

Review

Immune monitoring of transplant patients in transient mixed chimerism tolerance trials

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ABSTRACT

This review focuses on mechanistic studies performed in recipients of non-myeloablative bone marrow transplant regimens developed at Massachusetts General Hospital in HLA-identical and HLA-mismatched haplo-identical combinations, initially as a platform for treatment of hematologic malignancies with immunotherapy in the form of donor leukocyte infusions, and later in combination with donor kidney transplantation for the induction of allograft tolerance. In patients with permanent mixed chimerism, central deletion may be a major mechanism of long-term tolerance. In patients in whom donor chimerism is only transient, the kidney itself plays a significant role in maintaining long-term tolerance. A high throughput sequencing approach to identifying and tracking a significant portion of the alloreactive T cell receptor repertoire has demonstrated biological significance in transplant patients and has been useful in pointing to clonal deletion as a long-term tolerance mechanism in recipients of HLA-mismatched combined kidney and bone marrow transplants with only transient chimerism.

1. Introduction

The studies of combined kidney/bone marrow transplantation (CKBMT) across HLA barriers in patients without malignant disease at MGH [1,2] (see article by Tatsuo Kawai et al.) built on a series of translational studies in murine models, non-human primates and patients with hematological malignancies. The translational aspect of these studies is schematized in Fig. 1. Studies in the murine model for the purpose of two different goals each involved initial induction of mixed chimerism. The original mixed chimerism model upon which all of the studies are based was designed for the purpose of inducing allograft tolerance [3] (Fig. 1C). It involved treatment of mice with depleting anti-CD4 and anti-CD8 mAbs along with low-dose (3 Gy) TBI in order to overcome allograft rejection and make “space” for marrow engraftment, respectively. The addition of thymic irradiation was found to be necessary for the achievement of T cell chimerism, which correlated with durable chimerism [3] and was later shown to be required to eliminate intrathymic alloreactivity that otherwise rejected donor progenitors as they entered the thymus, precluding a donor

contribution to central deletional tolerance of newly-developing thymocytes [4–6]. Later, using the model represented in Fig. 1A, we utilized mixed chimerism induction in mice as a platform for delayed donor lymphocyte infusion (DLI), which we had shown could mediate potent graft-vs-leukemia (GVL) effects without causing graft-vs-host disease (GVHD) when inflammatory stimuli induced by conditioning had been given sufficient time to subside before DLI administration [7–12]. This “lymphohematopoietic GVH response (LGVHR)” remained confined to the lymphohematopoietic system due to the absence of local inflammatory stimuli in the epithelial GVHD target tissues. We had found that such inflammation provided a critical checkpoint for trafficking of GVH-reactive T cells to these tissues, where they caused disease [11,12]. The conditioning regimen in these mice was tailored to the treatment of indolent lymphoid malignancies by replacing the low-dose TBI in Fig. 1C with pre-transplant cyclophosphamide [10,13], which has cytoreductive effects on lymphoid malignancies.

The protocol in Fig. 1A was translated directly into clinical studies in patients with refractory hematologic malignancies in whom all other treatment modalities had failed (Fig. 1B). While this protocol excluded

Abbreviations: APC, antigen-presenting cell; BMT, bone marrow transplantation; CKBMT, combined kidney and bone marrow transplantation; CML, cell-mediated lympholysis; DLI, donor leukocyte infusions; GVHD, graft-vs-host disease; GVL, graft-vs-leukemia/lymphoma; LDA, limiting dilution analysis; LGVHR, lymphohematopoietic graft-vs-host response; MiHA, minor histocompatibility antigen; MLR, mixed lymphocyte reaction; TBI, total body irradiation; TCR, T cell receptor; Treg, regulatory T cell

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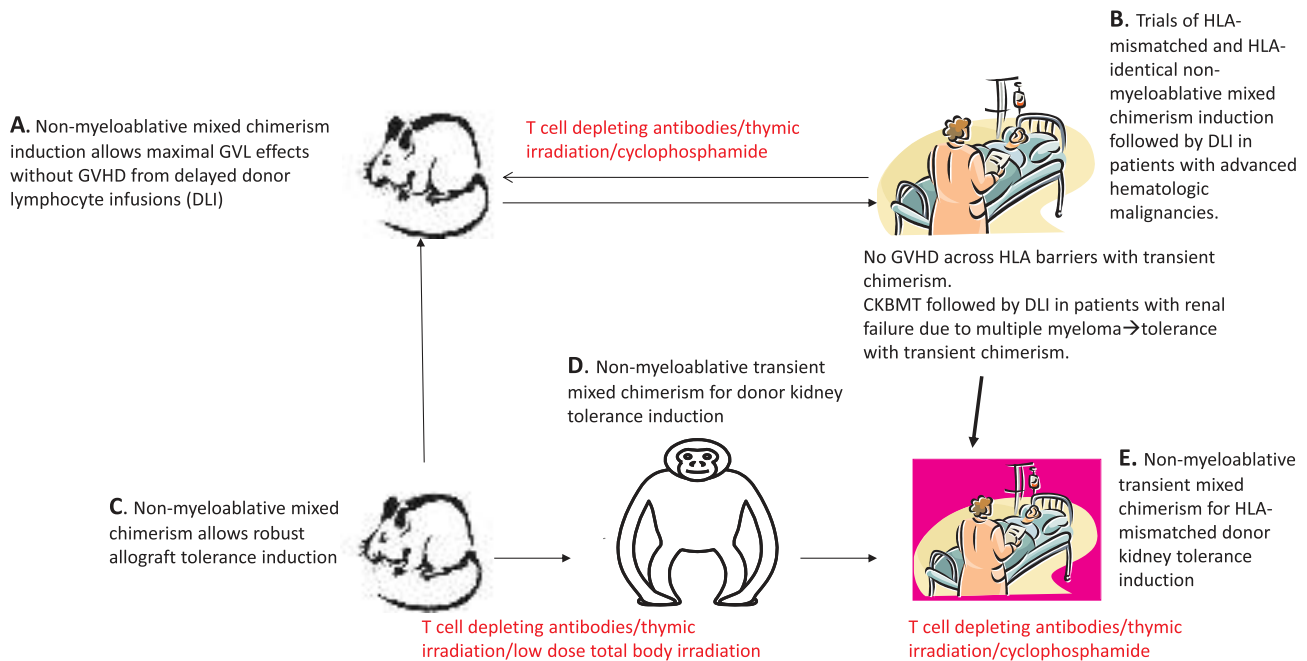


Fig. 1. Schematic representation of translational studies that led to the MGH ITN CKBMT trials. A. A murine model for non-myeloablative mixed chimerism induction was established with a combination of pre-transplant cyclophosphamide, T cell-depleting antibodies and local thymic irradiation. Mixed chimerism was used as a platform for delayed donor lymphocyte infusions, which mediated potent GVL effects without causing GVHD; B. The murine model in A was translated into clinical trials in patients with advanced hematologic malignancies, aiming to achieve mixed chimerism with non-myeloablative conditioning in both HLA-identical and HLA-mismatched haploidentical related donor settings, followed by DLI to achieve anti-tumor effects. Conditioning was similar to that in A, except a short course of cyclosporine was given post-transplant to compensate for the incomplete donor and recipient T cell depletion achievable with the *in vivo* antibody treatments given; C. Murine models using low-dose total body irradiation (TBI), T cell-depleting antibodies and thymic irradiation were used to establish durable mixed chimerism across full MHC barriers; D. Efforts to translate the model in C to a non-human primate model were successful in achieving renal allograft tolerance if the donor kidney and bone marrow were given simultaneously, although only transient chimerism was achieved. Conditioning included low-dose TBI, thymic irradiation, peri-transplant T cell-depleting antibodies and a short course of cyclosporine following the transplant to compensate for incomplete T cell depletion; E. The achievement of transient chimerism without GVHD across HLA barriers in hematologic malignancy patients in B, combined with the renal allograft tolerance achieved in monkeys with transient chimerism in D, permitted evaluation of a regimen developed in B for the induction of renal allograft tolerance across HLA barriers in patients without malignant disease.

the indolent types of malignancies for which the approach was intended, it nevertheless was associated with remarkable tumor responses and even cures in patients with bulky, refractory lymphomas and myelomas who lacked any other hope for survival [14–20]. These potent anti-tumor effects most likely reflected the ability of recipient-derived professional antigen-presenting cells (APCs) to trigger GVH alloreactivity, which is associated with improved GVL effects compared to those elicited in full chimeras lacking recipient APCs [8,18,21–23]. Thus, the strategy of using mixed chimerism as a platform to achieve potent GVL effects without GVHD was successfully translated into the clinic, both in the HLA-identical and HLA-mismatched settings. In the case of HLA-identical transplants, stronger GVL effects in mixed chimeras [17,24] may be explained by the expectation that the number of recipient miHAs presented on host APCs likely exceeds the number that can be presented on donor APCs through the exogenous antigen processing pathway, especially for CD8 allorecognition. These studies provided seminal demonstrations that: 1) durable mixed chimerism could be achieved with non-myeloablative conditioning in humans, even with HLA-mismatched donors [14–20], albeit not as reliably as desired. An example of durable mixed chimerism in an HLA-mismatched transplant recipient is illustrated in Fig. 2; 2) durable or transient mixed chimerism achieved under these conditions could occur without GVHD [14–20], though not as reliably as desired. As is discussed below, transient chimerism achieved across HLA barriers with one of these regimens was reliably NOT associated with GVHD, providing a key safety feature that permitted exploration of this approach for CKBMT in patients without malignant disease; and 3) delayed DLI could convert mixed to full chimerism, even across HLA barriers, without inducing GVHD [14–20], though not as reliably as desired.

The bottom part of the translational scheme presented in Fig. 1 represents the studies of mixed chimerism solely for the purpose of

organ allograft induction, which were closely interrelated with those in the top part of the figure. Early efforts were made to translate the non-myeloablative tolerance protocol in Fig. 1C to a large animal, non-human primate model (Fig. 1D) [25]. This model is discussed in more detail in the paper in this issue by Kawai et al. In my view, intermediate large animal models are ethically necessary before embarking on clinical trials of early immunosuppression withdrawal, which denies the patient standard-of-care treatment. Non-human primates, for the most part, model the hurdles to tolerance induction in patients quite faithfully. As is discussed by Kawai et al., achievement of durable mixed chimerism was elusive in this non-human primate model, but key observations were made that permitted the clinical trials in Fig. 1E to be carried out. In particular, it was found that a high proportion (about 60–70%) of animals receiving non-myeloablative CKBMT achieved long-term tolerance to their kidney grafts, despite the transient nature of their chimerism [25,26].

Meanwhile, the non-myeloablative mixed chimerism/DLI studies in patients with lymphoid malignancies (Fig. 1B) had shown an acceptable safety profile and were extended to a group of patients who had renal failure due to multiple myeloma and an available HLA-identical sibling donor. These first human CKBMT studies revealed that both transient and durable mixed chimerism could be associated with tolerance to donor kidneys [27–29]. Remarkably, those patients with transient chimerism, like others in the lymphoma trials discussed above, often enjoyed remarkable tumor responses [19,27,28], suggesting that the initial engraftment followed by rejection of donor marrow could elicit anti-tumor immunity, a hypothesis that was tested and shown to be valid in the murine model [30–33].

Thus, clinical studies in patients with hematological malignancies provided key safety and efficacy data that permitted testing of HLA-mismatched related donor CKBMT in patients with renal failure and no

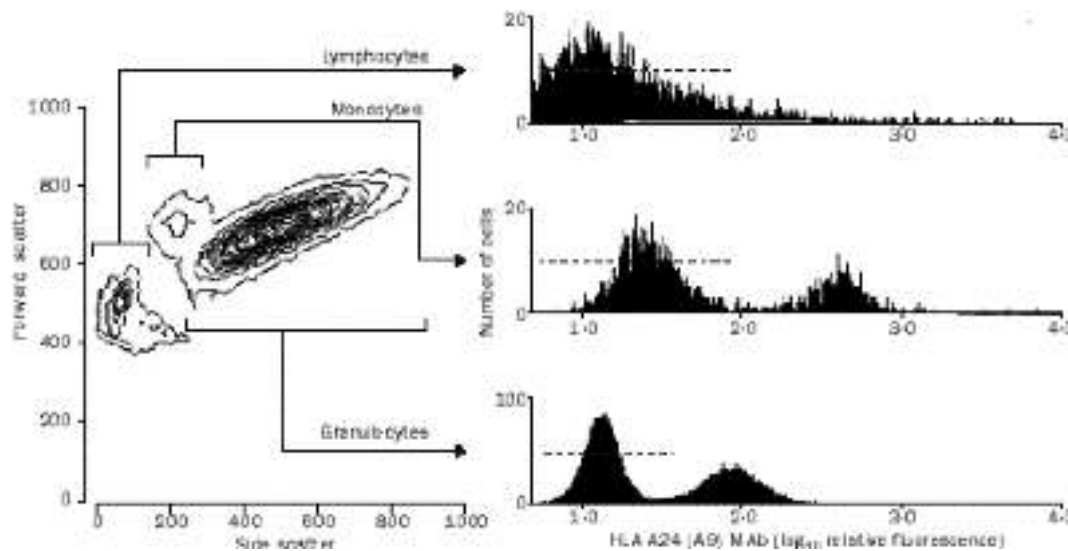


Fig. 2. Proof of principle that durable mixed chimerism can be achieved across HLA barriers with non-myeloablative conditioning in humans. A patient with an advanced, bulky non-Hodgkin lymphoma received one of the regimens tested in B in the scheme in Fig. 1, including pre-transplant cyclophosphamide, T cell depleting antibodies, thymic irradiation, and post-transplant cyclosporine. Chimerism of lymphocytes, monocytes and granulocytes at one year is shown. Reprinted with permission from Lancet 1999, 353:1755.

malignant disease [1,2,34]. These were the first trials to successfully achieve allograft tolerance in a high proportion of patients with HLA-mismatched donors and are discussed in detail in the article by Kawai et al. Mechanistic studies were carried out in each of these models and are the topic of this review.

2. Mechanisms of tolerance in murine mixed allogeneic chimeras

Before discussing the human studies, it will be useful to briefly review the mechanisms of tolerance that have been well-described in the murine models of mixed allogeneic chimerism upon which our preclinical and clinical trials were based. In these rodent models, mixed chimerism was permanent. T cells present the only major barrier to allogeneic donor marrow engraftment in naïve mice [35], and both intrathymic and peripheral alloreactivity must be overcome in order to permit both engraftment of donor hematopoietic stem cells in the bone marrow and a long-term contribution of donor APCs to negative selection in the recipient thymus [4–6,36,37]. Thymic alloreactivity can be overcome by thymic irradiation [3,6] or by other means, such as high-dose anti-CD4 and CD8 mAbs [5,6] or costimulatory blockade [38,39]. Likewise, peripheral T cell alloreactivity can be overcome by exhaustive T cell depletion [3], costimulatory blockade [38,40] or partial T cell depletion combined with costimulatory blockade [41,42]. BMT is a critical component of the peripheral deletional tolerance that develops in recipients of these costimulatory blockade-based regimens. In the regimens that were translated to the monkey model and human patients, thymic irradiation has been included for the depletion of pre-existing thymocytes and T cell depleting antibodies, along with cyclophosphamide in the clinical regimens, have been used to deplete peripheral T cells. However, because peripheral T cell depletion with these approaches is not as complete as that achieved in the murine models, it has been necessary to include a short course of a calcineurin inhibitor in all of these monkey and clinical trials.

In all of the above murine regimens, peripheral donor-reactive T cells are depleted, either as part of global T cell depletion or as a more donor-specific peripheral deletion that results from the combination of costimulatory blockade and engrafted donor marrow [38,40,41,43–51]. Newly developing thymocytes are tolerized to the donor and recipient by clonal deletion as a result of the presence of both types of APCs in the thymus throughout life [43]. In the presence of complete deletion of donor-reactive T cells in the periphery, regulatory T cells do not play a significant role in maintaining tolerance [45,48,52], and the only

location where ongoing chimerism is required is the thymus, where donor APCs are needed to assure intrathymic deletion of donor-reactive T cells that otherwise can develop throughout life, even in senescent mice [52]. In contrast, mixed chimerism regimens that are not associated with complete deletion of pre-existing donor-reactive T cells are associated with the expansion of donor-specific regulatory T cells (Tregs) that help to maintain tolerance [53–55].

3. Functional readouts to assess mechanisms of tolerance in clinical trials

Consistent with the robust tolerance achieved in the murine models described above, functional assays such as skin graft experiments *in vivo* and mixed lymphocyte responses (MLR) and cell-mediated lympholysis (CML) reactions *in vitro* demonstrate complete and specific unresponsiveness to the donor [6,37,38,40–48]. “Bulk” *in vitro* alloresponses of this kind are generally only measurable in naïve recipients in the setting of MHC mismatches, making them useful as a readout of tolerance in the analysis of clinical trials that cross HLA barriers, but not in the setting of HLA-identical transplantation. In the latter setting, more sensitive assays such as limiting dilution analysis (LDA) can be used in efforts to quantify responses to the donor and determine the possible role of regulatory cells in donor-specific tolerance. The presence of bulk MLR or CML responses to an HLA-identical donor is usually evidence of immune sensitization to that donor.

A patient with a hematologic malignancy receiving HLA-mismatched BMT with our non-myeloablative conditioning approach, who achieved durable mixed chimerism prior to DLI administration, showed donor-specific unresponsiveness *in vitro*, consistent with deletional tolerance. Others in this protocol, who achieved only transient chimerism, showed similar responses to donor and third party when their chimerism was lost [56]. In hematologic malignancy patients receiving the protocol upon which clinical trials of CKBMT using HLA-identical donors to replace destroyed kidneys and treat multiple myeloma were based, an association was observed between low (< 20%) T cell chimerism, increased recipient CD8 T cell recovery and sensitized anti-donor MLR and/or CML as well as LDA responses, and loss of chimerism [57]. In recipients of CKBMT with this protocol, loss of chimerism was associated in some cases with increased anti-donor cytotoxic T lymphocyte (CTL) activity in bulk CML and/or LDA analyses. These responses were restricted to donor hematopoietic cells and did not extend to donor renal tubular epithelial cells, suggesting that the lack of kidney

allograft rejection in association with loss of chimerism might have reflected tolerance to minor histocompatibility antigens shared by the kidney but not to those expressed by hematopoietic cells alone [28]. The one patient who had a kidney graft rejection episode (but eventually achieved tolerance) when immunosuppression was initially withdrawn developed increased GVH reactivity in LDA measuring helper T lymphocyte responses immediately prior to loss of chimerism, which was then associated with sensitized anti-donor CTL LDA when chimerism was lost [28]. This phenomenon was interesting, because our studies in the related murine model had demonstrated a similar phenomenon, in which CD4-mediated without CD8-mediated GVH alloreactivity could trigger a host-versus-graft CTL response that led to loss of donor chimerism [58].

In recipients of HLA-mismatched CKBMT in the absence of malignant disease, donor-specific unresponsiveness or hyporesponsiveness was always observed in bulk MLR and CML assays after the transplant [1,59,60]. LDA assays can be informative not only in quantifying an alloresponse but also in pointing to suppressive mechanisms via a “sawtooth” pattern, in which responses paradoxically increase as responder cell numbers are diluted, reflecting the presence of a suppressive cell population at lower concentration than the responding cells [61,62]. While this pattern was observed in some of the HLA-identical CKBMT recipients [28], it was not observed in long-term HLA-mismatched CKBMT recipients [60], arguing against a suppressive mechanism in the maintenance of donor-specific unresponsiveness and kidney allograft tolerance in these patients. Consistently, while some early anti-donor responsiveness could be revealed by T cell enrichment or Treg depletion in the first year in some HLA-mismatched CKBMT recipients, long-term (> 1 year) MLR and CML responses were not enhanced by depletion of Tregs [59], suggesting that other mechanisms were responsible for long-term tolerance. The persistent donor-specific unresponsiveness in CKBMT recipients contrasts sharply with the re-appearance of anti-donor reactivity *in vitro* after loss of chimerism in recipients of hematopoietic cell transplants alone with similar regimens, pointing to a role for the kidney allograft itself in maintaining tolerance in HLA-mismatched CKBMT with transient chimerism.

Thus, there is a difference in *in vitro* donor reactivity in recipients of HLA-identical vs HLA-mismatched CKBMT after chimerism is lost. HLA-identical recipients showed persistent renal allograft tolerance with evidence of sensitization to donor minor histocompatibility antigens (miHA) expressed on hematopoietic (but not renal tubular) cells *in vitro*, while HLA-mismatched CKBMT recipients showed specific unresponsiveness or hyporesponsiveness to donor hematopoietic antigens *in vitro* when chimerism was lost. The explanation for this disparity may lie with the nature of alloresponses to miHA versus HLA antigens. Responses to miHA are far less potent than those to HLA, as is evident from the requirement for sensitization to detect the former but not the latter in bulk T cell populations. In view of the apparent role of the kidney itself in maintaining tolerance in patients with transient chimerism after CKBMT (see below), it seems likely that recipients of both HLA-identical and HLA-mismatched grafts are tolerant of antigens expressed by the kidney. In the case of HLA-mismatching, tolerance to these myriad antigens, which are largely ubiquitous donor HLA/peptide complexes, results in a loss of measurable bulk MLR and CML responses to the donor. In the case of HLA-matched transplants, however, tolerance to the smaller number of miHA shared by the donor kidney and hematopoietic cells does not preclude detection of sensitization to the subset of miHA expressed by hematopoietic cells alone (and not renal parenchymal cells) when chimerism is lost. Overall, our observations, at least in the HLA-matched setting, are consistent with a kind of split tolerance that allows active rejection of donor hematopoietic cells while the kidney promotes tolerance to antigens that it expresses. It should be noted that chimerism was much more short-lived in the HLA-mismatched compared to the HLA-identical CKBMT protocols at MGH, and it is unknown whether the early loss of chimerism in the former group is due to immunologic rejection or failure of the hematopoietic cells to

engraft for other reasons.

In the HLA-mismatched CKBMT recipients, long-term allograft acceptance without immunosuppression was achieved in 7 of 8 evaluable patients ie those who did not have early kidney graft loss due to preformed donor-specific antibody or an early thrombomicroangiopathy (see article by Kawai et al.) [1,2,34]. One of these 8 patients, however, had acute cellular rejection after immunosuppression was withdrawn and eventually lost his allograft. Surprisingly, this patient showed robust donor-specific unresponsiveness in MLR and CML assays both before and after the rejection [60], indicating that these functional assays cannot be relied upon to specify a tolerant state. The mechanistic underpinning of these findings is discussed below.

4. Role of regulatory cells in donor-specific tolerance in CKBMT recipients

CKBMT recipients in both the HLA-identical and HLA-mismatched cohorts showed enrichment of CD25+ cells among the CD4 populations recovering early post-transplant [28,56]. Our studies of non-myeloablative, anti-CD2 (siplizumab)-based, HLA-mismatched hematopoietic cell transplant recipients without a kidney had revealed enrichment for FOXP3 + CD25 + CTLA4+ cells among the CD4+ T cells recovering in the first few months post-transplant [56]. Similar results were observed in CKBMT recipients with a similar siplizumab-based regimen, where the enriched CD25+ cells were shown to be CD127- and to correlate with Treg-specific demethylated region (TSDR) demethylation levels, indicating that they are Tregs [63]. Tregs were enriched to a level up to 80% of CD4 T cells in the first week post-transplant and only gradually declined to baseline percentages over the first year (Fig. 3A). High-throughput sequencing of T cell receptor beta chain hypervariable (CDR3) regions of post-transplant CD4 T cells and sorted CD25highCD127- CD4+ T cell (Treg) populations confirmed the enrichment post-transplant of Tregs [63]. Absolute concentrations of circulating Tregs recovered to near pre-transplant baseline levels within weeks in these patients, while non-Treg CD4 counts took well over a year to recover to baseline levels [63]. In one patient, sequencing of post-transplant CD4 + CD25highCD127- T cells suggested that significant conversion to Tregs of pre-transplant CD4 non-Tregs had occurred ie that Tregs had been induced from non-Tregs [63]. Phenotypic analyses following CKBMT revealed high levels of Treg proliferation in the early weeks post-transplant, as indicated by Ki67 expression (Fig. 3B), suggesting that they may have been expanding in response to peripheral lymphopenia induced by conditioning and/or to donor antigen. There was also substantial CD31 (Fig. 3D) and CD45RA (Fig. 3C) expression among Tregs at one week post-transplant, both of which declined markedly by two weeks, consistent with phenotypic “memory-like” conversion associated with lymphopenia-driven expansion of human T cells [64]. The majority converted to the “activated” Treg [65] phenotype by 2 weeks, losing CD45RA expression (Fig. 3C), gaining CD45RO and upregulating HLA-DR (Fig. 3E). Whether the initial presence of “resting”-type Tregs [65] expressing CD31 early post-transplant reflects an early wave of emigration from the thymus or simply reflects selective preservation of this Treg subset following conditioning requires further investigation. While full thymic recovery takes months to years in these patients, as shown by the marked delay in recovery of naïve-type CD4 T counts [59,63,66], it is possible that early peripheral inflammation is associated with early migration of APCs bearing donor antigen to the thymus and rapid intrathymic generation of Tregs specific for those antigens, as reported in a murine model [67]. We have observed very rapid, early activation of T cells in these patients [63], even on the day of transplant [68], consistent with a possible role for inflammation not only in the observed “engraftment syndrome”, a vascular leak phenomenon that occurs at the time of hematopoietic recovery in these patients, but also in tolerance induction.

We were able to assess the role of Tregs in the donor-specific hyporesponsiveness in some patients. As mentioned above, persistent

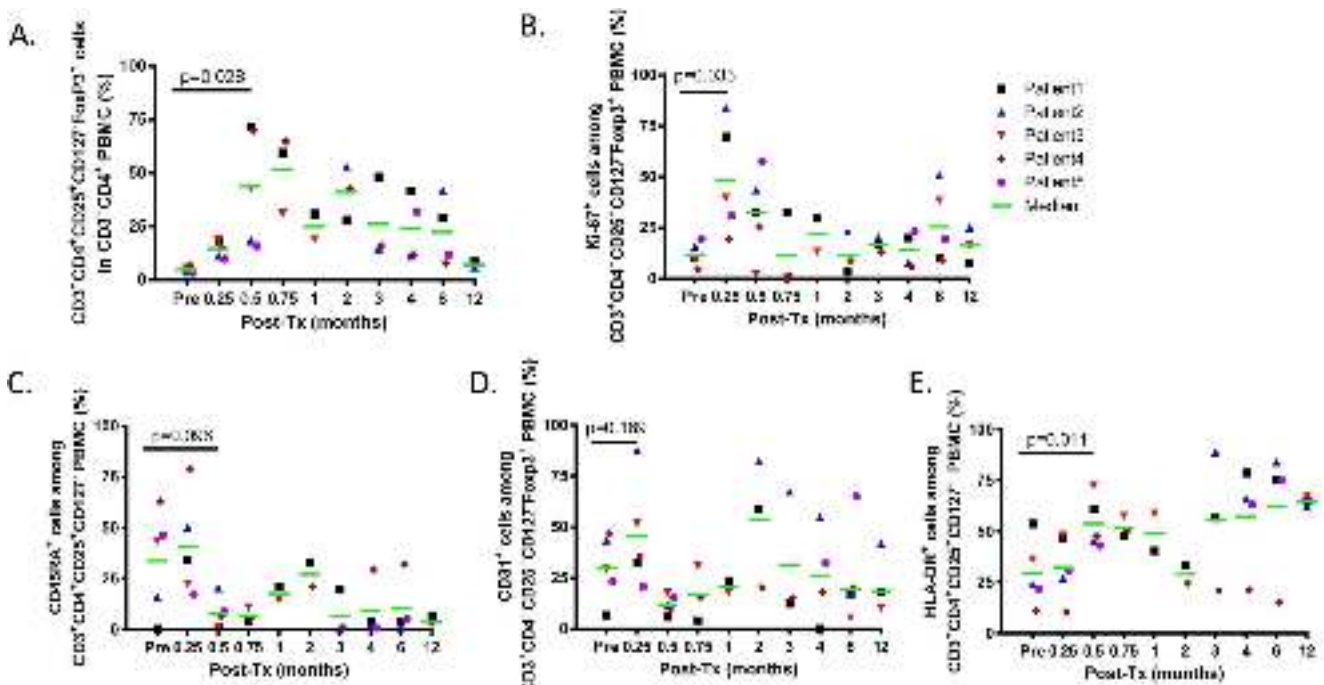


Fig. 3. Treg recovery in five CKBMT recipients on ITN trial ST036. A. Percentages of CD25 + CD127-FoxP3+ cells among circulating CD4+ T cells at the indicated time points post-transplant; B. Percentages of Ki67+ (proliferating) cells among circulating CD25 + CD127-FoxP3 + CD4+ T cells at the indicated time points post-transplant; C. Percentages of CD45RA+ cells among circulating CD25 + CD127- CD4+ T cells at the indicated time points post-transplant; D. Percentages of CD31+ cells among circulating CD25 + CD127-FoxP3 + CD4+ T cells at the indicated time points post-transplant; E. Percentages of HLA-DR+ cells among circulating CD25 + CD127- CD4+ T cells at the indicated time points post-transplant. Each symbol represents the indicated individual patient. Patients 1, 2 and 4 achieved tolerance, whereas Patient 5 rejected the graft following immunosuppression withdrawal and Patient 3 lost the graft early due to a tacrolimus-related thromboembolic microangiopathy [2,34]. Reprinted with permission from American Journal of Transplantation 2017, 17:2020.

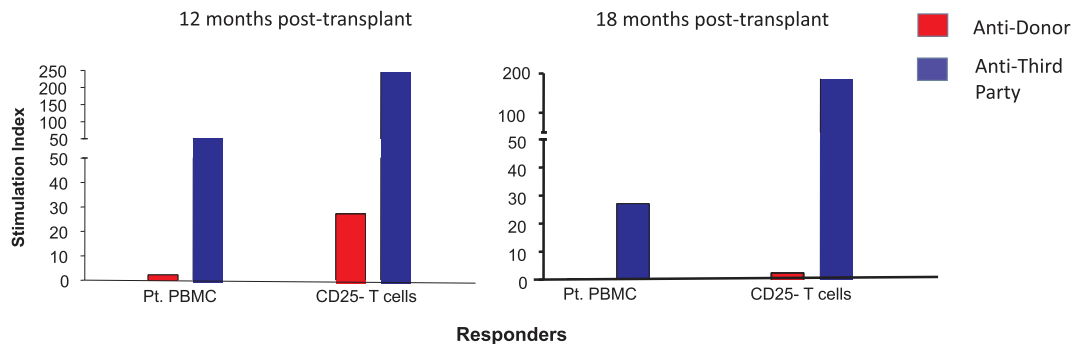


Fig. 4. Donor-specific unresponsiveness in MLR of a CKBMT patient at 12 and 18 months. While whole PBMCs were unresponsive to the donor (red bars) at both time points, an anti-donor response was revealed for purified Treg-depleted T cells at 12 months (left) but not 18 months (right), suggesting that a partially Treg-dependent form of tolerance may have evolved to a deletional mechanism. Third party responses (blue bars) were enhanced by Treg depletion at both time points.

anti-donor reactivity was revealed within the first year in some patient samples by depleting Tregs or non-T cells [59]. However, after this timepoint, depletion of Tregs did not enhance anti-donor reactivity in any patients (e.g. Fig. 4). This result suggested that either deletion or anergy of donor-reactive T cells had occurred over time.

5. Assessing the role of clonal deletion in tolerant patients: A new approach to analyzing the alloresponse

Distinguishing between anergy and deletion as mechanisms of tolerance in human recipients of HLA-mismatched transplants presented a major challenge, as the anti-MHC alloresponse appears to be very diverse, involving many different T cell clones and has shown no evidence of predictable immunodominance (reviewed in [69]). This situation has precluded the use, for example, of HLA-peptide tetramers, which have been used to track miHA-reactive T cells where the peptide specificity and presenting HLA molecule are well-defined [70]. Following the

development of commercially-available platforms for high-throughput sequencing of human TCRβ hypervariable (CDR3) regions, we hypothesized that a large component of the alloreactive T cell repertoire could in fact be identified by sequencing sorted recipient cells that divided in response to donor antigens in an MLR performed prior to transplant, then tracking post-transplant the pool of TCRs that expanded in the MLR compared to unstimulated pre-transplant T cells. Host-vs-Graft (HvG) alloreactive clones were identified as those that expanded at least 5-fold compared to unstimulated cells in this pre-transplant CFSE-MLR and CD4 and CD8 cells were sorted and sequenced separately in these samples. By sampling post-transplant blood and using a uniform frequency threshold for detection of clones to normalize for variability in sample size over time, we were able to assess the rate at which clones designated as donor-reactive prior to transplant were detected in the post-transplant circulating pools. In all 3 tolerant patients tested, a significant decline in the number of circulating donor-reactive CD4 and CD8 clones was detected with this

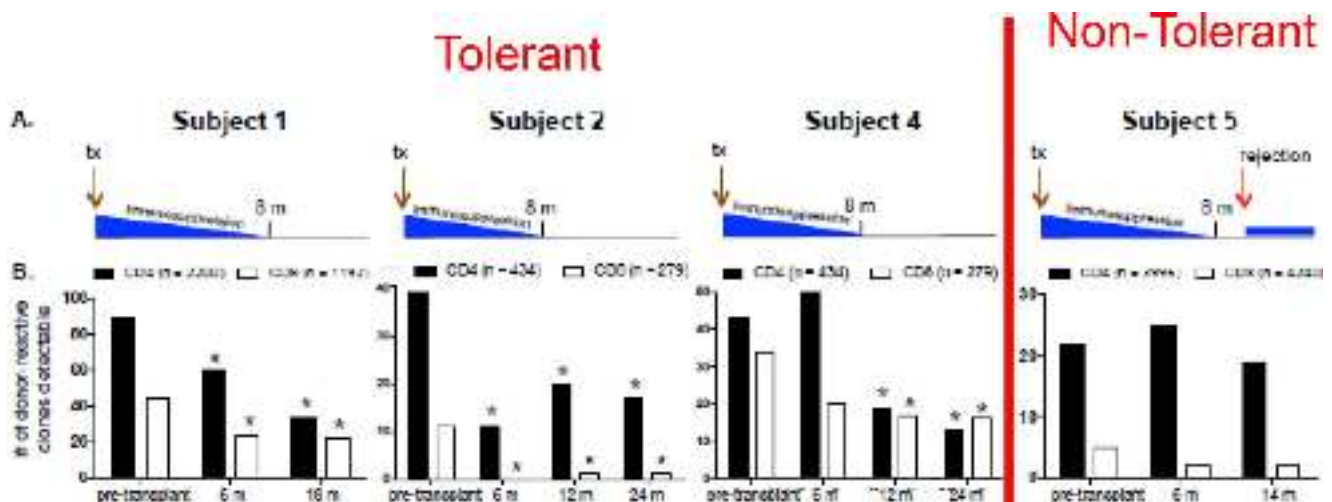


Fig. 5. Gradual loss of circulating donor-reactive T cell clones in 3 tolerant patients on ITN trial ST036 (Subjects 1, 2 and 4) but not in a patient who failed to achieve tolerance (Subject 5). A shows the time course of immunosuppression withdrawal in each patient and B shows the number of circulating donor-reactive CD4 (closed bars) and CD8 (open bars) TCRs detected at the indicated time points. The total number of circulating donor-reactive T cells prior to transplant is also shown. * indicates statistically significant reduction compared to pre-transplant value. Reprinted with permission from Science Translational Medicine 2015, 7:272ra10.

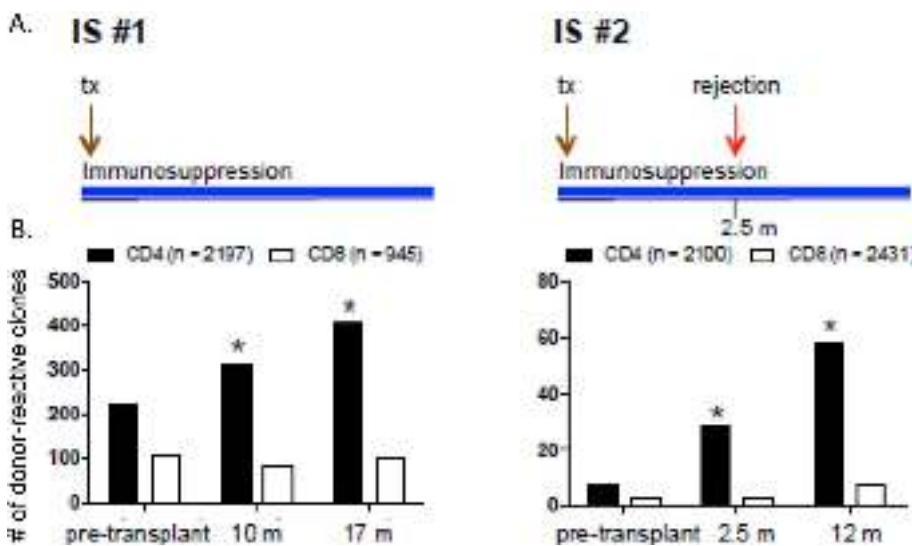


Fig. 6. No loss of circulating donor-reactive T cell clones over time in two conventional kidney allograft recipients. A shows the time course of immunosuppression in each patient and B shows the number of circulating donor-reactive CD4 (closed bars) and CD8 (open bars) TCRs detected at the indicated time points. The total number of circulating donor-reactive T cells prior to transplant is also shown. * indicates statistically significant increase compared to pre-transplant value. Reprinted with permission from Science Translational Medicine 2015, 7:272ra10.

method. In contrast, the patient who failed to achieve tolerance despite receiving the same CKBMT regimen did not show any reduction in the number of circulating donor-reactive T cell clones (Fig. 5) [60]. Since the patient who failed to achieve tolerance had shown donor-specific unresponsiveness in *in vitro* assays, these results suggested that the TCR tracking approach was more specific in identifying a tolerant state than post-transplant functional assays [60]. The functional assays suggested that the donor-specific T cells were “anergic” under the conditions of these assays, but that this anergy did not translate to a robustly tolerant state *in vivo*. Since the kidney graft rejection in this patient followed a urinary tract infection, it is possible that a fragile anergic state was broken by the inflammation induced by the infection. Furthermore, two conventional kidney transplant recipients studied in the same way showed significant increases in the number of circulating donor-reactive CD4 T cell clones [60] (Fig. 6). These findings suggest that tracking of donor-specific TCRs may have the potential to distinguish tolerant from non-tolerant patients, and deserves exploration as a biomarker of “spontaneous” allograft tolerance that would identify patients in whom weaning of immunosuppression could be safely performed.

Further analysis of the above tolerant and non-tolerant patients provided insight into the factors influencing the fate of donor-reactive T

cells in kidney transplant recipients. The kinetics of loss of circulating donor-specific T cells were somewhat variable from patient to patient (see Fig. 5) and in some cases suggested an initial expansion of donor-reactive clones before their eventual deletion. The regimen used for CKBMT in our studies involved extensive peripheral T cell depletion with siplizumab and cyclophosphamide. Comparison of pre- and post-transplant repertoires of these patients revealed considerable turnover of T cells, in marked contrast to untreated healthy controls, who showed strong repertoire stability over the same one-year period [60]. Conventional transplant recipients, one of whom had received ATG to treat rejection following the transplant, also showed considerable repertoire turnover over the period of one year. Thus, the loss of donor-reactive clones in tolerant patients might be considered to be part of a global T cell depletion process. When post-transplant detection of donor-reactive TCRs was compared to that of all clones detected prior to transplant, we found that the rate of disappearance of donor-reactive CD8 but not CD4 clones in some but not all cases exceeded that of non-donor-reactive clones. In some instances, donor-reactive CD4 clones initially showed selective expansion compared to all pre-transplant clones [60]. Given the high level of repertoire turnover in these patients, these results are consistent with the interpretation that donor antigen selectively drives the expansion of donor-reactive T cells under

the lymphopenic conditions produced by conditioning therapy. Thus, the loss of donor-reactive clones in tolerant patients may be regarded as the consequence of both global T cell depletion and the tolerance-inducing conditions that may prevent continued expansion of donor-reactive clones and cause their attrition over time.

Since the patients in our study underwent protocol biopsies of the renal allograft, we used the above sequencing method to interrogate them for the presence of donor-reactive TCR. No enrichment of donor-reactive TCR was seen in these specimens, which were free of rejection and did not contain significant infiltrates. Detailed analysis of the TCR sequences from the biopsies revealed similar repertoires to those detected in post-transplant peripheral blood, suggesting that T cell sequences detected in these biopsy specimens represented cells within the graft microcirculation. The only exception was a post-rejection specimen from the CKBMT recipient who failed to achieve tolerance, in which the ratio of donor-reactive CD8 to CD4 T cells was elevated compared to those in the circulation, suggesting that residual graft-infiltrating T cells may have been present [63].

Overall, the association of results of T cell clonal analysis by our method with clinical events in CKBMT and conventional transplant recipients strongly suggested that biologically relevant clones are identified as donor-reactive with this approach. Further validation of the biological relevance of this method was obtained through serial surveillance biopsies of intestinal transplants, in which TCRs identified as donor-reactive dominated among recipient-mappable TCRs during rejections [71] and donor TCRs identified pre-transplant as GVH-reactive were expanded in association with recipient APC replacement of donor APCs within the graft [71]. Thus, our technique has considerable potential to track the fate of alloreactive T cells in a variety of transplant settings, providing insight into events within the graft itself. The approach deserves exploration in both additional solid organ transplants and in hematopoietic cell transplantation, where it could enhance the tissue diagnosis and provide insight into the pathogenesis of GVHD. The approach may also have the potential to predict tolerance and rejection, a possibility currently being explored in our laboratory.

6. Conclusions

Our studies have strongly implicated an initial role for expanded donor-reactive Tregs in promoting tolerance in CKBMT recipients with transient chimerism. Studies are in progress in our laboratory to use the high throughput TCR sequencing approach to identify the alloreactive Treg repertoire and examine this hypothesis directly. We also hope to determine the contribution of natural vs induced Tregs in this way. The possible role of new Treg emigrants from the thymus must also be considered in light of the phenotype observed very early post-transplant within this cell population. Additionally, our studies suggest that a gradual attrition of pre-existing donor-reactive T cells takes place after tolerance is achieved, perhaps after an initial period of expansion of these clones during the lymphopenic period of exposure to donor antigen. The mechanisms leading to donor-specific Treg expansion and ultimate deletion of donor-reactive effector T cells are not currently understood. However, the Treg expansion may be driven by the donor-specific effector cell activation and expansion that occurs early and that may also promote the observed “engraftment syndrome”. Anti-CD2 mAb has costimulatory blocking properties in addition to T cell depletion, and this may play a role in these early events. The incomplete deletion of donor-reactive T cells by the conditioning protocol is undoubtedly important in driving the expansion of donor-reactive Tregs, as seen in murine mixed chimerism regimens [53–55]. In view of the greater ease in inducing tolerance to renal allografts than to other organs with transient chimerism [72,73] and the failure to achieve donor-specific hyporesponsiveness via induction of transient chimerism without a kidney allograft [56], it is reasonable to assume that the kidney itself plays a role in this long-term tolerance. We speculate that the unique circulation of the kidney, in which lymphocytes encounter

donor endothelial cells as they flow through the vast capillary microcirculation of non-inflamed renal glomeruli, results in chronic high-level antigenic exposure that may lead to senescence, anergy or exhaustion that ultimately culminates in deletion, as observed in some murine studies [74,75]. Indeed, while we were not able to detect enrichment for Tregs in small renal allograft biopsy specimens from tolerant patients lacking significant infiltrates [63], Tregs and other regulatory cells may contribute to T cell exhaustion [75] and deletion of alloreactive T cells [48] and we may simply not have had sufficient early biopsy samples to detect such a mechanism.

Our studies have been limited by the lack of detailed functional information on residual T cell responses, which could include global RNA sequencing studies or more specific analyses of cytokine and chemokine responses in the future. Additionally, we not have separated indirect and direct alloresponses in our alloreactive TCR studies. The inclusion of both donor and recipient APCs in the CFSE-MLRs used for sorting alloreactive T cells that undergo TCR sequencing allows the potential detection of both directly and indirectly alloreactive T cells, though directly reactive cells are assumed to dominate the response. Challenges in isolating indirect responses for TCR analyses include the expected low frequency of such T cell clones in non-sensitized recipients and the difficulty distinguishing semi-direct from true indirect presentation when donor cell lysates or apoptotic cells are used as a source of antigen. These problems could be avoided by the use of purified donor proteins such as HLA molecules as the stimulus for indirect allorecognition, but such an approach would need to be tailored for the donor specificities to which the recipient is likely to be sensitized, perhaps as determined by alloantibody responses, and would not capture all possible specificities of an indirect response. Further functional studies of alloreactive T cells at various stages as tolerance evolves, as well as identification and analysis of the donor-specific Treg and indirectly alloreactive TCR repertoires, should help to elucidate mechanisms in future patients. On a practical note, it should be pointed out that the extensive mechanistic and TCR analyses on our CKBMT patients were made possible by the availability of large numbers of lymphocytes obtained by leukapheresis. While sufficient cells for assays already established (eg the TCR analyses) are available from conventional phlebotomy, the ability to develop new assays and evaluate new mechanistic hypotheses is greatly facilitated by the availability of archived patient and donor specimens.

Ultimately, we aim to achieve durable mixed chimerism without GVHD to induce long-term deletional tolerance to all types of organs and to islet allografts. In view of the proof of principle that this can be achieved in a handful of patients with haploidentical donors [14,20], this is a realistic goal.

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