Translational Studies in Hematopoietic Cell Transplantation: Treatment of Hematologic Malignancies as a Stepping Stone to Tolerance Induction

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Abstract

Allogeneic hematopoietic cell transplantation (HCT) has most commonly been used to treat hematologic malignancies, where it is often the only potentially curative option available. The success of HCT has been limited by transplant-associated toxicities related to the conditioning regimens used and to the common immunologic consequence of donor T cell recognition of recipient alloantigens, graft-vs-host disease (GVHD). The frequency and severity of GVHD observed when extensive HLA barriers are transgressed has essentially precluded the routine use of extensively HLA-mismatched HCT. Allogeneic HCT also has potential as an approach to organ allograft tolerance induction, but this potential has not been previously realized because of the toxicity associated with traditional conditioning. In this paper we review two approaches to HCT involving reduced intensity conditioning regimens that have been associated with improvements in safety in patients with hematologic malignancies, even in the HLA-mismatched transplant setting. These strategies have been applied in the first successful pilot studies for the induction of organ allograft tolerance in humans. Thus, we summarize an example of vertical translational research between animal models and humans and horizontal translation between two separate goals that culminated in the use of HCT to achieve allograft tolerance in humans.

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Separation of GVHD and GVL is a major challenge in HCT.
Novel strategies for doing this that were developed at two centers are described here.
Chimerism induction can also lead to transplantation tolerance.
The strategies described here led to protocols for tolerance to organ allografts.

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Keywords
Transplantation; Tolerance; Graft-vs.-Host Disease; Graft-vs.-Leukemia; rejection; kidney transplantation

1. Introduction
Allogeneic hematopoietic cell transplantation (HCT) has most commonly been used to treat hematologic malignancies, where it is often the only potentially curative option available. While it has revolutionized the therapy of many of these diseases, the success of HCT has been limited by transplant-associated toxicities related to the conditioning regimens used and to the common immunologic consequence of donor T cell recognition of recipient alloantigens, graft-vs-host disease (GVHD). While GVHD can be effectively prevented by removing mature T cells from the donor graft, this maneuver is associated increased relapsed rates, demonstrating the beneficial graft-vs-leukemia/lymphoma (GVL) effect of the GVH alloresponse, as well as high rates of infectious complications due to poor immune reconstitution, and increased rates of graft rejection. A mild to moderate level of GVHD is considered acceptable when hematologic malignancies are treated, as GVHD is associated with enhanced anti-tumor effects [1]. However, the frequency and severity of GVHD observed when extensive HLA barriers are transgressed has essentially precluded the routine use of extensively HLA-mismatched HCT. In this review, we discuss two different approaches to HCT for the treatment of hematologic malignancies that, based on data on rodent models, aim to reduce the toxicity and GVHD rates associated with HCT and to thereby extend its availability to extensively HLA-mismatched donor-recipient pairs. We also describe how iterative translational studies between animals and humans have resulted in the extension of one of these strategies to a third, novel approach, in which hematopoietic cell engraftment is followed by intentionally induced marrow rejection in order to achieve anti-tumor effects without GVHD.

HCT also has potential as an approach to organ allograft tolerance induction. Immune tolerance denotes a state in which the immune system accepts donor organs or tissues but is capable of responding normally to foreign antigens. While recent improvements in immunosuppressive drugs have enhanced early organ allograft survival rates, they have had little impact on late graft loss due to chronic rejection. Moreover, malignancies, opportunistic infections and metabolic and organ-specific toxicities severely limit the tolerability of long-term chronic immunosuppressive therapy. The induction of donor-specific immune tolerance would avoid these toxicities while preventing chronic rejection. The association of mixed chimerism, a state in which donor and recipient hematopoietic elements coexist, with a state of donor-specific tolerance, was first demonstrated in the pioneering studies of Owen on bovine mixed chimerism generated in utero due to sharing of a placental circulation [2]. However, achievement of chimerism in adult recipients with pre-existing immune systems is far more challenging, largely due to the immune resistance imposed by recipient T cells [3;4]. The potential of tolerance induction via allogeneic HCT has not been realized because of the considerable toxicity that has been associated with conditioning traditionally used to achieve hematopoietic cell engraftment in recipients with established immune systems. In recent years, however, a variety of clinical HCT protocols have been developed for the treatment of hematologic malignancies that utilize reduced intensity conditioning. The two approaches to HCT discussed in this article involve reduced intensity conditioning regimens that have been associated with improvements in safety in patients with hematologic malignancies, even in the HLA-mismatched transplant setting. Efficacy data for the induction of transplantation tolerance were obtained initially in rodent models, followed by non-human primate studies. Critical safety data permitting the radical
departure from the standard of care for organ transplantation entailed by removal from immunosuppression was obtained in the development of these regimens as less toxic approaches to HCT in the treatment of hematologic malignancies. Thus, our review describes how vertical translational research between animal models and humans and horizontal translation between the two separate goals of organ allograft tolerance induction and optimization of HCT for the treatment of malignancies has culminated in the first successful pilot studies of intentional allograft tolerance induction in humans.

2. Approach I: Hematopoietic Cell Transplantation (HCT) for Organ Allograft Tolerance Induction and Treatment of Hematologic Malignancies Using Conditioning with Total Lymphoid Irradiation (TLI) and Anti-Thymocyte Globulin (ATG) to Prevent graft-versus-host disease (GVHD)

2.1 Studies in Preclinical Models of Tolerance Induction

The use of TLI in humans as a curative and safe treatment of Hodgkin’s disease has been reported extensively in clinical trials in the United States and Europe based on the initial development of this radiotherapy procedure by Henry Kaplan and his colleagues at the Stanford University School of Medicine more than 50 years ago[5–7]. The TLI procedure was adapted to laboratory animal studies by Stanford investigators as a conditioning regimen for bone marrow transplantation to achieve mixed chimerism without the major side effect of GVHD in MHC mismatched strains of mice and rats[8–12]. The recipients were found to be tolerant to skin and heart allografts that were transplanted at the same time as the donor bone marrow cells after pretransplant conditioning that targeted the spleen, thymus, and peripheral lymph nodes with multiple small fractions of radiotherapy over a period of two to three weeks[8–12]. Radiotherapy was non-myeloablative and all recipients survived without marrow transplants[8–12]. However, marrow transplantation was required to achieve tolerance to organ grafts[8–12].

In order to model clinical bone marrow transplantation in which the marrow cells are heavily contaminated with blood T cells that are potent inducers of acute GVHD, spleen or peripheral blood mononuclear cells from donors were added to the marrow cells in the animal model[13–16]. When myeloablative or non-myeloablative total body irradiation (TBI) was used to condition recipients with combined marrow and spleen or blood cells, all recipients given MHC mismatched transplants developed lethal GVHD[13–17]. Although conditioning with TLI significantly reduced the severity of GVHD, a proportion of murine recipients still died of this complication[13]. However, addition of injections of ATG (or anti-thymocyte serum; ATS) during the first week of TLI conditioning prevented the development of GVHD, and resulted in the survival of all recipients[13;16]. The number of donor T cells added to the bone marrow cells determined whether the TLI and ATS conditioned recipients became mixed or complete chimeras after transplantation[16]. When high numbers of splenic T cells were added (contained within 60×10⁸ spleen cells), then recipients became complete chimeras, whereas when marrow cells alone were used then all recipients became mixed chimeras[16]. Protection against GVHD was observed regardless of whether the recipients became mixed or complete chimeras.

Since mixed chimerism is desirable for the development of tolerance to organ transplants, and complete chimerism increases the risk of GVHD, the protocols used for tolerance were designed to induce mixed chimerism, and the hematopoietic cell grafts were manipulated to contain restricted numbers of donor T cells[17]. On the other hand, complete chimerism is desirable for the development of graft anti-leukemia/lymphoma (GVL) activity for the treatment of hematologic malignancies, and protocols were designed to contain high...
numbers of donor T cells in the hematopoietic cell grafts[16]. Accordingly, the studies of
tolerance and treatment of malignancies were performed along parallel but different paths
due to the different goals.

In the case of tolerance to organ transplants, the conditioning regimen was changed to
perform the TLI and ATG administration after the organ transplant was in place[18–21], in
order to accommodate to the clinical situation of deceased donor organ transplantation. The
timing of the availability of the latter organs is not predictable, and a posttransplant
conditioning regimen is desirable. Accordingly, the laboratory animal models started the
ATG on the day of organ transplantation, and the TLI one day later. After two weeks of
posttransplant conditioning, the donor bone marrow cells were injected[18–21]. The delayed
injection still resulted in uniform mixed chimerism in murine recipients, and tolerance to the
organ grafts[18–21].

The mechanisms of tolerance using the posttransplant conditioning regimen have been
studied in detail[22;23]. Both clonal deletion and immune regulation play required
roles[22;23]. Clonal deletion in the mixed chimeras was demonstrated by examining the Vβ
repertoire of T cells as compared to non-chimeric mice[22]. The requirement for regulatory
NKT cells of host origin was shown by the abrogation of tolerance in NKT cell deficient
CD1d−/− or Ja18−/− recipients[22;23]. An additional requirement for host Treg cells was
shown by abrogation of tolerance after depletion of the Treg cells with an anti-CD25
monoclonal antibody[23]. Both types of regulatory T cells become favored subsets among
surviving T cells due to resistance to apoptosis induced by the radiation and ATS[23;24].

2.2 Studies in Preclinical Models of Treatment of Lymphoma

The TLI and ATS conditioning regimen was adapted to treat the BCL1 B cell lymphoma in
murine studies[16]. These recipients were given the tumor cells along with a combination of
MHC mismatched marrow and spleen cells in order to induce complete chimerism[16].
About 80% of recipients survived the transplant procedure without GVHD, and became
complete chimeras with eradication of the tumor cells[16]. The tumor cell eradication in the
strain combination used was dependent on donor CD8+ T cells[25]. The prevention of
GVHD despite the potent GVL activity was due to the interaction of host NKT cells with
donor Treg cells such that the expansion and IL-10 secretion of the donor Treg cells was
markedly augmented[26]. Previous studies showed that donor Treg cells can suppress lethal
GVHD even in TBI conditioned hosts, and permit the donor CD8+ T cells to mediate potent
GVL activity[27;28].

In more recent studies, a model of lymphoma relapse was developed after bone marrow
transplantation that resulted in mixed chimerism, since the lymphoma relapse rate of human
mixed chimeras given non-myeloablative conditioning is significantly higher than that of the
complete chimeras[29]. The infusion of donor conventional T cells (DLI) induced acute
GVHD in the recipients as has been reported in the clinical situation[29]. However, the
infusion of purified donor CD8+CD45RA memory phenotype T cells resulted in conversion
from mixed to complete chimerism, and tumor eradication without GVHD[29;30]. This DLI
strategy may be desirable in the treatment of human mixed chimeras with tumor relapse,
since the risk of acute GVHD is reduced using “engineered” instead of unmanipulated T
cells as a source of DLI.

2.3 Stanford clinical studies of induction of tolerance to kidney transplants using TLI and
ATG conditioning

2.3.1 Use of Pre-Transplant TLI in the Induction of Tolerance to Deceased
Donor Kidney Transplants Without HCT—Preclinical models developed by
investigators at the Stanford University School of Medicine demonstrated that non-chimeric tolerance was achieved using pre-transplantation TLI combined with post transplantation ATG in canine recipients[11;12]. Therefore, the first clinical trial was designed to determine whether a similar regimen applied to humans with end stage renal disease resulted in a reduced need for chronic immunosuppressive drugs after deceased donor renal transplantation[31]. Twenty-eight patients received concurrent mantle and inverted Y fields of irradiation following techniques described for Hodgkin lymphoma yet using a mid-plane dose of 100 cGy per treatment, delivered three times per week until 2000 cGy TLI were delivered. Patients were selected for entry based on a low level of serum cytotoxic antibodies to HLA antigens (<25% positive on a panel of HLA typed cells). This low level of positive antibodies facilitated the rapid identification of a crossmatch negative ABO compatible deceased donor kidney as soon as possible after the completion of TLI. When an appropriate renal cadaver graft was not available within 7 days after completion of irradiation, patients were given 100 cGy to the inverted Y field once a week.

Transplant recipients were given six intramuscular injections of rabbit ATG (2mg/kg/dose) on alternate days that started on the day after surgery. Prednisone therapy was started on the day of surgery at 0.2mg/kg/day. If no rejection episodes occurred the daily dose was reduced to 0.15mg/kg at 6 weeks and followed a scheduled taper to 10mg daily by 8 months. Of the 27 patients that completed TLI and post transplant ATG, 25 were available for long-term follow-up, of whom 6 died of diabetes or heart disease complications with a normal functioning graft (median time 48 months), 4 had graft loss and 15 had long-term functioning graft survival. Twelve of these 15 patients (80%) were maintained with low dose (≤10mg daily) prednisone as the sole chronic immune suppressive drug[31]. Two of the latter patients were completely withdrawn from prednisone and one was followed for 7 years without evidence of rejection[32;33]. Graft and patient survival in this study appeared to be at least as good as those obtained with prednisone plus cyclosporine given to patients in the same institution or other institutions. Therefore TLI was effective in reducing the requirement for maintenance immunosuppressive drugs in cadaveric renal transplantation.

This study, however, highlighted two inadequacies in the clinical protocol. First, was the inability to predict the timing of cadaver transplantation which resulted in heterogeneity with the TLI preparation that ranged from 6 to 19 weeks[31]. Data from the preclinical models showed that delaying the timing of transplant after completion of TLI, and extending the duration of TLI beyond 17 days impaired the ability to develop transplantation tolerance[11]. It was decided that an entirely post transplantation TLI regimen would standardize the procedure and circumvent the inability to predict the timing of transplantation. The second issue related to the dose of TLI (20Gy), which caused neutropenia, thrombocytopenia, and/or mucositis in most patients, necessitating treatment interruptions[31]. These interruptions further compromised the ability to administer a uniform dose and schedule of TLI. Therefore a lower TLI dose was required.

### 2.3.2 Use of TLI in the Treatment of Cardiac Allograft Rejection—
Between 1986 and 1995, TLI was administered to 37 cardiac transplant patients for the treatment of intractable cardiac allograft rejection at the Stanford Medical Center [34]. The prescribed radiation dose was 800cGy delivered in 80 cGy fractions several times weekly to mantle and inverted-Y plus spleen fields. The heart was not specifically included or excluded and shielding followed the protocol as outlined above for the deceased donor renal transplant recipients. Using this lower dose TLI, mucositis was minimal and there were few treatment delays because of cytopenia. The ratio of CD4 to CD8 T-cells decreased during TLI from 1.33 to 0.89 and remained low, at 0.44, when measured more than 4 months after completing treatment. This prolonged altered T cell ratio was similar to that observed following the delivery of higher dose TLI (2000 cGy in the above mentioned clinical trial).
With a median follow up of 5.7 years the rejection rate dropped from 0.46 to 0.14 and to 0.06 episode/patient/month before, during, and after TLI (p < 0.0001)[34]. Therefore, TLI at 800 cGy was well tolerated, associated with prolonged alterations in CD4/CD8 T cell ratios, and was effective treatment for the control of intractable cardiac rejection.

2.3.3 Use of Post Transplant TLI and ATG Conditioning to Induce Tolerance to Combined HLA Mismatched Living Donor Kidney and HCT—In studies of tolerance induction in Lewis rats given vascularized heterotopic heart transplants from MHC-mismatched donors, the TLI protocol was modified such that hosts were conditioned with 10 daily doses of TLI starting on day 1 after transplantation combined with 5 post transplantation doses of rabbit ATG during the first week, starting on day 0[18–21]. Although the combination of post transplantation TLI and ATG markedly prolonged graft survival, none of the hosts developed tolerance and all rejected their grafts within a 100-day observation period. In contrast, hosts that received an infusion of donor bone marrow cells after TLI and ATG was completed had prolonged acceptance of the heart allografts such that almost all continued to beat beyond 150 days[18–21] Hosts given bone marrow cells were stable multi-lineage mixed chimeras throughout a 150-day observation period. Chimerism was associated with specific unresponsiveness of host peripheral blood mononuclear cells to donor stimulator cells in the MLR, whereas PBMC from non-chimeric hosts responded to donor cells in the MLR[19].

We adapted this preclinical model to human transplantation and in 2002 reported four HLA mismatched patients given combined hematopoietic cell and living donor kidney transplants who were conditioned with post transplantation TLI (10 doses of 80 cGy each) and ATG (5 doses of 1.5mg/kg/dose) administered over 2 weeks[35]. The donor hematopoietic cell infusion was given immediately after the completion of TLI. Harvesting of the donor cells was performed approximately 6 weeks before transplant surgery, after donors received a 5-day course of granulocyte colony-stimulating factor (G-CSF) to mobilize hematopoietic progenitor cells into the peripheral blood. CD34+ cells were purified from the mobilized blood mononuclear cells (collected by 1–2 high volume aphereses) using a CD34+ cell selection column and the purified cells were cryopreserved until infusion after TLI. The purified CD34+ cell inoculum contained approximately 1×10^5 CD3+ T cells/kg and approximately 3 ×10^6 CD34+ cells/kg. Thus, the number of T cells infused was at least 1,000-fold reduced compared to that infused into cancer patients who undergo allogeneic hematopoietic cell transplantation using unmanipulated cell grafts[30]. The T cell depleted, CD34+ cell selected donor inoculum was used to reduce the risk of acute GVHD in these mismatched combinations.

Of the four donors enrolled in the combined transplant protocol, two were unrelated and two were haploidentical relatives. The recipients of the combined transplants were given full doses of prednisone and cyclosporine in the early post-transplantation period. Steroids were to be completely withdrawn from 6 to 9 months and cyclosporine by 12 months if the patients met the following criteria: (1) development of macrochimerism in the first 3 months after transplantation; (2) no clinical rejection episodes and no evidence of rejection on protocol biopsies; and (3) specific unresponsiveness to donor cells in the MLR assay[35]. Three of four patients developed transient low level of macrochimerism (peak levels of between 1–16% donor cells) that was lost within the first 3 months. Two of these three showed a pattern of specific unresponsiveness to donor cells in MLR assays performed monthly, starting at 6 months after transplantation. These patients had no evidence of clinical or histologic rejection in the first year after transplantation and were completely withdrawn from immunosuppressive drugs at 12 months. However, both patients withdrawn from immunosuppressive drugs had mild (Banff grade 1A) rejection episodes approximately 5 months later (17 months after transplantation) that rapidly resolved with steroid therapy.
Two more patients were enrolled in the same protocol for a total of 6 transplanted from 2002 to 2004. Long-term (>6 years) follow up confirmed all 6 patients have normal graft function without evidence of chronic rejection and all are maintained on low maintenance doses of one or two immunosuppressive drugs (unpublished observations). None of the patients on this trial developed GVHD, pulmonary capillary leak syndrome or opportunistic infections[35]. The lack of mixed donor:recipient chimerism persisting beyond 90 days after transplantation was considered a contributing reason for the inability to achieve immune suppression drug withdrawal without incurring a rejection episode.

2.3.4 Use of Post Transplant TLI and ATG Conditioning to Induce Tolerance to Combined HLA Matched Living Donor Kidney and HCT—In preclinical models of transplantation tolerance the continued presence of the organ donor's bone marrow- derived cells in the recipient's thymus and peripheral lymphoid tissue promoted and maintained immune tolerance by eliminating T-cell clones that react to alloantigens of the graft[36;37]. To facilitate the establishment of persistent mixed hematopoietic cell chimerism, the Stanford group applied several modifications to the HLA mismatched living donor combined kidney and hematopoietic cell transplant protocol. First, the desired CD34+ cell dose was increased to >10×10⁶CD34+cells/kg because evidence supported that higher dose CD34+ cells facilitated donor hematopoietic cell engraftment[38]. Second the infused T cell dose was increased at least 10-fold to 1×10⁶ CD3+ T cells/kg. Third, the dose of TLI was increased from a total dose of 800 cGy to 1200 cGy to more effectively reduce residual host immune cells that may mediate resistance to donor hematopoietic cell engraftment. Finally, using HLA-matched donors would was expected to favor sustained hematopoietic cell engraftment. The detailed outcome of one patient and progress of five others who received combined HLA-matched kidney and hematopoietic cells using post-transplantation TLI and ATG that included the above modifications were reported [39]. Persistent mixed chimerism that extended beyond 180 days and ranged from 20–85% donor type among blood derived CD3+, CD19+, CD15+, and CD56+ cells was observed in 4 of 6 patients. Immunosuppressive drug withdrawal with maintenance of normal graft function was achieved in the 4 patients with persistent mixed chimerism. The two patients who failed to achieve mixed chimerism persisting beyond 6 months required low dose maintenance immune suppression medication for normal graft function. The conditioning regimen and transplant procedure were not associated with notable adverse events such as a pulmonary capillary leak syndrome, severe (<500 cells/mm³) neutropenia, or GVHD. The median time for discharge from the hospital was 5 days after kidney transplantation, and the donor-cell infusion was given in all cases in the outpatient clinic. In the patient reported in detail, evidence of adequate immune reconstitution was the absence of opportunistic infections, normal in vitro T-cell responses to tetanus toxoid and influenza antigen, and a vigorous mixed leukocyte reaction to third-party stimulator cells. There was a minimal response of post-transplantation T cells from the recipient to dendritic cells from the donor, as compared to significantly greater response of pre-transplant T cells[39]. Currently, 12 patients have received transplants at the Stanford Medical Center with follow up for at least 1 year using this protocol and 8 of whom achieved persistent mixed chimerism extending beyond 6 months have been withdrawn from immunosuppressive drugs[40]. These 8 patients continued with normal graft function with follow-up 1-year beyond the time of drug withdrawal. Therefore it is feasible to achieve persistent mixed chimerism and normal graft function in the absence of immunosuppressive drugs in HLA matched humans, without the development of GVHD. We have begun a clinical study applying the above approach to HLA haplotype-mismatched patients given combined kidney and hematopoietic cell transplants (HCT). A higher dose of donor T cells is being used in this study to facilitate persistent chimerism based on our preliminary studies of haplotype-mismatched cancer patients who received HCT only.
2.4. Stanford Clinical Studies of HCT for Treatment of Leukemia and Lymphoma Using TLI and ATG Conditioning

2.4.1 Protection against GVHD in HLA Matched Recipients—The Stanford Group also studied TLI (10 doses of 80 cGy) and ATG (5 doses of 1.5 mg/kg) conditioning to reduce acute GVHD in cancer patients given only hematopoietic cell transplants[30;41]. Recipients were given postgrafting immunosuppression of mycophenolate mofetil (MMF) for 1 month, and cyclosporine with tapered withdrawal at 6 months in a study of HLA-matched related (n=61) and unrelated (n=50) transplant recipients with lymphoma and leukemia[30]. Patients were infused with 1-2 leukapheresis products of G-CSF “mobilized” blood without CD34+ cell selection (whole mononuclear cell fraction) and received approximately 3–7×10^8 CD3+ T cells/kg with approximately 3–7×10^6 CD34+ cells/kg. The regimen was well tolerated and without significant regimen related toxicity and the cell infusion was performed in the outpatient clinic in all patients. From among the 111 transplant recipients the incidence of severe acute GVHD (≥ grade II) was less than 5%[30]. Donor chimerism was evaluated in peripheral blood CD3, CD19 and CD15 compartments and sustained high levels were achieved in almost all patients. Relapse of leukemia and lymphoma was comparable to previous reports of similar patients using other reduced intensity conditioning (RIC) regimens.

The effect of TLI and ATG on circulating host T-cell subsets was monitored by analysis of peripheral blood samples at 24-hours pre-TLI and immediately post-TLI, yet before infusion of donor cells, for changes in absolute numbers of T-cell subsets. The median decrease in CD3+, CD4+, and CD8+ T cells was 191-fold, 180-fold, and 268-fold, respectively. In contrast, the median-fold decrease (8.6-fold) in invariant NKT cells was significantly less than that observed for conventional T-cell subsets (P = .001). This resulted in a significant increase in the percentage of NKT cells in the blood among gated CD3+ (T-cell receptor [TCR] γδ+)+ cell; whereas NKT cells accounted for only 0.01% of all CD3+ cells before TLI/ATG administration, 0.5% of T cells expressed the invariant NKT-cell TCR Vα24/Vβ11 after TLI [30]. Based on our preclinical studies, the preponderance of NKT cells that developed after TLI and ATG was critical for GVHD protection, because NKT cells polarized donor T cells to a Th2 phenotype with expansion of donor T regulatory cells via an IL-4–dependent mechanism[13;14;16]. This polarization markedly reduced the ability of donor T cells to mediate GVHD.

2.4.2 Relationship between Tumor Relapse and Mixed versus Complete Chimerism—Allogeneic HCT remains the only potentially curative therapy for some cancer patients with advanced stages of lymphoma and leukemia. The curative potential derives from two sources; the contribution of anti-tumor activity associated with the transplant conditioning regimen, and, the contribution from the immunologic graft-versus-tumor (GVT) reactions mediated by cells contained in or derived from the donor graft. The use of RIC regimens has shifted the burden of tumor eradication to the alloimmune GVT reactions. We and others have shown that the attainment of complete (>95%) multi-lineage donor hematopoietic chimerism relatively soon (typically within 90 days) after RIC allogeneic HCT is associated with a lower rate of cancer progression compared to recipients who achieved mixed chimerism[30;42–44]. Following TLI/ATG the relapse free survival was more than 50% at 3 years in patients who developed complete chimerism[30]. In contrast, almost all patients with mixed chimerism had cancer relapse by 3 years with significantly lower survival, as in other RIC regimens[30;42–44]. As the rate of cancer relapse is increased in recipients with persistent mixed chimerism, some investigators used an infusion of donor lymphocytes (DLI) in an attempt to convert mixed to complete chimerism[45;46]. In studies of patients with persistent mixed chimerism, DLI therapy was successful in converting chimerism from mixed to complete chimerism, and effectively
reduced the risk of subsequent tumor relapse[45;46]. However, severe acute GVHD can occur after DLI therapy, albeit at reduced rates if the initial hematopoietic cell graft is T cell depleted (see below) [45–48]. From the aggregate literature it appears that the attainment of complete chimerism is clearly associated with increased alloimmune graft versus host reactions manifesting either as GVHD, GVT reactions or both. In recent preclinical studies the Stanford Group used an infusion of CD8 memory T cells as DLI therapy to convert mixed to complete chimerism and effectively treat lymphoma relapse without GVHD[29]. This approach is being translated to clinical studies.

3. Approach II: Hematopoietic Cell Transplantation Using Nonmyeloablative Conditioning for Intentional Mixed Chimerism Induction for Organ Allograft Tolerance Induction and Followed by DLI to Achieve GVL Effects

3.1 Studies in the Mouse Model

For the purpose of allograft tolerance induction, achievement of mixed rather than full donor hematopoietic chimerism is desirable. Mixed chimerism can be achieved with less toxic (non-myeloablative) conditioning regimens than those achieving full donor chimerism, so that life-threatening marrow failure does not occur if donor marrow is rejected. Improved immunocompetence has been observed in mixed compared to fully allogeneic chimeras when complete MHC barriers are crossed because host-type APCs in mixed chimeras allow optimal antigen presentation to T cells that have developed in the host thymus, and which therefore preferentially recognize peptide antigens presented by host MHC alleles [49–51]. Host hematopoietic cells present in mixed but not full chimeras also ensure complete intrathymic deletion of host-reactive cells [52;53].

A non-myeloablative conditioning regimen consisting of low dose (3 Gy) TBI (instead of TLI), T cell depleting mAbs and thymic irradiation [54] reliably achieves a state of mixed chimerism in mice. Intrathymic deletion is the major mechanism maintaining donor-specific tolerance in this model[52;55], in which T cell alloreactivity pre-existing in both the thymus and periphery is completely eliminated in order to permit allogeneic stem cell engraftment and early seeding of the thymus with allogeneic APCs. Intrathymic alloreactivity in this model is eliminated by thymic irradiation [54;56]. This can also be achieved by BMT in combination with high doses of T cell depleting antibodies [57], or costimulatory blockers such as anti-CD154 or CTLA4Ig [58;59]. Elimination of peripheral anti-donor T cell alloreactivity can be achieved with global T cell depletion with mAbs [54;60] or with costimulatory blockers combined with BMT [59].

In addition to the induction of transplantation tolerance, achievement of initial mixed chimerism also has advantages in the treatment of hematologic malignancies. Since established mixed chimeras are immunologically tolerant of their original marrow donor, GVH reactions resulting from administration of non-tolerant T cells in donor leukocyte infusions (DLI) are not opposed by a host-vs-graft response. When DLI are administered to such animals, the unopposed GVH response results in conversion of mixed hematopoietic chimerism to full donor chimerism, which is associated with potent graft-vs-leukemia/lymphoma (GVL) effects [61;62]. Remarkably, this powerful GVH alloresponse against lymphohematopoietic cells is not associated with clinical or histological GVHD, even though administration of similar donor T cell inocula causes rapidly lethal GVHD in freshly conditioned recipients [61;63]. This separation of GVL from GVHD by giving DLI to mixed chimeras became the basis of clinical trials of mixed chimerism induction at Massachusetts General Hospital/Harvard Medical School (MGH), as described below.
Studies to understand this resistance of established mixed chimeras to GVHD when DLI are administered, despite the clear presence of an alloresponse that resulted in GVL effects and conversion of mixed to full donor chimerism, led to the conclusion that conditioning-induced tissue inflammation plays an important role in promoting GVHD. In established mixed chimeras receiving DLI, MHC-directed GVH alloreactivity is confined to the lymphohematopoietic system. DLI-derived GVH-reactive T cells are clearly activated and expand when given to established mixed chimeras [64;65], but do not migrate to the epithelial GVHD target tissues, skin, intestines and liver. This failure to traffic is due to the absence of inflammatory signals in those tissues [64], which include chemokines and adhesion molecules, are induced in target tissues by conditioning treatment and subside over time [66]. The most potent GVL effects are mediated by T cells recognizing host MHC alloantigens [62;65] presented by recipient hematopoietically-derived APCs. The requirement for recipient hematopoietic APCs for induction of GVL in such non-inflammatory conditions is a significant advantage of mixed over fully allogeneic chimerism. Fully allogeneic chimeras lack host APCs within the lymphoid tissues and therefore fail to induce activation or expansion of T cells in DLI, resulting in markedly reduced GVL effects of delayed DLI [62;65].

3.2 Clinical Strategies for Inducing Mixed Lymphohematopoietic Chimerism Followed by Delayed DLI

The MGH group began a series of clinical trials based on the above pre-clinical discoveries, in which mixed lymphohematopoietic chimerism was reliably induced following nonmyeloablative chemotherapy, in vivo T-cell depletion, thymic irradiation and MHC-mismatched bone marrow transplantation with conversion of the chimerism to full donor hematopoiesis after delayed DLI and optimization of a graft vs. tumor (GVT) effect without graft vs. host disease (GVHD) [62;63:65;67]. Conditioning therapy initially consisted of cyclophosphamide, equine anti-thymocyte globulin (ATG) and thymic irradiation [48;68–70]. Patients with a variety of advanced, refractory hematologic malignancies received either HLA-matched or haploidentical bone marrow, or in a subsequent trial, in order to reduce the risk of graft rejection, peripheral blood stem cell transplants. A short course of cyclosporine was given post-transplant for GVHD prevention. Mixed chimerism was reliably induced and “prophylactic” DLI, given to convert chimerism to full donor hematopoiesis, were given to approximately half of the initial cohort of patients beginning 5–6 weeks after the transplant. Potent and, in some cases, sustained anti-tumor responses were seen, providing proof of principle that meaningful GVT effects can be achieved following nonmyeloablative conditioning therapy and BMT, induction of mixed chimerism and conversion to full donor hematopoiesis, either spontaneously or after DLI. Prolonged mixed chimerism with minimal GVHD (which precluded the use of a prophylactic DLI) and the remission of a chemoradiotherapy refractory aggressive non-Hodgkin lymphoma following a haploidentical BMT was for the first time demonstrated on this protocol [68]. A subsequent analysis from the MGH group showed that a trend towards a higher response rate and improved progression-free survival occurred in patients with advanced lymphoma treated with the same preparative regimen who achieved late (≥ day 45) conversion of chimerism (often in response to DLI) compared to patients who achieved early conversion of their chimerism (which included patients who, in a subsequent trial, received peripheral blood stem cells instead of bone marrow) [71]. These data mirror the observation in the murine model that mixed chimerism is associated with optimal GVL effects following delayed DLI [62].

Because of a high risk of GVHD following haploidentical transplantation, the MGH group developed an alternative nonmyeloablative approach involving vigorous in vivo and ex vivo T-cell depletion of G-CSF mobilized peripheral stem cells[48]. Cyclophosphamide, pre-
transplant thymic irradiation, and short course post-transplant cyclosporine were also utilized with this approach. Monoclonal anti-CD2 antibody therapy (MEDI-507; MedImmune) was substituted for ATG for in vivo T-cell depletion. With this approach, mixed chimerism was reliably achieved with minimal or no GVHD as a platform for delayed DLI. Potent anti-tumor responses were observed in some patients with advanced lymphoma.

3.3 Clinical Tolerance Induction Studies

3.3.1 Combined HLA-Matched Bone Marrow and Kidney Transplantation for Kidney Failure Secondary to Multiple Myeloma—Patients with multiple myeloma often have impairment of kidney function. Myeloma patients with kidney failure are not candidates for kidney transplantation because of their multiple myeloma and are not usually candidates for potentially curative allogeneic HCT because of the prohibitive risk of performing such transplants in the presence of kidney failure. Based on the results of nonmyeloablative transplant protocols for advanced hematologic malignancy, the MGH group initiated a series of clinical trials intended to induce specific tolerance for the kidney graft, and in the case of myeloma, to induce potent anti-tumor responses [72;73]. Using a similar cyclophosphamide, equine ATG and thymic irradiation preparative approach to that used in other patients with hematologic malignancy, nine patients have received a combined HLA-matched kidney and bone marrow transplant for myeloma with kidney failure. The long term outcomes of the first 7 patients were recently reported [74]. Mixed chimerism was initially achieved in all 7 patients. Five of 7 patients are alive, four without recurrent myeloma from more than 4+ to 12+ years post-transplant. Three patients have normal or near normal function off of systemic immunosuppression, indicating successful tolerance induction. These encouraging results, which demonstrated the feasibility of performing combined bone marrow and kidney transplants to induce specific tolerance for the renal allograft, and to achieve sustained anti-myeloma responses, were the foundation of subsequent tolerance induction approaches.

Chimerism in the above patients has been transient in some, mixed in others (before and even following DLI) and fully donor in still others. The observation of durable remissions in these and other patients with transient chimerism has raised some interesting questions (see below). Studies of the mechanisms of tolerance in the above series of patients suggested that the renal allograft tolerance in patients with only transient chimerism was specific for minor histocompatibility alloantigens expressed by the kidney, but did not extend to antigens expressed only on hematopoietic cells. In fact, loss of chimerism was associated with increased reactivity to donor hematopoietic cells in some of the patients, despite the achievement of renal allograft tolerance [73].

3.3.2 Combined Haploidentical Bone Marrow and Kidney Transplantation for Kidney Failure (Without Underlying Malignancy)—Given the advantages of donor-specific tolerance induction (i.e. the avoidance of long-term immunosuppressive therapy), the induction of mixed chimerism following nonmyeloablative conditioning for tolerance induction was extended to patients with renal failure without an underlying malignancy by the MGH group. Preparative therapy for the transplants consisted of cyclophosphamide, MEDI-507 and thymic irradiation, a regimen that had already been shown to achieve only transient chimerism in patients with hematopoietic malignancies, and to be free of the risk of GVHD, despite crossing full haplotype HLA barriers[48]. Transient chimerism had been associated with renal allograft tolerance in the HLA-identical myeloma study described above [72;73]. Moreover, non-human primate studies performed by the MGH group had revealed that renal allograft tolerance could be achieved across MHC barriers when non-myeloablative conditioning was used with combined kidney and bone marrow
transplantation that led to only transient chimerism [75]. The lack of GVHD in the haploidentical transplant recipients with hematologic malignancies provided safety data that allowed the MGH group to try this MEDI-507-based regimen with combined kidney and BMT for tolerance induction. Despite only transient chimerism in all cases, sustained donor-specific tolerance was achieved in four of the first five patients who enrolled on this trial, with discontinuation of systemic immunosuppression from 9 to 14 months post-transplant and the demonstration of donor-specific unresponsiveness in vitro [76]. Complete donor-specific unresponsiveness in proliferative and cytotoxic assays, and in limiting dilution analyses of IL-2-producing and cytotoxic cells, developed and persisted for the 3-year follow-up in all patients, and extended to donor renal tubular epithelial cells. The T cell recovery in these patients was characterized by increased proportions of CD4⁺CD25⁺CD127⁻FOXP3⁺ Treg during the lymphopenic period [77]. Assays in 2 of 4 patients were consistent with a role for a suppressive tolerance mechanism in the first year post-transplant, but later (≥18 months) studies provided no evidence for a suppressive mechanism in any patient. These studies demonstrate the achievement of long-term, systemic donor-specific unresponsiveness in patients with HLA-mismatched allograft tolerance. While regulatory cells may play an early role and further studies are needed to address this hypothesis, long-term tolerance appears to be maintained by a deletion or anergy mechanism.

3.4 Hematopoietic Cell Graft Rejection and Sustained Anti-Tumor Responses

In initial nonmyeloablative hematopoietic cells transplant trials for hematologic malignancy performed by the MGH group, approximately one third of the patients had loss of their graft following initial mixed chimerism. Among this group of patients, anti-tumor responses were achieved in 41% of the cases, some of which were durable [78]. Included in these patients are two of the five long-term survivors of combined HLA-matched bone marrow and kidney transplantation for myeloma with kidney failure. In an effort to define the mechanism of the anti-tumor responses that occurred in association with graft rejection, nonmyeloablative transplants were performed in mice to induce mixed chimerism followed by intentional rejection of the hematopoietic graft using recipient lymphocyte infusions (RLI) [79–82]. The importance of the induction of mixed chimerism followed by RLI-induced graft rejection was demonstrated by a survival advantage of the mice who had intentional graft rejection with RLI when challenged with host strain- specific tumor cell lines, compared to mice who received conditioning alone, conditioning with RLI, or conditioning and BMT without a subsequent RLI. A clinical trial has been initiated at the MGH for patients with advanced hematologic malignancies who are not candidates for conventional hematopoietic cell transplants, using low dose total body irradiation preparative therapy, and ex vivo T-cell depleted haploidentical transplants, followed by RLI in those who achieve stable mixed chimerism. Correlative studies will be performed in an effort to define the mechanism of the graft rejection mediated anti-tumor responses.

4. Concluding Remarks

This article has reviewed studies in animals and patients that involve two different, unique approaches for optimizing GVL effects while minimizing GVHD in HCT recipients. Both approaches have successfully achieved engraftment without severe GVHD while traversing extensive HLA barriers. These achievements provide hope that more patients with hematologic malignancies lacking curative options other than HCT may have access to this therapy. While additional work is needed to lead to further improvements, both approaches have enjoyed success in avoiding the most significant complication of allogeneic HCT, namely GVHD, even while transgressing HLA barriers. Safety data in the HLA-identical setting has allowed both of these approaches to be applied successfully to a novel application of HCT, the induction of specific allograft tolerance in small groups of patients.
with HLA-identical living related donors. In addition, the approach developed by the MGH group, involving in vivo T cell depleting mAb therapy, thymic irradiation and cyclophosphamide, has successfully allowed allograft tolerance induction across full haplotype HLA barriers. Both the toxicities associated with the initial transplant protocol and the less than 100% success rate in achieving tolerance suggest that there is further room for improvement. It is expected that further advances in both of the approaches described here will ultimately permit the routine use of HCT to achieve transplantation tolerance in all recipients of organ allografts from both living and deceased donors.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>ATG</td>
<td>anti-thymocyte globulin</td>
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<tr>
<td>ATS</td>
<td>anti-thymocyte serum</td>
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<tr>
<td>BMT</td>
<td>bone marrow transplantation</td>
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<tr>
<td>DLI</td>
<td>donor leukocyte infusion</td>
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<tr>
<td>GVHD</td>
<td>graft-versus-host disease</td>
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<tr>
<td>GVL</td>
<td>graft-versus-leukemia/lymphoma</td>
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<tr>
<td>GVT</td>
<td>graft-versus-tumor</td>
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<tr>
<td>HCT</td>
<td>hematopoietic cell transplantation</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>NKT</td>
<td>natural killer T</td>
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<tr>
<td>TLI</td>
<td>total lymphoid irradiation</td>
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<td>TBI</td>
<td>total body irradiation</td>
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