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Path to Clinical Transplantation Tolerance and Prevention of Graft versus Host Disease

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Abstract

Although organ and bone marrow transplantation are life saving procedures for patients with terminal diseases, the requirement for the lifelong use of immunosuppressive drugs to prevent organ graft rejection and the development of graft versus host disease (GVHD) remain important problems. Experimental approaches to solve these problems, first in preclinical models and then in clinical studies, developed at Stanford during the past 40 years are summarized in this article. The approaches use fractionated radiation of the lymphoid tissues, a procedure initially developed to treat Hodgkin's disease, to alter the immune system such that tolerance to organ transplants can be achieved and GVHD can be prevented after the establishment of chimerism. In both instances, the desired goal was achieved when the balance of immune cells was changed to favor regulatory innate and adaptive immune cells that suppress the conventional immune cells that ordinarily promote inflammation and tissue injury.

Keywords

transplantation; immunology; haplotype; chimerism

Choosing a Career in Immunology

The path began in elementary school, Public School 92, in Brooklyn, New York. By far the most famous person to go to this school was the great opera singer, Beverly Sills (Silverman). However, I was interested in science instead of singing because the dawning of the atomic age occurred just as I entered first grade, and the mystery of the atom was a national theme; some elementary school students even knew that $E=MC^2$. I started a science club, and after graduation entered a "science and math" examination school, Stuyvesant High School, in Manhattan. Although I was devoted to a career in nuclear physics, I entered the Manhattan Science Fair by building a paper electrophoresis device that detected the abnormal gamma globulin "spike" in the serum of a patient with multiple myeloma. I did not associate that project with immunology, and was not thinking of a career in immunology. Shortly thereafter, I competed in the National Science Fair in Los Angeles, but did not imagine that I would emigrate to California at a later date. I entered Columbia College as a physics major, and graduated as a pre-med student after I realized that I had greater talents in medical research than in nuclear physics.

Working with Joseph Murray at Harvard University

When I entered the Harvard Medical School in 1961, I wanted to replace failed human organs with new ones by means of surgical transplantation. I joined the Surgical Research Laboratory of Joseph Murray, who went on to receive the Nobel Prize for performing the first kidney transplants in humans and starting the field of clinical organ transplantation. That laboratory had begun the use of immunosuppressive drugs to prevent rejection, first in dogs and then in humans with a combination of steroids and azathioprine.

However, the “Holy Grail” at that time and up to the present (2013) was to adapt the approach of Medawar and his group[1] to induce immune tolerance to prevent rejection and eliminate the need for lifelong immunosuppressive drugs. During my 4 years in the Surgical Research Laboratory, I realized that the goal of inducing tolerance in humans was a long way off. Instead, I decided to focus on understanding the biology of the rejection process, and, in particular, how the immune system of the host first recognizes the antigens of the organ transplant. There were two theories; in one, the antigens of the transplant were carried by lymphatics to the local lymph nodes where they stimulated immune cells. In another, immune cells in the blood, circulated through vascularized organ transplants such as the kidney, encountered the transplant alloantigens in the organ vasculature, and then returned to the lymphoid tissues. Evidence for the latter process, ”peripheral sensitization” , was obtained in dogs given kidney transplants encased in plastic bags to prevent lymphatic drainage, since these transplants were rejected as rapidly as non-encased transplants.[2] I obtained some preliminary data to support the latter theory by continuously circulating the white blood cells from one dog through the kidney of another, *ex vivo*, and then returning the white blood cells intravenously to the blood donor. The blood donor rejected skin grafts from the kidney donor in an accelerated fashion indicating that sensitization had occurred after the reinfusion of the blood cells. I decided to try to repeat these experiments in inbred rats using pure populations of thoracic duct lymphocytes instead of white blood cells after joining the laboratory of James Gowans at Oxford University as a research fellow between my second and third year of medical school.

Working with James Gowans at Oxford University

When I arrived in the Gowans laboratory in the William Dunn School of Pathology (Oxford), I met another American student, Irving Weissman, who was also there for 1 year. Irv and I spent a great deal of time discussing scientific themes in immunology, and we both recognized that Gowans was the first scientist to determine the type of cell in the lymphoid tissues, the small lymphocyte, that initiated immune responses.[3] In particular, he showed that small lymphocytes which are highly enriched in the thoracic duct lymph of rats were able to initiate the immune response that caused graft versus host disease (GVHD). Gowans demonstrated that the latter cells continuously recirculated from the blood to the thoracic duct lymph, and identified the pathway of recirculation via the post capillary high endothelial venules in the lymph nodes.[4, 5] These discoveries were milestones in immunology.

My project at Oxford was to cannulate the rat thoracic duct, harvest the enriched small lymphocyte from one inbred rat strain (HO), and continuously circulate the cells through the kidney of another strain (HOxAO) *ex vivo*, return the cells intravenously to the HO strain, and determine whether the infused recipients rejected skin grafts from the HOxAO strain in an accelerated fashion. After appropriate controls were performed, the results showed that the grafts were rejected as if the recipients had been sensitized to the HOxAO alloantigens.[6] This indicated that recipient small lymphocytes can recognize alloantigens and initiate the immune response while they are circulating through the donor kidney.

About the time I was at Oxford, and shortly thereafter, important advances were made in immunology that built on the Gowans observations including the division of small lymphocytes into T and B cells, the division of T cells into CD4 and CD8 subsets as well as new technology to identify the lymphocyte subsets by surface markers. In addition, the structure of immunoglobulin molecules was determined by another faculty member at Oxford, Rodney Porter, who was an associate of Gowans.

Joining the faculty at Stanford University

After my fellowship at Oxford, I returned to Harvard Medical School and to the Murray laboratory, wrote a medical school thesis based on my Harvard and Oxford research, completed my internship training at the Massachusetts General Hospital, and joined the laboratory of Lloyd Law for 3 years at the NIH while I was in the Public Health Service. Thereafter, I visited Stanford Medical School to look at job opportunities in immunology. My link to Stanford was based on my friendship with Irv Weissman that developed at Oxford, and Irv introduced me to immunologists Hugh McDevitt, and Halsted Holman (Department of Medicine Chairman). The power of immunology at Stanford was very attractive including the innovative work of Len and Lee Herzenberg, and I worked out a plan to complete my clinical training and join the Division of Immunology and Rheumatology as a faculty member immediately thereafter. McDevitt was the Division Chief and my mentor at the time I joined the faculty in 1971.

My laboratory at Stanford initially studied the characteristics and differences between naive (virgin) and memory B cells. The work was started in rats and then continued in mice. An important issue was whether memory B cells switched from expression of IgM to IgG on the cell surface, and Lee Herzenberg and I differed in this area, whereas I claimed that memory B cells expressed IgM[7], Lee claimed that they expressed IgG. Fifty years later, we agree that both subsets exist.

The B cell studies extended to the discovery by Shimon Slavin (a postdoctoral fellow in my laboratory) of the BCL_1 B cell leukemia/lymphoma arising spontaneously in a control BALB/c mouse with an exceptionally high white blood cell count.[8] This discovery led to long term collaboration with IgD experts, John Uhr and Ellen Vitetta at the University of Texas, since the BCL_1 tumor expressed both IgM and IgD on the cell surface.[9] This tumor was the subject of collaborative studies on tumor dormancy,[10] and to eventual DNA rearrangement studies by a graduate student in my laboratory, Michael Knapp, in collaboration with Frederick Blattner at the University of Wisconsin. Knapp showed that the

mu and delta immunoglobulin constant region genes were associated with a single rearrangement of the heavy chain variable region genes.[11]

Working with Henry Kaplan; Link between the immunodeficiency of Hodgkin's Disease and the discovery of AIDS

During the rodent B cell studies, I began a collaboration with Henry Kaplan, the Chairman of the Stanford Department of Radiology. Kaplan was considered the father of modern radiotherapy, and he was best known for the development of a radiotherapy technique, total lymphoid irradiation (TLI), that was the first reported curative treatment for patients with early stage Hodgkin's disease.[12] Kaplan had been studying the immunological abnormalities of untreated patients with Hodgkin's disease, and had discovered that there were impairments in lymphocyte function, and cell mediated immunity including anergy to delayed hypersensitivity skin tests.[12, 13] I hypothesized that the cell mediated immune impairments would resolve after cure with TLI, and examined both the immune function of treated and untreated patients as well as the numbers of T cells in the blood. We used the newly developed assay for T cell enumeration, the sheep red blood cell rosette assay, as well as a cytotoxicity assay with our own anti-Tcell antibodies. The results of the studies showed that the immune impairment within the first year after TLI was even more profound than that seen with untreated patients, and the T cell numbers were dramatically reduced as compared to untreated patients for the first few months after TLI.[14] Thus, the patients were cured, immunosuppressed, and T cell depleted by the TLI therapy.

Michael Gottlieb, a fellow in my laboratory who participated in laboratory animal studies of immune suppression and tolerance induction after TLI, also learned the human T cell enumeration assays. After Gottlieb left the laboratory he took a faculty position at the UCLA Medical Center, and began studying a group of homosexual men who had been hospitalized with severe pneumocystis infections.[15] Using the e-rosette T cell enumeration technique, he found that a common feature in all these patients was an extremely low absolute number of T cells in the blood that was associated with a dramatic immunodeficiency. He reported this patient group in the New England Journal of Medicine in 1981, and identified these patients as victims of an Acquired Immunodeficiency Disease Syndrome in the first description of AIDS.[15]

Link between treatment of Hodgkin's Disease and conditioning for bone marrow transplantation that protects against GVHD

After observing that the TLI treatment of Hodgkin's Disease was immunosuppressive and lymphodepletive for several months without severe reduction of white blood cell and platelet counts, I theorized that the TLI procedure could be adapted as a non-myeloablative conditioning regimen for bone marrow transplantation that would allow for acceptance of the transplant, the induction of chimerism, and ultimately the induction of transplantation tolerance. The goal to achieve tolerance was my stimulus to join the Murray laboratory, but the goal was dropped because I could not identify a path to achieve that goal.

In order to study TLI as a conditioning regimen, I worked with Kaplan, and a new radiotherapy faculty member, Zvi Fuks, to make a mouse model in which the thymus, spleen, and lymph nodes were targeted with radiation while shielding other vital organs and tissues such as the lungs, brain, and bone marrow with lead. After designing the lead shields, multiple small doses of radiation were delivered daily as in the clinical regimen in order to achieve a total dose of 3,400 cGy after 17 doses of 200cGy each.[16–19] These experiments were carried out by Shimon Slavin, who returned to Israel after his training, and went on to found the first institute devoted to bone marrow transplantation in the Middle East.

The most surprising outcome of the mouse experiments was ability to achieve stable mixed chimerism in fully MHC mismatched donor /recipient pairs without the development of GVHD.[19] When total body irradiation (TBI) was used to condition mice for bone marrow transplantation instead of TLI, the majority developed complete chimerism and lethal GVHD.

In a series of studies, we performed combined bone marrow and organ transplantation in mice and rats, and showed that the stable mixed chimeras accepted the organs from the bone marrow donors.[16–18] The chimeric recipients were clearly tolerant, since they rejected organs from third party strains. Other laboratories had shown previously that radiation chimeras established with TBI and bone marrow transplantation were able to accept organs from the marrow donor; however, GVHD was a frequent problem. The advance using TLI was the prevention of GVHD even in a fully mismatched MHC combination such that the approach was clinically applicable. The prevention of GVHD was enhanced by the addition of anti-thymocyte serum (ATS) injections during the first few days of TLI such that 100% of recipients given bone marrow transplants were protected from acute GVHD and became mixed chimeras.[20] In order to make the TLI/ATS procedure applicable to deceased donor transplants in humans in which organ availability timing is uncertain, the protocol was modified to a completely posttransplant conditioning regimen .[21–24] In the latter modification, the organ was transplanted on day 0 , and the first of 5 daily doses of anti-thymocyte serum in combination with 5 daily doses of TLI was followed by 5 additional doses of TLI. The infusion of the donor bone marrow cells was performed immediately after the completion of TLI at day 15. [21–24]

Different goals for chimerism after bone marrow transplantation; mixed chimerism for tolerance induction and complete chimerism for lymphoma eradication

The goal of the TLI/ATS tolerance induction regimen was to establish stable mixed chimerism, since mixed chimerism reduced the risks of GVHD and immunodeficiency as compared to complete chimerism. An alternative mixed chimerism approach to transplantation tolerance was developed later by Sachs and his co-workers by administration of lethal TBI followed by the infusion of a mixture of recipient and donor marrow cells in initial studies.[25] Thereafter administration of a sublethal dose of TBI, radiation of the thymus, administration of anti-T cell antibodies, and infusion of only donor bone marrow cells was used to achieve this goal in a variety of inbred and outbred laboratory animals.[26]

In order to treat the BCL_1 lymphoma with bone marrow transplantation using the TLI/ATS conditioning regimen, we combined donor spleen cells with bone marrow cells to achieve complete chimerism,[27] since our previous studies showed that eradication of the tumor cells after marrow transplantation with TBI required complete rather than mixed chimerism.[28] Interestingly, the TLI/ATS conditioning regimen protected against GVHD even after the development of complete chimerism in fully MHC mismatched donor/recipient pairs, and the BCL_1 tumor was eradicated by the graft anti-lymphoma (GVL) effect.[27, 29]

Cellular and molecular mechanisms of GVHD protection and tolerance after TLI/ATS conditioning: the role of suppressor cells

Initial observations in my laboratory made first by Slavin, and then by Okada and King, showed that the spleen cells obtained from marrow transplant recipients shortly after conditioning with TLI were able to suppress any mixed leukocyte reaction (MLR) regardless of the MHC type of the responder or stimulator cells.[30–33] This lack of specificity of the suppressor cells for the marrow donor alloantigens, and the lack of requirement of MHC restriction of the suppressor cells with the responder cells in the MLR ran contrary to immunological concepts at that time. The findings could explain the acceptance of donor marrow cells, but could not explain the specificity of tolerance.

The discoveries of regulatory adaptive immune and innate immune cells had not been made yet, and awaited the future identification of immune suppressive natural killer (NK) T cells that had no MHC restriction because they recognized CD1d instead of MHC,[34, 35] naturally occurring regulatory $CD25^+CD4^+$ T cells that suppressed immune responses to a wide variety of antigens,[36, 37] and innate immune regulatory cells such as myeloid derived suppressor cells (MDSCs).[38, 39] As it turned out, the percentage of all three of the latter regulatory cells were increased in the spleen after TLI/ATS conditioning, and NKT cells [40–42], Treg cells [43–45], and suppressive myeloid cells[46] were required for the induction of tolerance and protection against GVHD.

In the case of protection against GVHD, the host NKT cells interacted with donor Treg cells in the marrow transplant in an IL-4 dependent manner to suppress donor conventional T cells from mediating anti-host immune injury.[45] In the case of tolerance induction, the host NKT cells interacted with host Treg cells and MDSCs in an IL-4 dependent manner to suppress host conventional T cells from rejecting the donor marrow cells and organs.[44] Thus, the discovery of the three types of regulatory cells was able to explain the initial *in vitro* observations of lack of antigen specificity and MHC restriction of suppressor cells after the tolerance conditioning regimen. Although Shimon Sakaguchi, the discoverer of the important role of the $CD4^+CD25^+FoxP3^+$ regulatory T cells[36, 37], was a postdoctoral fellow in my laboratory for 2 years, we had not linked these T cells to TLI tolerance induction at that time.

NKT cells and Treg cells in bone marrow transplants can prevent GVHD

Early studies of the cause of acute GVHD after bone marrow transplantation in rodent recipients conditioned with TBI concluded that the mature T cells in the marrow transplant

mediated the immune attack on the allogeneic recipient tissues .[47] It was unclear whether, in addition to the injury inducing T cells in the transplant, there were regulatory T cells that suppressed GVHD. Our studies of marrow transplant T cells separated by density gradients indicated that such regulatory T cells were present in the low density fractions, and $CD4^{-}CD8^{-}$ T cells within these fractions were suppressive.[48, 49] We determined that the latter cells were almost entirely NKT cells that suppressed GVHD in an IL-4 dependent manner. [50] In further studies, we showed that naturally occurring $CD4^{+}CD25^{+}$ Treg cells in the marrow transplant also suppressed GVHD induced by conventional T cells.[51] The latter cells had the desirable profile of preventing GVHD activity without suppressing the graft versus lymphoma/leukemia (GVL) activity that eradicates tumor cells.[29] These rodent studies led to the current interest by several groups to initiate clinical trials of the use of regulatory T cells to prevent GVHD.

Interestingly, clinical transplant groups in London and Paris that studied multiple pretransplant co-variate parameters that predict GVHD in HLA matched recipients (including the type of conditioning regimen, gender and age of donor and recipient, total number of donor CD4 and CD8 T cells or hematopoietic progenitor cells infused) found that the single most important predictor was the number of donor NKT cells infused or the number present in recipient blood shortly after infusion.[52, 53] In concert with our rodent findings, the clinical studies showed that increased numbers of donor NKT cells were associated with a decreased risk of GVHD despite the rarity of these cells among all T cells (<1%).

Clinical application of the TLI based conditioning regimen to hematopoietic cell transplantation for treatment of leukemia and lymphoma at Stanford

Since the TLI/ATS conditioning regimen was able to protect against GVHD in mice, and at the same time eliminate the BCL_1 lymphoma via GVL activity, the application to the treatment of hematologic malignancies in humans was developed at Stanford in collaboration with Robert Lowsky, a new faculty member in the Division of Blood and Marrow Transplantation, starting in 2000. By the end of 2013, over 450 Stanford patients were given transplants using this regimen by enrolling those who did not qualify for standard high intensity conditioning regimens due to either co-morbid medical conditions or older age. Thus, a potentially curative treatment was made available to a larger number of patients with acute myelogenous leukemia or non-Hodgkin’s lymphoma.

Patients given the TLI/ATG regimen had a very low (<5%) incidence of severe acute GVHD associated with minimal regimen related toxicity.[54, 55] Protection against GVHD was associated with an increased percentage of recipient NKT cells among all T cells after conditioning as in the studies with laboratory animals. 53, 54 About half of the patients had durable complete remissions, and as in the studies with laboratory animals, relapse rates were markedly lower after the development of complete rather than mixed chimerism.[55] The low toxicity allowed application of the regimen to patients who developed relapse of lymphoma after auto-transplants with results similar to those who had relapse after chemotherapy.[55] Multi-center trials in Europe confirmed the marked reduction in GVHD and low toxicity.[56]

Clinical application of the TLI based conditioning regimen to the induction of tolerance to combined kidney and hematopoietic cell transplantation at Stanford

The goals of immune tolerance in clinical organ transplantation are to eliminate the lifelong need for immunosuppressive drugs while preventing the loss of grafts due to acute and chronic rejection. Side effects of immunosuppressive drugs include increased risks of infection, diabetes, hypertension, cancer, heart disease, liver and kidney toxicity, and osteoporosis. Despite the improvement in the incidence of early rejection episodes by more effective immunosuppressive drugs, about half of kidney transplants from living donors are lost in about 15 years while patients are maintained on these drugs.[57]

In view of the achievement of tolerance using the combination of TLI and anti-thymocyte serum or globulin in laboratory animals[58, 59], we applied TLI based regimens to both heart and kidney transplant patients. In the case of heart transplant patients, I collaborated with Norman Shumway's pioneering heart transplant group at Stanford to administer TLI to transplant recipients who had recalcitrant acute rejection , since all of the standard anti-rejection medications had failed to control a rejection process that had potentially lethal consequences.[60] Fortunately, a course of ten TLI treatments markedly attenuated the rejection process, and the majority of patients could return to maintenance immunosuppressives with resolution of the ongoing tissue injury.[60] The approach was adapted in many other centers with observations for up to 18 years.[61]

In the case of deceased donor kidney transplantation in humans, the goal of the first studies was to use minimal maintenance immunosuppression with only low dose steroids (prednisone; 10mg/day), or to completely withdraw immunosuppressive drugs from patients who had received the deceased donor transplants. These studies were performed in the early and mid-1980's before the widespread use of cyclosporine replaced azathioprine. Standard practice was to use high dose steroids in combination with azathioprine as maintenance therapy. The initial studies of TLI without donor cell infusions in the kidney transplant patients were based on the ability of pretransplant TLI in combination with ATG to induce tolerance in outbred dogs without the infusion of donor bone marrow cells.[62] The clinical studies were initiated as collaborations first with Oscar Salvatierra at the University of California San Francisco (UCSF), and then with Barry Levin and Derek Samson at the California Pacific Medical Center (CPMC).

In the case of UCSF, the first patient was given the pretransplant TLI/ATG conditioning regimen to avoid the use of standard high dose steroids because the patient developed severe osteoporosis while on dialysis, developed bone fractures with severe weight loss, and became wheel chair bound. The TLI/ATG regimen allowed the patient to undergo kidney transplantation with only maintenance low dose steroids to avoid worsening of osteoporosis associated with the use of high dose steroids. The patient had no rejection episodes after transplantation, resolved the osteoporosis and weight loss, and returned to normal daily activities after an extensive physical therapy program. At the end of the first year after

transplantation, the patient took up residence in Hawaii, was lost to follow up, and was not the subject of a published report.

In collaboration with CPMC, a study of 28 recipients of deceased donor transplants given pretransplant TLI/ATG were given only low dose steroids as the maintenance anti-rejection regimen.[63, 64] About half of these patients were free of rejection episodes after transplantation, and the remainder were treated with high dose steroids and azathioprine after rejection episodes. [62, 63] The patients without rejection episodes developed a pattern of specific unresponsiveness to donor alloantigens in the MLR that was indicative of the development of tolerance.[65] In particular, during the first 12 months after conditioning the patient's PBMCs lost the ability to proliferate in response to both third party and donor alloantigenic stimulation. During the second year the response to third party alloantigens returned, but the response to donor antigens did not.[65] PBMC's obtained pretransplant responded well to both third party and donor alloantigens; thus the specific unresponsiveness was an acquired pattern.

In view of this pattern, two patients given pretransplant TLI/ATG conditioning at CPMC and a patient of B. Myburgh at the Wittwatersrand Hospital in Johannesburg were completely withdrawn from maintenance drugs without subsequent rejection episodes.[66] These patients showed the pattern of specific unresponsiveness to the donor alloantigens, and were reported in 1989 to be the first documented examples of acquired immune tolerance to human allogeneic kidney transplants. [66] A follow-up report showed that one of these patients maintained good graft function without maintenance anti-rejection drugs for 12 years.[67]

In view of the uncertain timing of the availability of deceased donor organ transplants, and the difficulty of logistics of the use of pretransplant TLI in this setting, a completely posttransplant TLI/ATG conditioning regimen was developed in laboratory animals to better accommodate the use of deceased donor transplants in humans. Accordingly, we found that posttransplant TLI/ATG conditioning was successful in establishing tolerance to MHC mismatched heart transplants in rats as long as donor bone marrow cells were infused at the end of the TLI conditioning regimen at about day 15 after organ transplantation.[20, 21] The recipients became stable mixed chimeras, and developed the expected pattern of donor specific unresponsiveness that was associated with clonal deletion. [19, 20]

We adapted the posttransplant regimen to HLA mismatched living donor kidney and hematopoietic cell transplantation in humans in 2000, at about the same time the use of the same TLI/ATG regimen was applied to patients with hematologic malignancies who were treated with hematopoietic cell transplants.[68] In both the cancer patients and the kidney transplant patients, 10 doses of TLI were administered over 2 weeks and 5 doses of ATG were administered in the first week, and patients were discharged from the hospital after the first 5 days to complete the TLI in clinics.

Patients with kidney transplants received the grafts one day before starting TLI, and received the first dose of ATG intra-operatively. Immediately after the completion of TLI, the patients received an infusion of G-CSF "mobilized" PBMCs that were harvested from

the donor and cryopreserved a few weeks before infusion. In the case of cancer patients, all of whom were HLA matched, it was desirable to achieve complete chimerism, and unmanipulated PBMCs containing 200–300x10^6 T cells/kg were administered.[54, 55] In the case of the organ transplant patients, the first 6 of whom received grafts from HLA mismatched related or unrelated donors in 2000–2004, it was desirable to achieve mixed chimerism to reduce the risk of GVHD and immunodeficiency. Accordingly, the donor PBMCs were manipulated by enriching for $CD34^+$ hematopoietic progenitor cells on magnetic bead columns to achieve an infused dose of T cells of 0.01–0.1x10^6 cells/kg.[68] The latter dose of T cells was more than 1,000 fold lower than that used in cancer patients.

The first 6 mismatched kidney transplant patients failed to achieve chimerism for more than 90 days, and none were successfully withdrawn from immunosuppressive drugs due to the lack of persistent chimerism.[68] In a follow-up protocol started in 2005, 22 HLA matched patients were enrolled through 2013. These patients received a donor cell infusion containing enriched $CD34^+$ cells as before, but the added back T cell dose was increased to 1x10^6/kg. Almost all of the latter patients developed persistent mixed chimerism for at least 12 months. Sixteen of the first 20 patients (80%) were withdrawn from maintenance immunosuppressive (IS) drugs within the first 12 months without subsequent rejection episodes with a follow-up period of up to 5 years (median 3 years) after withdrawal.[69, 70] The criteria for drug discontinuation was persistence of chimerism for at least 6 months, lack of clinical rejection episodes and GVHD, and lack of microscopic rejection on protocol biopsy.[68, 69] Patients who successfully discontinued drugs showed a pattern of specific unresponsiveness to donor cells in the MLR.[68, 69] Graft survival in this cohort of HLA matched patients was 100% and was increased as compared to conventionally treated HLA matched patients at Stanford. Thus, the tolerance protocol is likely to be the treatment of choice for matched patients.

A third protocol enrolled combined kidney and hematopoietic cell transplant patients who received grafts from HLA mismatched related donors (haplotype matched). The TLI and ATG conditioning regimen was the same as used for HLA matched patients, but the donor cell content was changed to perform a dose escalation study of infused T cells starting at 3x10^6/kg to determine the level that can be used to achieve persistent mixed chimerism for at least 6 months without development of GVHD. Persistent mixed chimeras were scheduled to have immunosuppressive drugs withdrawn between 12 and 18 months. Currently 9 patients have been enrolled in this protocol, and persistent mixed chimerism required the infusion of at least 10x10^6 T cells/kg (about 100 fold higher than that used in the first cohort of mismatched patients enrolled beginning in 2000). The ability to withdraw IS drugs from these chimeras remains to be determined.

In conclusion, the TLI and ATG conditioning regimen protected both laboratory animals and humans from GVHD, and was used to achieve tolerance to organ transplants. The protocol for organ transplantation was safe and there have been no graft losses in the 37 kidney transplant patients enrolled with up to13 years of follow up. Thus, the two key goals of tolerance induction have been achieved: elimination of immunosuppressive drugs and their attendant side effects, as well as prevention of graft loss due to acute and chronic rejection. The challenge in the cancer patients is to reduce the relapse rate that is currently about 50%

after transplantation, and to apply the success in tolerance induction observed in HLA matched patients to those who are mismatched.

Did the transplant group at Stanford including myself, John Scandling, the kidney transplant physician, Stephan Busque, the organ transplant surgeon, Judith Shizuru and Robert Lowsky, the bone marrow transplant physicians, and Edgar Engleman, the supervisor of immune tolerance assays, find the "Holy Grail" of organ transplantation? In the case of HLA matched kidney transplant recipients, who represent a small fraction of all patients receiving kidney transplants, the results indicate the "Grail" was found. Among the last 12 of the 22 matched patients observed at Stanford, 11 were successfully withdrawn from IS drugs and the twelfth is in the process of withdrawal. The results suggest uniform capacity to induce tolerance in this group, after learning from some early failures. Whether the success of this protocol in matched patients can be exported to other transplant centers, and whether modifications of the protocol can achieve success in mismatched patients such that the "Grail" is enlarged will require the continuing enrollment of patients in studies at Stanford and in multi-center trials over the next several years.

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