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Chimerism-based tolerance in organ transplantation: preclinical and clinical studies

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Summary

Induction of allograft tolerance has been considered the ultimate goal in organ transplantation. Although numerous protocols to induce allograft tolerance have been reported in mice, a chimerism-based approach through donor haematopoietic stem cell transplantation has been the only approach to date that induced allograft tolerance reproducibly following kidney transplantation in man. Renal allograft tolerance has been achieved by induction of either transient mixed chimerism or persistent full donor chimerism. Although the risk of rejection may be low in tolerance achieved via durable full donor chimerism, the development of graft-*versus*-host disease (GVHD) has limited the wider clinical application of this approach. In contrast, tolerance induced by transient mixed chimerism has not been associated with GVHD, but the risk of allograft rejection is more difficult to predict after the disappearance of haematopoietic chimerism. Current efforts are directed towards the development of more clinically feasible and reliable approaches to induce more durable mixed chimerism in order to widen the clinical applicability of these treatment regimens.

Keywords: chimerism, cyclophosphamide, durable mixed chimerism, full donor chimerism, GVHD, haematopoietic stem cell transplantation, living-donor kidney transplantation, NHP, persistent mixed chimerism, TBI, TI, TLI, transient mixed chimerism, transplantation tolerance induction

Introduction

As the development of highly efficacious immunosuppressive agents has prevented or treated acute allograft rejection successfully, the short-term survival of organ transplants has improved significantly, making solid organ transplantation the therapy of choice for most end-stage organ diseases [1,2]. However, the current requirement for lifelong immunosuppression results in significantly increased risks of cardiovascular disease [3–5], *de-novo* diabetes [6–8], dyslipidaemia [9–12] and malignancies [13–15], which lead to patient death with functioning graft as high as 25% by 10 years after kidney transplantation (KTx) [16]. Unfortunately, despite these toxicities, the development of chronic rejection is not prevented consistently by currently available immunosuppressive regimens. Immune tolerance induction is the ultimate solution to these limitations associated with long-term immunosuppression in transplanted patients. Except for a limited report of induction of liver allograft tolerance through infusion of regulatory cells [17], induction of chimerism through donor haematopoietic stem cell transplantation (HSCT) has been the only approach to date that reproducibly achieved allograft tolerance in clinical KTx.

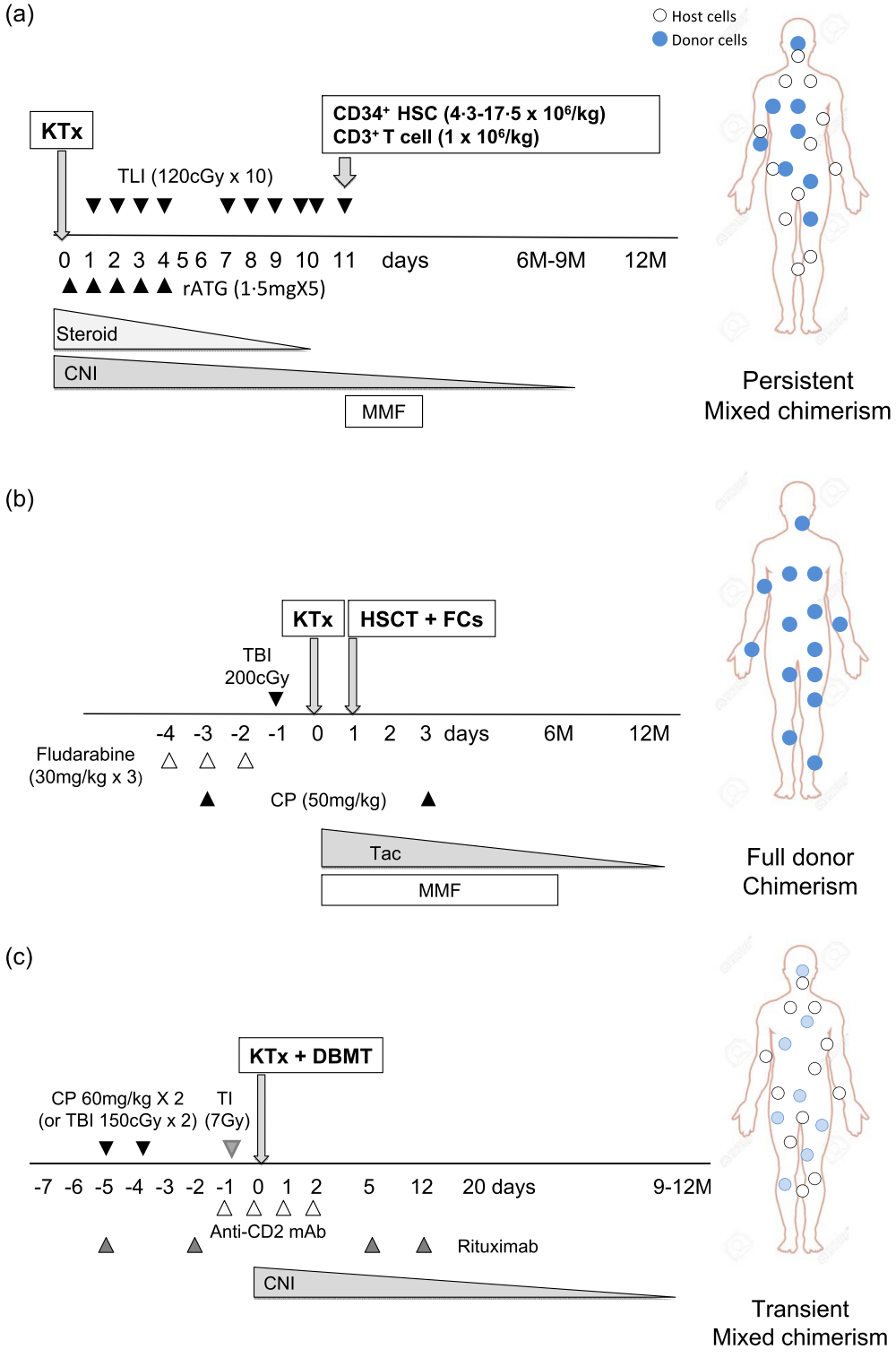
Preclinical studies

Since Owen and Medawar's discoveries of mixed chimerism and allograft tolerance in Freemartin Cattle, extensive efforts have been directed towards induction of persistent mixed chimerism in adult experimental animals. Although induction of persistent mixed chimerism has been achieved readily in small animal models, it has been extremely difficult to achieve this in non-human primates (NHPs) or

humans. If the conditioning regimen is intensive, donor haematopoietic cells overwhelm recipient haematopoietic cells, which lead to full donor chimerism. Conversely, if the conditioning regimen is less intensive, donor haematopoietic cells are rejected. These contrasting results observed in rodent *versus* primate studies may be attributed to the presence of heterologous memory T cells (T_{MEM}) in primates, as Adams *et al.* reported failure of chimerism induction in mice in which alloreactive T_{MEM} were augmented by multiple lymphocytic choriomeningitis (LCMV) infections [18,19]. Nevertheless, we demonstrated in NHPs that induction of only transient mixed chimerism can induce renal allograft tolerance in major histocompatibility complex (MHC)-mismatched transplant recipients [20–22]. Continued survival of the kidney allograft despite the loss of chimerism suggested that peripheral mechanisms were involved primarily, and induction of renal allograft tolerance has been improved by adding a short course of co-stimulatory blockade, such as anti-CD154 monoclonal antibody (mAb) [21] or cytotoxic T lymphocyte (CTLA4) immunoglobulin (Ig) (belatacept) [22]. As our original conditioning regimen required initiation of conditioning 1 week before transplantation, the protocol was applicable only to living donor transplant recipients. To extend our approach to deceased donor transplant recipients, we subsequently developed a 'delayed tolerance' strategy in which kidney transplantation is performed first with conventional immunosuppression, followed by conditioning and donor bone marrow transplantation (DBMT) several months later using cryopreserved donor bone marrow cells in NHPs [23,24]. 'Delayed tolerance' has the theoretical disadvantage of enhanced donor-specific T_{MEM} responses elicited despite administration of potent immunosuppressive medications during the interval prior to the DBMT. Indeed, more substantial depletion of $CD8^+$ T cells with anti-CD8 mAb was necessary to induce mixed chimerism in the delayed tolerance conditioning protocol. Nevertheless, the delayed tolerance approach expands the potential applicability of tolerance induction protocol significantly, as it can be used not only for deceased donor transplant recipients but also for any previous living donor transplant recipient, if their donor is available to provide the haematopoietic stem cells. Although the exact mechanistic pathways leading to tolerance induction via transient mixed chimerism remain to be defined, studies to date have provided a number of important observations. We found in NHPs that tolerant recipients consistently lost anti-donor $CD8^+$ T cell responses while retaining substantial anti-donor $CD4^+$ T cell responses *in vitro*. The majority of these $CD4^+$ T cell responses appeared to be from regulatory T cells (T_{regs}) which expand significantly more to stimulation with donor antigens than to third-party antigens. When sorted T_{regs} and non-T_{regs} from tolerant recipients were stimulated with donor antigens in the presence of interleukin (IL)-2, T_{reg} expansion was observed only from non-T_{regs}. Furthermore, the expansion of T_{regs} in tolerant recipients was inhibited by blocking transforming growth factor (TGF)-β, which resulted in restoration of anti-donor $CD8^+$ T cell responses. These observations suggest that specific loss of anti-donor $CD8^+$ T cell responses are maintained by donor-specific induced T_{regs} [25]. We also found that T_{regs} were enriched significantly in the kidney allograft of tolerant recipients. Further studies are in progress to clarify the mechanisms of local enrichment of T_{regs} in the kidney allograft.

A limitation of our tolerance approach with transient mixed chimerism has been inconsistent stability of allograft tolerance. Approximately 20–30% of NHP recipients who were apparently withdrawn successfully from immunosuppression developed antibody-mediated chronic rejection later [21,26]. Therefore, improving the stability of tolerance is critically important to widen the application of this approach. One strategy to improve the stability of tolerance, therefore, was to modify the conditioning regimen to achieve more robust mixed chimerism. It is possible that reduced-intensity conditioning regimens permit a substantial proportion of heterologous T_{MEM} to survive, which may contribute to the loss of allogeneic haematopoietic stem cell engraftment [18,19,27]. Because T_{regs} have been reported to be capable of suppressing T_{MEM} function effectively [28–30], we sought modalities to expand T_{regs} *in vivo*. We evaluated IL-6 blockade with anti-IL-6R mAb to determine whether we could expand T_{regs} *in vivo*. Although IL-6 blockade alone failed to expand T_{regs} in this setting, the expansion was successful when combined with anti-thymocyte globulin (ATG) [31]. Subsequently, we included anti-IL-6R mAb in our DBMT conditioning regimen to induce mixed chimerism in a widely recognized 'tolerance-resistant' lung transplant model [32]. Interestingly, three of four lung transplant recipients developed prolonged mixed chimerism and achieved robust lung allograft tolerance [32]. Unfortunately, when this conditioning regimen was tested in kidney transplant recipients, robust chimerism was not induced (unpublished results). We suggest that IL-6 blockade may be especially effective in lung transplant recipients via suppression of rejection through Th17 [33], and this approach may not be applicable to other organ transplant recipients. Nevertheless, this success in lung transplantation emphasized that robust tolerance is inducible in NHP, even in this typically 'tolerance-resistant' lung allograft model by induction of prolonged mixed chimerism.

Kean's group, in Seattle, has reported successful induction of persistent mixed chimerism in NHP recipients of MHC-matched DBMT. Using a conditioning regimen that consisted of low-dose total body irradiation (TBI), basiliximab, anti-CD154 mAb, belatacept and sirolimus, three of nine recipients developed multi-lineage mixed chimerism for as long as 24 months. Those recipients also achieved prolonged specific acceptance of skin allografts from the



bone marrow (BM) donor. However, six of the nine recipients were euthanized because of cytomegalovirus (CMV) reactivation, which suggested that the protocol may be unacceptably immunosuppressive [34]. Sykes's group, at Columbia University, also achieved prolonged lymphoid chimerism successfully in a cynomolgus monkey recipient by infusion of *ex-vivo* expanded T_{regs}. However, in this model T_{reg} infusion was also associated with CMV reactivation in a significant number of recipients [35]. Thus, more specific suppression of alloimmunity while maintaining anti-viral immunity is required to develop a conditioning regimen for induction of persistent mixed chimerism.

Fig. 1. Three pilot studies of renal allograft tolerance induction in human living-donor kidney transplantation at Stanford, Northwestern and Massachusetts General Hospital (MGH). Stanford human leucocyte antigen (HLA)-matched conditioning protocol consists of total lymphoid irradiation (TLI) (120 cGy/day, 10 daily doses starting on postoperative day 1) and rabbit anti-thymocyte globulin (rATG) (1·5 mg/kg/day, five daily doses starting intra-operatively). Following the last dose of TLI, $CD34^+$-enriched donor peripheral blood stem cells are infused. The recipients are then maintained on calcineurin inhibitor (CNI)/mycophenolate mofetil (MMF)/steroid therapy until weaning was attempted several months later (a). Northwestern protocol consists of Fludarabine (30 mg/kg on days −4, −3 and −2), cyclophosphamide (CP) (50 mg/kg on days −3 and +3) and total body irradiation (TBI) (200 cGy) on day −1. This is followed by kidney transplantation (KTx), then donor haematopoietic stem cell transplantation (HSCT) on day +1. Immunosuppression consists of MMF and tacrolimus starting on day 0 and tapered off slowly by 1 year. In addition, the Northwestern regimen includes infusion of a unique 'facilitating cell' [a mixture of $CD8^+$/T cell receptor (TCR^-)] in the attempt to enhance engraftment and reduce further the risk of graft-*versus*-host disease (GVHD) (b). The initial MGH conditioning regimen for human leucocyte antigen (HLA)-mismatched KTx included CP, TI anti-CD2 monoclonal antibody (mAb) and post-transplant CNI administration. To prevent donor-specific antibody (DSA) development, we add rituximab therapy subsequently around the peri-transplant period. As acute kidney injury had not been observed in the non-human primate (NHP) studies that utilized TBI rather than CP in the conditioning regimen, a revised regimen in which low-dose TBI replaced CP, has been evaluated recently in three recipients (c). Open circles indicate host haematopoietic cells and closed circles indicate donor haematopoietic cells.

Clinical trials

Clinical trials to induce renal allograft tolerance through DBMT have been reported from three centres: Stanford, Northwestern and Massachusetts General Hospital (MGH) in the United States.

Stanford approach (Fig. 1a and Table 1)

In 1989, Strober *et al.* reported successful induction of renal allograft tolerance in three human leucocyte antigen (HLA)-mismatched kidney transplant recipients using total lymphoid irradiation (TLI) and rabbit ATG (rATG), but without DBMT [36]. However, two of these three recipients eventually lost renal allograft function due to chronic rejection and ureteral stricture [37]. Based on this initial experience with TLI, HSCT was combined with TLI and rATG to induce mixed chimerism. Their conditioning protocol consists of TLI (80–120 cGy/day 10 daily doses starting on post-operative day 1) and rATG (1·5 mg/kg/day, five daily doses starting on day 0). Following the last dose of TLI, $CD34^+$-enriched donor peripheral blood stem cells were infused. These cells were collected by one or two aphereses from the donor after treatment with granulocyte–colony-stimulating factor for 5–6 days. The recipients were maintained with calcineurin inhibitor (CNI), mycophenolate mofetil (MMF) and steroid immunosuppression. The advantage of this protocol is its clinical applicability to deceased donor transplantation, because all treatments are initiated after transplantation. This group has described three cohorts that underwent this protocol in a recent summary of their experience [38]. The first cohort (2000–03) included six HLA-mismatched renal allograft recipients. Only two of the six recipients developed transient chimerism for 2–3 months. Weaning of immunosuppression was attempted in these two recipients, but both developed mild rejection (Banff I) at 3 and 5 months after immunosuppression withdrawal, leading to reinstitution of immunosuppression [39]. The second cohort (2005–13) included only HLA-matched allograft recipients. Immunosuppression withdrawal criteria were modified in this group, requiring persistent chimerism for at least 6 months, absence of rejection on protocol biopsy and no evidence of graft-*versus*-host disease (GVHD) (Fig. 1a and Table 1). Chimerism was induced initially in 21 of 22 subjects studied, and 18 patients met the immunosuppression withdrawal criteria. Among these 18 recipients, 16 (seven with stable chimerism and nine with transient chimerism) continued to be off immunosuppression for 2–66 months. Immunosuppression was reinstituted in one recipient due to lupus flare, and one recipient is still in the midst of immunosuppression withdrawal. Three did not meet the immunosuppression withdrawal criteria despite development of chimerism, because of clinical or biopsy-proven rejection [38]. The third cohort included 10 recipients of HLA-haplotype-matched kidneys. An escalating dose of infused $CD34^+$ and $CD3^+$ T cells (3, 10, 20 and 50×10^6/kg compared to 1×10^6/kg in the prior two cohorts) was used in the effort to promote mixed chimerism induction. Persistent chimerism for at least 12 months was achieved in two patients. In these two patients MMF was discontinued at 9 months, after which the patients remained on tacrolimus monotherapy which continued at the time of this report. The remaining eight recipients developed transient chimerism or no chimerism, and their immunosuppression was not tapered [38]. In summary, with the Stanford protocol, durable or transient chimerism was induced in the majority of HLA-matched transplant recipients and immunosuppression was discontinued successfully in approximately 70% of the patients. Induction of chimerism has been more difficult in HLA-mismatched transplant recipients, and none of these recipients has achieved complete withdrawal of immunosuppression to date.

Northwestern approach (Fig. 1b and Table 1)

Until recently HLA-mismatched allogeneic DBMT (complete chimera) was associated with a mortality rate exceeding 50%, mainly as a result of GVHD [40]. The Johns Hopkins group developed a novel conditioning regimen for HLA-haploidentical allogeneic DBMT, which provided

Table 1. Outcomes of pilot studies of tolerance induction for living donor kidney transplantation in Stanford, Northwestern and Massachusetts General Hospital (MGH)

	Stanford	Northwestern	MGH
HLA	Matched	Mismatched	Mismatched
Number	22	31	12*
Off immunosuppression	17†	16	8
Death (related directly to the regimen)	0	1‡	0
Rejection	3	3	3§
GVHD	0	2	0
Chimerism			
Induction	21	30	11
Transient	9	5	11
Stable mix	7	3	0
Full	0	16	0

*10 patients received the cyclophosphamide-based conditioning regimen and two received total body irradiation (TBI)-based regimen. †One patient is in the midst of immunosuppressive drug tapering. ‡One patient died due to graft-*versus*-host disease (GVHD). §Two patients developed chronic rejection after 5 and 8 years. One patient developed acute rejection at 9 months. HLA = human leucocyte antigen.

no incidence of GVHD among 13 sickle cell disease patients [41]. The most important component of this regimen is post-transplant cyclophosphamide (CP) on days 3 or 4, designed to delete alloreactive T cells elicited after DBMT [41]. The conditioning regimen developed by the Northwestern group also includes post-Tx CP. Their regimen consisted of fludarabine (30 mg/kg on days −4, −3 and −2), CP (50 mg/kg on day −3) and TBI (200 cGy on day −1), followed by KTx on day 0 and HSCT on day +1. Post-Tx CP is administered on day +3, but they also added infusion (day + 1 with HSCT) of a unique recipient cell population named 'facilitating cells', which have tolerogenic features of $CD8^+$/T cell receptor (TCR^-) and a heterogeneous population composed predominantly of plasmacytoid DC [42,43], on day +1 in an effort to enhance engraftment of haematopoietic stem cells and to reduce further the risk of GVHD [44]. MMF and tacrolimus are administered after transplant and are tapered off by 6 and 12 months, respectively. A total of 31 patients have been enrolled into their clinical trial and 30 exhibited donor chimerism at 1 month after KTx. Nineteen recipients achieved durable chimerism, 16 were completely withdrawn from immunosuppressant for 3–65 months and the remaining three were weaning with all stable chimeric conditions. Two of 30 subjects lost their allograft due to infectious complications. Although the incidence of GVHD was reduced significantly, even in HLA-mismatched transplantation, two of 30 patients developed GVHD and one was dead [45]. The state of full chimerism has been considered immuno-incompetent [46–48], and two serious infectious complications that resulted in graft loss have been reported. Another death due to malignancy has also been reported [45], but its relevance to the state of full chimerism is not known.

The Northwestern group is pursuing another tolerance induction strategy for HLA-matched living donor KTx recipients. This regimen includes alemtuzumab induction, donor HSC infusion, MMF and tacrolimus, with tacrolimus being converted to sirolimus after 3 months and then tapered off slowly by 24 months post-transplantation. Twenty recipients were enrolled originally, but five recipients were excluded due to positive pre-Tx cross-match ($n = 1$), non-compliance ($n = 1$) and disease recurrences ($n = 3$). Among the 15 remaining recipients who completed 36 months post-Tx follow-up, six recipients achieved successful immunosuppression withdrawal for 32–64 months. Immunosuppression was not discontinued in the other nine recipients due to rejection detected in the protocol biopsies [49].

MGH approach (Fig. 1c and Table 1)

Based on decades-long studies in NHPs [20–22], we have performed clinical trials to induce allograft tolerance in HLA-matched [50] and -mismatched living donor KTx [51–53]. The initial conditioning regimen for HLA-mismatched KTx included CP, thymic irradiation (TI), anti-CD2 mAb and post-transplant CNI administration. Because of humoral responses observed in the second and third patients, perioperative administration of rituximab was added after the fourth recipient. Of the 10 recipients enrolled into the studies, all developed transient mixed chimerism and immunosuppression was discontinued in eight recipients by 9–14 months post-transplant. One of the eight developed acute rejection and required retransplantation 2 years later despite reinstitution of immunosuppression. After a follow-up period of 7–14 years, four of the remaining seven remained immunosuppression free for 14, 7, 6 and 6 years, while three resumed immunosuppression at 5, 7 and 8 years after KTx as a result of original kidney disease recurrence or chronic rejection [52,53]. An unexpected adverse event, acute kidney injury, was observed between 10 and 20 days post-transplant in nine of these 10 subjects. It was associated with haematopoietic cell recovery and then rapid loss of chimerism. As acute kidney injury had not been observed in the NHP studies that received low-dose TBI rather than CP, we

performed a clinical pilot study more recently using a conditioning regimen in which CP was replaced with low dose TBI. Both recipients have done well, without evidence of the acute kidney injury, and immunosuppression in the first patient has been discontinued for $>$ 3 years. As anti-CD2 mAb (MEDI-507 mAb) included in the initial conditioning regimen is not a Food and Drug Administration (FDA)-approved drug and its future clinical availability is uncertain, further clinical trials are planned using a new regimen with belatacept, which is developed based on an NHP study [22].

We compared postoperative complications and quality of life (QoL) of five tolerance recipients (tolerant group) with 31 comparable live donor kidney recipients on conventional immunosuppression (conventional group). Patients in the tolerant group required significantly less treatment after transplant for hypertension and no medications for diabetes ($P < 0{\cdot}01$). There was no diabetes, dyslipidaemia or malignancy in the tolerant group, while these were observed in 12·5, 40·6 and 11·8% of the conventional group, respectively. Tolerant patients experienced better overall health ($P < 0{\cdot}01$) and scored higher on kidney transplant-targeted scales and healthy survey scales than patients in the conventional group according to the KDQOL SF-36 ($P < 0{\cdot}05$). Tolerant patients were less likely to experience depression, dyspnoea, excessive appetite/thirst, flatulence, hearing loss, itching, joint pain, lack of energy, muscle cramps and lack of libido than conventional patients, according to the MTSOSD-59R ($P < 0{\cdot}05$) [54]. These observations provide the proof of principle that induction of tolerance is ideal for maintenance of overall QOL.

Conclusion

Tolerance induction is now a clinical reality in humans, at least for patients undergoing living donor KTx. The three major centres performing these studies continue to obtain promising results, and hopefully can soon expand their tolerance approaches to deceased donor transplants or non-renal organs. Improving the consistency and safety of tolerance induction will be a next crucial step to bringing tolerance to a wider range of clinical applications.

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Disclosure

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