhibitor sparing strategies in renal transplantation, part one: Late sparing strategies. World J Transplant 2014; 4:57–80.

McIntire JJ, Umetsu DT, DeKruyff RH. TIM-1, a novel allergy and asthma susceptibility gene. Springer Semin Immunopathol 2004; 25:335–348.

Medawar PB. The behavior and fate of skin autografts and skin homografts in rabbits. J Anat 1944; 78:176–199.

Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. Am J Transplant. 2004; 4:378–383.

Merrill JP, Murray JE, Harrison JE, Guild WR. Successful homotransplantation of the human kidney between identical twins. J Am Med Assoc 1956; 160:277–282.

Meyers JH, Chakravarti S, Schlesinger D, Illes Z, Waldner H, Umetsu SE, Kenny J, Zheng XX, Umetsu DT, DeKruyff RH, Strom TB, Kuchroo VK. TIM-4 is the ligand for TIM-1, and the TIM-1-TIM-4 interaction regulates T cell proliferation. Nat Immunol 2005; 6:455–464.

Mohiuddin MM, Singh AK, Corcoran PC, Hoyt RF, Thomas ML 3rd, Lewis BG, Eckhaus M, Dabkowski NL, Belli AJ, Reimann KA, Ayares D, Horvath KA. Role of anti-CD40 antibody-mediated costimulation blockade on non-Gal antibody production and heterotopic cardiac xenograft survival in a GTKO.hCD46Tg pig-to-baboon model. Xenotransplantation 2014; 21:35–45.

Mohiuddin MM, Singh AK, Corcoran PC, Thomas Iii ML, Clark T, Lewis BG, Hoyt RF, Eckhaus M, Pierson Iii RN, Belli AJ, Wolf E, Klymiuk N, Phelps C, Reimann KA, Ayares D, Horvath KA. Chimeric 2C10R4 anti-CD40 antibody therapy is critical for long-term survival of GTKO.hCD46.hTBM pig-to-primate cardiac xenograft. Nat Commun 2016; 7:11138. doi: 10.1038/ncomms11138.

Moingeon P, Chang HC, Sayre PH, Clayton LK, Alcover A, Gardner P, Reinherz EL. The structural biology of CD2. Immunol Rev 1989; 111:111–144.

Morales JM, Marcén R, del Castillo D, Andres A, Gonzalez-Molina M, Oppenheimer F, Serón D, Gil-Vernet S, Lampreave I, Gainza FJ, Valdés F, Cabello M, Anaya F, Escuin F, Arias M, Pallardó L, Bustamante J. Risk factors for graft loss and mortality after renal transplantation according to recipient age: a prospective multicentre study. Nephrol Dial Transplant 2012; 27 Suppl 4:iv39–46.

Moreau A, Varey E, Anegon I, Cuturi MC. Effector mechanisms of rejection. Cold Spring Harb Perspect Med 2013; 3:a015461.

Morel Y, Schiano de Colella JM, Harrop J, Deen KC, Holmes SD, Wattam TA, Khandekar SS, Truneh A, Sweet RW, Gastaut JA, Olive D, Costello RT. Reciprocal expression of the TNF family receptor herpes virus entry mediator and its ligand LIGHT on activated T cells: LIGHT down-regulates its own receptor. J Immunol 2000; 165:4397–4404.

Moxham VF, Karegli J, Phillips RE, Brown KL, Tapmeier TT, Hangartner R, Sacks SH, Wong W. Homeostatic proliferation of lymphocytes results in augmented memory-like function and accelerated allograft rejection. J Immunol 2008; 180:3910–3918.

Murray JE, Merrill JP, Dammin GJ, Dealy JB, Alexandre GW, Harrison JH. Kidney transplantation in modified recipients. Ann Surg 1962; 156:337–355.

Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcRIII-positive and negative natural killer cells. J Immunol 1989; 143:3183–3191.

Nakae S, Iikura M, Suto H, Akiba H, Umetsu DT, Dekruyff RH, Saito H, Galli SJ. TIM-1 and TIM-3 enhancement of Th2 cytokine production by mast cells. Blood 2007; 110:2565–2568.

Nakae S, Saijo S, Horai R, Sudo K, Mori S, Iwakura Y. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. Proc Natl Acad Sci USA 2003; 100:5986–5990.

Nakayama M, Akiba H, Takeda K, Kojima Y, Hashiguchi M, Azuma M, Yagita H, Okumura K. Tim-3 mediates phagocytosis of apoptotic cells and the cross-presentation. Blood 2009; 113:3821–3830.

Nankivell BJ, Alexander SI. Rejection of the kidney allograft. N Engl J Med 2010; 363:1451–1462.

Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. N Engl J Med 2003; 349:2326–2333.

Neujahr DC, Chen C, Huang X, Markmann JF, Cobbold S, Waldmann H, Sayegh MH, Hancock WW, Turka LA. Accelerated memory cell homeostasis during T cell depletion and approaches to overcome it. J Immunol 2006; 176:4632–4639.

Niblack G, Johnson K, Williams T, Green W, Richie R, MacDonell R. Antibody formation following administration of antilymphocyte serum. Transplant Proc 1987; 19:1896–1897.

Nicolls MR, Coulombe M, Yang H, Bolwerk A, Gill RG. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. J Immunol 2000; 164:3627–3634.

Noelle RJ, Snow EC. T helper cell-dependent B cell activation. FASEB J 1991; 5:2770–2776.

Nollet F, Kools P, van Roy F. Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. J Mol Biol 2000; 299:551–572.

Oderup C, Cederbom L, Makowska A, Cilio CM, Ivars F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on DCs in CD4+ CD25+ regulatory T-cell-mediated suppression. Immunology 2006; 118:240–249.

Okimura K, Maeta K, Kobayashi N, Goto M, Kano N, Ishihara T, Ishikawa T, Tsumura H, Ueno A, Miyao Y, Sakuma S, Kinugasa F, Takahashi N, Miura T. Characterization of ASKP1240, a fully human antibody targeting human CD40 with potent immunosuppressive effects. Am J Transplant 2014; 14:1290–1299.

Olsson C, Michaëlsson E, Parra E, Pettersson U, Lando PA, Dohlsten M. Biased dependency of CD80 versus CD86 in the induction of transcription factors regulating the human IL-2 promoter. Int Immunol 1998; 10:499–506.

Oshima H, Nakano H, Nohara C, Kobata T, Nakajima A, Jenkins NA, Gilbert DJ, Copeland NG, Muto T, Yagita H, Okumura K. Characterization of murine CD70 by molecular cloning and mAb. Int Immunol. 1998; 10:517–526.

Oshima S, Karrer EE, Kawato Y, Maeda M, Fukahori H, Tsujimoto S, Hirose J, Nakamura K, Marui T, Takamura F, Noto T, Chapin SJ, Fujii Y, Neighbors M, Viswanathan S, Devens BH, Higashi Y. The Effect of ASP2409, a Novel CD86-Selective Variant of CTLA4-Ig, on Renal Allograft Rejection in Nonhuman Primates. Transplantation 2016b; 100:2611–2620.

Oshima S, Karrer EE, Paidhungat MM, Neighbors M, Chapin SJ, Fan RA, Reed MA, Wu K, Wong C, Chen Y, Whitlow M, Anderson FA, Bam RA, Zhang Q, Larsen BR, Viswanathan S, Devens BH, Bass SH, Higashi Y. ASP2408 and ASP2409, novel CTLA4-Ig variants with CD86-selective ligand binding activity and improved immunosuppressive potency, created by directed evolution. Protein Eng Des Sel 2016a; 29:159–167.

Ossevoort MA, Ringers J, Kuhn EM, Boon L, Lorré K, van den Hout Y, Bruijn JA, de Boer M, Jonker M, de Waele P. Prevention of renal allograft rejection in primates by blocking the B7/CD28 pathway. Transplantation 1999; 68:1010–1018.

Oura T, Yamashita K, Suzuki T, Fukumori D, Watanabe M, Hirokata G, Wakayama K, Taniguchi M, Shimamura T, Miura T, Okimura K, Maeta K, Haga H, Kubota K, Shimizu A, Sakai F, Furukawa H, Todo S. Long-term hepatic allograft acceptance based on CD40 blockade by ASKP1240 in nonhuman primates. Am J Transplant 2012; 12:1740–1754.

Page A, Srinivasan S, Singh K, Russell M, Hamby K, Deane T, Sen S, Stempora L, Leopardi F, Price AA, Strobert E, Reimann KA, Kirk AD, Larsen CP, Kean LS. CD40 blockade combines with CTLA4Ig and sirolimus to produce mixed chimerism in an MHC-defined rhesus macaque transplant model. Am J Transplant 2012; 12:115–125.

Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, Kumar V, Wu L, Kaer LV. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. J Immunol 2009; 182:2816–2826.

Parker DC, Greiner DL, Phillips NE, Appel MC, Steele AW, Durie FH, Noelle RJ, Mordes JP, Rossini AA. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. Proc Natl Acad Sci U S A 1995; 92:9560–9564.

Paterson AM, Vanguri VK, Sharpe AH. SnapShot: B7/CD28 costimulation. Cell 2009; 137:974–974.e1.

Paul LC, Davidoff A, Benediktsson H, Issekutz TB. The efficacy of LFA-1 and VLA-4 antibody treatment in rat vascularized cardiac allograft rejection. Transplantation 1993; 55:1196–1199.

Peach RJ, Bajorath J, Brady W, Leytze G, Greene J, Naemura J, Linsley PS. Complementarity determining region 1 (CDR1)- and CDR3-analogous regions in CTLA-4 and CD28 determine the binding to B7-1. J Exp Med 1994; 180:2049–2058.

Pearl JP, Parris J, Hale DA, Hoffmann SC, Bernstein WB, McCoy KL, Swanson SJ, Mannon RB, Roederer M, Kirk AD. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. Am J Transplant 2005; 5:465–474.

Pearl JP, Xu H, Leopardi F, Preston E, Kirk AD. CD154 blockade, sirolimus, and donor-specific transfusion prevents renal allograft rejection in cynomolgus monkeys despite homeostatic T-cell activation. Transplantation 2007; 83:1219–1225.

Pearson TC, Trambley J, Odom K, Anderson DC, Cowan S, Bray R, Lin A, Hollenbaugh D, Aruffo A, Siadak AW, Strobert E, Hennigar R, Larsen CP. Anti-CD40 therapy extends renal allograft survival in rhesus macaques. Transplantation 2002; 74:933–940.

Pellat-Deceunynck C, Bataille R, Robillard N, Harousseau JL, Rapp MJ, Juge-Morineau N, Wijdenes J, Amiot M. Expression of CD28 and CD40 in human myeloma cells: a comparative study with normal plasma cells. Blood 1994; 84:2597–2603.

Pennycuik PR. Unresponsiveness of nude mice to skin allografts. Transplantation 1971; 11:417–418.

Pentcheva-Hoang T, Egen JG, Wojnoonski K, Allison JP. B7-1 and B7-2 selectively recruit CTLA-4 and CD28 to the immunological synapse. Immunity 2004; 21:401–413.

Pletinckx K, Döhler A, Pavlovic V, Lutz MB. Role of DC maturity/costimulation for generation, homeostasis, and suppressive activity of regulatory T cells. Front Immunol 2011; 2:39. doi: 10.3389/fimmu.2011.00039. eCollection 2011.

Poirier N, Dilek N, Mary C, Ville S, Coulon F, Branchereau J, Tillou X, Charpy V, Pengam S, Nerriere-Daguin V, Hervouet J, Minault D, Le Bas-Bernardet S, Renaudin K, Vanhove B, Blancho G. FR104, an antagonist anti-CD28 monovalent fab' antibody, prevents alloimmunization and allows calcineurin inhibitor minimization in nonhuman primate renal allograft. Am J Transplant 2015; 15:88–100.

Pollok KE, Kim SH, Kwon BS. Regulation of 4-1BB expression by cell-cell interactions and the cytokines, interleukin-2 and interleukin-4. Eur J Immunol 1995; 25:488–494.

Popoola J, Greene H, Kyegombe M, MacPhee IA. Patient involvement in selection of immunosuppressive regimen following transplantation. Patient Prefer Adherence 2014; 8:1705–1712.

Pot C, Apetoh L, Kuchroo VK. Type 1 regulatory T cells (Tr1) in autoimmunity. Semin Immunol. 2011; 23:202–208.

Pradet-Balade B, Medema JP, López-Fraga M, Lozano JC, Kolfschoten GM, Picard A, Martínez-A C, Garcia-Sanz JA, Hahne M. An endogenous hybrid mRNA encodes TWE-PRIL, a functional cell surface TWEAK-APRIL fusion protein. EMBO J 2002; 21:5711–5720.

Prasad KS, Andre P, Yan Y, Phillips DR. The platelet CD40L/GP IIb–IIIa axis in atherothrombotic disease. Curr Opin Hematol 2003; 10:356–361.

Prasad KV, Ao Z, Yoon Y, Wu MX, Rizk M, Jacquot S, Schlossman SF. CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein. Proc Natl Acad Sci U S A 1997; 94:6346–6351.

Preston EH, Xu H, Dhanireddy KK, Pearl JP, Leopardi FV, Starost MF, Hale DA, Kirk AD. IDEC-131 (anti-CD154), sirolimus and donor-specific transfusion facilitate operational tolerance in non-human primates. Am J Transplant 2005; 5:1032–1041.

Prin Mathieu C, Renoult E, Kennel De March A, Béné MC, Kessler M, Faure GC. Serum anti-rabbit and anti-horse IgG, IgA, and IgM in kidney transplant recipients. Nephrol Dial Transplant 1997; 12:2133–2139.

Puttarajappa C, Shapiro R, Tan HP. Antibody-mediated rejection in kidney transplantation: a review. J Transplant 2012; 2012:193724.

Pype S, Declercq W, Ibrahimi A, Michiels C, Van Rietschoten JG, Dewulf N, de Boer M, Vandenabeele P, Huylebroeck D, Remacle JE. TTRAP, a novel protein that associates with CD40, tumor necrosis factor (TNF) receptor-75 and TNF receptor-associated factors (TRAFs), and that inhibits nuclear factor-kappa B activation. J Biol Chem 2000; 275:18586–18593.

Qi S, Xu D, Peng J, Vu MD, Wu J, Bekersky I, Fitzsimmons WE, Peets J, Sehgal S, Daloze P, Chen H. Effect of tacrolimus (FK506) and sirolimus (rapamycin) mono- and combination therapy in prolongation of renal allograft survival in the monkey. Transplantation 2000; 69:1275–1283.

Qin L, Guan HG, Zhou XJ, Yin J, Lan J, Qian HX. Blockade of 4-1BB/4-1BB ligand interactions prevents acute rejection in rat liver transplantation. Chin Med J (Engl) 2010; 123:212–215.

Quill H, Schwartz RH. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. J Immunol 1987; 138:3704–3712.

Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, Baker J, Jeffery LE, Kaur S, Briggs Z, Hou TZ, Futter CE, Anderson G, Walker LS, Sansom DM. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science 2011; 332:600–603.

Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P, Gaber LW, Gibson IW, Glotz D, Goldberg JC, Grande J, Halloran PF, Hansen HE, Hartley B, Hayry PJ, Hill CM, Hoffman EO, Hunsicker LG, Lindblad AS, Marcussen N, Mihatsch MJ, Nadasdy T, Nickerson P, Olsen TS, Papadimitriou JC, Randhawa PS, Rayner DC, Roberts I, Rose S, Rush D, Salinas-Madrigal L, Salomon DR, Sund S, Taskinen E, Trpkov K, Yamaguchi Y. The Banff 97 working classification of renal allograft pathology. Kidney Int 1999; 55:713–723.

Reinitz DM, Mansfield JM. T-cell-independent and T-cell-dependent B-cell responses to exposed variant surface glycoprotein epitopes in trypanosome-infected mice. Infect Immun 1990; 58:2337–2342.

Ridge JP, Di Rosa F, Matzinger P. A conditioned DC can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature 1998; 393:474–478.

Riella LV, Liu T, Yang J, Chock S, Shimizu T, Mfarrej B, Batal I, Xiao X, Sayegh MH, Chandraker A. Deleterious effect of CTLA4-Ig on a Treg-dependent transplant model. Am J Transplant 2012; 12:846–855.

Robles-Carrillo L, Meyer T, Hatfield M, Desai H, Dávila M, Langer F, Amaya M, Garber E, Francis JL, Hsu YM, Amirkhosravi A. Anti-CD40L immune complexes potently activate platelets in vitro and cause thrombosis in FCGR2A transgenic mice. J Immunol 2010; 185:1577–1583.

Rocha PN, Plumb TJ, Crowley SD, Coffman TM. Effector mechanisms in transplant rejection. Immunol Rev 2003; 196:51–64.

Rogers PR, Song J, Gramaglia I, Killeen N, Croft M. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. Immunity 2001; 15:445–455.

Roos A, Schilder-Tol EJ, Weening JJ, Aten J. Strong expression of CD134 (OX40), a member of the TNF receptor family, in a T helper 2-type cytokine environment. J Leukoc Biol 1998; 64:503–510.

Rosenberg JC, Lekas N, Lysz K, Morrell R. Effect of antithymocyte globulin and other immune reactants on human platelets. Surgery 1975; 77:520–529.

Rostaing L, Vincenti F, Grinyó J, Rice KM, Bresnahan B, Steinberg S, Gang S, Gaite LE, Moal MC, Mondragón-Ramirez GA, Kothari J, Pupim L, Larsen CP. Long-term belatacept exposure maintains efficacy and safety at 5 years: results from the long-term extension of the BENEFIT study. Am J Transplant 2013; 13:2875–2883.

Sabbagh L, Snell LM, Watts TH. TNF family ligands define niches for T cell memory. Trends Immunol 2007; 28:333–339.

Safford M, Collins S, Lutz MA, Allen A, Huang CT, Kowalski J, Blackford A, Horton MR, Drake C, Schwartz RH, Powell JD. Egr-2 and Egr-3 are negative regulators of T cell activation. Nature Immunol. Nat Immunol 2005; 6:472–480.

Sakaguchi S, Fukuma K, Kuribayashi K, Masuda T. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. J Exp Med. 1985; 161:72–87.

Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004; 22:531–562.

Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 2000; 12:431–440.

Salvadori M, Bertoni E. Is it time to give up with calcineurin inhibitors in kidney transplantation? World J Transplant 2013; 3:7–25.

Sánchez-Fueyo A, Strom TB. Immunologic basis of graft rejection and tolerance following transplantation of liver or other solid organs. Gastroenterology 2011; 140:51–64.

Sansom DM, Walker LS. The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. Immunol Rev 2006; 212:131–148.

Saoulli K, Lee SY, Cannons JL, Yeh WC, Santana A, Goldstein MD, Bangia N, DeBenedette MA, Mak TW, Choi Y, Watts TH. CD28-independent, TRAF2-dependent costimulation of resting T cells by 4-1BB ligand. J Exp Med 1998; 187: 1849–1862.

Saunders AE, Johnson P. Modulation of immune cell signalling by the leukocyte common tyrosine phosphatase, CD45. Cell Signal 2010; 22:339–348.

Saunders RN, Metcalfe MS, Nicholson ML. Rapamycin in transplantation: a review of the evidence. Kidney Int 2001; 59:3–16.

Schneider H, Cai YC, Prasad KV, Shoelson SE, Rudd CE. T cell antigen CD28 binds to the GRB-2/SOS complex, regulators of p21ras. Eur J Immunol 1995; 25:1044–1050.

Schneider H, Smith X, Liu H, Bismuth G, Rudd C. CTLA-4 expression disrupts ZAP-70 microcluster formation, T-cell/APC conjugation and calcium mobilization. Eur J Immunol 2007; 38:40–47.

Schönbeck U, Libby P. The CD40/CD154 receptor/ligand dyad. Cell Mol Life Sci 2001; 58:4–43.

Schuler W, Bigaud M, Brinkmann V, Di Padova F, Geisse S, Gram H, Hungerford V, Kleuser B, Kristofic C, Menninger K, Tees R, Wieczorek G, Wilt C, Wioland C, Zurini M. Efficacy and safety of ABI793, a novel human anti-human CD154 monoclonal antibody, in cynomolgus monkey renal allotransplantation. Transplantation 2004; 77:717–726.

Schwartz MA, Schaller MD, Ginsberg MH. Integrins: emerging paradigms of signal transduction. Annu Rev Cell Dev Biol 1995; 11:549–599.

Schwartz RH. A cell culture model for T lymphocyte clonal anergy. Science 1990; 248:1349–1356.

Schwartz RH. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. Cell 1992; 71:1065–1068.

Seki M, Oomizu S, Sakata KM, Sakata A, Arikawa T, Watanabe K, Ito K, Takeshita K, Niki T, Saita N, Nishi N, Yamauchi A, Katoh S, Matsukawa A, Kuchroo V, Hirashima M. Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. Clin Immunol (Orlando, FL) 2008; 127:78–88.

Serfling E, Avots A, Neumann M. The architecture of the interleukin-2 promoter: a reflection of T lymphocyte activation. Biochim Biophys Acta 1995; 1263:181–200.

Setoguchi K, Schenk AD, Ishii D, Hattori Y, Baldwin WM 3rd, Tanabe K, Fairchild RL. LFA-1 antagonism inhibits early infiltration of endogenous memory CD8 T cells into cardiac allografts and donor-reactive T cell priming. Am J Transplant 2011; 11:923–935.

Shahinian A, Pfeffer K, Lee KP, Kündig TM, Kishihara K, Wakeham A, Kawai K, Ohashi PS, Thompson CB, Mak TW. Differential T cell costimulatory requirements in CD28-deficient mice. Science 1993; 261:609–612.

Shankar G, Pendley C, Stein KE. A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs. Nat Biotechnol 2007; 25:555–561.

Shankarkumar U. The human leukocyte antigen (HLA) system. Int J Hum Genet 2004; 4:91–103.

Sharpe AH, Abbas AK. T-cell costimulation--biology, therapeutic potential, and challenges. N Engl J Med 2006; 355:973–975.

Sharpe AH, Freeman GJ. The B7-CD28 superfamily. Nat Rev Immunol 2002; 2:116–126.

Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol 2007; 8:239–245.

Shiina T, Hosomichi K, Inoko H, Kulski JK. The HLA genomic loci map: expression, interaction, diversity and disease. J Hum Genet 2009; 54:15–39.

Shimizu T, Toma H, Hayakawa N, Shibahara R, Ishiyama R, Hayashida A, Fujimori D, Tsunoyama K, Ikezawa E, Kitajima S, Iida S, Ishida H, Tanabe K, Honda K, Koike J. Clinical and pathological analyses of interstitial fibrosis and tubular atrophy cases after kidney transplantation. Nephrology (Carlton) 2016; 21 Suppl 1:26–30.

Shimizu Y, Van Seventer GA, Horgan KJ, Shaw S. Role of adhesion molecules in T cell recognition—fundamental similarities between four integrins on resting human T cells (LFA-1, VLA-4, VLA-5, VLA-6) in expression, binding and costimulation. Immunol Rev 1990; 114:109–143.

Shuford WW, Klussman K, Tritchler DD, Loo DT, Chalupny J, Siadak AW, Brown TJ, Emswiler J, Raecho H, Larsen CP, Pearson TC, Ledbetter JA, Aruffo A, Mittler RS. 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. J Exp Med 1997; 186:47–55.

Simeoni L, Lindquist JA, Smida M, Witte V, Arndt B, Schraven B. Control of lymphocyte development and activation by negative regulatory transmembrane adapter proteins. Immunol Rev 2008; 224:215–228.

Simeoni L, Posevitz V, Kölsch U, Meinert I, Bruyns E, Pfeffer K, Reinhold D, Schraven B. The transmembrane adapter protein SIT regulates thymic development and peripheral T-cell functions. Mol Cell Biol 2005; 25:7557–7568.

Sizing ID, Bailly V, McCoon P, Chang W, Rao S, Pablo L, Rennard R, Walsh M, Li Z, Zafari M, Dobles M, Tarilonte L, Miklasz S, Majeau G, Godbout K, Scott ML, Rennert PD. Epitope-dependent effect of anti-murine TIM-1 monoclonal antibodies on T cell activity and lung immune responses. J Immunol 2007; 178: 2249–2261.

Smith KM, Brewer JM, Webb P, Coyle AJ, Gutierrez-Ramos C, Garside P. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nat Immunol 2009; 10:167–175.

Smith KM, Brewer JM, Webb P, Coyle AJ, Gutierrez-Ramos C, Garside P. Inducible costimulatory molecule-B7-related protein 1 interactions are important for the clonal expansion and B cell helper functions of naive, Th1, and Th2 T cells. J Immunol 2003; 170:2310–2315.

Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annu Rev Immunol 2009; 27:591–619.

Smyth LA, Afzali B, Tsang J, Lombardi G, Lechler RI. Intercellular transfer of MHC and immunological molecules: molecular mechanisms and biological significance. Am J Transplant 2007; 7:1442–1449.

Sollinger HW, Belzer FO, Deierhoi MH, Diethelm AG, Gonwa TA, Kauffman RS, Klintmalm GB, McDiarmid SV, Roberts J, Rosenthal JT, Tomlanovich SJ. RS-61443 (mycophenolate mofetil). A multicenter study for refractory kidney transplant rejection. Ann Surg 1992; 216:513–519.

Sollinger HW. MMF for the prevention of acute rejection in primary cadaveric renal allograft recipients. U.S. Renal Transplant Mycophenolate Mofetil Study Group. Transplantation 1995; 60:225–232.

Song L, Qi S, Dun H, Hu Y, Ma A, Yu G, Xiong Z, Zhu S, Wang X, Xu D, Li G, Shang Y, Kinugasa F, Sudo Y, Bai J, Zeng L, Daloze P, Chen H. Surgical complications in kidney transplantation in nonhuman primates. Microsurgery 2010; 30:327–331.

Sperling AI, Auger JA, Ehst BD, Rulifson IC, Thompson CB, Bluestone JA. CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. J Immunol 1996; 157:3909–3917.

Stamenkovic I, Clark EA, Seed B. A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J 1989; 8:1403–1410.

Stärck L, Scholz C, Dörken B, Daniel PT. Costimulation by CD137/4-1BB inhibits T cell apoptosis and induces Bcl-xL and c-FLIP(short) via phosphatidylinositol 3-kinase and AKT/protein kinase B. Eur J Immunol 2005; 35:1257–1266.

Starzl TE, Klintmalm GB, Weil R 3rd, Porter KA, Iwatsuki S, Schroter GP, Fernandez-Bueno C, MacHugh N. Cyclosporin A and steroid therapy in sixty-six cadaver kidney recipients. Surg Gynecol Obstet 1981; 153:486–494.

Starzl TE, Marchioro TL, Brittain RS, Holmes JH, Waddell WR. Problems in Renal Homotransplantation. JAMA 1964; 187:734–740.

Starzl TE, Marchioro TL, Hutchinson DE, Porter KA, Cerilli GJ, Brettschneider L. The clinical use of antilymphocyte globulin in renal homotransplantation. Transplantation 1967a; 5 (Suppl 4):1100–1105.

Starzl TE, Marchioro TL, Porter KA, Iwasaki Y, Cerilli GJ. The use of heterologous antilymphoid agents in canine renal and liver homotransplantation and in human renal homotransplantation. Surg Gynecol Obstet 1967b; 124:301–308.

Starzl TE, Marchioro TL, Waddell WR. The reversal of rejection in human renal homografts with subsequent development of homograft tolerance. Surg Gynecol Obstet 1963; 117:385–395.

Starzl TE, Todo S, Fung J, Demetris AJ, Venkataramanan R, Jain A. FK 506 for human liver, kidney and pancreas transplantation. Lancet 1989; 2:1000–1004.

Starzl TE, Weil R 3rd, Iwatsuki S, Klintmalm G, Schröter GP, Koep LJ, Iwaki Y, Terasaki PI, Porter KA. The use of cyclosporin A and prednisone in cadaver kidney transplantation. Surg Gynecol Obstet 1980; 151:17–26.

Starzl TE, Weil R 3rd, Koep LJ, Iwaki Y, Terasaki PI, Schröter GP. Thoracic duct drainage before and after cadaveric kidney transplantation. Surg Gynecol Obstet 1979; 149:815–821.

Stepkowski SM, Erwin-Cohen RA, Behbod F, Wang ME, Qu X, Tejpal N, Nagy ZS, Kahan BD, Kirken RA. Selective inhibitor of Janus tyrosine kinase 3, PNU156804, prolongs allograft survival and acts synergistically with cyclosporine but additively with rapamycin. Blood 2002; 99:680–689.

Stratta P, Canavese C, Quaglia M, Balzola F, Bobbio M, Busca A, Franchello A, Libertucci D, Mazzucco G. Posttransplantation chronic renal damage in nonrenal transplant recipients. Kidney Int 2005; 68:1453–1463.

Stüber E, Neurath M, Calderhead D, Fell HP, Strober W. Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. Immunity 1995; 2:507–521.

Sugamura K, Ishii N, Weinberg AD. Therapeutic targeting of the effector T cell costimulatory molecule OX40. Nat Rev Immunol 2004; 4:420–431.

Suvas S, Singh V, Sahdev S, Vohra H, Agrewala JN. Distinct role of CD80 and CD86 in the regulation of the activation of B cell and B cell lymphoma. J Biol Chem 2002; 277:7766–7775.

Swallow MM, Wallin JJ, Sha WC. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. Immunity 1999; 11:423–432.

Tafuri A, Shahinian A, Bladt F, Yoshinaga SK, Jordana M, Wakeham A, Boucher LM, Bouchard D, Chan VS, Duncan G, Odermatt B, Ho A, Itie A, Horan T, Whoriskey JS, Pawson T, Penninger JM, Ohashi PS, Mak TW. ICOS is essential for effective T-helper-cell responses. Nature 2001; 409:105–109.

Tai X, Van Laethem F, Pobezinsky L, Guinter T, Sharrow SO, Adams A, Granger L, Kruhlak M, Lindsten T, Thompson CB, Feigenbaum L, Singer A. Basis of CTLA-4 function in regulatory and conventional CD4(+) T cells. Blood 2012; 119:5155–5163.

Takeichi M. Morphogenetic roles of classic cadherins. Curr Opin Cell Biol 1995; 7:619–627.

Takeichi M. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development 1988; 102:639–655.

Takemoto SK, Zeevi A, Feng S, Colvin RB, Jordan S, Kobashigawa J, Kupiec-Weglinski J, Matas A, Montgomery RA, Nickerson P, Platt JL, Rabb H, Thistlethwaite R, Tyan D, Delmonico FL. National conference to assess antibody-mediated rejection in solid organ transplantation. Am J Transplant 2004; 4:1033–1041.

Tamkun JW, DeSimone DW, Fonda D, Patel RS, Buck C, Horwitz AF, Hynes RO. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. Cell 1986; 46:271–282.

Tan CD, Baldwin WM 3rd, Rodriguez ER. Update on cardiac transplantation pathology. Arch Pathol Lab Med 2007; 131:1169–1191.

Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, Zheng XX, Strom TB, Bluestone JA. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. J Immunol 2003; 171:3348–3352.

Taraban VY, Rowley TF, Al-Shamkhani A. Cutting edge: a critical role for CD70 in CD8 T cell priming by CD40-licensed APC. J Immunol 2004; 173:6542–6546.

Taylor HE, Ackman CF, Horowitz I. Canadian clinical trial of antilymphocyte globulin in human cadaver renal transplantation. Can Med Assoc J 1976; 115:1205–1208.

Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded $CD4^{+}CD25^{+}$ immune regulatory cells inhibits graft-versus-host disease lethality. Blood 2002; 99:3493–3499.

Terada N, Lucas JJ, Szepesi A, Franklin RA, Domenico J, Gelfand EW. Rapamycin blocks cell cycle progression of activated T cells prior to events characteristic of the middle to late G1 phase of the cycle. J Cell Physiol 1993; 154:7–15.

Tesselaar K, Gravestein LA, van Schijndel GM, Borst J, van Lier RA. Characterization of murine CD70, the ligand of the TNF receptor family member CD27. J Immunol 1997; 159:4959–4965.

Testi R, Lanier LL. Functional expression of CD28 on T cell antigen receptor gamma/delta-bearing T lymphocytes. Eur J Immunol 1989; 19:185–188.

Thistlethwaite JR Jr, Cosimi AB, Delmonico FL, Rubin RH, Talkoff-Rubin N, Nelson PW, Fang L, Russell PS. Evolving use of OKT3 monoclonal antibody for treatment of renal allograft rejection. Transplantation 1984; 38:695–701.

Thistlethwaite JR Jr, Gaber AO, Haag BW, Aronson AJ, Broelsch CE, Stuart JK, Stuart FP. OKT3 treatment of steroid-resistant renal allograft rejection. Transplantation 1987; 43:176–184.

Thompson CB, Lindsten T, Ledbetter JA, Kunkel SL, Young HA, Emerson SG, Leiden JM, June CH. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. Proc Natl Acad Sci U S A 1989; 86:1333–1337.

Thompson P, Cardona K, Russell M, Badell IR, Shaffer V, Korbutt G, Rayat GR, Cano J, Song M, Jiang W, Strobert E, Rajotte R, Pearson T, Kirk AD, Larsen CP. CD40-specific costimulation blockade enhances neonatal porcine islet survival in nonhuman primates. Am J Transplant 2011; 11:947–957.

Truitt KE, Hicks CM, Imboden JB. Stimulation of CD28 triggers an association between CD28 and phosphatidylinositol 3-kinase in Jurkat T cells. J Exp Med 1994; 179:1071–1076.

Turka LA, Ledbetter JA, Lee K, June CH, Thompson CB. CD28 is an inducible T cell surface antigen that transduces a proliferative signal in CD3+ mature thymocytes. J Immunol 1990; 144:1646–1653.

Turka LA, Linsley PS, Lin H, Brady W, Leiden JM, Wei RQ, Gibson ML, Zheng XG, Myrdal S, Gordon D. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. Proc Natl Acad Sci U S A 1992; 89:11102–11105.

Umetsu SE, Lee WL, McIntire JJ, Downey L, Sanjanwala B, Akbari O, Berry GJ, Nagumo H, Freeman GJ, Umetsu DT, DeKruyff RH. TIM-1 induces T cell activation and inhibits the development of peripheral tolerance. Nat Immunol 2005; 6:447–454.

Valdez-Ortiz R, Bestard O, Llaudó I, Franquesa M, Cerezo G, Torras J, Herrero-Fresneda I, Correa-Rotter R, Grinyó JM. Induction of suppressive allogeneic regulatory T cells via rabbit antithymocyte polyclonal globulin during homeostatic proliferation in rat kidney transplantation. Transpl Int 2015; 28:108–119.

Valujskikh A, Pantenburg B, Heeger PS. Primed allospecific T cells prevent the effects of costimulatory blockade on prolonged cardiac allograft survival in mice. Am J Transplant 2002; 2:501–509.

Valujskikh A. The challenge of inhibiting alloreactive T-cell memory. Am J Transplant 2006; 6:647–651.

van Besouw NM, Zuijderwijk JM, Vaessen LM, Balk AH, Maat AP, van der Meide PH, Weimar W. The direct and indirect allogeneic presentation pathway during acute rejection after human cardiac transplantation. Clin Exp Immunol 2005; 141:534–540.

van der Pol W, van de Winkel JG. IgG receptor polymorphisms: risk factors for disease. Immunogenetics 1998; 48: 222.

van Kooten C, Banchereau J. CD40-CD40 ligand. J Leukoc Biol 2000; 67:2–17.

van Kooten C, Banchereau J. Functions of CD40 on B cells, DCs and other cells. Curr Opin Immunol 1997; 9:330–337.

van Lier RA, Borst J, Vroom TM, Klein H, Van Mourik P, Zeijlemaker WP, Melief CJ. Tissue distribution and biochemical and functional properties of Tp55 (CD27), a novel T cell differentiation antigen. J Immunol 1987; 139:1589–1596.

VanBuskirk AM, Wakely ME, Sirak JH, Orosz CG. Patterns of allosensitization in allograft recipients: long-term cardiac allograft acceptance is associated with active alloantibody

production in conjunction with active inhibition of alloreactive delayed-type hypersensitivity. Transplantation 1998; 65:1115–1123.

Veillette A, Bookman MA, Horak EM, Bolen JB. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. Cell 1988; 55:301–308.

Vella JP, Spadafora-Ferreira M, Murphy B, Alexander SI, Harmon W, Carpenter CB, Sayegh MH. Indirect allorecognition of major histocompatibility complex allopeptides in human renal transplant recipients with chronic graft dysfunction. Transplantation 1997; 64:795–800.

Veronese F, Rotman S, Smith RN, Pelle TD, Farrell ML, Kawai T, Benedict Cosimi A, Colvin RB. Pathological and clinical correlates of FOXP3+ cells in renal allografts during acute rejection. Am J Transplant 2007; 7:914–922.

Vijayakrishnan L, Slavik JM, Illés Z, Greenwald RJ, Rainbow D, Greve B, Peterson LB, Hafler DA, Freeman GJ, Sharpe AH, Wicker LS, Kuchroo VK. An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells. Immunity 2004; 20:563–575.

Villa A, Notarangelo LD, Di Santo JP, Macchi PP, Strina D, Frattini A, Lucchini F, Patrosso CM, Giliani S, Mantuano E. Organization of the human CD40L gene: Implications for molecular defects in X chromosome-linked hyper-IgM syndrome and prenatal diagnosis. Proc Natl Acad Sci U S A 1994; 91:2110–2114.

Vinay DS, Kwon BS. Role of 4-1BB in immune responses. Semin Immunol 1998; 10:481–489.

Vincenti F, Charpentier B, Vanrenterghem Y, Rostaing L, Bresnahan B, Darji P, Massari P, Mondragon-Ramirez GA, Agarwal M, Di Russo G, Lin CS, Garg P, Larsen CP. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). Am J Transplant 2010; 10:535–546.

Vincenti F, Larsen C, Durrbach A, Wekerle T, Nashan B, Blancho G, Lang P, Grinyo J, Halloran PF, Solez K, Hagerty D, Levy E, Zhou W, Natarajan K, Charpentier B; Belatacept Study Group. Costimulation blockade with belatacept in renal transplantation. N Engl J Med 2005; 353:770–781.

Vincenti F, Larsen CP, Alberu J, Bresnahan B, Garcia VD, Kothari J, Lang P, Urrea EM, Massari P, Mondragon-Ramirez G, Reyes-Acevedo R, Rice K, Rostaing L, Steinberg S, Xing J, Agarwal M, Harler MB, Charpentier B. Three-year outcomes from BENEFIT, a randomized, active-controlled, parallel-group study in adult kidney transplant recipients. Am J Transplant 2012; 12:210–217.

Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. Science 1996; 273:104–106.

Viola A, Schroeder S, Sakakibara Y, Lanzavecchia A. T lymphocyte costimulation mediated by reorganization of membrane microdomains. Science 1999; 283:680–682.

Vu MD, Amanullah F, Li Y, Demirci G, Sayegh MH, Li XC. Different costimulatory and growth factor requirements for CD4+ and CD8+ T cell-mediated rejection. J Immunol 2004; 173:214–221.

Vu MD, Clarkson MR, Yagita H, Turka LA, Sayegh MH, Li XC. Critical, but conditional, role of OX40 in memory T cell-mediated rejection. J Immunol 2006; 176:1394–1401.

Vu MD, Xiao X, Gao W, Degauque N, Chen M, Kroemer A, Killeen N, Ishii N, Li XC. OX40 costimulation turns off Foxp3+ Tregs. Blood 2007; 110:2501–2510.

Wada J, Kanwar YS. Identification and characterization of galectin-9, a novel beta-galactoside-binding mammalian lectin. J Biol Chem 1997; 272:6078–6086.

Wadhwa M, Thorpe R. Unwanted immunogenicity: lessons learned and future challenges. Bioanalysis 2010; 2:1073–1084.

Wagner DH Jr, Hagman J, Linsley PS, Hodsdon W, Freed JH, Newell MK. Rescue of thymocytes from glucocorticoid-induced cell death mediated by CD28/CTLA-4 costimulatory interactions with B7-1/B7-2. J Exp Med 1996; 184:1631–1638.

Walker LS, Gulbranson-Judge A, Flynn S, Brocker T, Raykundalia C, Goodall M, Förster R, Lipp M, Lane P. Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. J Exp Med 1999; 190:1115–1122.

Walunas TL, Bakker CY, Bluestone JA. CTLA-4 ligation blocks CD28-dependent T cell activation. J Exp Med 1996; 183:2541–2550.

Wang HC, Klein JR. Multiple levels of activation of murine CD8(+) intraepithelial lymphocytes defined by OX40 (CD134) expression: effects on cellmediated cytotoxicity, IFN-gamma, and IL-10 regulation. J Immunol 2001; 167:6717–6723.

Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol 2007; 37:129–138.

Wang YM, Ghali J, Zhang GY, Hu M, Wang Y, Sawyer A, Zhou JJ, Hapudeniya DA, Wang Y, Cao Q, Zheng G, Harris DC, Alexander SI. Development and function of $Foxp3^+$ regulatory T cells. Nephrology (Carlton) 2016; 21:81–85.

Wasowska BA. Mechanisms involved in antibody- and complement-mediated allograft rejection. I mmunol Res 2010; 47:25–44.

Watanabe M, Yamashita K, Suzuki T, Kamachi H, Kuraya D, Koshizuka Y, Ogura M, Yoshida T, Sakai F, Miura T, Todo S. Long-term acceptance of islet allografts by ASKP1240 (4D11), a fully human anti-CD40 monoclonal antibody in cynomolgus monkeys [abstract]. Am J Transplant 2011; 11 Suppl 2:80.

Watanabe M, Yamashita K, Suzuki T, Kamachi H, Kuraya D, Koshizuka Y, Ogura M, Yoshida T, Aoyagi T, Fukumori D, Shimamura T, Okimura K, Maeta K, Miura T, Sakai F, Todo S. ASKP1240, a fully human anti-CD40 monoclonal antibody, prolongs pancreatic islet allograft survival in nonhuman primates. Am J Transplant 2013; 13:1976–1988.

Waterman PM, Cambier JC. The conundrum of inhibitory signaling by ITAM-containing immunoreceptors: potential molecular mechanisms. FEBS Lett 2010; 584:4878–4882.

Weaver TA, Charafeddine AH, Agarwal A, Turner AP, Russell M, Leopardi FV, Kampen RL, Stempora L, Song M, Larsen CP, Kirk AD. Alefacept promotes costimulation blockade based allograft survival in nonhuman primates. Nat Med 2009; 15:746–749.

Weiss A, Littman DR. Signal transduction by lymphocyte antigen receptors. Cell 1994; 76:263–274.

Whitby EH, Sparshott SM, Bell EB. Allograft rejection in athymic nude rats by transferred T-cell subsets. I. The response of naive CD4+ and CD8+ thoracic duct lymphocytes to complete allogeneic incompatibilities. Immunology 1990; 69:78–84.

Wiesner RH, Demetris AJ, Belle SH, Seaberg EC, Lake JR, Zetterman RK, Everhart J, Detre KM. Acute hepatic allograft rejection: incidence, risk factors, and impact on outcome. Hepatology 1998; 28:638–645.

Williams BL, Irvin BJ, Sutor SL, Chini CC, Yacyshyn E, Bubeck Wardenburg J, Dalton M, Chan AC, Abraham RT. Phosphorylation of Tyr319 in ZAP-70 is required for T-cell antigen receptor-dependent phospholipase C-gamma1 and Ras activation. EMBO J 1999; 18:1832–1844.

Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. CTLA-4 control over Foxp3+ regulatory T cell function. Science 2008; 322:271–275.

Wittenbecher F, Rieger K, Dziubianau M, Herholz A, Mensen A, Blau IW, Uharek L, Dörken B, Thiel A, Na IK. Rabbit antithymocyte globulin induces rapid expansion of effector memory CD8 T cells without accelerating acute graft versus host disease. Leuk Res Rep 2013; 2:82–85.

Womer KL, Stone JR, Murphy B, Chandraker A, Sayegh MH. Indirect allorecognition of donor class I and II major histocompatibility complex peptides promotes the development of transplant vasculopathy. J Am Soc Nephrol 2001; 12:2500–2506.

Won EY, Cha K, Byun JS, Kim DU, Shin S, Ahn B, Kim YH, Rice AJ, Walz T, Kwon BS, Cho HS. The structure of the trimer of human 4-1BB ligand is unique among members of the tumor necrosis factor superfamily. J Biol Chem 2010; 285:9202–9210.

Wood KJ, Goto R. Mechanisms of rejection: current perspectives. Transplantation 2012; 93:1–10.

Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DA. The development and function of regulatory T cells. Cell Mol Life Sci. 2009; 66:2603–2622.

Wu Y, Zhou Q, Zheng P, Liu Y. CD28-independent induction of T helper cells and immunoglobulin class switches requires costimulation by the heat-stable antigen. J Exp Med 1998; 187:1151–1156.

Xu H, Elster EA, Blair PJ, Burkly LC, Tadaki DK, Harlan DM, Kirk AD. Effects of combined treatment with CD25- and CD154-specific monoclonal antibodies in non-human primate allotransplantation. Am J Transplant 2003; 3:1350–1354.

Yamada A, Salama AD, Sho M, Najafian N, Ito T, Forman JP, Kewalramani R, Sandner S, Harada H, Clarkson MR, Mandelbrot DA, Sharpe AH, Oshima H, Yagita H, Chalasani G, Lakkis FG, Auchincloss H Jr, Sayegh MH. CD70 signaling is critical for CD28-independent CD8+ T cell-mediated alloimmune responses in vivo. J Immunol 2005; 174:1357–1364.

Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, Shin T, Tsuchiya H, Pardoll DM, Okumura K, Azuma M, Yagita H. Expression of programmed death 1 ligands by murine T cells and APC. J Immunol 2002; 169:5538–5545.

Yang H, Issekutz TB, Wright JR Jr. Prolongation of rat islet allograft survival by treatment with monoclonal antibodies against VLA-4 and LFA-1. Transplantation 1995; 60:71–76.

Yang J, Brook MO, Carvalho-Gaspar M, Zhang J, Ramon HE, Sayegh MH, Wood KJ, Turka LA, Jones ND. Allograft rejection mediated by memory T cells is resistant to regulation. Proc Natl Acad Sci U S A 2007; 104:19954–19959.

Yang Y, Wilson JM. CD40 ligand dependent T cell activation: requirement of B7-CD28 signaling through CD40. Science 1996; 273:1862–1864.

Yellin MJ, Brett J, Baum D, Matsushima A, Szabolcs M, Stern D, Chess L. Functional interactions of T cells with endothelial cells: The role of CD40L-CD40-mediated signals. J Exp Med 1995; 182:1857–1864.

Yoshinaga SK, Whoriskey JS, Khare SD, Sarmiento U, Guo J, Horan T, Shih G, Zhang M, Coccia MA, Kohno T, Tafuri-Bladt A, Brankow D, Campbell P, Chang D, Chiu L, Dai T, Duncan G, Elliott GS, Hui A, McCabe SM, Scully S, Shahinian A, Shaklee CL, Van G, Mak TW, Senaldi G. T-cell co-stimulation through B7RP-1 and ICOS. Nature 1999; 402:827–832.

Yuan X, Paez-Cortez J, Schmitt-Knosalla I, D'Addio F, Mfarrej B, Donnarumma M, Habicht A, Clarkson MR, Iacomini J, Glimcher LH, Sayegh MH, Ansari MJ. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. J Exp Med 2008; 205:3133–3144.

Zhai Y, Meng L, Gao F, Busuttil RW, Kupiec-Weglinski JW. Allograft rejection by primed/memory $CD8^+$ T cells is CD154 blockade resistant: Therapeutic implications for sensitized transplant recipients. J Immunol 2002; 169:4667–4673.

Zhang G. Tumor necrosis factor family ligand-receptor binding. Curr Opin Struct Biol 2004; 14:154–160.

Zhang Q, Chen Y, Fairchild RL, Heeger PS, Valujskikh A. Lymphoid sequestration of alloreactive memory CD4 T cells promotes cardiac allograft survival. J Immunol 2006; 176:770–777.

Zhang W, Kernstock RM, Karrer EE, Cohen SB, Chindalore VL, Kivitz AJ, Blahunka PC, Delgado-Herrera L, Zeiher BG, Samberg NL, Garg JP. A phase 1 dose-escalation study of

ASP2409, a selective T-Cell costimulation inhibitor, in stable rheumatoid arthritis patients on methotrexate therapy. Clinical Pharm in Drug Dev. 2016; doi: 10.1002/cpdd.237

Zhang W, Sloan-Lancaster J, Kitchen J, Trible RP, Samelson LE. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell 1998; 92:83–92.

Zhang X, Voskens CJ, Sallin M, Maniar A, Montes CL, Zhang Y, Lin W, Li G, Burch E, Tan M, Hertzano R, Chapoval AI, Tamada K, Gastman BR, Schulze DH, Strome SE. CD137 promotes proliferation and survival of human B cells. J Immunol 2010; 184:787–795.

Zheng SG, Wang JH, Stohl W, Kim KS, Gray JD, Horwitz DA. TGF-β requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells. J Immunol 2006; 176:3321–3329.

Zheng Y, Zha Y, Gajewski TF. Molecular regulation of T-cell anergy. EMBO Rep 2008; 9:50–55.

Zhong X, Tumang JR, Gao W, Bai C, Rothstein TL. PD-L2 expression extends beyond DCs/macrophages to B1 cells enriched for V(H)11/V(H)12 and phosphatidylcholine binding. Eur J Immunol 2007; 37:2405–2410.

Zhong XP, Guo R, Zhou H, Liu C, Wan CK. Diacylglycerol kinases in immune cell function and self-tolerance. Immunol Rev 2008; 224:249–264.

Zhou SY, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, Neel BG, Birge RB, Fajardo JE, Chou MM, Hanafusa H, Schaffhausen B, Cantley LC. SH2 domains recognize specific phosphopeptide sequences. Cell 1993; 72:767–778.

Zhou Z, Kim S, Hurtado J, Lee ZH, Kim KK, Pollok KE, Kwon BS. Characterization of human homologue of 4-1BB and its ligand. Immunol Lett 1995; 45:67–73.

Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB, Kuchroo VK. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol 2005; 6:1245–1252.

Zinkernagel RM. On cross-priming of MHC class I-specific CTL: rule or exception? Eur J Immunol 2002; 32:2385–2392.