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The Bidirectional Paradigm of Transplant Immunology[a]

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Until 1992, the conventional view of transplantation immunology was what we have referred to as the one-way paradigm (FIG. 1A and B), a conceptual framework that had been extrapolated from the neonatal tolerance model of Billingham, Brent, and Medawar.[1,2] In Medawar's defenseless recipient experiments, and in the parent to offspring F_1 hybrid and recipient cytoablation models (FIG. 1A), it was learned in the 1950s that the risk of lethal graft-versus-host disease (GVHD) after splenocyte or bone marrow transplantation was directly proportional to the degree of MHC incompatibility.

As early as 1959, it was known that all the same rules applied when whole organs containing immunologically active cells, such as the intestine, were transplanted. Thus, any kind of hematolymphopoietic transplantation was conceived to be an essentially one-way cellular transaction, yielding either GVHD, rejection, or tolerance.

THE DEFECTIVE ONE-WAY PARADIGM

In this context, it was perfectly logical to view solid organ transplantation as a mirror image of the bone marrow experiments, the difference being that the graft was the defenseless victim instead of being the aggressor (FIG. 1B). This one-way paradigm in the opposite direction became the disorienting dogma upon which most clinically directed transplantation research was based for the next third of a century.

The One-Way In Vitro *Tests*

Ironically, the introduction of *in vitro* models beginning with the one-way mixed lymphocyte reaction (MLR) in 1963[3,4] further supported this dogma. These so-called

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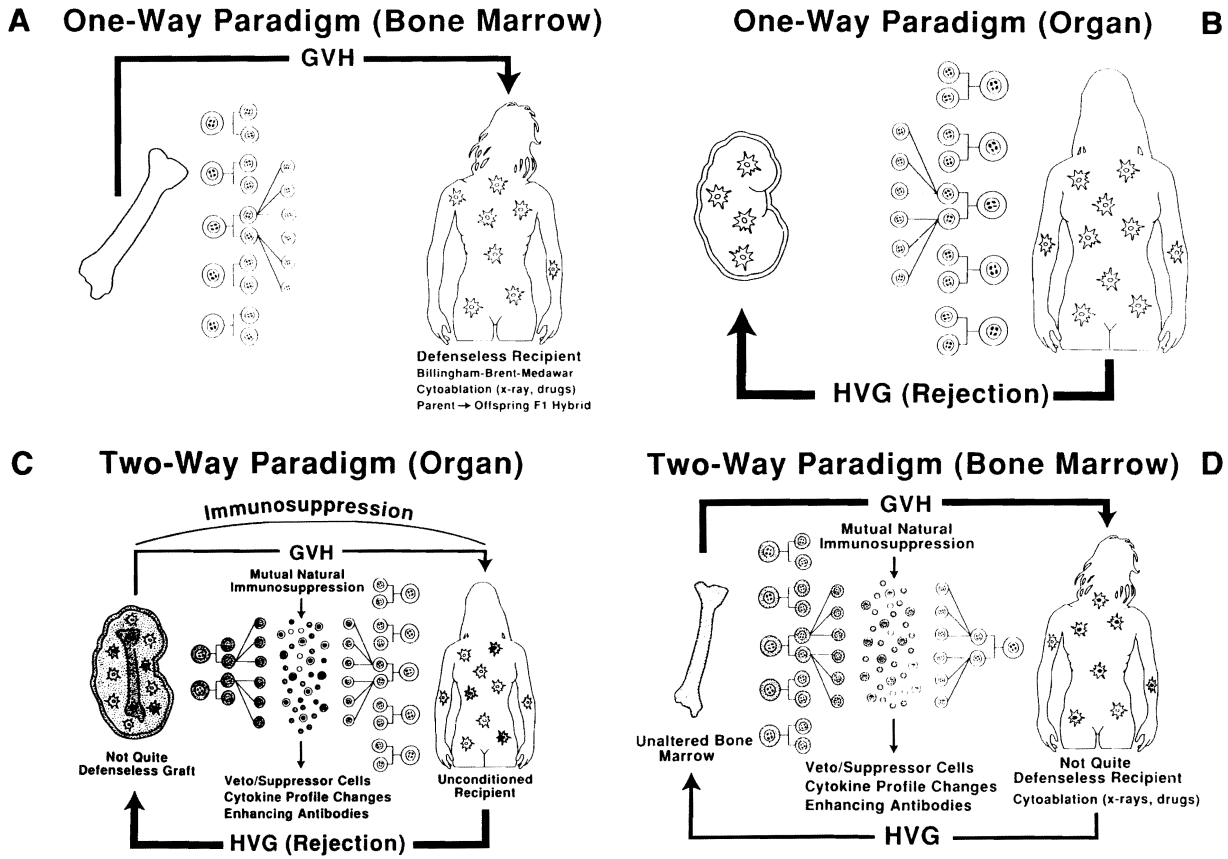

FIGURE 1. Transplantation immunology as seen with the conventional one-way paradigm (**A** for bone marrow and **B** for whole organ transplantation). The two-way paradigm is shown in **C** (whole organ) and **D** (bone marrow transplantation).

"minitransplant models" generated thousands of increasingly sophisticated cellular and ultimately molecular studies of immunologic interactions, the full understanding and clinical exploitation of which was hampered by the restrictive context of the one-way paradigm.

With Clinical Bone Marrow Transplantation

Aside from the overwhelming support provided by the unidirectional *in vitro* tests, the one-way paradigm was ostensibly strengthened in 1968 when the strategy of recipient cytoablation before bone marrow transplantation finally was extended from the mouse model to successful clinical application, emphasizing at every step the need for HLA compatibility if stable engraftment (called tolerance) was to be accomplished without the complication of GVHD.[5,6]

With Whole Organ Transplantation

However, the one-way paradigm never explained what was being observed and accomplished with organ transplantation without dependence on MHC matching, without host preconditioning, and with no GVHD. Because of these striking "violations of rules," organ transplantation was dissociated from the kind of rational scientific base enjoyed by those involved in bone marrow transplantation. In fact, the tumultuous development of the whole organ field can only be described as empirical. Treatment was based on the assumption that continuous immunosuppression would be required for life to maintain adequate graft function.

The avalanche of clinical whole organ cases began in 1962-1963 when kidney recipients were treated with the combination of azathioprine and prednisone at the University of Colorado.[7] A characteristic postoperative pattern was recognized in which rejection was found surprisingly to be easily reversed with augmented doses of prednisone. More importantly, maintenance immunosuppression could later be progressively reduced and even stopped in some cases. The same sequence of immunologic crisis and resolution has since been seen with all other organs successfully transplanted and with all of the clinical immunosuppressive regimens (FIG. 2). Something appeared to have changed in the host, the graft, or both. But what?

THE DISCOVERY OF CHIMERISM

A plausible answer was found in 1992, when a group of the original still surviving Colorado kidney recipients (then approaching 31 years posttransplantation) and more than 2 dozen liver recipients (10-23 years posttransplantation) were restudied.[8-13] A low level of donor leukocyte chimerism was ubiquitously found in biopsies obtained of the graft, of multiple host tissue sites, and in blood.

The chimerism was thought to be multilineage, but the dominant cell population had the morphologic characteristics of dendritic cells. Because the number of donor cells was small, skeptics claimed, and perhaps some still do, that these cells were

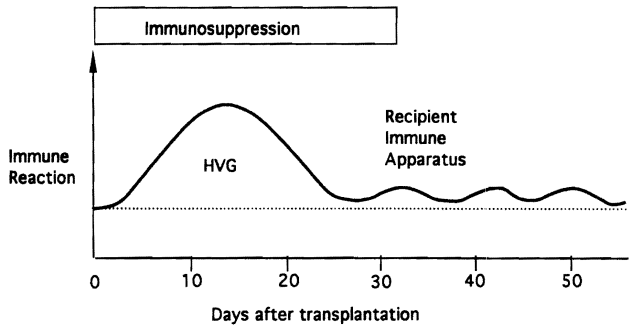

FIGURE 2. Postoperative events using the one-way paradigm to interpret events after successful whole organ transplantation.

merely an epiphenomenon of tolerance (or graft acceptance), not the cause of it. However, this position has become increasingly difficult to justify.

Confirmatory evidence of donor HLA alleles was obtained with polymerase chain reaction (PCR). Karyotyping in patients with opposite-sex donors, with either *in situ* hybridization or PCR, yielded similar results. All 30 of the chronically surviving kidney and liver recipients studied in 1992 were chimeras. The leukocytes of bone marrow origin, which are resident in all tissues, apparently had migrated and been assimilated by the overwhelmingly larger immunologic network of the host. In essence, a small fragment of disseminated extramedullary donor bone marrow, depicted in FIGURE 1C as a bone silhouette, had accidentally been engrafted. These observations provided the basis for the formulation of the two-way paradigm.

THE TWO-WAY PARADIGM

In the two-way paradigm, the immunologic confrontation following whole organ transplantation involved a graft-versus-host (GVH) as well as host-versus-graft (HVG) component in which the two cell populations were somehow reciprocally modulating, provided that both could survive (FIG. 3). Veto cells, suppressor cells, cytokine profile changes, and the development of enhancing antibodies seemingly had an accessory and ultimately crucial role in the development of reciprocal nonreactivity (FIG. 1C). However, these were derivative from the primary event of the David versus Goliath mutual cell engagement. The umbrella of immunosuppression that equally covered both in the empirically developed clinical protocols had permitted these changes.

It could be seen that the vast gap between the fields of bone marrow and whole organ transplantation merely reflected entrenched differences of treatment strategy, leaving intact the mutually censoring immunologic limbs with organ transplantation and deliberately trying to remove one of the limbs for bone marrow grafting procedures, following a recipient cytoablation.

However, one detail remained before the linkage was seamless. Although complete donor chimerism had long been assumed to be the objective of bone marrow trans-

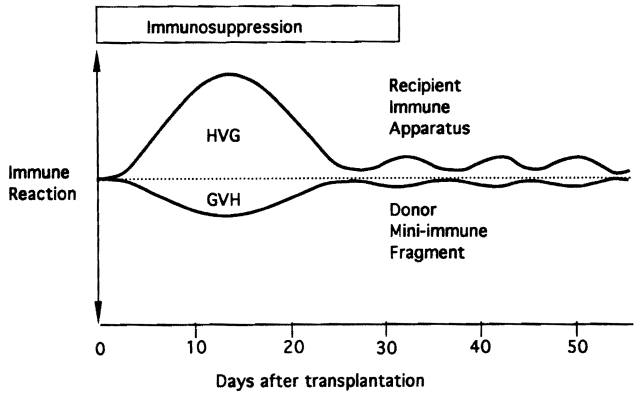

FIGURE 3. Postoperative events in the two-way paradigm after whole organ transplantation. The *small lower curve* is the graft-versus-host reaction which usually is silent.

plantation, Thomas and others[14,15] recently showed that in recipients of bone marrow from opposite sex donors, a trace population of recipient leukocytes can always be detected with sensitive molecular techniques. Thus, the veto and other accessory events at the cellular interface are the same in principle with bone marrow as with organ transplantation (FIG. 1D).

With either approach to treatment (that for whole organs versus that for bone marrow), the resulting reciprocal nonreactivity of the two populations is a natural event that apparently is only permitted, not caused, by the drugs we use. Chaperoned would be a better term, because it does not seem to matter where these agents interdict the allogeneic response: from the second signal transduction proximally (i.e., with CTLA4Ig fusion proteins) to the most distal inhibition of p70 S6 kinase activity by rapamycin or any site in between. By choosing an "easy" strain combination of rodents, these agents as well as dozens of less potent molecules sometimes have been made to look like Hercules, when a single dose or multiple doses were shown to induce permanent donor-specific tolerance, when in fact the model (not the drug) was the principal factor.

EXPERIMENTAL CLARIFICATION

A Rat Model

In an illustrative experiment of tolerance induction across a moderately difficult barrier,[16] Brown Norway rat recipients were transplanted with four different fully allogeneic Lewis organs (liver, heart, kidney, and intestine) or with a standard dose of Lewis cell suspensions from four different sources (bone marrow, thymus, spleen, and lymph node), with or without immunosuppression with tacrolimus.

No Treatment. As expected without immunosuppression, all Lewis cell suspensions were rejected by the Brown Norway recipients. There was no mortality, but

after 100 days, there was no chimerism. The hearts, kidneys, and intestines were rejected after 7.5-12 days and the livers in 28 days.

Transient Tacrolimus (FK 506) Treatment. A 2-week course of treatment with tacrolimus with two supplementary single doses at days 20 and 27 dramatically changed the outcome.[16] All hearts, kidneys, and livers now survived to 100 days, drug free for the last three quarters of this time. Bone marrow engrafted silently with no overt evidence of GVHD. The intestines were not rejected. However, all of the bowel recipients died of GVHD at about 45 days. Spleen and lymph node cell suspensions behaved like intestine, always causing GVHD. Thymocytes failed to engraft.

The striking divergence of outcome with different allografts reflected their cellular composition.[16] The lack of some essential factor(s) may account for the failure of engraftment of T-cell-rich adult thymocytes. The infusion of T- and B-cell-rich splenocytes and lymph node cells led to GVHD, whereas engraftment of the clinically inocuous bone marrow correlated with a large component of immature cells of undetermined phenotype.

At 100 days, the animals successfully engrafted with bone marrow, heart, or liver underwent examination with *in vitro* mixed lymphocyte reaction and cytotoxic assays. These revealed consistent anti-donor reactivity (so-called split tolerance). Now drug free, the animals accepted challenge livers from donor, but not third-party animals. The hepatic allografts went through spontaneously resolving rejection in rats primed originally with either donor-strain hearts or bone marrow. After passing through these crises, all of the orthotopically transplanted challenge livers permanently supported life and thereafter were completely normal histopathologically.

Challenge heart grafts that normally are rejected in 8 days were also accepted by the drug-free animals primed with liver, bone marrow, or heart, with no clinical failures in any of these groups. However, when examined histologically, the challenge hearts appeared completely normal at 100 days posttransplantation only in rats primed originally with donor-strain liver. In rats that had been primed with bone marrow, challenge hearts had the subendothelial infiltration of recipient lymphoid cells (called Quilty lesions) that are generally considered as very early premonitors of chronic rejection.[16]

Animals primed originally with hearts were at the lowest end of the tolerance scale. Challenge hearts in these rats also escaped clinical rejection and continued to function along with the priming hearts, but at the 200-day milestone both first and second hearts showed the classic proliferative arterial lesions of chronic rejection as well as low grade cellular infiltration.

The Tolerance/Chimerism Relation. The spectrum of tolerogenicity defined by histopathologic outcome was liver best –> bone marrow next –> heart least. These results correlated with the chimerism produced by the priming transplant. The lineage composition of the chimerism caused by the tolerogenic priming allografts included T and B lymphocytes and was qualitatively similar to that caused by the transplantation of GVHD-inducing allografts such as intestine, lymph node cells, or splenocytes. However, there were fewer total number of donor leukocytes in the recipient tissues, a smaller proportion of T cells, and a more prominent population of cells of myeloid lineage, notably dendritic cells.[16]

Mouse Liver Transplantation

The use of drugs like tacrolimus in such models could obscure the search for fundamental mechanisms of natural tolerance. Consequently, the spontaneous tolerance induced by orthotopic liver transplantation in the mouse model has presented unique opportunities for investigation, particularly because so much about mammalian immunology is learned from this species. Qian *et al.*[17] have shown that in virtually all strain combinations, the majority of mouse liver recipients survive permanently without immunosuppression.

As in the rats, low levels of donor cell chimerism were typically observed in animals followed for more than 300 days posttransplantation. As expected, the liver recipients accepted subsequently transplanted donor heart and skin despite retention of donor specific MLR and CML reactivity (again split tolerance). It was noteworthy that the induction of donor-specific nonreactivity by primary heterotopic heart transplants in some strain combinations precluded completion of these experiments. This induced tolerance by the mouse heart as well as the observations made in rats showed that such tolerogenicity that is most readily induced with hepatic grafts is not organ-specific. Hepatic tolerogenicity is only an extreme illustration of a cardinal principle operational with all tissues and organs, based on donor leukocyte migration and chimerism.[17]

The Nature of Cell Migration

This trafficking of donor leukocytes after whole organ transplantation was described in the classic 1981 article by Nemlander and coworkers[18] of Helsinki. This was in the context of allosensitization as emphasized a decade later in the equally classic studies of dendritic leukocyte migration after heterotopic cardiac transplantation in mice by Larsen et al.[19] However, our investigations placed the phenomenon of donor cell migration squarely in the context of tolerance, both in transiently immunosuppressed rats[16,20] and in drug-free mice.[17] With both species, the migratory donor cells began to home to the central and secondary lymphoid organs within minutes after transplantation. After pausing there for 1 or 2 weeks,[17,20] they broke out and became generalized, including the skin where they can so easily be found in patients.

THE DONOR/RECIPIENT CELLULAR INTERFACE

The question of how the chimeric cells survive and induce donor-specific tolerance was examined by a team that included Angus Thomson, Anthony J. Demetris, and the husband and wife team of Drs. Lina Lu and Shiguang Qian. The hepatic leukocytes were the target of their investigation because of overwhelming evidence that the liver was more tolerogenic than any other organ. After discarding the hepatocytes and duct cells, approximately 10^7 nonparenchymal cells (NPCs) could be obtained from one mouse liver.[21]

The technology with which the suspect tolerogenic cells were studied was described in 1992 by Inaba and co-workers.[22,23] Following in their tracks, Thomson and

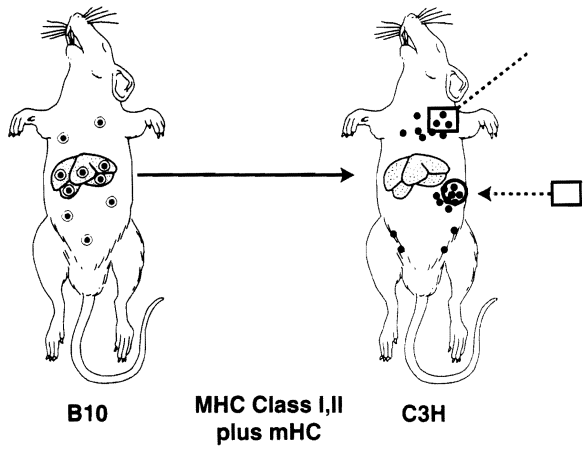

FIGURE 4. Experimental design in mice in which peripheralized donor leukocytes in whole organ allografts were shown to migrate and form self-perpetuating peripheral cellular oases. (See text for discussion.)

Lu cultured the NPCs in GM-CSF enriched medium. After 4 or 5 days of culture, approximately 2×10^6 cells with dendritic morphology and surface phenotype were identified.[21] A subpopulation of these cells formed clusters on the bottom of the culture wells. Loosely adherent cells were harvested and further cultured and studied according to the methods described by Inaba and co-workers.[22,23] Although they had the phenotype and function of precursor dendritic cells, it was difficult at first to prove their dendritic leukocyte origin because it was impossible to drive them to maturation, even with the addition of gamma-interferon and tumor necrosis factor. They had poor allostimulatory function, expressed low levels of MHC class II antigen, and were avidly phagocytic.[21]

This impasse was broken when the culture wells were coated with Type I collagen, thus stimulating the natural microenvironment of liver where mature dendritic cells are normally known to reside. Under these conditions, the precursor cells in the wells promptly assumed the properties of mature dendritic cells, now expressed high levels of MHC class II antigen, and acquired potent allostimulatory activity. The question of whether these unusual cells would mature and express class II antigen *in vivo* was investigated by injecting the purified precursor cells from B10.BR livers into the footpad of fully allogeneic B10 mouse recipients. The cells migrated promptly to the T-cell areas of the central lymphoid organs where they were easily phenotyped as donor and shown to express high levels of class II antigen.[21]

In the crucial next step, liver transplantation was carried out in the fully allogeneic but nonrejecting mouse stain combination (B10 to C3H).[24,25] The recipient animals were of course chimeric, and samples were collected from their bone marrow, spleen, thymus, and lymph nodes (FIG. 4). Donor as well as recipient precursor dendritic cells, at variable stages of maturation, were demonstrated in these samples, using the same culture techniques as had been used previously for study of liver-derived

NPCs. These observations suggest that these cells are derived from precursor dendritic cells and presumably pluripotent stem cells that have migrated from the graft and have widely distributed throughout the recipient tissues.[24,25] The profile and the ease with which these peripheralized cells (both donor and recipient) could be identified were much the same 4, 14, or 150 days after liver transplantation.

Although the same events occurred in heart recipients who rejected their allografts, the peripheralized donor cells were transient and could no longer be found 30 days after cardiac transplantation.[25] Although the cellular beachhead was the same, it was too feeble to be self-sustaining.

Thus, it appears that after transplantation, allografts export immature dendritic and probably stem cells, which establish residence in many preactive niduses within the recipient tissue, creating widespread and persistent cellular oases, presumably swimming in cytokines and other growth factors. Lu *et al.*[25] have obtained evidence that the dendritic precursor cells may be tolerogenic. These remarkable findings have suggested not only a mechanism for perpetuation of the migratory dendritic cells, but also a means by which the chimeric cells can exert a tolerogenic effect.

CLINICAL DONOR LEUKOCYTE AUGMENTATION

If our hypothesis of the mechanism of graft acceptance is correct, it should be possible to safely facilitate this process by adding unaltered donor bone marrow perioperatively to the minimal dose of the so-called passenger leukocytes contained in a whole organ allograft. Such a trial is well underway in Pittsburgh[26–28] and now includes 89 patients entered between December 1992 and February 1995. Donor bone marrow cells, obtained from cadaveric vertebral bodies, were not T-cell depleted or modified prior to infusion. Subsequent to organ placement, $3\text{-}5 \times 10^8$ cells/kg were infused into nonconditioned recipients who were then maintained on routine immunosuppression with tacrolimus and prednisone. No complication of bone marrow infusion was observed in any of the 89 primary whole organ transplant recipients, and their convalescence was rapid. The cumulative risk of rejection was similar in both bone marrow augmented and nonaugmented recipients and there was no incidence of serious GVHD. The results of this study are summarized in TABLE 1.

CONCLUSION

In FIGURE 1, the bottom panels (C, D) portray what transplantation immunology looks like after eliminating the blindfold of the one-way paradigm, which is depicted in the upper panels. A third of a century ago, Simonsen[29] and Michie, Woodruff, and Zeiss[30] challenged the one-way paradigm. While sound, their views were not accepted because the ideas could not be proved. In experiments prior to this, Martinez, Shapiro, and Good[31] described, without recipient cytoablation or immunosuppression, mutual immunologic tolerance in parabiotic mice, that could only be explained by the two-way paradigm. In retrospect, the resemblance its obvious of Good's mutually tolerogenic parabiotic partners, the allograft/host cellular relationship of our experiments, and for that matter the observations of chimerism in Freemartin cattle by Owen in 1945.[32]

TABLE 1. Function and Actuarial One-Year Graft Survival of Bone Marrow Augmented and Nonaugmented Whole Organ Transplant Recipients

			Function ($\bar{x}$ ± SD)			
Organs Transplanted	*n*	Graft Survival[a]	Bilirubin (mg/dl)	Creatinine (mg/dl)	Cardiac Output (L/min)	FEV_1 (L)
Bone Marrow Augmented						
Liver	34[b]	31 (91%)	0.8 ± 0.6	—	—	—
Kidney	40[c]	39 (98%)	—	1.7 ± 0.6	—	—
Heart	10	08 (80%)	—	—	6.0 ± 1.7	—
Lungs	05	05 (100%)	—	—	—	2.0 ± 2.0
Controls						
Liver	33	26 (79%)	0.7 ± 0.3	—	—	—
Kidney	21[d]	18 (86%)	—	2.2 ± 1.5	—	—
Heart	04	06 (100%)	—	—	6.3 ± 3.2	—
Lung	01	0	—	—	—	—

[a] Actuarial 1-year graft survival.

[b] One type I diabetic also received pancreatic islets; not insulin-free.

[c] Nineteen type I diabetics also received either whole organ pancreas (n = 13) or isolated pancreatic islets (n = b); 11 patients (all recipients of pancreases) are insulin-free.

[d] Four type I diabetics also received whole organ pancreas transplants; two are currently off insulin.

The continuity of this theme was interrupted in the early 1960s and not restored until 1992, when our observation regarding the persistence of donor cells in long-term allograft recipients exposed the cellular events that transpire after organ transplantation. The therapeutic implications of the two-way paradigm, including the iatrogenic augmentation of spontaneous chimerism with perioperative unaltered donor bone marrow, are obvious. In our clinical trials, adjuvant bone marrow under conventional tacrolimus/prednisone immunosuppression has never caused clinically significant GVHD. Levels of chimerism 1,000 times greater than that occurring spontaneously have been regularly produced.

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