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REVIEW

Clinical experience with mixed chimerism to induce transplantation tolerance

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Summary

Lymphohematopoietic chimerism was first shown to be associated with donor-specific allograft tolerance more than 60 years ago. However, early clinical experience with bone marrow transplantation soon revealed that conventional, myeloablative approaches were far too toxic and the risk of graft-versus-host disease too great to justify using this technology for the purpose of organ allograft tolerance induction in the absence of malignant disease. In this review, we discuss a step-wise approach that has been applied by several centers to establish less toxic approaches to using hematopoietic cell transplantation (HCT) for tolerance induction. These steps include (i) feasibility and efficacy data for tolerance induction in large animal models; (ii) safety data in clinical trials for patients with hematologic malignancies; and (iii) pilot trials of combined HCT and kidney transplantation for tolerance induction. Thus far, only one published trial conducted at the Massachusetts General Hospital in Boston has achieved long-term acceptance of human leukocyte antigen-mismatched kidney allografts without chronic immunosuppressive therapy. Alternative protocols have been successful in large animals, but long-term organ allograft tolerance has not been reported in patients. Thus, proof-of-principle that nonmyeloablative induction of mixed chimerism can be used intentionally to induce organ allograft tolerance has now been achieved. Directions for further research to make this approach applicable for a broader patient population are discussed.

General introduction

Donor-specific tolerance in clinical transplantation: why is it needed?

Uremia – the syndrome of fluid, metabolic and circulatory changes associated with end-stage renal failure – was universally lethal before 1960, and it is one of the important medical successes of the 20th century that most of these patients nowadays survive. Two major achievements allowed for this advance: the establishment of chronic hemodialysis as a routine treatment for uremia, and introduction of renal allotransplantation into clinical application (Fig. 1). Life expectancy is greatly prolonged by renal allotransplantation compared to chronic dialysis [1], making transplantation the treatment of choice.

The first successful renal transplant was performed in Boston in 1954 as a living-donor transplant between identical twins [2]. Although this served as proof-of-principle for the feasibility of this procedure, it was only the introduction of potent immunosuppressive drugs that allowed for widespread clinical application of renal allotransplantation. With a broad armamentarium of immunosuppressive drugs, the problem of allograft loss due to acute rejection has been mainly overcome. The major remaining challenge is late allograft failure. It is frustrating for all transplant physicians to recognize the

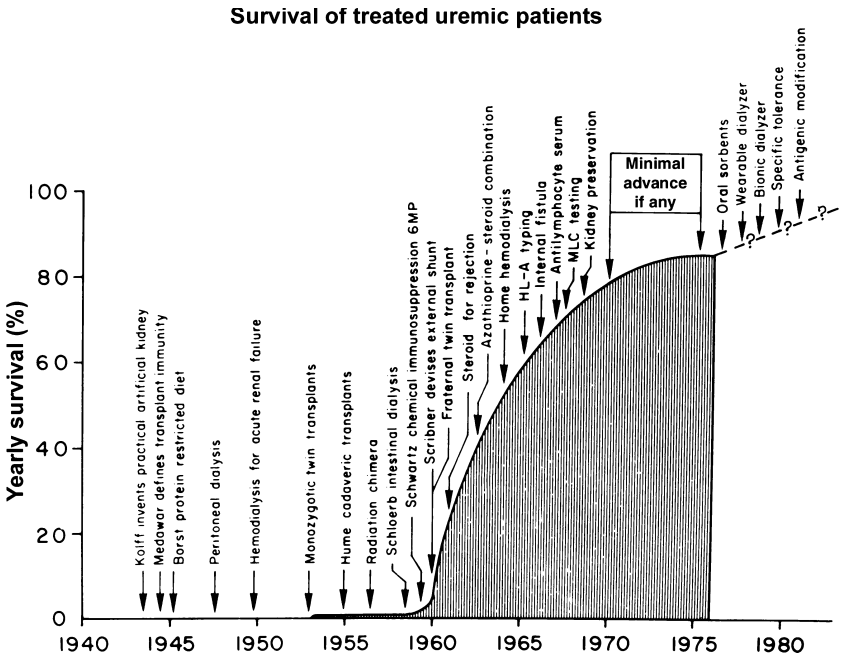

Figure 1 Survival of treated uremic patients 1940–1980. In the second half of the 20th century, the syndrome of uremia has changed from a universally lethal disease to a mostly treatable condition. This has been achieved by two major advances: introduction of maintenance dialysis and of renal allotransplantation (figure kindly provided by Prof. U. Binswanger, Prof. Emer. for Nephrology, University Hospital, Zurich, Switzerland).

unchanging downslope of late allograft loss despite modern immunosuppressive treatment. A similar situation has been encountered for all transplanted organs (Fig. 2). 'Chronic allograft nephropathy' for kidneys and 'chronic allograft vasculopathy' for hearts can be caused by both immunologic (including true chronic rejection and recurrence of immunologic diseases) and nonimmunologic mechanisms (including longer term effects of ischemia/reperfusion injury and immunosuppressive drug toxicity and side effects). In addition, one half of kidney recipients die with a functioning graft, mainly due to cardiovascular events which are often due to immunosuppressive drug side effects [3].

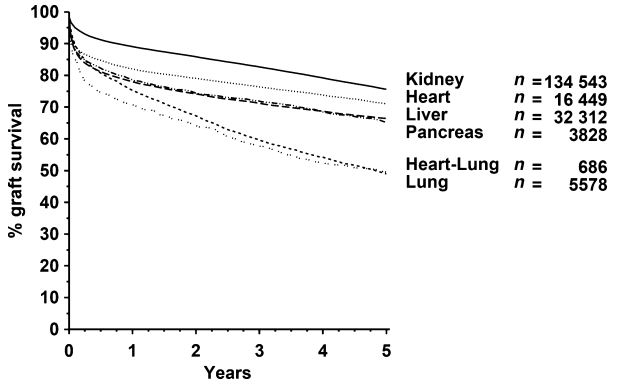

Figure 2 Five-year survival of kidney, heart, liver and lung allografts (1996–2005). From the European Collaborative Transplant Study, 5-year survival of various allografts under modern immunosuppression (postmycophenolate era) is depicted. A universal chronic allograft loss over time is evident for all types of allografts (details: see text). Reference for the diagram: E-11011-0207.

In view of this chronic loss of successfully transplanted allografts, the establishment of immunologic tolerance is of great interest because: (i) it prevents chronic rejection [4]; (ii) it obviates the need for long-term immunosuppressive drug treatment and therefore limits direct toxicity and metabolic side effects; (iii) although the initial ischemia/reperfusion injury inherent in any organ transplant procedure would not be changed by tolerance induction, tolerance would avoid the additional damage that often occurs through acute rejection episodes that are promoted by ischemic injury [5]; (iv) if combined with bone marrow transplantation (BMT), it might prevent recurrence of the primary disease in certain cases (e.g. systemic lupus erythematosus [6,7], type I diabetes [8] or certain glomerulonephritides [9]). Clinical studies demonstrating the association between donor-specific unresponsiveness and allograft survival [10,11] suggest that immunologic tolerance might indeed solve the problem of chronic allograft failure.

Definition of donor-specific tolerance for clinical purposes

'Immune tolerance' to a set of antigens can be defined as a state in which the immune system does not mount a destructive response to organs or tissues expressing those antigens, but is capable of responding normally to foreign antigens. In a clinical context, tolerance can be called 'operational' when an allograft is accepted long-term

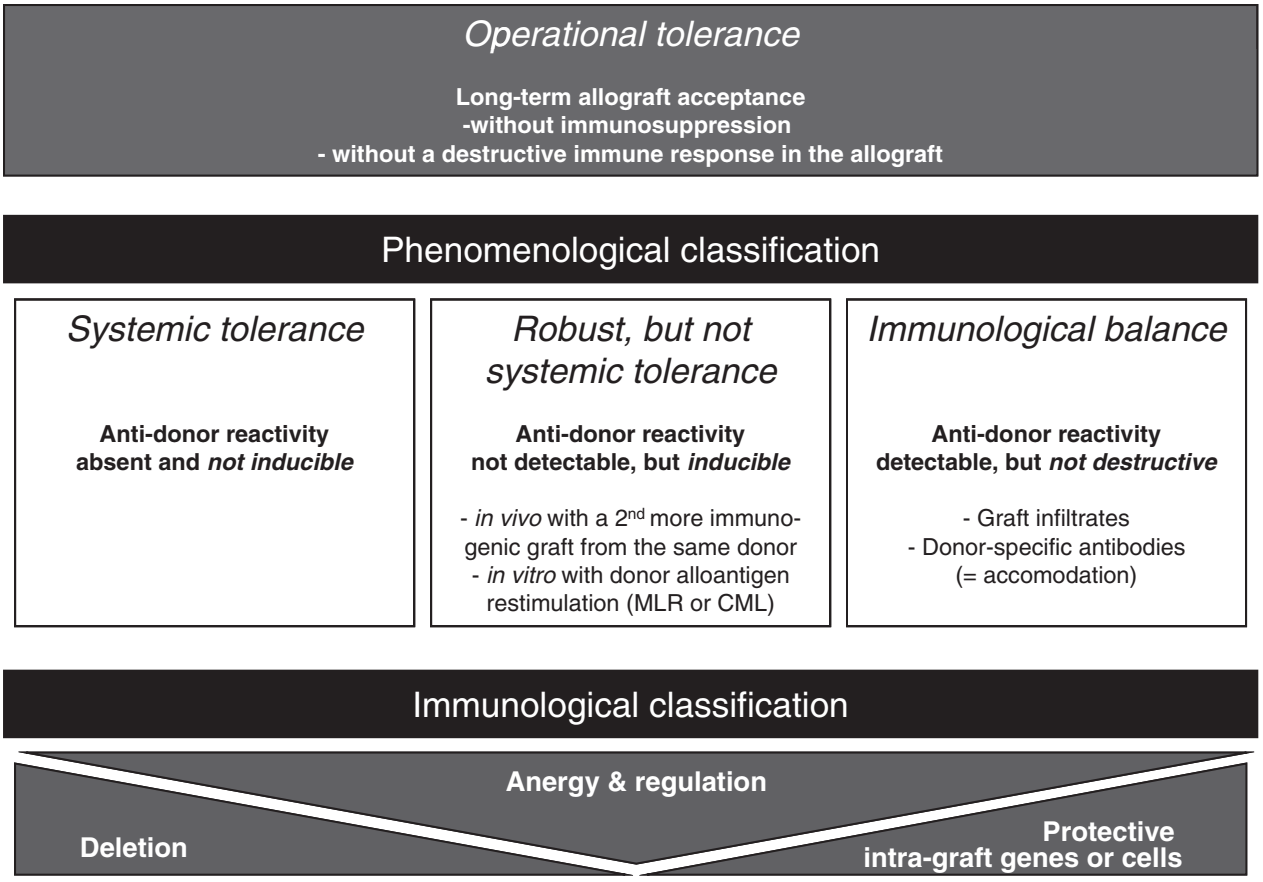

Figure 3 Definition of various states of allograft tolerance. Operational tolerance in the clinical context means long-term allograft acceptance without chronic immunosuppression and without evidence of a destructive immune response in the graft. This status includes various immunologic mechanisms, which determine the robustness of tolerance to a secondary rechallenge *in vivo* and *in vitro* (marked in blue underneath; details: see text).

without immunosuppressive therapy. This definition includes various states of tolerance (Fig. 3): the most robust form is systemic tolerance, meaning that the organism's entire immune system is tolerant to a set of alloantigens of a given donor, so that a response cannot be induced to them either *in vivo* or *in vitro*; a somewhat less robust form of tolerance is achieved when a certain allograft is accepted without immunosuppression, but a second more immunogenic graft from the same donor may be rejected. This state would also apply to the situation of graft acceptance with inducible anti-donor alloreactivity *in vitro*; finally, a more fragile state of 'immunologic balance' may be observed, in which long-term graft acceptance is achieved without immunosuppression, despite evidence for persistent anti-donor immunologic reactivity (e.g. presence of graft infiltrates and/or anti-donor antibodies; the latter is also referred to as 'accommodation'). Systemic tolerance often involves central and peripheral deletion of donor-reactive clones [12], whereas immunologic balance may include the upregulation of protective genes in the graft itself to control the destructive immune response [13]. Evidence for systemic tolerance in the preclinical and clinical setting might therefore include: (i) the absence of donor-specific alloantibodies; (ii) the absence of destructive lymphocyte infiltration in allograft biopsies; and (iii) donor-specific unresponsiveness with recovery of third party responses in functional assays *in vitro* [14].

Two recent reports have advocated the use of donor skin grafting as a measure of tolerance in the context of mixed or full chimerism induced with hematopoietic cell transplantation (HCT) [15,16]. Although skin allograft tolerance is considered to be the most stringent test of tolerance in the experimental setting, caution should be applied in concluding that skin graft acceptance would allow for kidney allograft acceptance in every situation. In the case that a minor histocompatible antigen is shared by skin and kidney but not the hematopoietic cell

graft, placing a skin graft could sensitize the host and jeopardize the success of a subsequent kidney transplant from the same living donor. Evidence for 'split' tolerance to minor histocompatibility antigens expressed on the kidney with sensitized responses to donor hematopoietic cells has been described in a recent clinical human leukocyte antigen (HLA)-identical combined kidney–BM transplant protocol [17], suggesting that tissue-specific antigen distribution is immunologically relevant in humans. In addition, as clinical HCT is mostly performed in the HLA-identical setting, a postulated kidney allograft rejection would have to be directed against minor histocompatibility antigens. In this case, a skin graft would also not help to assure a tolerant state towards a kidney allograft of the same donor, because minor antigen expression may differ between the skin and the kidney. For these reasons, we and others do not recommend using skin grafting as a test of tolerance in this clinical situation [18,19].

Measuring tolerance is still a major problem, as many of the approaches commonly used in experimental models are not applicable in a clinical setting. In patients rendered tolerant with HLA-identical combined kidney–BM transplants (see below), the absence of donor-reactive helper T lymphocytes and of CTL reacting to renal tubular epithelial cells was associated with kidney allograft tolerance, whereas the presence of CTL recognizing donor hematopoietic cells was detected in several operationally tolerant patients [17]. In a patient achieving durable mixed hematopoietic chimerism across a haplotype barrier with nonmyeloablative conditioning, a state of donor- and recipient-specific unresponsiveness of T-helper cells was recently demonstrated [20]. Recently, Brouard *et al.* reported on a new approach using gene expression microarrays on peripheral blood lymphocytes. They identified a transcriptional biomarker panel of 49 genes that was highly associated with a clinical state of operational tolerance in renal allograft recipients, who had successfully withdrawn their own immunosuppression, and stable patients on immuosuppression [21]. Martinez-Llordella *et al.* [22] applied a similar strategy for operationally tolerant liver allograft recipients and could also identify a gene profile specifically associated with the tolerance status. Although it remains to be determined whether or not this microarray approach can prospectively identify patients who will not reject their allograft after immunosuppression withdrawal, this result provides a promising approach to identifying a 'tolerance footprint' for individual patients.

Clinical considerations concerning the use of HCT for tolerance induction

Many different experimental approaches have been evaluated for the achievement of donor-specific tolerance to organ allografts. These can broadly be divided into approaches based on HCT and those that are not (reviewed in Ref. 23). As the former are the only strategies that have thus far led to successful tolerance induction in the clinical context, this review will focus on HCT-based approaches. HCT-based approaches to achieve immunologic tolerance differ from non-HCT-based approaches in at least one major respect: donor-derived hematopoietic cells may reach the recipient thymus and promote negative selection of newly generated donor-reactive T cells. In rodents, HCT-based approaches have indeed been shown to induce robust tolerance by central T-cell deletion (reviewed in Ref. 12). The clear understanding of the tolerance mechanisms involved with the achievement of durable-mixed chimerism via successful engraftment of allogeneic hematopoietic stem cells makes this a desirable approach for clinical application.

In the clinical setting, proof-of-principle for successful tolerance induction by HCT was provided by several reports of sequential allogeneic HCT followed much later by a solid organ allograft from the same donor for a new indication. Two patients confirming this finding in the clinical setting were reported by Sayegh *et al.* [24]. Both patients received a conventional HLA-identical BMT for therapy of acute leukemia. Several years later they developed chronic renal failure and subsequently received a renal allograft from the original BM donor. These grafts were accepted without immunosuppressive therapy. Subsequently, several small studies reported similar findings for kidney [25–30], lung [31] and liver [32] transplantation. Interestingly, three recent case reports showed that tolerance can also be achieved when an organ transplant is followed by BMT from the same donor [33–35]. Eight additional patients receiving BM after solid organ transplant from the same donor have been summarized [36]. These findings are encouraging, as sensitization to the donor by the allograft and consequent failure of BM engraftment might have been expected. However, the feasibility of this approach was very recently demonstrated in a nonhuman primate model, in which depletion of T cells, including CD8 memory cells, allowed successful tolerance induction to a previously transplanted kidney allograft using an established protocol for the induction of mixed chimerism and tolerance with combined BM and kidney transplantation [37].

These cases illustrate two important points: (i) Sequential organ and HCT leads to immunologic tolerance in the clinical setting, an important 'proof-of-principle' that the results from animal studies can be applied and (ii) this procedure may be a suitable option for two types of patients. These include patients needing HCT for treatment of their primary disease who subsequently develop organ failure due to graft-versus-host disease (GVHD) or

a complication of therapy (HCT itself, GVHD therapy or others) and those who need an allograft for therapy of their primary disease but have contraindications to chronic immunosuppression. For example, a patient with cholangiocarcinoma received a living-donor liver transplant for treatment of this malignancy from a donor of BMT 14 years earlier to treat lymphoblastic leukemia [32]. However, this approach is not feasible and acceptable for the vast majority of organ allograft recipients because of the high complication rate, lethality and cost of allogeneic BMT and the high risk of GVHD, even in the setting of HLA-identical HCT. Therefore, the efforts have been undertaken to develop clinical protocols to establish mixed lymphohematopoietic chimerism with reduced intensity and therefore less toxic conditioning with reduced GVHD risk. In conjunction with several additional steps, including establishment of efficacy in large animal models, the evaluation of reduced intensity conditioning protocols in patients with hematologic malignancies may provide the safety and toxicity data in humans needed for application of such protocols for clinical tolerance induction to kidney allografts. The status of various protocols with respect to these steps is discussed in the ensuing sections.

Preclinical and clinical tolerance through mixed chimerism induction with reduced intensity conditioning

Step 1: efficacy data from large animal models

All successful protocols for the induction of mixed chimerism in the preclinical and clinical settings involve three major elements: (i) a myelosuppressive treatment to promote donor hematopoietic engraftment; (ii) an immunosuppressive treatment (often involving T-cell depletion) to prevent rejection and GVHD; and (iii) a source of allogeneic hematopoietic stem cells.

Based on their previous mouse data (for review see Ref. 38), Strober *et al.* at Stanford and Myburgh *et al.* in South Africa established protocols for tolerance induction in dogs [39] and nonhuman primates [40] involving total lymphoid irradiation (TLI) and T-cell depletion with anti-thymocyte globulin (ATG). With this approach, Myburgh *et al.* observed prolonged survival of kidney and liver allografts in about one-third of the animals, some with graft survival >10 years [41,42]. Replacing ATG with anti-CD3 or anti-CD4 conjugated to idarubicin permitted operational tolerance of kidney allografts in three of six baboons [43]. Based on their dog data, Strober *et al.* [44] applied a similar regimen combining TLI and ATG to 28 patients receiving a kidney allograft, but only three of 28 developed tolerance. One of these patients was recently reported to have normal allograft function, no evidence of microchimerism in peripheral blood, no anti-HLA antibodies, but a vigorous anti-donor response in mixed leukocyte reaction (MLR), suggesting that clonal deletion was not the mechanism of tolerance induction [45]. These studies prove that immunologic tolerance of an allograft can be achieved by TLI combined with anti-T-cell antibodies. However, as the protocol led to tolerance in only a low percentage of patients and the complications of high doses of TLI (especially infectious complications and secondary malignancies) are not acceptable for routine transplantation, this approach has not been pursued further. The same group then developed a protocol based on TLI and ATG combined with donor BMT to achieve donor-specific tolerance via mixed chimerism induction. Compared to the rodent experience, the TLI regimen had to be modified due to toxicity in large animals: radiation fields were narrowed and radiation doses were reduced and fractionated. These necessary changes in the TLI regimen decreased its efficacy, as reported by Myburgh in baboons (nine of 28 monkeys achieved long-term kidney allograft acceptance [46]) and Strober in dogs (0 of 12 dogs achieved long-term heart allograft acceptance [39]). In fact, adding BMT to the TLI/ATG protocol reduced rather than increased the percentage of tolerant animals.

A different approach has been taken by Storb *et al.* from the Fred Hutchinson Cancer Research Center in Seattle. They developed a canine dog leukocyte antigen (DLA)-matched model of nonmyeloablative BMT first using cyclophosphamide and later low-dose total body irradiation (TBI) for induction of mixed hematopoietic chimerism followed by a short course of immunosuppression (mycophenolate mofetil or cyclosporine for 28–35 days) to prevent GVHD and graft rejection [47,48]. They recently reported on five mixed chimeric dogs who accepted kidney allografts from their DLA-identical hematopoietic cell donors long-term without immunosuppression, whereas kidney allografts transplanted in the opposite direction were promptly rejected. In this study, two dogs received BM cells, two dogs received mobilized peripheral stem cells and one dog received both [49]. In a very recent study, they used the same protocol for DLA-identical BMT using two marrow donors per recipient, as multiple donors have been used clinically to enhance engraftment in the context of umbilical cord grafts. In this study, five of eight dogs were stable trichimeras, two were stable chimeras from one donor and one rejected both grafts. Five of the seven chimeric dogs received kidney allografts from their HCT donors at least 6 months after BMT, and four of five grafts were accepted long-term without immunosuppression [50].

Another approach has been evaluated by the group of Sachs, Sykes, Cosimi and Kawai at Massachusetts General

Hospital in Boston, who have had a long-standing interest in nonmyeloablative induction of mixed chimerism for tolerance induction. They developed a protocol to induce mixed chimerism in the mouse, which consisted of T-cell depletion with monocloncal anti-CD4 and -CD8 antibodies, low dose TBI (3 Gy), thymic irradiation (TI) and BMT [51]. The protocol was successfully translated into two large animal models: in MGH miniature swine, stable mixed chimerism and long-term donor-type skin graft acceptance was achieved with 3 Gy TBI divided into two fractions, 7 Gy TI, T-cell depletion with CD3-immunotoxin and BMT followed by a 30-day course of cyclosporine [52]. A similar protocol using ATG instead of CD3-immunotoxin was used for combined kidney and BM transplantation in fully major histocompatibility complex (MHC)-mismatched cynomolgus monkeys. With this protocol, long-term survival of fully mismatched kidney allografts was achieved in eight of 13 (62%) monkeys overall and in eight of 11 (73%) chimeric monkeys [53]. Of note, only animals that achieved mixed chimerism developed tolerance; however, most of them lost mixed chimerism later without rejection of the kidney allograft. The reason for this 'split tolerance' has not been elucidated, though a similar phenomenon has now been reported in patients (see below). Second, splenectomy was a necessary part of the protocol to prevent alloantibody production. Splenectomy was later successfully replaced with anti-CD154 monoclonal antibody (mAb) [54]. The same protocol used in the context of fully mismatched heart instead of kidney transplantation led to a prolongation of graft survival, but tolerance was not achieved [55].

In the above-mentioned nonhuman primate model, Kawai *et al.* showed that each of the elements of this protocol was necessary to achieve tolerance to a fully mismatched kidney allograft. The TI serves mainly to deplete donor-reactive thymic mature T cells, which are not depleted or tolerized by circulating anti-T-cell depleting antibodies, thereby permitting intrathymic engraftment of tolerogenic donor-derived dendritic cells [56–58]. As TI might be associated with delayed T-cell recovery in people, especially older individuals, efforts have been made to replace it with other modalities. In the mouse model, we have demonstrated that the need for TI can be overcome by the introduction of co-stimulatory blockade with one injection of either CTLA4-Ig or anti-CD154 mAb [59] or by a more intense course of T-cell depleting mAbs, which also inactivates alloreactive thymocytes [58]. However, when anti-CD154 was utilized in a mixed chimerism protocol in nonhuman primates, thromboembolic complications occurred [60]. Thus, based on the mouse model, the replacement of TI with either CTLA4Ig, which is currently being evaluated in clinical trials, or with a more clinically applicable agent for blocking CD40–CD154 interactions, can be envisioned. In the meantime, however, it is significant that the MGH group now has experience with over 200 combined kidney/BM transplants in nonhuman primates, 12 combined kidney/BM transplants in humans and over 60 BM transplants in patients with hematologic malignancies using a protocol that includes 7 Gy TI, and no undesirable side effects (such as thymoma, hypothyroidism or hypoparathyroidism, thyroid carcinoma or other adverse effects) were observed, although some of the patients and monkeys now have been followed for more than 10 years.

Step 2: safety and toxicity data from clinical trials in patients with hematologic malignancies

A large number of protocols using reduced intensity conditioning for HCT in the setting of malignant disease have been published. In the following section, we only present results from three groups who have established reduced intensity HCT protocols in large animal models (see Step 1) not only with the goal of developing less toxic treatments for patients with hematologic malignancies, but also with the intention to potentially apply such protocols for benign conditions such as induction of transplantation tolerance. While the terms 'reduced intensity' and 'nonmyeloablative' have been used interchangeably by some authors, we restrict the term 'nonmyeloablative' to conditioning regimens that have been shown to leave sufficient hematopoietic progenitors and stem cells intact to allow robust host hematopoiesis to occur in the absence of a marrow graft or following rejection of donor hematopoietic cells.

Strober *et al.* recently reported successful translation of their reduced intensity conditioning regimen based on TLI (10 doses of 80 cGy each) and T-cell depletion with ATG (five doses of 1.5 mg/kg each) followed by HLA-matched mobilized peripheral-blood mononuclear cells to 37 patients suffering from either lymphoma or acute leukemia. The patients were either >50 years old, had preexisting medical conditions or had received prior therapy and were therefore considered to be at too high risk for a conventional myeloablative HCT. This regimen showed a high safety profile, as only one of 37 patients developed acute GVHD ≥ grade II. However, 29 of 37 patients became full donor chimeras and sevem of them developed extensive chronic GVHD. Of the 10 patients who died, three died of treatment-related causes (acute GVHD, sepsis, thrombotic thrombocytopenic purpura), six of progressive disease and one due to suicide (Table 1 [61]). In a report on the follow-up this group, >100 patients treated with this protocol had a very low rate of acute GVHD (4% [62]).

Table 1. Induction of mixed chimerism with reduced intensity HCT protocols for treatment of hematologic malignancies.

Center	Protocol	Patients	Follow-up	Engraftment	GVHD	Disease status/outcome	Ref.
Stanford University School of Medicine	TLI (10 × 80 cGy) ATG (5 × 1.5 mg/kg) Mobilized peripheral blood mononuclear cells, all *HLA-identical* *GVHD prophylaxis*: cyclosporine day −3 to 180, MMF day +1 to 28	*N* = 37 13 acute leukemias 2 Hodgkin 22 NHL/CLL	Mean 482 days (222–1069)	100% initial mixed chimerism *Follow-up*: 6/37 graft loss, 2 stable mixed chimeras, 29 full donor chimeras	Acute ≥ Gr II: 1/37 Chronic extensive: 7/37	Acute leukemias: • Entry: 13/13 in CR • Follow-up: 10/13 alive; 9 CR, 4 relapse Lymphomas: • Entry: 4 CR, 18 PR, 2 PD • Follow-up: 17/24 alive; 15 CR, 2 PR, 5 PD, 2 early deaths	61
Fred Hutchison Cancer Research Center, Seattle	TBI (2 Gy; all patients) Fludarabine (3 × 30 mg/m^2, 75/120 patients) Mobilized peripheral blood mononuclear cells (*n* = 110) or bone marrow (*n* = 10), all *HLA-identical* *GVHD prophylaxis*: cyclosporine day −3 to 35/100, MMF day 0 to 27/96	*N* = 120 16 acute leukemias 14 CML 11 Hodgkin 32 NHL/CLL 27 myeloma/ Waldenström 20 MDS	Mean 199 days	100% initial mixed chimerism *Follow-up*: 12/120 graft loss, most mixed chimeras with increasing chimerism up to 180 days post-transplant	Acute ≥ Gr II: 67/120 Chronic: 42/120	Measurable disease at entry: 93/120 Follow-up: • 1 year survival 63/120 • 1 year progression-free survival 50/120	63
Massachusetts General Hospital, Boston	Cyclophosphamide (3–4 × 50 mg/kg) Equine ATG (3–4 × 15–30 mg/kg) TI (7 Gy) Bone marrow cells, *HLA-identical* *GVHD prophylaxis*: cyclosporine day −1 to ≥35 *DLI* for patients with mixed chimerism and no GVHD (individual decision)	*N* = 42 5 acute leukemias 7 Hodgkin 27 NHL/CLL 2 myeloma 1 MDS	>300 days	100% initial mixed chimerism *Follow-up*: • 16 patients with mixed chimerism received DLI → 10/16 full donor and 2/16 mixed chimeras, 4 graft losses • 26 patients without DLI → 5/26 full donor & 7/26 mixed chimeras, 10 graft losses, 4 not evaluable	Acute ≥ Gr II: 12/26 without DLI, 7/16 with DLI Chronic: not reported	Patients with DLI (*n* = 16): 7 alive; 8 CR, 3 PR, 5 PD Patients without DLI (*n* = 26): 4 alive; 7 CR, 2 PR, 17 PD	69

Table 1. continued

Center	Protocol	Patients	Follow-up	Engraftment	GVHD	Disease status/outcome	Ref.
Massachusetts General Hospital, Boston	Cyclophosphamide (3 × 50 mg/kg) Siplizumab (total of 2 mg/kg in 4 doses) TI (7 Gy) Bone marrow cells (cohort 1 and 2), mobilized $CD34^+$ peripheral stem cells (cohort 3), *HLA-mismatched haploidentical* *GVHD prophylaxis:* cyclosporine day −1 to ≥35 *DLI* for patients with mixed chimerism and no GVHD (individual decision)	*N* = 12 (3 cohorts of 4) 1 acute leukemia 2 Hodgkin 9 NHL	Up to 800 days	100% initial mixed chimerism *Follow-up:* • Cohort 1: 4 graft losses • Cohort 2: 2 full donor chimeras, 2 graft losses • Cohort 3: 1 full donor and 1 mixed chimera, 2 graft losses	Cohort 1: no GVHD Cohort 2: 2 acute ≥ Gr II, 1 chronic extensive Cohort 3: 1 acute ≥ Gr II post-DLI	• Cohort 1: 1/4 alive; 1 CR, 3 PD • Cohort 2: 2/4 alive; 2 CR, 1 PD, 1 early death • Cohort 3: 1/4 alive; 2 PD, 2 PR	73

Storb *et al.* recently reported on 120 patients treated with their reduced intensity regimen for HLA-matched allogeneic HCT established in dogs, which consisted of low dose TBI (2 Gy) with or without additional fludarabine [63]. As for the Stanford protocol, patients were included when they suffered from a hematologic malignancy, but were ineligible for conventional allogeneic HCT because of age, comorbidities or previous therapies. All were HLA-matched grafts (71% related). The rate of initial engraftment was 100%, and most patients (90%) achieved durable chimerism. However, and in contrast to the large animal data, a high rate of acute (56%) and chronic (35%) GVHD was observed. This resulted in good anti-tumor responses (44% of patients having measurable disease at study entry achieved complete remission), but the high rate of GVHD renders this protocol unsuitable for tolerance induction to solid organ allografts in patients without malignant disease, despite the success of the canine model (see above). These data highlight the importance of assessing safety of HCT protocols in an appropriate group of patients with malignant disease before extending them to the induction of organ allograft tolerance in patients without malignancy, even if the protocol has been successfully tested in a large animal allograft tolerance model.

In contrast to other reduced intensity regimens for HCT for the treatment of hematologic malignancies, in which full donor chimerism is sought, the approach used by Spitzer *et al.* involves the intentional induction of mixed lymphohematopoietic chimerism. Truly non-myeloablative protocols that include cyclophosphamide, TI, ATG or MEDI-507 (siplizumab) have been developed for HLA-matched and haploidentical HLA-mismatched HCT. These protocols are based on the results of rodent studies showing that induction of mixed chimerism without an initial GVH response can be followed >5 weeks later by donor lymphocyte infusions (DLI), which mediate potent graft-versus-tumor effects without inducing GVHD, while converting mixed to full donor chimerism. In this mouse model, DLI-induced GVH responses have been shown to be confined to the lymphohematopoietic system when GVH reactivity from the initial HCT is avoided [64–67]. The initial presence of recipient professional antigen-presenting cells in mixed chimeras is key in allowing this graft-versus-leukemia response to occur [68]. Spitzer *et al.* reported on 42 patients who received HLA-identical BMT for refractory lymphohematopoietic malignancies [69] using a protocol derived from the above rodent studies, involving cyclophosphamide, TI, equine ATG and BMT. This protocol also shared many features with the protocol used for induction of tolerance through combined kidney/BM transplantation by Kawai *et al.* in cynomolgus monkeys (see above). The major

difference was the use of cyclophosphamide instead of TBI for cytoreduction. The results are summarized in Table 1: a total of 19/42 (45%) patients developed ≥grade II GVHD, with or without DLI. Among those patients receiving DLI (16/42), eight achieved a complete remission and three achieved a partial remission. A total of seven patients achieved durable complete remissions, which was quite surprising in this group of patients with particularly poor prognoses. While the incidence of GVHD in this trial group was acceptable for patients with a hematologic malignancy, this regimen could not be broadly applied to HLA-matched kidney transplantation for the sole purpose of tolerance induction. However, the safety data achieved in the above trial allowed its later application in a trial of combined kidney/BM transplantation in patients with multiple myeloma and end-stage renal failure (see below).

The above protocol led to an unacceptably high incidence of acute GVHD when applied in patients with haploidentical, HLA-mismatched donors, but it provided the first proof that mixed chimerism could be intentionally achieved across extensive HLA barriers in humans [70]. Because of the greater incidence of GVHD observed with the equine ATG-based regimen in HLA-mismatched haploidentical transplants, the same group has been developing a similar protocol for HLA-mismatched HCT using a more potent T-cell depleting agent, namely a humanized anti-CD2 mAb, siplizumab (MEDI-507 [71]). In addition to inducing more extensive T-cell depletion than ATG, siplizumab also depletes CD2-bearing natural killer cells when used in this regimen [72]. In the initial study, three cohorts of four patients each received variations in the protocol in relation to the timing of siplizumab and the source of HCT (BM cells in cohort 1 and 2, mobilized peripheral stem cells in cohort 3) [73]. The results of cohort 1 in this study were appropriate for evaluation in a protocol for organ tolerance induction, as initial mixed chimersim was achieved in all patients and no GVHD was observed.

Step 3: clinical trials for induction of tolerance to solid organ allografts via induction of mixed lymphohematopoietic chimerism

Beginning with the observations of Owen [74] more than 60 years ago, it has been known that mixed hematopoietic chimerism, when established in the fetus or neonate, leads to transplantation tolerance. It was later demonstrated that mixed allogeneic chimeras produced by reconstitution of lethally irradiated adult mice with a mixture of T-cell-depleted allogeneic and host-type marrow were specifically tolerant of donor tissue grafts, with full immunocompetence that was superior to that of full allogeneic chimeras [75]. Unfortunately, the potential toxicity of this regimen and the greater difficulty achieving engraftment of T-cell-depleted MHC-mismatched marrow in humans made it unsuitable for the clinical application of tolerance induction. In the mid-1970s Monaco *et al.* for the first time used anti-lymphocyte globulin (ALG) for induction therapy in renal transplantation, which was followed by conventional immunosuppression with prednisone, azathioprine and donor BM cell infusion. This so-called 'Monaco model' was then tested in a landmark trial by Barber *et al.* who analyzed 57 cadaveric renal allograft recipients who received quadruple immunosuppression with ALG, cyclosporine, azathioprine and prednisone. These patients were compared to 54 kidney recipients receiving the contralateral organ of the same donors with identical immunosuppression, but no BM infusion [76]. The differences were striking: three graft losses and one chronic rejection in the BM group, 13 graft losses and five chronic rejections in the control group. Many more patients in the BM group could be tapered off prednisone, but operational tolerance with the ability to discontinue all immunosuppression was not demonstrated. Since then, and nurtured by the microchimerism theory of Starzl *et al.* [77], many trials have been performed in attempts to induce donor-specific hyporesponsiveness via donor BM infusion, not only in kidney, but also liver, heart, lung and pancreas transplantation. In general, good graft survival has been achieved, perhaps with some reduction in chronic rejection, but clear-cut benefits of additional BM or peripheral stem cell infusion have not been demonstrated [76,78].

Currently, we are aware of three centers who systematically applied a reduced intensity conditioning regimen followed by HCT for tolerance induction to living-donor kidney allografts as well as one center using a similar approach for living-donor liver allografts. The results are described below and summarized in Table 2.

Strober *et al.* expanded their experience with the TLI protocol to HLA-mismatched combined kidney and HCT using mobilized $CD34^+$ peripheral stem cells instead of BM, together with TLI and ATG. This treatment was followed by maintenance immunosuppression with cyclosporine and prednisone, which was gradually tapered over time. In 2008, this group reported on six patients treated with this protocol, five of whom achieved multilineage macrochimerism with up to 16% donor-derived cells in peripheral blood mononuclear cells and no GVHD [62,79]. Chimerism was lost in all five patients within the first 3 months. However, two of them developed donor-specific unresponsiveness as measured by MLR. These two patients were weaned of all immunosuppression and developed Banff grade I cellular rejection within 5 months

Table 2. Induction of mixed chimerism with reduced intensity HCT protocols for achievement of organ allograft tolerance.

Center	Protocol	Patients	Follow-up	Engraft-ment	GVHD	Outcome	Ref.
Stanford University School of Medicine	ATG (5–6 × 1.5 mg/kg rabbit ATG) TLI (10 × 0.8 Gy) $CD34^+$ mobilized peripheral stem cells, *HLA-mismatched* *Immunosuppression and GVHD prophylaxis:* cyclosporine, prednisone up to 12 months	*N* = 4 *Kidney grafts* 1 lupus nephritis, 1 polycystic kidney disease, 2 glomerulonephritis	Up to 3 years	3/4 with macrochimerism All lost by 3 months post-transplant	None	2/4 weaned of all immunosuppression, both with subsequent Banff I rejection No tolerance achieved	79,80
Stanford University School of Medicine	ATG (5 × 1.5 mg/kg rabbit ATG) TLI (10 × 0.8 Gy) $CD34^+$ mobilized peripheral stem cells, *HLA-identical* *Immunosuppression and GVHD prophylaxis:* cyclosporine (stopped after 6 months), MMF (stopped after 1 month), prednisone (stopped after 10 days)	*N* = 3 *Kidney grafts* 1 focal and segmental glomerulosclerosis (FSGS), 2 unknown	Up to 2 years	2/3 with macrochimerism (one stable and one transient mixed chimera)	None	1/3 weaned of all immunosuppression and tolerant 1/3 weaned with subsequent rejection Banff I 1/3 not weaned because of recurrent FSGS	81
Massachusetts General Hospital, Boston	Cyclophosphamide (2 × 60 mg/kg) Equine ATG (3–4 × 15–30 mg/kg) TI (7 Gy) Bone marrow cells, *HLA-identical* *Immunosuppresion and GVHD prophylaxis:* cyclosporine day −1 to 60–90 *DLI* for patients with mixed chimerism and no GVHD (individual decision)	*N* = 6 *Kidney grafts* All myeloma kidney	Up to 9 years	6/6 with initial macrochimerism	2/6 GVHD (full donor chimeras, 1 following early DLI to treat progressive myeloma)	6/6 tolerant (2/6 as full donor chimeras) 1/6 cellular rejection (resolved under standard treatment, now weaned of all immunsuppression) Myeloma: 3/6 complete remission (follow-up 3.5–9 years), 2 partial remission (later progressive), 1 PD	17,82,83

Table 2. continued

Center	Protocol	Patients	Follow-up	Engraft-ment	GVHD	Outcome	Ref.
Massachusetts General Hospital, Boston	Cyclophosphamide (2×60 mg/kg) Rituximab (2 doses of 375 mg/m^2; patients 4 and 5) Siplizumab (total of 2 mg/kg in 4 doses) Prednisone 2 mg/kg day 0, tapering until day 10, then discontinued (patients 4 and 5) TI (7 Gy) Bone marrow cells, *HLA-mismatched* *Immunosuppression and GvHD prophylaxis:* cyclosporine day −1 to 9 months	*N* = 5 *Kidney grafts* 2 Alport, 2 polycystic kidney disease, 1 glomerulonephritis	Up to 5 years	5/5 with initial macrochimerism All lost by 3 weeks	None	4/5 tolerant, one graft loss due to antibody-mediated rejection	85
Institute of kidney diseases, Ahmedabad, India	Two donor-specific transfusions Cyclophosphamide (2×10 mg/kg) ATG (1×1.5 mg/kg) Intrathymic transplantation of donor renal tissue High-dose HCT (BMC day 0, PBSCs day 3 and 6; to BM, portal and systemic circulation), 5 *HLA-matched,* 28 *HLA-mismatched* *Immunosuppression and GVHD prophylaxis:* cyclosporine day 4 to 12 months, prednisone	*N* = 33 (*and* 33 *controls*) *Kidney grafts* 16 glomerulonephritis, 8 obstructive uropathy, 5 diabetic nephropathy, 2 polycystic kidney diseases, 1 reflux, 1 lupus	Up to 210 days (weaned patients)	Rate and stability of engraft-ment not reported	Not reported	4 patients weaned from all immunosuppression and rejection-free by day 210	87,95

Table 2. continued

Center	Protocol	Patients	Follow-up	Engraft-ment	GVHD	Outcome	Ref.
Université libre de Bruxelles, Belgium	Cyclophosphamide (4 × 50 mg/kg) ATG (3 × 3.75 mg/kg) $CD34^+$ mobilized peripheral stem cells, *HLA-mismatched* Live-donor liver grafts 40–55 days after HCT *Immunosuppression and GVHD prophylaxis:* tacrolimus (pt 1: day 5–day 90) or rapamycin (pt 2: day 1–day 28), prednisone (pt 1: day 0–day 12, pt 2: day 1–day 3)	*N* = 2 *Liver grafts* 1 hepatocellular carcinoma with alcoholic cirrhosis, 1 cholangiocarcinoma	370 and 270 days, respectively	Transient chimerism in pt 1, no chimerism in pt 2	None	Donor-specific hyporesponsiveness in pt 1, global hyporesponsiveness in pt 2 No rejections during follow-up Pt 1 died on day 370 from tumor recurrence; pt 2 alive with suspected tumor relapse	88,94

after discontinuation of immunosuppressive drugs. They were subsequently maintained on low dose immunosuppression. Thus, although maintenance immunosuppression was reduced compared to standard kidney transplantation, operational tolerance was not achieved with this regimen [62,80]. Based on this experience, this group tested a similar protocol for HLA-matched combined kidney and BMT. Three patients were involved in this study so far, and one of them achieved stable mixed chimerism and operational tolerance for 2 years after transplantation. Another patient developed only transient mixed chimerism and suffered from mild rejection after weaning of immunosuppression. The third patient did not develop any chimerism and had recurrent focal and segmental glomerulosclerosis in the transplant. Therefore, this patient was not weaned from immunosupression [62,81].

Based on their nonhuman primate data and the clinical data in patients with hematologic malignancies discussed above, the team at Massachusetts General Hospital launched two tolerance trials for combined kidney and BM transplantation, both sponsored by the Immune Tolerance Network (ITN). The first trial involved patients with end-stage renal disease due to multiple myeloma – a patient group that is usually not considered for HCT due to poor general health – based on the idea that concomitant BMT might not only allow establishment of immunologic tolerance, but might also produce a 'graft-versus-myeloma' effect, as suggested by the results in patients with refractory hematologic malignancies described above. The tolerance protocol included myelosuppressive treatment with cyclophosphamide, T-cell depletion with equine ATG and 7 Gy TI followed by combined renal allograft and BM transplantation from the same HLA-identical related donor, with a very short course of post-transplant cyclosporine (ca. 60 days). Results from six patients have been published so far, with follow-ups from 3.5 to 9 years. Four of these patients were transplanted under a protocol sponsored by the ITN. All six patients achieved initial macrochimerism. Two of six developed full donor chimerism (one spontaneously, one after DLI) and both developed GVHD. One of them returned to dialysis after 2 years due to recurrence of myeloma kidney. The other four patients lost their BM grafts within 3 months, but retained the kidneys between 2.5 and 9 years of follow-up [17,82,83]. Detailed *in vitro* studies performed on these patients revealed a state of 'split tolerance', in which recipient T cells did not respond to donor renal tubular epithelial cells, but in some cases showed sensitized responses to minor histocompatibility antigens expressed on donor hematopoietic cells during and following their rejection [17]. These intriguing results

support a role for the renal allograft itself in the maintenance of operational tolerance, as has been reported in the monkey model [84].

Although these results were very encouraging, they were not widely applicable, as only very few patients in need of an allograft have a suitable HLA-identical living donor, and GVHD is not an acceptable risk in patients who do not have malignant disease. However, in the meantime the MGH group published the above-mentioned trial of haploidentical HCT using the anti-CD2 mAb siplizumab [73]. The first cohort of four patients reported in this trial all lost their BM graft and had no GVHD. Therefore, this protocol was considered safe for evaluation in a clinical trial for organ tolerance and was used as the basis a for second ITN clinical tolerance trial, in this case for combined kidney/BM transplantation from haploidentical related donors in recipients without malignant disease [85]. This was further justified by the large animal data discussed above, in which renal allograft tolerance was achieved in the presence of only transient chimerism. In this trial, five patients have been transplanted. The follow-up is about 2 to more than 5 years after transplant. Four of five patients have been successfully taken off their initial immunosuppressive monotherapy with calcineurin inhibitor, and have had stable, normal graft function off immunosuppression for 1–4.5 years. One patient lost his graft early to a severe antibody-mediated rejection which occurred within 2 weeks after the combined transplant. Because of the experience with this patient and with three of the other patients, who developed an engraftment syndrome [86] that was associated with allograft dysfunction in two, pretransplant rituximab and postoperative corticosteroids (for 10 days) were added to the protocol. *In vitro* analyses revealed the progressive development of donor-specific unresponsiveness in both MLR and CML assays (with robust third party responses) in all four patients who have accepted their grafts without immunosuppression, suggesting a state of systemic tolerance. While the exact mechanism of tolerance is not currently understood, comparison of these results with *in vitro* assays on patients with hematologic malignancies who received BMT without a kidney transplant, with a similar conditioning regimen, strongly implicates the kidney as playing a role in the achievement of tolerance. The patients who received BMT without a kidney transplant showed stronger anti-donor than anti-third party responses following the loss of chimerism [20]. This is the first trial with successful intentional induction of tolerance to an organ allograft across HLA barriers. Intragraft levels of FoxP3 relative to Granzyme B mRNA were increased in tolerant patients compared to patients on immunosuppression, raising the possibility that regulatory T cells might play a role in tolerance [85].

A third group, led by Trivedi *et al.* at the Institute of Kidney Diseases & Research Centre in Gujarat/India, has reported on successful tolerance induction with a protocol including intrathymic transplantation of donor renal tissue, two donor-specific transfusions and high-dose HCT (applied to peripheral and portal circulation as well as intra-bone injection). They used a reduced intensity conditioning regimen including cyclophosphamide, T-cell depletion with ATG and localized low dose irradiation (abdominal and inguinal lymph nodes, thoracolumbar vertebrae and part of the pelvis). Renal transplantation was performed after a documented negative cross-match. If donor-specific antibodies were detected, a desensitization protocol including intravenous immunoglobulin and plasmapheresis was applied. Sixty-six consecutive patients scheduled for a living-donor kidney transplant were randomized to receive either this tolerance protocol or standard kidney transplantation followed by triple immunosuppression [87]. The tolerance group achieved significantly better kidney function. By the time of the report, four patients were completely weaned of all immunosuppression with no rejection up to 210 days. However, no details on chimerism and GVHD or any functional immunologic assays were reported. The lack of detail in reporting this trial is of some concern, as this regimen is quite invasive. Specific information on therapy-related side effects (morbidity or mortality) or on the nature of the 'tolerance status' achieved was not included in the report and the possibility of chronic allograft failure remains.

Recently, Donckier *et al.* [88] at the Université Libre de Bruxelles/Belgium reported on the first two patients treated with a nonmyeloablative protocol for tolerance induction to living-donor liver allografts. The rationale for this treatment was a recurrent and unresectable tumor (one hepatocellular and one cholangiocarcinoma) in both cases with the intention to remove the tumor by total liver resection and then allow for an anti-tumor response against residual tumor cells by using a tolerance protocol with early withdrawal of all immunosuppression. The protocol included cyclophosphamide as myelosuppressive treatment, T-cell depletion with ATG and HCT with mobilized $CD34^+$ peripheral blood stem cells (PBSCs) followed by a liver allograft 40 and 55 days later, respectively. Immunosuppression was stopped early (day 90 in patient 1 and day 28 in patient 2). Patient 1 had transient chimerism and developed donor-specific hyporesponsiveness when assessed by interleuin-2 transcription in MLR. In contrast, patient 2 had no measurable chimerism and showed global hyporesponsiveness, so specific tolerance could not be assessed. Unfortunately, both patients devel-

oped tumor relapse, from which patient 1 died 1 year after transplant. To the best of our knowledge, this is the first patient intentionally made tolerant to a liver allograft by a nonmyeloablative mixed chimerism protocol.

Conclusions and outlook

More than 60 years after Owen's observation that spontaneous mixed lymphohematopoietic chimerism leads to donor-specific tolerance of tissue allografts, this approach to tolerance induction has now been used intentionally to achieve clinical organ allograft tolerance. A large body of immunologic studies in rodents and in large animal models has laid the foundations for an understanding of tolerance mechanisms associated with mixed chimerism, but much remains to be learned. In several centers, the safety data obtained through the successful treatment of patients with hematologic malignancies with nonmyeloablative reduced intensity HCT protocols have allowed their application in attempts at tolerance induction. In general terms, the protocols evaluated in the clinical setting share the following three elements:

1 Myelosuppressive treatment to allow for donor hematopoietic cell engraftment (either by chemotherapy, TLI or TBI).

2 Immunosuppressive treatment to prevent GVHD and rejection (pretransplant with TLI, TBI and T-cell depletion via TI, polyclonal Ab such as ATG or mAb such as siplizumab; post-transplant with further antibody treatment and/or a limited course of nonspecific immunosuppressive therapy).

3 HCT (either with BM, $CD34^+$ PBSCs or a combination of both).

This research has led to the following conclusions: (i) Clinical tolerance induction by induction of mixed chimerism using reduced intensity conditioning is feasible; (ii) a reliable regimen to induce stable mixed chimerism across HLA barriers in humans has not yet been established; and (iii) to achieve tolerance to kidney (and probably also liver) allografts, chimerism is required, but transient chimerism is sufficient and is associated with a low risk of GVHD. However, a tolerant state fully relying on peripheral and, at least for a time, nondeletional tolerance mechanisms, might be expected to be susceptible to perturbation by events associated with immune activation, such as infections.

Therefore, future developments on this promising approach for tolerance induction may include advances in the following areas:

1 Development of even less toxic conditioning regimens would allow a broader applicability of such protocols. A more detailed understanding of peripheral tolerance mechanisms and the role of HCT in their development should allow the design of more efficient and less toxic conditioning regimens (e.g. by inclusion of mAb treatments to block costimulation and/or cell adhesion).

2 Establishment of a protocol to achieve stable mixed chimerism would be desirable, as only this approach will lead to life-long central deletion of donor-reactive cells in the thymus, achieving a robust and well-understood donor-specific tolerant state. Animal experiments investigating the influence of inflammation on allograft tolerance suggest that a tolerance mechanism relying purely on peripheral tolerance mechanisms may be more susceptible to disturbances, e.g. in the context of infections [89–91]. Furthermore, the persistence of a population of donor hematopoietic cells comes with the risk that host-restricted virus-specific CTL will be unable to clear virally infected donor cells, which may lead to significant adverse effects [92]. However, the presence of shared MHC alleles between donor and recipient can overcome this problem [93].

3 Recent successes in tolerance induction by HCT after organ transplantation would allow the use of such an approach in a large population of already-transplanted patients for whom a live donor is still available [37,94].

4 Development of new approaches to assess the tolerant state in a biologically meaningful way to know precisely when immunosuppression can be safely reduced and eventually stopped.

Taken together, mixed chimerism induction using reduced intensity HCT protocols has been the first and so far the only successful approach to inducing donor-specific tolerance to organ allografts in humans, and research in the coming years will most likely lead to increased safety and therefore broader applicability of such protocols for other patient populations and also for other organs besides the kidney.

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References

1. Wolfe RA, Ashby VB, Milford EL, *et al.* Comparison of mortality in all patients on dialysis, patients on dialysis

awaiting transplantation, and recipients of a first cadaveric transplant. *N Engl J Med* 1999; **341**: 1725.
2. Murray JE, Merrill JP, Harrison JH. Kidney transplantation between seven pairs of identical twins. *Ann Surg* 1958; **148**: 343.
3. Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB. Strategies to improve long-term outcomes after renal transplantation. *N Engl J Med* 2002; **346**: 580.
4. Womer KL, Lee RS, Madsen JC, Sayegh MH. Tolerance and chronic rejection. *Philos Trans R Soc Lond B Biol Sci* 2001; **356**: 727.
5. Mikhalski D, Wissing KM, Ghisdal L, *et al.* Cold ischemia is a major determinant of acute rejection and renal graft survival in the modern era of immunosuppression. *Transplantation* 2008; **85**: S3.
6. Kushida T, Inaba M, Hisha H, *et al.* Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. *Blood* 2001; **97**: 3292.
7. Traynor AE, Schroeder J, Rosa RM, *et al.* Treatment of severe systemic lupus erythematosus with high-dose chemotherapy and haemopoietic stem-cell transplantation: a phase I study. *Lancet* 2000; **356**: 701.
8. Nikolic B, Takeuchi Y, Leykin I, Fudaba Y, Smith RN, Sykes M. Mixed hematopoietic chimerism allows cure of autoimmune diabetes through allogeneic tolerance and reversal of autoimmunity. *Diabetes* 2004; **53**: 376.
9. Imasawa T, Nagasawa R, Utsunomiya Y, *et al.* Bone marrow transplantation attenuates murine IgA nephropathy: role of a stem cell disorder. *Kidney Int* 1999; **56**: 1809.
10. Reinsmoen NL, Matas AJ. Evidence that improved late renal transplant outcome correlates with the development of in vitro donor antigen-specific hyporeactivity. *Transplantation* 1993; **55**: 1017.
11. Reinsmoen NL, Bolman RM, Savik K, Butters K, Matas AJ, Hertz MI. Improved long-term graft outcome in lung transplant recipients who have donor antigen-specific hyporeactivity. *J Heart Lung Transplant* 1994; **13**: 30.
12. Sykes M. Mixed chimerism and transplant tolerance. *Immunity* 2001; **14**: 417.
13. Heslan JM, Renaudin K, Thebault P, Josien R, Cuturi MC, Chiffoleau E. New evidence for a role of allograft accommodation in long-term tolerance. *Transplantation* 2006; **82**: 1185.
14. Thomas JM, Hubbard WJ, Sooudi SK, Thomas FT. STEALTH matters: a novel paradigm of durable primate allograft tolerance. *Immunol Rev* 2001; **183**: 223.
15. Ravanan R, Dudley CR, Smith RM, Burton CJ, Lear PA, Unsworth DJ. Use of skin grafting to demonstrate tolerance before kidney transplantation without immunosuppression in the recipient of a previous bone marrow transplant. *Transplantation* 2005; **79**: 375.
16. Mache CJ, Schwinger W, Spendel S, Zach O, Regauer S, Ring E. Skin transplantation to monitor clinical donor-related tolerance in mixed hematopoietic chimerism. *Pediatr Transplant* 2006; **10**: 128.
17. Fudaba Y, Spitzer TR, Shaffer J, *et al.* Myeloma responses and tolerance following combined kidney and nonmyeloablative marrow transplantation: in vivo and in vitro analyses. *Am J Transplant* 2006; **6**: 2121.
18. Fehr T, Bardwell PD, Sykes M. Use of skin grafting to demonstrate tolerance before kidney transplantation without immunosuppression in the recipient of a previous bone marrow transplant. *Transplantation* 2005; **80**: 1359.
19. Deeg HJ. Should skin grafts be used to test for tolerance? *Pediatr Transplant* 2006; **10**: 755.
20. Shaffer J, Villard J, Means TK, *et al.* Regulatory T-cell recovery in recipients of haploidentical nonmyeloablative hematopoietic cell transplantation with a humanized anti-CD2 mAb, MEDI-507, with or without fludarabine. *Exp Hematol* 2007; **35**: 1140.
21. Brouard S, Mansfield E, Braud C, *et al.* Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A* 2007; **104**: 15448.
22. Martinez-Llordella M, Puig-Pey I, Orlando G, *et al.* Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant* 2007; **7**: 309.
23. Fehr T, Sykes M. Tolerance induction in clinical transplantation. *Transpl Immunol* 2004; **13**: 117.
24. Sayegh MH, Fine NA, Smith JL, Rennke HG, Milford EL, Tilney NL. Immunologic tolerance to renal allografts after bone marrow transplants from the same donors. *Ann Intern Med* 1991; **114**: 954.
25. Helg C, Chapuis B, Bolle JF, *et al.* Renal transplantation without immunosuppression in a host with tolerance induced by allogeneic bone marrow transplantation. *Transplantation* 1994; **58**: 1420.
26. Jacobsen N, Taaning E, Ladefoged J, Kristensen JK, Pedersen FK. Tolerance to an HLA-B,DR disparate kidney allograft after bone-marrow transplantation from same donor. *Lancet* 1994; **343**: 800.
27. Sorof JM, Koerper MA, Portale AA, Potter D, DeSantes K, Cowan M. Renal transplantation without chronic immunosuppression after T cell-depleted, HLA-mismatched bone marrow transplantation. *Transplantation* 1995; **59**: 1633.
28. Butcher JA, Hariharan S, Adams MB, Johnson CP, Roza AM, Cohen EP. Renal transplantation for end-stage renal disease following bone marrow transplantation: a report of six cases, with and without immunosuppression. *Clin Transplant* 1999; **13**: 330.
29. Sellers MT, Deierhoi MH, Curtis JJ, *et al.* Tolerance in renal transplantation after allogeneic bone marrow transplantation-6-year follow-up. *Transplantation* 2001; **71**: 1681.
30. Hamawi K, Magalhaes-Silverman M, Bertolatus JA. Outcomes of renal transplantation following bone marrow transplantation. *Am J Transplant* 2003; **3**: 301.

31. Svendsen UG, Aggestrup S, Heilmann C, *et al.* Transplantation of a lobe of lung from mother to child following previous transplantation with maternal bone marrow. *Eur Respir J* 1995; **8**: 334.
32. Kadry Z, Mullhaupt B, Renner EL, *et al.* Living donor liver transplantation and tolerance: a potential strategy in cholangiocarcinoma. *Transplantation* 2003; **76**: 1003.
33. Gajewski JL, Ippoliti C, Ma Y, Champlin R. Discontinuation of immunosuppression for prevention of kidney graft rejection after receiving a bone marrow transplant from the same HLA identical sibling donor. *Am J Hematol* 2002; **71**: 311.
34. Matthes-Martin S, Peters C, Konigsrainer A, *et al.* Successful stem cell transplantation following orthotopic liver transplantation from the same haploidentical family donor in a girl with hemophagocytic lymphohistiocytosis. *Blood* 2000; **96**: 3997.
35. Ringden O, Soderdahl G, Mattsson J, *et al.* Transplantation of autologous and allogeneic bone marrow with liver from a cadaveric donor for primary liver cancer. *Transplantation* 2000; **69**: 2043.
36. Dey B, Sykes M, Spitzer TR. Outcomes of recipients of both bone marrow and solid organ transplants. A review. *Medicine (Baltimore)* 1998; **77**: 355.
37. Koyama I, Nadazdin O, Boskovic S, *et al.* Depletion of CD8 memory T cells for induction of tolerance of a previously transplanted kidney allograft. *Am J Transplant* 2007; **7**: 1055.
38. Strober S, Slavin S, Gottlieb M, *et al.* Allograft tolerance after total lymphoid irradiation (TLI). *Immunol Rev* 1979; **46**: 87.
39. Strober S, Modry DL, Hoppe RT, *et al.* Induction of specific unresponsiveness to heart allografts in mongrel dogs treated with total lymphoid irradiation and antithymocyte globulin. *J Immunol* 1984; **132**: 1013.
40. Myburgh JA, Meyers AM, Thomson PD, *et al.* Total lymphoid irradiation – current status. *Transplant Proc* 1989; **21**: 826.
41. Myburgh JA, Smit JA, Hill RR, Browde S. Transplantation tolerance in primates following total lymphoid irradiation and allogeneic bone marrow injection. II. Renal allografts. *Transplantation* 1980; **29**: 405.
42. Smit JA, Hill RR, Myburgh JA, Browde S. Transplantation tolerance in primates after total lymphoid irradiation and allogeneic bone marrow injection. III. Lymphocyte responsiveness and suppressor cell activity. *Transplantation* 1980; **30**: 107.
43. Myburgh JA, Smit JA, Pieterz GA, Ling S, Sandrin MS, McKenzie IF. Tolerance in baboon kidney transplantation with total lymphoid irradiation (TLI) and anti-CD3/CD4-idarubicin conjugates. *Transplantation* 2001; **72**: 1150.
44. Strober S, Dhillon M, Schubert M, *et al.* Acquired immune tolerance to cadaveric renal allografts. A study of three patients treated with total lymphoid irradiation. *N Engl J Med* 1989; **321**: 28.
45. Strober S, Benike C, Krishnaswamy S, Engleman EG, Grumet FC. Clinical transplantation tolerance twelve years after prospective withdrawal of immunosuppressive drugs: studies of chimerism and anti-donor reactivity. *Transplantation* 2000; **69**: 1549.
46. Myburgh JA, Smit JA, Browde S. Transplantation tolerance in the primate following total lymphoid irradiation (TLI) and bone marrow (BM) injection. *Transplant Proc* 1981; **13**: 434.
47. Storb R, Yu C, Wagner JL, *et al.* Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood* 1997; **89**: 3048.
48. Storb R, Yu C, Barnett T, *et al.* Stable mixed hematopoietic chimerism in dog leukocyte antigen-identical littermate dogs given lymph node irradiation before and pharmacologic immunosuppression after marrow transplantation. *Blood* 1999; **94**: 1131.
49. Kuhr CS, Allen MD, Junghanss C, *et al.* Tolerance to vascularized kidney grafts in canine mixed hematopoietic chimeras. *Transplantation* 2002; **73**: 1487.
50. Graves SS, Hogan WJ, Kuhr CS, *et al.* Stable trichimerism after marrow grafting from two DLA-identical canine donors and nonmyeloablative conditioning. *Blood* 2007; **110**: 412.
51. Sharabi Y, Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. *J Exp Med* 1989; **169**: 493.
52. Huang CA, Fuchimoto Y, Scheier-Dolberg R, Murphy MC, Neville DM Jr, Sachs DH. Stable mixed chimerism and tolerance using a nonmyeloablative preparative regimen in a large-animal model. *J Clin Invest* 2000; **105**: 173.
53. Kawai T, Poncelet A, Sachs DH, *et al.* Long-term outcome and alloantibody production in a non-myeloablative regimen for induction of renal allograft tolerance. *Transplantation* 1999; **68**: 1767.
54. Kawai T, Sogawa H, Boskovic S, *et al.* CD154 blockade for induction of mixed chimerism and prolonged renal allograft survival in nonhuman primates. *Am J Transplant* 2004; **4**: 1391.
55. Kawai T, Cosimi AB, Wee SL, *et al.* Effect of mixed hematopoietic chimerism on cardiac allograft survival in cynomolgus monkeys. *Transplantation* 2002; **73**: 1757.
56. Tomita Y, Khan A, Sykes M. Role of intrathymic clonal deletion and peripheral anergy in transplantation tolerance induced by bone marrow transplantation in mice conditioned with a nonmyeloablative regimen. *J Immunol* 1994; **153**: 1087.
57. Tomita Y, Sachs DH, Khan A, Sykes M. Additional monoclonal antibody (mAB) injections can replace thymic irradiation to allow induction of mixed chimerism and tolerance in mice receiving bone marrow transplantation after conditioning with anti-T cell mABs and 3-Gy whole body irradiation. *Transplantation* 1996; **61**: 469.

58. Nikolic B, Khan A, Sykes M. Induction of tolerance by mixed chimerism with nonmyeloblative host conditioning: the importance of overcoming intrathymic alloresistance. *Biol Blood Marrow Transplant* 2001; **7**: 144.
59. Wekerle T, Sayegh MH, Ito H, *et al.* Anti-CD154 or CTLA4Ig obviates the need for thymic irradiation in a non-myeloablative conditioning regimen for the induction of mixed hematopoietic chimerism and tolerance. *Transplantation* 1999; **68**: 1348.
60. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* 2000; **6**: 114.
61. Lowsky R, Takahashi T, Liu YP, *et al.* Protective conditioning for acute graft-versus-host disease. *N Engl J Med* 2005; **353**: 1321.
62. Strober S. Protective conditioning against GVHD and graft rejection after combined organ and hematopoietic cell transplantation. *Blood Cells Mol Dis* 2008; **40**: 48.
63. Baron F, Baker JE, Storb R, *et al.* Kinetics of engraftment in patients with hematologic malignancies given allogeneic hematopoietic cell transplantation after nonmyeloablative conditioning. *Blood* 2004; **104**: 2254.
64. Sykes M, Sheard MA, Sachs DH. Graft-versus-host-related immunosuppression is induced in mixed chimeras by alloresponses against either host or donor lymphohematopoietic cells. *J Exp Med* 1988; **168**: 2391.
65. Pelot MR, Pearson DA, Swenson K, *et al.* Lymphohematopoietic graft-vs.-host reactions can be induced without graft-vs.-host disease in murine mixed chimeras established with a cyclophosphamide-based nonmyeloablative conditioning regimen. *Biol Blood Marrow Transplant* 1999; **5**: 133.
66. Mapara MY, Pelot M, Zhao G, Swenson K, Pearson D, Sykes M. Induction of stable long-term mixed hematopoietic chimerism following nonmyeloablative conditioning with T cell-depleting antibodies, cyclophosphamide, and thymic irradiation leads to donor-specific in vitro and in vivo tolerance. *Biol Blood Marrow Transplant* 2001; **7**: 646.
67. Chakraverty R, Cote D, Buchli J, *et al.* An inflammatory checkpoint regulates recruitment of graft-versus-host reactive T cells to peripheral tissues. *J Exp Med* 2006; **203**: 2021.
68. Mapara MY, Kim YM, Wang SP, Bronson R, Sachs DH, Sykes M. Donor lymphocyte infusions mediate superior graft-versus-leukemia effects in mixed compared to fully allogeneic chimeras: a critical role for host antigen-presenting cells. *Blood* 2002; **100**: 1903.
69. Dey BR, McAfee S, Colby C, *et al.* Impact of prophylactic donor leukocyte infusions on mixed chimerism, graft-versus-host disease, and antitumor response in patients with advanced hematologic malignancies treated with nonmyeloablative conditioning and allogeneic bone marrow transplantation. *Biol Blood Marrow Transplant* 2003; **9**: 320.
70. Sykes M, Preffer F, McAfee S, *et al.* Mixed lymphohaemopoietic chimerism and graft-versus-lymphoma effects after non-myeloablative therapy and HLA-mismatched bone-marrow transplantation. *Lancet* 1999; **353**: 1755.
71. Branco L, Barren P, Mao SY, *et al.* Selective deletion of antigen-specific, activated T cells by a humanized MAB to CD2 (MEDI-507) is mediated by NK cells. *Transplantation* 1999; **68**: 1588.
72. Koenecke C, Shaffer J, Alexander SI, *et al.* NK cell recovery, chimerism, function, and recognition in recipients of haploidentical hematopoietic cell transplantation following nonmyeloablative conditioning using a humanized anti-CD2 mAb, Medi-507. *Exp Hematol* 2003; **31**: 911.
73. Spitzer TR, McAfee SL, Dey BR, *et al.* Nonmyeloablative haploidentical stem-cell transplantation using anti-CD2 monoclonal antibody (MEDI-507)-based conditioning for refractory hematologic malignancies. *Transplantation* 2003; **75**: 1748.
74. Owen RD. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science* 1945; **102**: 400.
75. Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature* 1984; **307**: 168.
76. Barber WH, Mankin JA, Laskow DA, *et al.* Long-term results of a controlled prospective study with transfusion of donor-specific bone marrow in 57 cadaveric renal allograft recipients. *Transplantation* 1991; **51**: 70.
77. Starzl TE, Demetris AJ, Murase N, Trucco M, Thomson AW, Rao AS. The lost chord: microchimerism and allograft survival. *Immunol Today* 1996; **17**: 577.
78. Corry RJ, Chakrabarti PK, Shapiro R, *et al.* Simultaneous administration of adjuvant donor bone marrow in pancreas transplant recipients. *Ann Surg* 1999; **230**: 372.
79. Millan MT, Shizuru JA, Hoffmann P, *et al.* Mixed chimerism and immunosuppressive drug withdrawal after HLA-mismatched kidney and hematopoietic progenitor transplantation. *Transplantation* 2002; **73**: 1386.
80. Strober S, Lowsky RJ, Shizuru JA, Scandling JD, Millan MT. Approaches to transplantation tolerance in humans. *Transplantation* 2004; **77**: 932.
81. Scandling JD, Busque S, Dejbakhsh-Jones S, *et al.* Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* 2008; **358**: 362.
82. Spitzer TR, Delmonico F, Tolkoff-Rubin N, *et al.* Combined histocompatibility leukocyte antigen-matched donor bone marrow and renal transplantation for multiple myeloma with end stage renal disease: the induction of allograft tolerance through mixed lymphohematopoietic chimerism. *Transplantation* 1999; **68**: 480.
83. Buhler LH, Spitzer TR, Sykes M, *et al.* Induction of kidney allograft tolerance after transient lymphohematopoietic chimerism in patients with multiple myeloma and end-stage renal disease. *Transplantation* 2002; **74**: 1405.

84. Kimikawa M, Sachs DH, Colvin RB, Bartholomew A, Kawai T, Cosimi AB. Modifications of the conditioning regimen for achieving mixed chimerism and donor-specific tolerance in cynomolgus monkeys. *Transplantation* 1997; **64**: 709.
85. Kawai T, Cosimi AB, Spitzer TR, *et al.* HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med* 2008; **358**: 353.
86. Spitzer TR. Engraftment syndrome following hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2001; **27**: 893.
87. Trivedi HL, Vanikar AV, Modi PR, *et al.* Allogeneic hematopoietic stem-cell transplantation, mixed chimerism, and tolerance in living related donor renal allograft recipients. *Transplant Proc* 2005; **37**: 737.
88. Donckier V, Troisi R, Toungouz M, *et al.* Donor stem cell infusion after non-myeloablative conditioning for tolerance induction to HLA mismatched adult living-donor liver graft. *Transpl Immunol* 2004; **13**: 139.
89. Stapler D, Lee ED, Selvaraj SA, *et al.* Expansion of effector memory TCR Vbeta4+ CD8+ T cells is associated with latent infection-mediated resistance to transplantation tolerance. *J Immunol* 2008; **180**: 3190.
90. Thornley TB, Brehm MA, Markees TG, *et al.* TLR agonists abrogate costimulation blockade-induced prolongation of skin allografts. *J Immunol* 2006; **176**: 1561.
91. Forman D, Welsh RM, Markees TG, *et al.* Viral abrogation of stem cell transplantation tolerance causes graft rejection and host death by different mechanisms. *J Immunol* 2002; **168**: 6047.
92. Rüedi E, Sykes M, Ildstad ST, *et al.* Antiviral T cell competence and restriction specificity of mixed allogeneic (P1+ P2——P1) irradiation chimeras. *Cell Immunol* 1989; **121**: 185.
93. Koehn BH, Williams MA, Borom K, *et al.* Fully MHC-disparate mixed hemopoietic chimeras show specific defects in the control of chronic viral infections. *J Immunol* 2007; **179**: 2616.
94. Donckier V, Troisi R, Le Moine A, *et al.* Early immunosuppression withdrawal after living donor liver transplantation and donor stem cell infusion. *Liver Transpl* 2006; **12**: 1523.
95. Trivedi HL, Vanikar AV, Vakil JM, Shah VR, Modi PR, Trivedi VB. A strategy to achieve donor-specific hyporesponsiveness in cadaver renal allograft recipients by donor haematopoietic stem cell transplantation into the thymus and periphery. *Nephrol Dial Transplant* 2004; **19**: 2374.

