Published in final edited form as:

J Heart Lung Transplant. 2017 December ; 36(12): 1299–1305. doi:10.1016/j.healun.2017.10.003.

Advances in the immunology of heart transplantation

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Overall, the science of heart transplantation, and transplantation writ large, has seen major advances over the last 50 years. Some of these advances have already benefited patients, whereas others are poised to do so. Unexpectedly, in some areas, namely immunosuppression, progress has slowed. Herein we review the advances, or lack thereof, made in understanding the alloimmune response, immunosuppression, tolerance and xenotransplantation.

Immune response to an allograft: Discoveries with clinical implications

There remains much to learn about immune system's response to an allograft. However, significant advances have been made in our understanding of regulatory T cells, costimulatory signals and exosomes, all of which have clinical implications.

Regulatory T cells

Cells capable of suppressing an alloresponse were postulated to exist over 30 years ago.¹ However, it was not until 2003 that the identity of these elusive and mysterious cells (previously known as “suppressor cells”) was revealed to be CD4⁺ T cells expressing the FOXP3 transcription factor.² These cells play a critical role in normal immune homeostasis, maintaining self-tolerance and preventing autoimmunity, as exemplified by patients with mutations in *Foxp3* who develop IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) syndrome.^{3,4} Importantly, for transplantation, the potential for these cells to contribute to clinical immunosuppression minimization and/or tolerance induction has also now been recognized.⁵

Although several types of regulatory lymphocyte populations have been described, regulatory T cells (Tregs) are the most studied and best understood. Small-animal studies, using strategies of target cell depletion or adoptive transfer, have demonstrated that these cells downregulate effector T-cell responses, prevent transplant arteriopathy, and contribute to allograft tolerance.^{5–8} Of note, recent data support the hypothesis that specific induction

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Disclosure statement

The author has no conflicts of interest to disclose. This work was supported in part by the National Heart, Lung, and Blood Institute (PO1HL018646) and the National Institute of Allergy and Infectious Disease (PO1AI123086 and UO1AI131470) of the National Institutes of Health. The author thanks Drs. Jon Kobashigawa and A. Benedict Cosimi for critical review of the manuscript.

of Tregs by donor antigens plays a key role in tolerance induced by transient mixed chimerism in non-human primate recipients of kidney, lung and heart allografts (see later).⁹

Studies evaluating the administration of Tregs as a cellular immunotherapy have been initiated in human liver and kidney transplant recipients. In one approach, recipient Tregs were isolated, purified and expanded, and then reinfused as antigen non-specific Tregs. Phase 1 studies show that antigen–non-specific Tregs are safe, but Phase 2 studies, currently underway, are needed to make a determination of efficacy. Although more difficult to manufacture than non-specific Tregs, antigen-specific Tregs may be attractive as they are more potent in preventing rejection/inducing tolerance in experimental models and, theoretically, they should have less risk of non-specific immunosuppression.⁵ In a pilot liver transplant study, antigen-specific Tregs were generated by culturing recipient peripheral-blood mononuclear cells with irradiated donor cells. Seven of 10 patients treated with antigen-specific Treg infusion were successfully weaned from immunosuppression, with 4 of the 7 patients off drugs for >2 years.¹⁰

An important, ongoing, clinical study in the area of immune regulation is the ONE Study (www.onestudy.org). In this international trial, 7 centers all agreed on using the same immunosuppression regimen and testing cellular immunotherapy with either regulatory T-cell, regulatory macrophages or regulatory dendritic cells, for safety and their ability to promote kidney survival.¹¹ The results of this study will be available in the near future.

Although there are cautionary notes that need to be considered with Treg immunotherapy,¹² 50 years from now, when this article is rewritten for the 100th anniversary of the first heart transplant, the future author will likely recall this era in transplantation as the dawn of the age of cellular immunotherapy, with Tregs leading the way.

Costimulatory blockade

T-cell activation is central to allograft rejection. It is now clear that T cells require at least 2 signals to become optimally activated and develop full effector function: antigen-specific signals delivered through the T-cell receptor (Signal 1), and antigen–non-specific signals delivered through costimulatory molecules (Signal 2). The first costimulatory molecules described were CD28, expressed on resting T cells, and its ligands B7.1 (CD80) and B7.2 (CD86), expressed on antigen-presenting cells (APCs).¹³ The demonstration in the early 1990s that blocking this pathway inhibited T-cell proliferation and T-dependent B-cell antibody production¹⁴ ushered in the era of using costimulatory blockade to prevent rejection and potentially induce tolerance. Early results were encouraging. Long-term murine cardiac graft survival was achieved when blockade of the CD28/B7 pathway (through CTLA4-Ig, a CD28 antagonist) was combined with blockade of the CD40/CD154 costimulatory pathway (through anti-CD154 monoclonal antibody).^{15,16} However, in translating these findings to non-human primates (NHPs), it became apparent that, although agents of costimulatory blockade were effective in prolonging allograft survival, they alone were not capable of inducing tolerance, as most grafts were lost after cessation of treatment.^{17–22} Thus, the clinical focus turned to developing a drug for continuous maintenance immunosuppression. This culminated in belatacept, a CTLA-4 fusion protein that has been used in kidney transplant recipients as a calcineurin-sparing agent.^{23,24} It has only been used

anecdotally in heart recipients. Although the role for belatacept as a long-term immunosuppressive agent remains to be determined,²⁵ it is now clear that costimulatory blockade is an important component of experimental protocols to achieve cardiac allograft tolerance and to prolong cardiac xenograft survival (see below). As such, these agents will likely play an important role in the future of heart transplantation.

Exosomes

The release of membrane-enclosed vesicles (exosomes) by cells into bodily fluids has been known for decades. However, interest in exosomes has soared in the last few years with the realization that cells use these vesicles as a means of extracellular communication and exchange of proteins, lipids, carbohydrates and nucleic acids, perhaps as a primordial mechanism for cell-to-cell messaging.²⁶ How do exosomes relate to transplantation? In 3 ways. First, exosomes derived from an allograft are able to transfer donor-type major histocompatibility complex (MHC) molecules to the surface of antigen-presenting cells (APCs) in the recipient through a process called trogocytosis or “cross-dressing.”²⁷ They can also transfer activating mediators (microRNAs, surface molecules) to recipient APCs. These activated recipient APCs (usually dendritic cells), cross-dressed with donor MHC antigens, now appear to be the primary cells responsible for the initiating the adaptive immune response leading to acute rejection.^{28,29} The finding that donor exosomes rather than passenger leukocytes initiate alloreactive T-cell responses after transplantation suggests that the classic passenger leukocyte theory of allograft rejection needs to be revised. It also may afford new approaches to the prevention of acute and chronic rejection by limiting the production or altering the trafficking of vesicles. Second, the RNA and protein content of exosomes reflect that of the parent cells, thus extracellular vesicles isolated from blood, urine, or bronchoalveolar secretions could be used as non-invasive diagnostic and prognostic biomarkers in transplantation. Indeed, recent experimental findings have demonstrated that transplant exosomes can be quantified and profiled non-invasively, and that the RNA and proteomic signatures of transplant exosomes are tissue-specific and change as a result of rejection.³⁰ In human lung recipients, exosomes expressing the auto-antigen, collagen V isolated from recipient serum and bronchoalveolar lavage were predictive biomarkers of acute and chronic rejection.³¹ Finally, identification of exosomes opens new possibilities for the development of therapies to prevent rejection and/or induce tolerance. Given the immunomodulatory capacity of Tregs, exosomes derived from regulatory cells could contribute to tolerance.³² For instance, exosomes isolated from activated Tregs expanded ex vivo could be further modified to express specific inhibitory microRNAs, cytokines or surface molecules to be used in conjunction with adoptive Treg therapy to promote donor-specific unresponsiveness.³³ Although the field of exosome therapy is still in its infancy, it has real potential to benefit our transplant patients in time.

Immunosuppression: Advances and the lack thereof

Overall, the most significant advance in the history of heart transplantation was the approval of cyclosporine (CyA) for heart transplantation 34 years ago.³⁴ It resulted in acceptable post-transplant outcomes that legitimized the field. Although subsequent advances have been important, most have been iterative, following on the heels of progress made in renal

transplantation. Examples are the demonstration that substituting mycophenolate mofetil (MMF) for azathioprine reduced rejection and mortality in the first year after transplantation,³⁵ that tacrolimus (Tac)-based immunosuppression was non-inferior or better than CyA-based immunosuppression in preventing rejection,^{36–38} and that a Tac/MMF combination was more advantageous than a CyA/MMF combination in terms of rejection and side effects.³⁹ Arguably, the most novel clinical advance specific to heart transplantation in the last 35 years has been the demonstration that statin therapy diminished severe rejection and cardiac allograft vasculopathy (CAV).⁴⁰ The use of everolimus, a proliferation signal inhibitor, has also been shown to reduce intimal proliferation in de-novo heart recipients,⁴¹ but it is currently used in <20% of recipients⁴² due to adverse events such as renal insufficiency.

Important questions remain unanswered. Attempts to address the value of induction therapy have been made,⁴³ but the issue is not resolved. The role of calcineurin inhibitor (CNI)-free therapy is unclear because the Heart Spare-the-Nephron study, the first attempt to wean off CNIs early post-transplant, was stopped prematurely due to excess rejection. An effective treatment to fully prevent CAV remains sorely needed. Hopes rose when compelling data in non-human primates (NHPs) suggested that pre-emptive depletion of CD20⁺ B cells with rituximab attenuated CAV.⁴⁴ This led to the CTOT-11 (ClinicalTrials.gov NCT01278745) study, which questioned whether early rituximab therapy to decrease B cells and diminish donor-specific antibody production would prevent CAV in heart recipients treated with conventional immunosuppression. Disappointingly, compared with the placebo group, rituximab-treated patients demonstrated *an increase*, not a decrease, in intimal thickness (manuscript submitted), although the trial did not complete enrollment as planned.

Better therapies are needed to prevent/treat antibody-mediated rejection (AMR) resulting from donor-specific antibodies (DSA), which are either pre-formed in sensitized pre-transplant patients or arise de novo after transplantation. Over the past decade, AMR has been increasingly acknowledged as a significant cause of adverse outcomes, including hemodynamic compromise rejection, CAV, graft loss and death.⁴⁵ The guiding principles for the management of AMR comprise removing circulating alloantibodies, reducing production of additional alloantibodies, suppressing T- and B-cell responses, and inhibiting complement.⁴⁶ However, these current therapies remain inadequate. Recently, tocilizumab (TCZ), a first-in-class, humanized, interleukin-6 (IL-6) receptor monoclonal antibody (MAb), was shown to safely and effectively reduce DSA levels in highly sensitized kidney allograft recipients⁴⁷ and to improve graft and patient survival in kidney recipients with the most severe form of chronic AMR.⁴⁸ In addition, a *Streptococcus pyogenes*-derived endopeptidase that degrades immunoglobulin G (IgG), thereby inhibiting complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity, was shown to reduce or eliminate DSAs, permitting HLA-incompatible renal transplantation in 24 of 25 patients.⁴⁹ These novel agents may soon contribute to the prevention/treatment of AMR in heart recipients.

Tolerance: All organs are not created equal

The best immunosuppression is no immunosuppression. That is the promise of immune tolerance—a state in which indefinite graft survival is achieved in a fully immunocompetent host without long-term immunosuppression. Tolerance has been achieved in NHPs^{50–52} and now in human^{53–56} *kidney* allograft recipients using a combination of non-myeloablative conditioning and donor bone marrow transplantation that results in transient donor chimerism. However, the same mixed chimerism-based protocol that was successful in NHP kidney recipients failed to induce tolerance in *heart* recipients despite comparable levels of chimerism.⁵⁷ The reasons for this organ-specific difference are not clear. However, it is clear that all transplanted organs are *not* created equally in the eyes of the immune system. Not only does the strength of the immune response to a particular organ vary with the organ transplanted but it also varies with the nature of response—that is, rejection vs tolerance. Some organs, such as kidney and liver, are tolerance-prone, whereas others, such as heart and lung, are tolerance-resistant.⁵⁸

The organ-specific nature of tolerance is conserved across species. In miniature swine, the same tolerance protocol that led to 100% indefinite kidney allograft survival resulted in rejection of all heart allografts transplanted across the same MHC barrier (Figure 1A).⁵⁹ An attempt was made to exploit the inherent tolerogenicity of kidney allografts by co-transplanting heart allografts with kidney allografts from the same donor. Surprisingly, co-transplantation of the donor kidney led to long-term, stable tolerance of the heart allografts, which, if transplanted alone, would have rejected acutely (Figure 1B).⁶⁰ This finding has been extended to NHPs by combining donor kidney co-transplantation with a mixed chimerism conditioning regimen that included costimulatory blockade. The result was long-term tolerance of MHC-mismatched heart allografts.⁶¹ Notably, each recipient who successfully completed its protocol achieved indefinite allograft survival without evidence of CAV. These animals represent the first non-human primates to become tolerant of cardiac allografts. In doing so, they confirmed that tolerance induction of resistant, primate heart allografts is biologically possible and that tolerance can be achieved in a consistent manner. This is an essential requirement of tolerance protocols being considered for human heart recipients.

The compelling and clinically relevant question these studies beg is how a renal allograft is able confer tolerance upon a co-transplanted heart allograft? One theory is that cells or cell products intrinsic to a kidney and not a heart (e.g., renal tubular epithelial cells,⁶² plasmacytoid dendritic cells⁶³ or erythropoietin⁶⁴) are particularly effective at activating and expanding host Tregs, which support a robust state of tolerance. Understanding these mechanisms could lead to novel therapeutic agents that would substitute for the donor kidney and make possible tolerance of *isolated* heart allografts.

Tolerance and ABO-incompatible heart transplantation

Tolerance has already benefited heart transplant recipients. In the most significant advance in pediatric heart transplantation over the last 50 years, tolerance has been harnessed to enlarge the donor pool of pediatric hearts through ABO-incompatible heart transplantation.⁶⁵ This has been achieved by exploiting a window of immaturity in the developing immune

system of infants and young children, similar to that used in Medawar's original studies of neonatal tolerance in mice.⁶⁶ Subsequent immune assays have shown a persistent lack of antibody production against donor blood group antigens together with an absence of donor-specific, antigen-specific B cells, indicating the induction of a unique form of antigen-specific B-cell tolerance.⁶⁷ Moreover, ABO tolerance in this setting is associated with absence of de-novo donor-specific HLA antibodies.⁶⁸ This unexpected finding suggests that the immune system may be more malleable than previously anticipated, which bodes well for bringing tolerance to heart transplant recipients.

The future of transplantation is xenotransplantation, and it will be

Xenotransplantation research has been the butt of many jokes over the years, but no one is laughing now that a pig heart has survived for over 2.5 years in a baboon with excellent echocardiographic function and no histologic evidence of graft injury or CAV.⁶⁹ How was this achieved when, only 15 years ago, heterotopic pig-to-NHP transplantation resulted in graft survival times measured in days⁷⁰? It was achieved, in part, by using genetically engineered donor pigs that had genes for deleterious porcine antigens removed and genes for beneficial human proteins added. For instance, the heart that survived over 2.5 years was taken from a *GTKO.hCD46.hTBM* donor in which the carbohydrate antigen, galactose- α 1,3-galactose (Gal), which causes hyperacute rejection, had been eliminated, and in which the human proteins CD46 (membrane co-factor protein), which downregulates complement, and thrombomodulin (TBM), which prevents coagulation, were expressed. Recipients of these *GTKO.hCD46.hTBM* hearts were treated with an anti-CD40 MAb-based immunosuppression regimen. The 5 recipients in the study achieved a median survival of 298 days with the longest survival being 945 days.⁶⁹ Remarkably, there was no evidence of humoral rejection or coagulation pathway dysregulation. However, reduction of the anti-CD40 MAb dose on Day 100 or after 1 year resulted in the appearance of anti-pig antibodies and graft failure.⁶⁹ Thus, blockade of the CD40/CD154 costimulation pathway also appears to play a major role in successful prevention of xenograft rejection, at least for hearts.

The field of xenotransplantation has been reinvigorated further by the revolution in genome editing.⁷¹ Whereas the generation of a homozygous single-gene knockout pig using a traditional homologous recombination could take 3 years, the newly discovered clustered, regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has the capacity to generate new donor pigs with multiple protective genetic modifications and with much greater precision and scale *in months*.⁷² The potential and promise of this system was recently demonstrated in a dramatic fashion. Addressing one of the major concerns of the Food and Drug Administration with regard to xenotransplantation, namely the transmission of porcine endogenous retroviruses (PERVs) to humans, healthy pigs have been generated in which all 62 PERV loci were simultaneously inactivated using the CRISPR/Cas9 systems (Figure 2).⁷³ Not only was this a gene editing "tour de force," but the availability of pigs with a PERV-free genome makes moot any argument to limit clinical xenotransplantation based on the unlikely risk of pig-to-human viral transmission.⁷⁴ The goal now is using CRISPR/Cas9 to generate pigs with increased biologic compatibility with humans, perhaps using PERV-free pigs as a foundation strain.

These encouraging developments are balanced by several remaining challenges, notably the heavy and often clinically unacceptable non-specific immunosuppression still required to abrogate the formidable immune response to a xenograft. This challenge may ultimately be met by inducing tolerance across xenogeneic barriers. Presently, 2 approaches are being studied for the induction of pig-to-primate tolerance: (1) the simultaneous transplantation of vascularized donor thymus with the xenograft; and (2) mixed hematopoietic chimerism, similar in concept to that described previously.⁷⁵ In a kidney xenograft model, transfer of vascularized donor thymus has been achieved by either implanting donor (i.e., autologous) thymic tissue under the renal capsule and waiting 1 or 2 months for thymic revascularization⁷⁶ or transplanting an independent vascularized thymic lobe (VTL).⁷⁷ The survival of composite GalT-KO “thymokidney” and the VTL plus kidney were prolonged (83 days and 65 days, respectively) compared with the survival of GalT-KO kidney alone (34 days) in an initial study.⁷⁸ More recently, thymokidney survival was extended to 193 days without evidence of rejection.⁷⁹ Attempts to induce mixed hematopoietic chimerism in primates using GalT-KO bone marrow have been advanced recently by generating hCD47 transgenic GalT-KO miniature swine. CD47 expression has prolonged the duration of porcine hematopoietic chimerism in baboons by inhibiting the phagocytosis of donor cells by primate macrophages.⁸⁰ Encouragingly, such prolongation has been associated with markedly prolonged donor swine skin xenografts.⁸¹

Translating the exciting results achieved in the heterotopic model to an orthotopic, life-supporting setting may also be challenging. The maximal survival of a pig-to-NHP orthotopic heart is 57 days.⁸² According to the International Society for Heart and Lung Transplantation guidelines, a clinical trial should only be considered when approximately 60% survival of life-supporting pig organs in non-human primates has been achieved for a minimum of 90 days, with at least 10 animals surviving for this minimum period. Furthermore, evidence should suggest that longer survival (>6 months) can be achieved. These results should be achieved in the absence of life-threatening complications caused by the immunosuppressive regimen.⁸³ What group of patients would be the first to benefit from a cardiac xenograft? Perhaps those with complex congenital heart disease, who may have undergone previous palliative surgical procedures and are unsuitable for ventricular assist device implantation.⁸⁴

In conclusion, remarkable advances have been made in the science of transplantation over the last 50 years. In the next 50 years these advances will be creatively integrated with advances in other emerging technologies, such as genome editing, synthetic biology and bioengineering. The result will be novel therapies that will combine with heart transplantation (or even replace transplantation) to provide a normal and productive life for *all* patients with end-stage heart disease.

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Figure 1.

(A) MHC inbred miniature swine treated with a 12-day course of high-dose tacrolimus were transplanted with isolated heart or isolated kidney allografts across a full MHC disparity.⁵⁹

(B) MHC inbred miniature swine treated with a 12-day course of high-dose tacrolimus were co-transplanted with hearts and kidneys from the same fully MHC-disparate donors.⁶⁰

Figure 2.
Image of the first born PERV-inactivated pig.⁷³