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MANIPULATING THE IMMUNE SYSTEM FOR ANTI-TUMOR RESPONSES AND TRANSPLANT TOLERANCE VIA MIXED HEMATOPOIETIC CHIMERISM

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Summary

Stem cells (SCs) with varying potentiality have extensive potential to repair injured tissues. While promising animal data have been obtained, allogeneic SCs and their progeny are subject to immune-mediated rejection. Here, we review the potential of hematopoietic stem cells (HSCs) to promote immune tolerance to allogeneic and xenogeneic organs and tissues, to reverse autoimmunity and to be used optimally to cure hematologic malignancies. We also review the mechanisms by which hematopoietic cell transplantation (HCT) can promote anti-tumor responses and establish donor-specific transplantation tolerance. We discuss the barriers to clinical translation of animal studies and describe some recent studies indicating how they can be overcome. The recent achievements of durable mixed chimerism across HLA barriers without graft-versus-host disease and of organ allograft tolerance through combined kidney and bone marrow transplantation suggest that the potential of this approach for use in the treatment of many human diseases may ultimately be realized.

Keywords

hematopoietic cell transplantation; mixed chimerism; graft-versus-host disease; graft-versus-tumor; tolerance

Introduction

Stem cells (SCs) have extensive potential to repair injured tissues, including myocardium, nerves, kidney, liver, lung, and pancreatic islets (1;2) and thereby treat human disease. Organ replacement might be achieved through *ex vivo* differentiation into organs or tissues from stem cells followed by transplantation, or repair could be achieved by transplantation of the stem cells themselves, which could then differentiate *in vivo*. Many different types of SCs with varying potentiality have been described. While the field is still in its infancy, transplantation of some SC types has entered early clinical trials. Mesenchymal stem cells (MSCs), for example, have the potential to differentiate into bone, cartilage, adipose, tendon, and muscle tissue. Because of their low immunogenicity and suppressive properties *in vitro* (3;4;5;6), they have been administered to allogeneic recipients. In experimental models, MSCs have been shown to promote bone marrow engraftment by enhancing hematopoietic cell expansion and differentiation and to suppress immune activation and, therefore, rejection and graft-versus-host disease (GvHD) (7;8). While MSCs clearly are more immunogenic *in vivo* than originally

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hoped (9) and therefore may have limited potential to achieve tissue regeneration in the allogeneic setting, their broad immunosuppressive properties can reverse GvHD (10). Furthermore, there is some evidence to suggest that intracardiac injection of MSCs may enhance myocardial repair processes (11). Neural stem cells have also begun to be evaluated clinically (12).

While promising animal studies provide the basis for the clinical studies underway, the animal data often involve syngeneic transplants and do not address a key issue related to the use of allogeneic SCs for therapy, namely immune-mediated rejection. This problem would be avoided if SCs derived from autologous cells could be used in patient-specific therapies. For example, if truly pluripotent stem cells, such as embryonic stem (ES) cells, could be derived from more differentiated, adult-derived cells via “de-differentiation”, tissues and organs that are autologous to the recipient could be generated. In recent studies (13;14), retroviral transduction of a few key transcription factors (Oct3/4, Sox2, and Klf4 with or, less efficiently, without the proto-oncogene c-Myc) into human dermal fibroblasts generated “induced” pluripotent stem cells capable of differentiating into cell types of all three germ layers *in vitro* and in *in vivo* teratomas (13;14). Furthermore, ES cells can be directed to differentiate into hematopoietic stem cells that might subsequently be used for hematopoietic cell transplantation (15). This approach could then be used for induction of tolerance to other organs derived from the same pluripotent stem cells. However, there are major barriers to allogeneic HCT that must be overcome before HCT can be used for tolerance induction, as is discussed below. Moreover, we are still many years away from being capable of generating functioning organs *ex vivo* from ES cells. Finally, for successful clinical application of ES cell-based therapy, the unacceptable risk of teratoma or teratocarcinoma formation due to contamination by undifferentiated cells and the potentially deleterious mutagenesis induced by random integration of foreign DNA into the genome via retroviral vectors must be addressed. The focus of this review is transplantation of a particular type of stem cell, namely the hematopoietic stem cell (HSC). These cells are present throughout life and can generate all cell types of the lymphohematopoietic system. They are self-renewing and thereby capable of sustaining hematopoiesis long-term. The practice of HSC transplantation is well established in experimental models and in man. We review here the mechanisms by which hematopoietic cell transplantation (HCT) can induce anti-tumor responses and establish donor-specific transplantation tolerance.

Experimental HCT in animals began in 1951 with the demonstration that the lethal effect of irradiation injury could be avoided by infusing BMCs (16). This experiment was preceded by observations that shielding of the spleen or femur of animals during experimental irradiation was protective against lethality (17). Initial attempts to translate HCT into the clinic began in the late 1950s and early 1960s (18). The commonest indication for HCT is the treatment of malignant disease. Other indications include congenital immunodeficiency diseases and aplastic anemia. The original concept behind using HCT in the context of malignant disease was to rescue the recipient from hematopoietic failure following high-dose chemo/radiotherapy aimed at tumor eradication. Both allogeneic and autologous HCT have been explored for the treatment of hematologic malignancies and solid tumors with this goal in mind. Allogeneic HCT was considered to be advantageous over autologous transplantation because the former lacked the potential to reinfuse tumor cells into the recipient. However, as clinical data accumulated, it became apparent that allogeneic HCT had a major immunotherapeutic benefit, termed a graft-versus-leukemia (GvL) or graft-versus-tumor (GvT) effect. This observation, which is discussed in more detail below, led to the development in recent years of reduced-intensity conditioning regimens. This approach relies on the GvT effect via the allogeneic HCT to eradicate the tumor. Reduced-intensity conditioning regimens are often referred to as “non-myeloablative”. The use of this term here is reserved for regimens that have been shown to

leave sufficient recipient hematopoiesis intact to allow recovery of normal hematopoietic function if no HCT is given or if an allogeneic HCT is rejected.

A unique feature of HCT compared to other types of transplantation, in which immune rejection is usually considered only in the host-versus-graft (HvG) direction, is the ability of T cells in the hematopoietic allograft to mount an immunological attack on the recipient's tissues, resulting in graft-versus-host (GvH) responses and the associated disease, GvHD, which is primarily a disease of the skin, intestines, and liver. GvHD is a major cause of morbidity and mortality following allogeneic HCT. GvHD rates and severity are reduced using prophylaxis with non-specific immunosuppressive drugs. Nonetheless, acute GvHD still afflicts up to 50% of human leukocyte antigen (HLA)-identical, related-donor HCT recipients, and chronic GvHD is also a major problem. The frequency and severity of the GvHD that develops when extensive HLA barriers are transgressed has essentially precluded the routine performance of HLA-mismatched transplants, making HCT unavailable to many for whom no other curative treatment exists. Despite the use of large donor registries that identify closely matched unrelated donors, a high incidence of severe GvHD occurs due to the presence of HLA mismatches that are undetected by conventional HLA typing (19) and possibly to increased minor histoincompatibilities. However, the GvL effect of allogeneic T cells is due largely to recognition by donor T cells of host alloantigens, which are also expressed on malignant cells. Because of the remarkable strength of the immune response against MHC alloantigens, the strongest GvL effects are observed in the setting of donor-host MHC disparity. Therefore, a major challenge in the HCT field is to develop methods of separating the GvL- and GvHD-inducing effects of GvH-reactive donor T cells.

HCT-associated toxicities consist of not only GvHD but also infectious complications and organ toxicities associated with the conditioning treatment. If the morbidity of HCT were reduced to sufficiently low levels, its applications would be even broader than they are currently. HCT could be used for induction of donor-specific tolerance to solid organ grafts. Although prevention of acute rejection has been increasingly successful, these improvements have not been associated with a reduction in late graft loss due to chronic rejection. Donor-specific tolerance would prevent both acute and chronic graft rejection while avoiding the toxicities associated with the chronic immunosuppressive therapies currently used with all allogeneic organ transplants. Tolerance is readily achieved in murine models using HCT to induce a state of mixed hematopoietic chimerism, in which both donor and recipient hematopoietic systems coexist. This state confers mutual tolerance of recipient and donor graft without graft rejection or GvHD for the life of the animal. As is discussed below, this approach has begun to be evaluated clinically. HCT regimens with minimal toxicity could also greatly improve the treatment of hemoglobinopathies and other congenital hematopoietic abnormalities, inborn errors of metabolism, and autoimmune diseases. HCT is currently reserved for patients at advanced stages of these diseases because of the toxicity associated with the procedure.

This review will cover the following topics: (1) approaches to separating GvHD and GvL; (2) development of HCT regimens appropriate for tolerance induction; (3) mechanisms by which mixed chimerism achieves tolerance; (4) translation of HCT-mediated GvL and transplantation tolerance to the clinic; and (5) use of HCT for treatment of autoimmune diseases.

Approaches to Separating GvHD and GvL

Since non-tolerant donor T cells are critical for the induction of acute GvHD, this complication can be prevented by T cell depletion of the donor graft. However, this benefit of T cell depletion is offset by increased relapse rates (20) and an increased risk of graft rejection (21). Rejection can be prevented by more intensive host conditioning and increased HSC doses, even with

extensively (one haplotype) HLA-mismatched stem cell grafts (22), but at the expense of delayed immune reconstitution (22).

An elusive goal has been to separate the GvL effect of donor T cells from GvHD. Many new approaches for inhibiting GvHD have been developed, most of which are broadly and non-specifically immunosuppressive. These include the use of costimulation blockade, new immunosuppressive drugs, transfer of regulatory cells, immunosuppressive cytokines, and many others. However, these approaches globally suppress donor-anti-host responses, including those that eliminate residual leukemia, raising the concern that they would also impair GvL responses. Since GvH reactivity also eliminates residual host alloreactive lymphocytes that mediate HSC rejection, treatments that selectively impair GvH responses without inhibiting recipient immunity, like graft T cell depletion, also increase the likelihood of graft rejection. GvL may be preserved when NKT cells are used to suppress GvHD, apparently by promoting Th2 responses (23;24), or when Treg are administered. However, tumor titration studies were not performed to assess a potential reduction in the strength of anti-tumor effects using Treg (25). Administration of polyclonal Treg, while non-specifically immunosuppressive, can protect the recipient lymphoid tissues from GvH-associated injury, resulting in improved recovery of pathogen-specific immunity (26). Administration of type 2 “immune deviated” T cells (secreting IL-4, IL-5, IL-9, and/or IL-13) to achieve GvL without GvHD has also been explored (27), but a pilot clinical study did not show any obvious reduction in GvHD (28).

While T cell-mediated GvL effects are diminished with T cell-depleted (TCD) haploidentical HCT in patients receiving very intense conditioning, this drawback may be compensated for by the ability of NK cells to mediate GvL when the host lacks inhibitory MHC ligands recognized by the donor, at least for patients with acute myelogenous leukemia (29). Such alloreactive NK cells may also help to promote donor engraftment by eliminating alloresistant host T cells (29).

In general, broadly immunosuppressive approaches to preventing GvHD might have their greatest utility in the treatment of non-malignant diseases. In contrast, a number of immunostimulatory cytokines, including IL-2, IFN-γ, IL-12, and IL-18, which can promote anti-tumor immunity, have been shown, paradoxically, to inhibit GvHD in mouse models (30;31;32;33). Several of these cytokines have been shown to preserve or enhance GvL effects while GvHD is inhibited (34;35;36;37;38). The inhibitory effect of IL-12 and of IL-18 on GvHD is mediated by induction of an early surge of IFN-γ, which also promotes the GvL effect of CD8 T cells (39;40). The mechanism by which IFN-γ inhibits GvHD has not been fully established but is Fas-dependent and associated with reduced expansion of host-reactive T cells (41). The potential roles of Tregs, whose activity may depend on IFN-γ (42), and of inhibition of Th17 differentiation by IFN-γ in this phenomenon have not been elucidated. The protective effect of giving exogenous IL-2 at the time of transplant is associated with reduced IFN-γ production but normal expansion of host-reactive T cells (43) and might also involve Tregs. Indeed, increased numbers of Tregs were detected in peripheral blood mononuclear cells (PBMCs) from patients who received IL-2 to induce anti-tumor immunity following HCT (44) or to treat solid tumors (45).

Both cytotoxic T lymphocytes (CTLs) and cytokines contribute to GvHD. Some data suggest that certain effector functions of T cells are more important in inducing GvHD than GvL and vice versa, suggesting approaches to preserving GvL while preventing GvHD (46). Overall, the Fas-mediated cytotoxic pathway appears to be of greater importance than the perforin pathway in the induction of GvHD, while the perforin/granzyme pathway plays a predominant role in anti-leukemic effects, especially of CD8 T cells. Thus, selective blockade of the Fas/FasL pathway may ameliorate CD8 T cell-mediated GvHD without eliminating GvL effects

(47). TNF-related apoptosis-inducing ligand (TRAIL) may contribute to GvL without making a major contribution to GvHD (48). GvHD still occurs in the absence of the perforin and Fas pathways, suggesting that cytokines alone can mediate significant GvHD (49). Blockade of Fas/FasL, perforin, TNF-α, and IL-1 pathways have shown some efficacy in animal models, and the latter two have been evaluated in clinical trials (reviewed in (50)). Although efficacy was limited, newer biological agents or protocols may still demonstrate benefit.

Since leukemias and lymphomas reside largely in the lymphohematopoietic tissues, GvL could be achieved without GvHD by confining the GvH response to the lymphohematopoietic system if T cell trafficking to the epithelial GvHD target tissues were blocked. Blockade of adhesion molecules and chemokine pathways has shown some efficacy in animal models, but the incompleteness of the effects may reflect the considerable redundancy in the pathways that permit T cell trafficking to GvHD target tissues. FTY720, a sphingosine-1-phosphate receptor agonist that traps lymphocytes within the lymphoid tissues, effectively inhibited GvHD while preserving GvL (51). However, the mechanisms involved appear to be more complex than simply trapping lymphocytes in the lymphoid tissues (W. Asavageroenchi and M. Sykes, manuscript in preparation) and may include inhibition of Th17 development (52) and reductions in host antigen presenting cell (APC) numbers (53).

Conditioning-induced tissue inflammation plays an important role in promoting GvHD (Figure 1). Indeed, anti-MHC alloreactivity can be confined to the lymphohematopoietic system when donor T cells are given after host recovery from the initial conditioning regimen has occurred in established mixed hematopoietic chimeras. These animals are immunologically tolerant of their original marrow donor's antigens. Therefore, a GvH reaction occurs after administration of non-tolerant donor lymphocyte infusions (DLIs) without any significant host-versus-graft response, resulting in conversion of mixed hematopoietic chimerism to full donor chimerism and strong GvL effects (54;55). However, this powerful GvH alloreaction against lymphohematopoietic cells is not associated with GvHD in established mixed chimeras, even though the number of donor T cells given causes rapidly lethal GvHD in freshly conditioned recipients (56;57). Anti-host MHC alloreactivity mediates the most potent GvL effects (55; 58), and GvH-reactive T cells in the DLI can clearly be shown to be activated and proliferating in mixed chimeras receiving DLI (59;58). Expression of both class I and class II MHC on recipient hematopoietically-derived APCs plays a critical role in inducing this anti-host reactivity and maximal GvL. DLI T cells do not become activated or expand in full allogeneic chimeras that lack host APCs, precluding GvL effects (55;58). Paradoxically, despite converting to the "effector/memory" phenotype, characterized by expression of chemokine receptors and adhesion molecules that promote tissue trafficking, DLI-derived T cells given to established mixed allogeneic chimeras do not migrate to the epithelial GvHD target tissues. This failure to traffic reflects the absence of inflammatory signals in those tissues (59). Such inflammatory signals are induced by the tissue injury caused by conditioning treatment and subside over time (60).

Eradication of relapsed leukemia following delayed DLI is somewhat variably associated with GvHD in patients but generally to a lesser degree than would be expected in freshly conditioned recipients of similar cell numbers (61;62). In attempts to apply the above approach to separating GvHD from GvL clinically, proof of principle has been obtained that GvH responses can be confined to the lymphohematopoietic system and thereby fail to induce GvHD in lymphoma patients receiving non-myeloablative HCT with an initially T cell-depleted graft, followed by delayed DLI (63). However, some patients who are apparently "quiescent" mixed chimeras when they receive their DLI do subsequently develop GvHD. One of the major differences between these patients and the mouse model is that T cell recovery in patients is generally poor, resulting in opportunistic infections. In contrast, mice have excellent T cell recovery due to *de novo* thymopoiesis by the time DLIs are given. This, combined with their maintenance in a

specific pathogen-free facility, makes them extremely unlikely to have infections at the time of DLI administration. Even in such "quiescent" established mixed chimeras, activation of toll-like receptors (TLRs), as occurs in infection, promotes the trafficking of DLI-derived T cells to the GvHD target tissues (59). If the TLR stimulus was provided systemically (as in a systemic infection), GvHD affecting skin, liver, and intestines developed. In contrast, when TLR stimuli were applied locally to the skin, the GvHD resulting from DLI administration was confined to the treated area of skin (59). Together, these results have several important implications: 1) They indicate that regulatory cells, which also recover in mixed chimeric mice by the time of DLI administration, are insufficient to prevent the development of GvHD when an inflammatory stimulus is provided by TLR activation; 2) they indicate that inflammatory stimuli in the skin play a critical role in promoting the trafficking of GvH-reactive T cells into that tissue and only that tissue; and 3) they suggest that improved immune recovery with better control of post-transplant infections to prevent TLR-dependent immune activation may be needed to optimize this approach to separating GvHD and GvL in patients.

Increasing resistance to GvHD with time may also be conferred by recovery of T cell populations that down-regulate GvH reactions. Consistent with this notion, lethally irradiated mice receiving MHC-matched bone marrow (64;65) experience GvL effects from delayed DLI without developing GvHD (66) due to the effect of donor marrow-derived regulatory cells (64;67).

Additional strategies for separating GvHD from allogeneic GvT effects include the transduction of donor T cells with suicide genes so that the alloresponse can be turned off at will, hopefully after residual tumor has been eradicated (68;69). Another approach is to avoid the GvH alloresponse while targeting the donor immune response to tumor-specific antigens (70), including idiotypic determinants associated with unique immunoglobulin receptors and T cell receptors (TCRs) expressed by B and T cell malignancies, respectively (71;72). However, this approach is limited by the low frequency of tumor antigen-specific T cells pre-existing in a given T cell repertoire. These frequencies are lower than those against multiple minor histocompatibility antigens and orders of magnitude lower than those against MHC alloantigens. Meaningful tumor-specific responses might be achieved with donor immunization with tumor antigens, with or without *in vitro* expansion of tumor-specific effector cells, but both manipulations may be impractical. Minor histocompatibility alloantigens expressed by lymphohematopoietic cells (including leukemias and lymphomas) but not by the epithelial GvHD target tissues may also be targeted using *in vitro*-expanded CTL (73). Primed T cells specific for a single immunodominant class I-restricted minor histocompatibility antigen, without additional GvH specificities, mediated GvL without GvHD in mice (74). While the less risky strategy of generating tumor-specific responses from autologous T cells could theoretically achieve similar outcomes, T cell immunity is markedly impaired in tumor-bearing hosts (75), making the use of immunologically unimpaired allogeneic donors attractive. Non-myeloablative conditioning can deplete regulatory cells and create space for lymphopenia-driven expansion of autologous lymphocytes, which may then mediate significant anti-tumor effects (76).

Some unexpected observations in patients receiving non-myeloablative HCT suggested a novel approach to using autologous T cells to achieve anti-tumor effects. Non-myeloablative HCT was performed with the intention of inducing mixed chimerism to be followed by DLI in patients with advanced hematologic malignancies (77;63). All 82 patients that could be evaluated achieved initial myeloid and T cell donor chimerism. However, 22 patients (27%) ultimately lost their graft (<1% donor cells), and chimerism could not be rescued by DLI(s). A major mechanism of graft loss in these recipients was immunological rejection, as evidenced by increased numbers of circulating host T cells and sensitized anti-donor T cell responses (78). Graft rejection was thought to be undesirable with respect to an immune-mediated anti-

tumor response. However, nine of 22 (41%) patients who lost chimerism achieved a response, including six (27%) who achieved a complete response of advanced chemotherapy-refractory disease.

These results prompted development of a mouse model to address the hypothesis that rejection of an established donor marrow graft might induce tumor antigen-specific immune responses by the host. Recipient lymphocyte infusions (RLI) administered to established mixed chimeras indeed induced significant anti-tumor effects against several different hematologic malignancies (79;80) in association with complete loss of chimerism. In the absence of pre-existing chimerism, RLI did not mediate a measurable anti-tumor effect, demonstrating a requirement for an anti-donor alloresponse in this phenomenon. Spontaneous loss of chimerism was also associated with a measurable anti-tumor effect (79), paralleling the clinical observation that patients who reject their marrow grafts sometimes enjoy striking tumor responses despite their loss of chimerism.

No tumor protection was observed in naïve, non-chimeric mice receiving and rejecting allogeneic leukocytes (80). Therefore, mere rejection of allogeneic cells is insufficient to mediate anti-tumor effects; i.e., pre-establishment of mixed chimerism is required. Donor APCs engrafted throughout the lymphohematopoietic system may play an important role in inducing alloresponses at each site, allowing these responses to promote the generation of systemic tumor antigen-specific immunity. RLI from tumor-bearing mice were as effective in mediating anti-tumor effects as RLI from non-tumor-bearing mice (80), indicating that the rejection process overcomes any global immunoincompetence resulting from the presence of tumor in the animals. Recipient CD4 T cells and RLI-derived CD8 T cells collaborate in these anti-tumor effects, with no demonstrable role for donor T cells (81). IFN-γ also plays a requisite role in this anti-tumor effect (79), and the production of this cytokine is dependent on CD8 T cells (81). However, IFN-γ does not directly inhibit tumor growth (79), suggesting other mechanisms for its *in vivo* anti-tumor effects.

Chimeras receiving RLI lose donor-specific tolerance, as evidenced by cytolytic activity against donor lymphoblasts. Importantly, the same CTL that killed donor targets did not kill tumor cells, arguing against “bystander killing” of tumor cells by anti-donor alloreactive CTL induced by RLI (81). Mice receiving RLI and tumor demonstrated tumor-specific cytolytic activity (81). Studies are in progress to identify the precise interactions involved in the anti-tumor effect of RLI, which may involve both direct and indirect alloreactivity (82). This approach is of potential clinical interest because the use of RLI is not associated with any risk of GvHD.

Development of Regimens for Applying HCT to Tolerance Induction

Despite improvements in short-term graft survival, little progress has been made in preventing or treating chronic organ allograft rejection. Moreover, the immunosuppressive therapy used for all allograft recipients has serious organ-specific toxicities and is associated with a high incidence of opportunistic infection and malignant disease. Thus, a major goal in transplantation is to achieve robust donor-specific tolerance of organ grafts in order to permit life-long acceptance while avoiding global, non-specific immunosuppression. A major impediment to achieving this goal is the considerable redundancy in rejection mechanisms, involving numerous cell types and multiple pathways of immune recognition (direct, indirect, and possibly “semi-direct” via expression of donor MHC on recipient hematopoietic cells) (83). MHC barriers are crossed in most organ allograft recipients, and the extraordinary strength of the T cell response to allogeneic MHC molecules, combined with the multiple minor histoincompatibilities between allogeneic individuals, present a formidable barrier. The inability of T cell-deficient animals to reject allografts demonstrates that T lymphocytes are

required to induce allograft rejection. While other cell types such as natural killer (NK) cells or macrophages can contribute to rejection (84;85;86) and their role may be more central when T cell function is impaired (87;88), these cells have not been shown to cause allograft or xenograft rejection in the absence of T cells. Furthermore, anti-donor antibodies (Abs), which are usually T cell-dependent, pose a formidable challenge to solid organ graft acceptance since they are the major inducers of chronic graft vasculopathy.

Given the robustness of the alloresponse, a robust tolerance mechanism must be established in order to assure lifelong allograft acceptance without immunosuppression. This is apparent in the ease with which fragile tolerant states, such as those established with costimulation blockade (without HCT) combined with solid organ grafting are broken by infection or TLR stimulation (89;90;91;92). This infection-induced loss of tolerance is also observed in tolerant states that depend on T cell anergy or suppression (93;94). Moreover, vascularized heart, liver, and kidney grafts are not as tolerogenic in large animals or humans as they are in rodents, so precarious states of tolerance achieved in rodents will not be sufficient for successful translation into clinical protocols.

A promising approach to inducing robust transplantation tolerance involves induction of mixed hematopoietic chimerism in the recipient. Engraftment of donor HSCs in the recipient assures that donor-type dendritic cells (DCs) contribute to the DC pool in the thymus for the life of the animal, where they participate in T cell education by promoting negative selection of any newly developing recipient or donor T cell that recognizes donor antigens. DCs in the host thymus that are derived from the recipient contribution to the HSC pool assure that the same is true for anti-host reactivity (95). When the T cell repertoire is centrally tolerized in this manner, any donor-type graft is accepted without immunosuppression, and GvHD does not occur. This form of tolerance is systemic, as evidenced by the specific unresponsiveness observed *in vitro* to both the donor and the recipient (96).

The major hurdle to overcome in order to permit donor HSC engraftment and allow central tolerance to be achieved is the T cell immune barrier to allogeneic HSC engraftment. This barrier includes both the large alloreactive T cell repertoire pre-existing in the peripheral lymphoid tissues and the smaller number of mature alloreactive T cells present in the thymus (96;97;98;99). For clinical applicability, this hurdle must be overcome with minimal toxicity, ideally with non-myeloablative conditioning without global T cell depletion. Although such a regimen has not yet been developed for clinical use, the ensuing paragraphs describe the stepwise development of such a protocol in animal models.

Owen and colleagues first reported the association of naturally occurring mixed hematopoietic chimerism with tolerance of transplanted grafts in 1945 (100). In dizygotic bovine twins, these investigators observed that, due to anastamoses of placental vessels, each individual had red blood cells from both twins circulating in the bloodstream. This was speculated to be due to permanent engraftment of precursor cells that were exchanged during fetal development, a time when the immature immune system is incapable of rejection. Subsequent studies by Medawar and colleagues revealed that these chimeric, dizygotic twins, but not siblings of separate birth, accepted skin grafts from their twin sibling (101;102). Subsequent studies using *in utero* transplantation of donor-type cell mixtures extended these findings to chickens, rabbits, and mice (103;104).

It wasn't until the late 1970s that Slavin, et al. published the first report of induction of mixed hematopoietic chimerism in adult animals with an established immune system (54). Prior to these studies, HCT was used in adults, as previously described, only for rescue after lethal irradiation, resulting in full donor chimerism and a high incidence of GvHD. Slavin and colleagues used fractionated, high-dose total lymphoid irradiation (TLI) followed by injection

of allogeneic BMCs (BMCs) to establish varying degrees of mixed hematopoietic chimerism in mice and rats (54;105;106). TLI had been used in humans for treatment of malignant lymphoma and was known to be immunosuppressive without the severe side effects of bone marrow aplasia caused by total body irradiation (TBI). These studies demonstrated that mixed hematopoietic chimerism could be established and sustained in adult animals.

Subsequently, Ildstad and Sachs showed that administration of a mixture of host-type (syngeneic) and allogeneic or xenogeneic BMCs could be used to reconstitute lethally irradiated adult recipients in order to establish mixed chimerism and donor-specific transplantation tolerance (107;108). T cell-depletion of bone marrow from both the syngeneic (B10 mouse) and the either allogeneic (B10.D2 mouse) or xenogeneic (F344 rat) donors (107) was essential in allowing mixed chimerism to occur without GvHD. Mixed allogeneic chimeras demonstrated superior survival and immunocompetence compared to full allogeneic chimeras, presumably due to the presence of host-type APCs in the periphery that effectively present antigens to T cells positively selected on host MHC in the thymus. Furthermore, these mixed chimeras specifically accepted donor but not third party skin grafts, a stringent assessment of immunological tolerance (108). Tolerance to donor and host was systemic, as reflected by donor- and host-specific unresponsiveness in mixed lymphocyte reaction (MLR) and cell-mediated lympholysis (CML) assays *in vitro*. Relative to allogeneic mixed chimeras, the xenogeneic mixed chimeras maintained lower levels of chimerism, but they showed marked hyporesponsiveness to the donor (107). When minor histocompatibility differences were present in addition to a full MHC disparity, donor-specific skin graft survival was prolonged, but the grafts were eventually rejected despite the persistence of high levels of mixed chimerism and donor-specific unresponsiveness *in vitro*. The eventual rejection of donor skin grafts in this setting was attributed to the presence of skin-specific minor antigen disparities, to which the recipients could not be rendered tolerant by a donor hematopoietic cell graft (108).

Both the TLI model and the lethal TBI and mixed reconstitution model involve highly toxic host conditioning. Furthermore, the high irradiation dose rates administered to rodents cannot be tolerated by humans, and, based on the observed difficulty achieving engraftment of T cell-depleted HLA-mismatched marrow in heavily myeloablated humans, it is highly improbable that T cell-depleted allogeneic marrow would engraft in lethally irradiated humans also receiving T cell-depleted autologous marrow. Thus, these regimens did not have clinical applicability as an approach to transplant tolerance induction. A subsequent advance toward the development of regimens for applying HCT to tolerance induction was establishment of mixed chimerism using a more targeted, non-myeloablative preparative conditioning. Cobbold, et al. showed that monoclonal antibody (mAb) treatment against CD4 and CD8 T cells could be used in combination with sub-lethal TBI to achieve engraftment of MHC-mismatched BMCs (109). However, relatively high doses of TBI (6 or 8.5 Gy) were required to achieve durable engraftment, leading to predominantly donor hematopoiesis with little recipient contribution (109). Sharabi and Sachs then successfully reduced the dose of TBI to 3 Gy by adding local irradiation to the thymus (thymic irradiation, TI) (96). This modification was added because extensive peripheral, but not thymic, T cell depletion was observed when anti-CD4 and CD8 mAbs were administered. Thus, the new preparative regimen consisted of anti-CD4 and anti-CD8 mAbs, 3 Gy TBI and 7 Gy TI, followed by bone marrow transplantation (BMT) on Day 0 (96). This regimen consistently produced a high incidence of mixed allogeneic chimerism and donor-specific skin graft tolerance. Tomita, et al. later showed that TI could be eliminated if additional injections of anti-CD4 and anti-CD8 depleting mAbs were given (98). The higher total doses of these mAbs still failed to completely deplete thymocytes, but downmodulated coreceptors and TCRs on mature alloreactive thymocytes. Functional (MLR and CML) studies showed that alloreactivity of thymocyte populations was eliminated by the addition of this second mAb injection to the conditioning regimen (99). These studies confirmed the importance of a maneuver to overcome pre-existing intrathymic alloreactivity

(in addition to peripheral T cell alloreactivity) in order to achieve durable engraftment of allogeneic marrow.

In the late-1990s, costimulation-blocking reagents, namely anti-CD154 (anti-CD40L) and cytotoxic T lymphocyte antigen 4 (CTLA4)-Ig, were shown to markedly prolong solid organ allograft survival (110;111;112;113). Wekerle, et al. then utilized costimulation blockade as a means to promote engraftment of allogeneic BMCs. Anti-CD154 (clone MR1) is a hamster anti-mouse mAb that blocks the interaction between CD154 (CD40L) expressed on activated T cells and the CD40 receptor expressed on APCs. This interaction promotes upregulation of costimulatory molecules on APCs, enhancing their immunogenic capacity. CTLA4Ig is a fusion protein that binds with high affinity to the B7 molecules (CD80 and CD86) expressed on activated APCs and thereby prevents costimulation of T cells by blocking binding of CD28 to the B7 molecules. The combination of anti-CD154 and CTLA4Ig with 3 Gy TBI successfully allowed engraftment of fully MHC-mismatched allogeneic BMCs (114). Thus, costimulation blockade was able to overcome both the peripheral and the intrathymic T cell barriers to allogeneic marrow engraftment, circumventing the requirement for both peripheral T cell depletion and either TI or repeated injections of T cell-depleting antibodies (114). Permanent donor-specific skin graft acceptance was observed, with rapid rejection of third party grafts. *In vitro* CML and MLR analyses showed that the tolerance to the donor was indeed systemic. This was the first regimen to succeed in achieving durable mixed chimerism and systemic donor-specific tolerance without global T cell ablation. Instead, donor-reactive T cells in the periphery were specifically deleted in response to the allogeneic BMT and costimulation blockade. This was revealed by analyses of TCR using Vβ that recognize endogenous superantigens that could only be presented by the donor class II MHC, which served as a window into the fate of pre-existing and newly developing donor-reactive T cells. Following transplantation of B10.A BMCs into B6 recipients with the regimen described above, deletion of peripheral $V\beta5^+$ and $V\beta11^+$ donor-reactive CD4 T cells occurred within the first four to five weeks post-BMT (114). This initial deletion took place in the periphery (i.e., was thymus-independent), as evidenced by thymectomy studies (115). Donor HSC engraftment and hematopoiesis led to the continuous presence in the thymus of donor APCs, permitting the subsequent intrathymic deletion of any newly developing thymocytes that recognized the donor (114). Later studies showed that the previous regimen involving a single injection of anti-CD4 and CD8 mAbs could be combined with a single injection of either anti-CD40L or CTLA4Ig and 3 Gy TBI to achieve durable mixed chimerism (116), demonstrating the capacity of either costimulation-blocking reagent, in combination with allogeneic BMT, to overcome intrathymic alloresistance.

Although the costimulation blockade regimen described above was robust, the incidence of durable chimerism was usually less than 100%. Further studies (117) revealed that $CD4^-$ but not CD8-mediated alloreactivity was reliably overcome using a regimen consisting of 3 Gy TBI, 0.5 mg anti-CD154, and allogeneic BMCs, all given on Day 0. The ability of anti-CD154 alone (i.e., without CTLA4Ig) to render CD4 T cells completely incapable of rejection was somewhat surprising, given the large number of costimulatory pathways and the ability of CD4 T cells to reject allogeneic BMCs when CD8 T cells are depleted (118). Donor-specific skin graft tolerance was demonstrable in chimeras, even when grafted one day post-BMT, demonstrating the rapidity with which tolerance is achieved with this approach and suggesting applicability for cadaveric organ transplantation (117).

A later modification of this regimen reliably permitted achievement of durable chimerism without recipient CD8 T cell depletion (119). The addition of either donor-specific transfusion (DST) or administration of 3 Gy TBI one or two days prior to BMT (rather than on Day 0) allowed recipient CD8 T cells to be tolerized in addition to CD4 T cells in mice receiving anti-CD154 alone. Early *in vitro* and *in vivo* unresponsiveness of both CD4 and CD8 T cells was

achieved, as measured by MLR and CML assays and acceptance of donor, but not third-party, skin grafted the day following BMT (119). This simple, minimally toxic regimen quite reliably established mixed chimerism and systemic donor-specific tolerance in mice.

Related but slightly different costimulation blockade protocols have also permitted engraftment of MHC-mismatched allogeneic BMT in mice. Some include non-myeloablative TBI (on Day -1 or Day 0) with repeated injections of anti-CD154 (120;121). One of these protocols (120) resulted in full donor chimerism in NOD mice. DST on Day -7 was used in combination with four injections of anti-CD154 to induce mixed chimerism and donor-specific tolerance in another protocol (122).

When the immune barriers are adequately overcome, allogeneic HSCs given intravenously home to the bone marrow compartment of the recipient. HSC engraftment is facilitated by recipient irradiation or other myelosuppressive treatment, which is required to achieve engraftment when relatively low (“conventional”) numbers of HSCs are given (123). Even local TBI to only a few bones promotes CD45 congenic donor HSC engraftment, probably by promoting the initial expansion of donor HSCs that home to irradiated sites (124). This requirement to create “space” can be overcome by administering very high doses of donor stem cells (125;126;127;128;129). When T cell-mediated resistance to allogeneic marrow engraftment was overcome with anti-T cell mAbs and TI or with costimulation blockade, high doses of allogeneic marrow were also able to engraft and induce tolerance without the requirement for host treatment with TBI or chemotherapy (130;131). While local TI was still required in order to achieve alloengraftment and tolerance when high marrow doses were given with mAbs depleting peripheral T cells (130), both requirements were obviated by the addition of a single injection of each of two costimulation-blocking agents, anti-CD154 and CTLA4Ig (131). Again, mixed chimerism conferred long-lived donor-specific skin graft tolerance, systemic tolerance, and deletion of donor-reactive T cells. This protocol was a significant advance, as it avoided irradiation, cytotoxic drugs, and T cell depletion. Similar results have been subsequently obtained with regimens that utilize repeated marrow injection and anti-CD154 (132) or costimulation blockade in combination with rapamycin (133) or donor-specific transfusion (122).

Attempts have been made to apply regimens that are successful in rodent models in large animal models. A non-myeloablative regimen involving TBI, TI, T cell depletion, and transplantation of minor antigen-mismatched BMCs achieved long-lived chimerism and specific donor skin and cardiac graft tolerance (134;135). Mixed chimerism was established in haploidentical miniature swine using non-myeloablative conditioning consisting of 1 Gy TBI, partial T cell depletion with an anti-CD3 immunotoxin, and a short course of cyclosporine. High doses of allogeneic hematopoietic cells are required to achieve tolerance with this non-myelosuppressive regimen, which was not associated with GvHD and permitted achievement of stable, multilineage mixed chimerism with minimal toxicity (136). These animals accepted donor-type renal allografts without immunosuppression (137). The mechanisms involved in the achievement of tolerance to donor and host with this model are more complex than the central deletion associated with durable mixed chimerism in murine models, probably because neither the donor nor the recipient pre-existing T cell repertoire is fully depleted by anti-CD3 immunotoxin. Regulatory mechanisms appear to play a role in this delicate state of mutual tolerance (137). However, persistent thymic chimerism and evidence for HSC engraftment correlate with the achievement of allograft tolerance in this model (138).

A non-myeloablative protocol involving 2 Gy TBI and peri-transplant immunosuppression (with cyclosporine and either methotrexate or mycophenolate mofetil) has allowed mixed chimerism to be achieved in dogs receiving MHC-matched marrow (139). These animals were shown to be tolerant of MHC-matched, donor kidney grafts (140). Subsequent protocols

consisted of 1 Gy TBI, costimulation blockade with infusion of donor PBMC, and injection of CTLA4Ig followed by a course of immunosuppression (141). However, durable chimerism across MHC barriers has not been successfully achieved without GvHD in the dog model.

Studies in non-human primates support the clinical applicability of non-myeloablative mixed chimerism induction for the achievement of transplantation tolerance. Early successful non-myeloablative regimens in cynomolgus monkeys involved partial recipient T cell depletion (using equine anti-thymocyte globulin, ATG), 7 Gy TI, 3 Gy TBI, splenectomy, and HCT from MHC disparate donors, followed by a short course of cyclosporine. Multilineage mixed chimerism, although transient, was associated with renal allograft tolerance, and GvHD did not occur (142). However, using a similar regimen, long-term heart allograft prolongation, but not permanent acceptance, was achieved in MHC-mismatched cynomolgus monkeys, suggesting that transient chimerism is not sufficient for robust, systemic tolerance induction (143). Consistent with these observations, *in vitro* assays sometimes revealed persistent anti-donor reactivity in animals accepting a renal allograft (144). As is discussed below, the kidney graft itself likely plays an important role in the tolerance achieved in this model. While recipient splenectomy was required to prevent anti-donor alloantibody responses from developing in these animals, a short course of anti-CD154 successfully replaced splenectomy for this purpose (145). Thromboembolic complications associated with anti-CD154 treatment in these animals were later successfully prevented by prophylactic ketorolac, a non-steroidal anti-inflammatory drug (146). Even with anti-CD154 treatment, chimerism was still only transient but, nonetheless, resulted in permanent survival of MHC-mismatched donor renal allografts. As is discussed below, these results supported clinical evaluation of transient mixed chimerism for renal allograft tolerance induction.

The immunological barriers to xenografts are even greater than those to allografts. Nevertheless, successful xenotransplantation could overcome the severe supply-demand imbalance that exists for allografts. Mixed chimerism induction also provides a promising approach to crossing xenogeneic barriers. Lethal irradiation followed by reconstitution of mice with a mixture of T cell-depleted recipient-type and high-dose xenogeneic rat BMC achieved low-level chimerism and donor-specific skin graft prolongation (107;147). Subsequently, a non-myeloablative preparative regimen consisting of depleting mAbs against NK1.1, Thy1.1, CD4, and CD8, followed by 3 Gy TBI and 7 Gy TI, was shown to permit engraftment of TCD rat BMCs, permitting mixed xenogeneic chimerism induction in the rat-to-mouse model (148). The NK1.1 mAb was needed to deplete NK cells and the anti-Thy1 mAb was required to deplete γδ T cells (149). In contrast, neither of these mAbs were required to achieve allogeneic marrow engraftment (96), indicating that these cells of the innate immune system pose a much greater barrier to xenogeneic than to allogeneic HSC engraftment. Mixed xenogeneic chimerism induced with this protocol resulted in systemic T cell tolerance and specific, long-term acceptance of xenogeneic donor skin grafts, despite gradually declining levels of chimerism (148). This gradual loss of chimerism was mediated by non-immune mechanisms related to the superior ability of host hematopoietic cells to compete with xenogeneic cells in a recipient hematopoietic environment (150;151). Natural antibodies (NAbs) against rat hematopoietic cells, which are present in normal mice, disappeared following the induction of mixed xenogeneic chimerism (152), suggesting that the cells (likely B-1b B cells; see below) producing them were also tolerized by mixed chimerism induction. Moreover, NK cells that would otherwise kill rat donor cells were also rendered unresponsive (153). Thus, mixed xenogeneic chimerism has the potential to tolerize not only T cells, but also B cells and NK cells to the xenogeneic source animal.

These rat-to-mouse studies were encouraging. However, the most suitable donor species for organ transplantation into humans is generally agreed to be the pig, whose organs encounter major barriers to engraftment in humans and non-human primates. The serum of such recipients

contains high levels of NAbs that predominantly recognize a carbohydrate epitope, Galα1-3Galβ1-4GlcNAc-R (αGal), which is ubiquitously expressed on pig cells. Mice with a targeted mutation in the gene encoding α1-3Gal transferase (GalT) produce anti-αGal Nabs, and can thereby model human NAb responses. Pre-existing B cells, as well as those developing after BMT, are tolerized by induction of mixed chimerism in GalT knockout mice using αGal-expressing donor BMCs (154;155;156).

Studies in humanized mice have documented that porcine mixed chimerism can lead to central tolerance of human T cells to donor porcine antigens (157). However, durable mixed chimerism has not yet been achieved in a pig-to-primate combination. The ability of recipient macrophages to rapidly engulf xenogeneic hematopoietic cells (158), reflecting a failure of inhibitory receptors to interact appropriately with xenogeneic variants of their ligands (159;160), as well as species incompatibilities in molecular interactions important for hematopioetic function, such as adhesion molecules (161) and cytokines (162;163), limit xenogeneic hematopoietic function. Approaches to overcoming these limitations have been recently reviewed (164).

Mechanisms of Tolerance Achieved With HCT

Central T cell tolerance

The major mechanism conferring long-term tolerance in mixed chimeras is intrathymic central deletion of newly developing anti-donor T cells. If peripheral or intrathymic T cell resistance prevents either engraftment of HSCs or survival of their intrathymic APC progeny, central tolerance cannot occur. Thus, there are two stages in which tolerance must be achieved. The first involves early tolerization of pre-existing donor-reactive T cells in the periphery and in the thymus, allowing donor engraftment in the bone marrow and the thymus. The second stage involves central, intrathymic deletion of newly developing T cells induced by donor APCs in the thymus. Sustained engraftment of donor HSCs in recipient marrow is required for the second phase to persist long-term because it continually replenishes donor APCs that regularly turn over in the thymus (Figure 2).

The above conditions are absolutely essential for achievement of durable tolerance in mixed chimeras prepared with regimens that globally deplete T cells and thymocytes or specifically delete pre-existing donor-reactive T cells and thymocytes. The dependence on central deletion to maintain tolerance reflects the failure to induce strong regulatory mechanisms. Clear evidence for the dependence on life-long intrathymic chimerism inducing deletion and for the absence of regulatory mechanisms was obtained in chimeras prepared with the globally T cell depleting regimen that includes T cell depleting mAbs and thymic irradiation. *In vivo* elimination of donor chimerism using depleting mAb against donor MHC class I led to loss of tolerance to the donor, resulting in rejection of donor skin grafts (165). If, however, chimeric animals were thymectomized prior to elimination of donor chimerism, donor skin grafts were specifically accepted, with robust rejection of third party grafts (166). In other words, the thymus was required for loss of tolerance in the absence of chimerism, and the peripheral T cell repertoire remained specifically tolerant of the donor, even without persistent donor chimerism. An increase in peripheral T cells with Vβ recognizing endogenous superantigens presented by donor MHC provided direct evidence that this loss of tolerance was mediated by newly developing donor-reactive T cells leaving the thymus after chimerism, and therefore donor APCs in the thymus, were eliminated. These donor-reactive T cells remained absent (due to central deletion) in the tolerant peripheral repertoire of the animals that had been thymectomized before elimination of chimerism (166). Injection of naïve donor splenocytes into euthymic mixed chimeras resulted in loss of chimerism and associated tolerance, arguing against a role for active suppression of donor-reactive cells (166). Furthermore, the persistence of tolerance in thymectomized mice after elimination of chimerism argues against any role for

anergy as a mechanism of long-term tolerance, since anergy requires persistent interactions with antigen (167;168).

When repeated injections of T cell-depleting mAbs are used to replace TI, the effect of these additional injections is to inactivate and deplete alloreactive $CD4^+$ and $CD8^+$ thymocytes that block thymic engraftment by donor cells (99;97). High doses of depleting mAbs induce coreceptor coating and downmodulation, promoting T cell inactivation (99). This maneuver allowed donor MHC class II^+ cells to appear in the thymus by Day 14 post-BMT and correlated with intrathymic deletion of donor (superantigen)-reactive T cells (97;95). These findings were later confirmed using alloreactive 2C TCR transgenic CD8 T cells with known specificity for donor MHC class I (L^d). Intrathymic deletion of 2C cells (169) and of donor endogenous superantigen-reactive thymocytes (95) correlated tightly with the presence of donor MHC class II^+ cells with dendritic morphology.

When global T cell depletion and/or TI was replaced with costimulation blockade, mechanisms of early peripheral and intrathymic tolerance of pre-existing anti-donor T cells required investigatation to determine how early rejection (prior to the central tolerance maintenance phase) was avoided. Peripheral, thymus-independent deletion of donor-reactive T cells was demonstrated in these animals (114). Furthermore, donor skin grafted the day after BMT was specifically accepted, demonstrating early unresponsiveness (prior to complete deletion of donor-reactive T cells) that is recapitulated in *in vitro* assays (119). As the molecular mechanisms of tolerance of peripheral CD4 and CD8 T cells differ, each subset has been investigated separately.

Peripheral CD4 T cell tolerance

As discussed earlier, peripheral CD4 T cells can be tolerized when CD8 T cells are depleted and 3 Gy TBI is given prior to a single injection of anti-CD154 mAb on the same day as allogeneic BMT (117). Studies of the mechanism of peripheral CD4 cell tolerance in these animals revealed gradual deletion of peripheral donor superantigen-reactive or truly alloreactive TCR transgenic CD4 T cells (117;170) over a period of four to five weeks post-BMT. This deletion was specific for alloantigen-reactive cells, as transgenic cells were not deleted in control mice receiving allogeneic marrow that did not express their allogeneic MHC ligand (170).

Studies in CD154 knockout mice as recipients of completely MHC-mismatched BMT with this protocol revealed that the absence of CD154-CD40 interactions is sufficient to allow CD4 cell tolerance, leading to HSC engraftment (171). Similar deletion of donor superantigen-reactive CD4 T cells was observed in CD154 knockout and wild-type mice receiving this regimen (171). These data are inconsistent with the possibility that the role of anti-CD154 in promoting alloreactive CD4 T cell deletion is to target activated $CD154^+$ cells for antibody-mediated depletion. Further investigation of the pathways involved in deletion of pre-existing alloreactive CD4 T cells in these mice were also inconsistent with a major role for activation-induced cell death (AICD) since both Fas deficiency and calcineurin inhibitors, both of which play a role in AICD (172;173), had no appreciable effect on CD4 T cell tolerance or chimerism induction (174). While studies in a slightly different model demonstrated a role for Fas in the initial (at one week) peripheral deletion of donor-reactive CD4 cells (175), those studies did not assess long-term tolerance or chimerism. Further support for the lack of a role for AICD is the IFN-γ-independence of tolerance, since this cytokine plays a critical role in AICD (170;176). Peripheral deletion and tolerance of CD4 T cells was blocked by constitutive Bcl-xL transgene expression, suggesting that the intrinsic pathway of cell death might be important in this setting (174).

Since complete deletion of donor-reactive CD4 T cells required several weeks and donor-specific skin graft tolerance was established by Day 1 post-BMT, other mechanisms must mediate initial tolerance. MLR assays revealed donor-specific unresponsiveness prior to deletion (171), and ELISPOT analysis revealed a specific lack of IL-2, IL-4, IL-5, and IFN-γ secretion in response to stimulation by donor antigens as early as two to four days post-BMT (174;170). When IL-2 was added to MLR assays, donor responsiveness was not restored (170).

Given that donor-specific T cells took four to five weeks to be deleted following BMT, it seemed possible that regulatory mechanisms might contribute to tolerance before deletion was complete. However, administration of modest numbers of naïve host-type splenocytes into either established chimeras prepared in wild-type mice or early chimeras prepared in CD154 knockout mice (so that anti-CD154 was not given, avoiding potential interference by anti-CD154 with the function of injected naïve T cells) led to a rapid loss of chimerism. These data argue against a role for robust suppressive mechanisms, even in the early induction phase of tolerance, since potent regulatory mechanisms confer resistance to breaking of tolerance with even much greater numbers of non-tolerant host lymphocytes (177;178;170;179;180). Coadoptive transfer of chimeric splenocytes with naïve cells to immunoincompetent mice grafted with donor and third party grafts also failed to provide evidence for potent suppressive mechanisms (170). Furthermore, linked suppression was not observed when either third party MHC class I or minor histocompatibility antigens were co-expressed on skin grafts with donor antigens (170). Thus, unresponsiveness of CD4 T cells prior to deletion appears to be via anergy that is not reversible by IL-2, with no major role for suppressive mechanisms (170). Consistent with a role for anergy in unresponsiveness of CD4 T cells (181) and with data obtained in other models (182;121), CTLA4 is required to achieve BM engraftment and donor-specific tolerance in this model (J. Kurtz et al, submitted).

In summary, peripheral CD4 T cell tolerance induced by allogeneic BMT with anti-CD154 is associated with early, specific unresponsiveness (by skin graft, MLR, and ELISPOT). The mechanisms appear to involve early, IL-2-refractory anergy followed by thymus-independent deletion within four to five weeks of BMT.

Peripheral CD8 T cell tolerance

As discussed above, pre-existing donor-reactive CD8 T cells can be tolerized in recipients of BMT with anti-CD154 by giving DST or moving the TBI to Day -1 or -2 (119). Depletion of CD4 T cells at any time within the first 10 days post-BMT either abrogated (when depleted on Day -1 or Day 3) or impaired (when depleted on Day 6 or Day 10) the ability to achieve mixed chimerism and tolerance (183). By Day 15 post-BMT, however, depletion of CD4 cells had no inhibitory effect on induction of chimerism or tolerance (183). Since recipient CD8 depletion prevented marrow rejection in CD4-depleted mice (183), these data implicated an early regulatory effect of recipient CD4 T cells on alloreactive CD8 T cells. Although “natural Tregs” are present in unimmunized animals, the development of potent regulatory CD4 T cell function in transplantation models usually requires several weeks, presumably to allow those with donor specificity to expand (184). Additionally, CD4 T cells distinct from “natural” Tregs can become regulatory IL-10^- or TGF-β-producing cells or $FoxP3^+$ Tregs after stimulation (185;186). The early, narrow window of time in which CD4 T cells are required to prevent CD8 cell-mediated rejection of allogeneic marrow in naïve mice argued against a role for any of these types of regulatory CD4 T cells in our model. Furthermore, *in vivo* administration of a mAb against CD25 that has been shown to deplete or block the function of natural, $CD25^+$ Tregs (187) and hence tolerance induction in other models (188) did not block tolerance in this model (183). Likewise, a neutralizing anti-IL-2 mAb that blocks tolerance in other models (189) by affecting the generation, expansion, or survival of regulatory T cells (190) did not

affect tolerance in our model (183). These results also argue against a role for conventional $CD4^+CD25^+$ regulatory T cells in promoting tolerance of CD8 T cells.

Although these findings are consistent with previous studies in this anti-CD154 monotherapy protocol, they contrast with results obtained using a protocol involving BMT with anti-CD154 and CTLA4Ig. Early (but not later) administration of neutralizing anti-IL-2 mAb to recipients of this regimen led to rejection of donor marrow and loss of donor-specific skin graft tolerance (189). Although administration of depleting anti-CD25 mAb immediately after BMT did not prevent long-term chimerism, it did result in loss of skin graft tolerance. Neither neutralizing anti-IL-2 nor depleting anti-CD25 interfered with deletion of peripheral donor superantigen-reactive T cells in this model. Studies involving adoptive transfer of naïve recipient-type splenocytes into early chimeras or co-transfer of naïve recipient-type splenocytes with splenocytes taken early (three weeks post-BMT) from mixed chimeras into immunodeficient mice also support a role for suppressive mechanisms in that model (189). Regulatory T cells have also been implicated in a model involving DST and anti-CD154 administration, since donor skin is rejected (but chimerism maintained) when depleting anti-CD25 is given and skin graft tolerance is maintained (while chimerism is lost) when naïve host-type splenocytes are administered (191).

The fate of peripheral donor-reactive CD8 T cells was investigated to determine if they, like CD4 cells, are deleted when BMT is performed with anti-CD154 and 3 Gy TBI. To do this, a traceable donor-reactive population of CD8 T cells bearing the 2C TCR, which is positively selected on K^b and alloreactive against L^d, was generated in syngeneic wild-type B6 recipients via transplantation of 2C marrow to 3 Gy-irradiated B6 mice (183). The reconstituted mice express the 2C TCR on 3-20% of all $CD8^+$ T cells but contain large polyclonal populations of non-transgenic CD4 and CD8 T cells. BMT using L^{d+} (B10.A) but not L^{d-} (A.SW) donors led to deletion of peripheral 2C CD8 T cells within approximately one week post-BMT (183). Early *in vitro* analyses (i.e., CML) revealed that donor-reactive CD8 T cells are specifically unresponsive by Day 4, prior to deletion (Haspot F, et al. manuscript submitted). Consistent with this observation, donor skin grafted on the day after BMT was specifically accepted. Moreover, complete deletion of donor-reactive CD8 cells was achieved within the early window of time (roughly 10-15 days) in which CD4 T cells were required to achieve mixed chimerism and tolerance (183). The complete deletion of peripheral donor-reactive CD8 cells obviates the need for CD4 cells to maintain tolerance. Thus, donor-reactive CD8 T cells are rendered unresponsive prior to deletion and this phenomenon depends on the presence of $CD4^+CD25^-$ T cells.

Distinct pathways to tolerance of CD4 versus CD8 T cells

Studies of CD8 T cell “exhaustion” due to chronic viral infection suggest that the inhibitory receptor programmed death-1 (PD-1) is required to maintain unresponsiveness (192;193; 194). The role of PD-1 in peripheral tolerance achieved with BMT with anti-CD154 and 3 Gy TBI was investigated. PD-1/PD-L1 interactions were found to be critical to permit development of mixed chimerism and tolerance only when recipient CD8 T cells are present (Haspot F, et al. manuscript submitted). No requirement for this pathway was demonstrated in the tolerization of CD4 cells with BMT and anti-CD154, indicating a dichotomy in the pathways leading to tolerance of the two T cell subsets in this model.

Further dichotomies have been detected in the pathways to CD4 versus CD8 T cell tolerance in this model. Recipient B cells and $CD11c^+$ cells, presumably DCs, are required for tolerance of peripheral CD8 but not CD4 T cells. Furthermore, recipient class II expression on hematopoietic cells is required to achieve CD8 cell tolerance (T. Fehr, manuscript submitted). The complex interactions between recipient CD4 cells, CD8 cells, and the various APC populations in this tolerance pathway are currently under investigation.

Differing mechanisms of tolerance using costimulation blockade with or without HCT

Prior to the investigation of costimulation blocking reagents in conjunction with allogeneic HCT, these reagents alone were shown to prolong allograft survival. DST with anti-CD154 achieves long-term skin graft survival only when animals are thymectomized prior to transplant, highlighting a key difference between protocols with and without HCT (195). In the absence of donor HSC engraftment, thymectomy is a necessity to prevent newly developing T cells with donor specificity from destroying the graft. By contrast, adding HCT to the regimen permits central, intrathymic deletion and precludes the requirement for thymectomy. When costimulation-blocking reagents are given without HCT, regulatory T cells may play an important role in suppressing alloreactivity since CD4 T cells are required for prolonged skin graft survival (195). Costimulation blockade alone has achieved tolerance to cardiac or islet allografts (196), but these protocols are unsuccessful at inducing tolerance when tested in stringent skin graft or large animal models (197;198;199). One model lacking HCT has been reported to achieve long-term acceptance of MHC-mismatched primary skin allografts. This protocol involves a short course of rapamycin with anti-CD154 and CTLA4Ig (200). In contrast to tolerance achieved via durable mixed chimerism, protocols lacking HCT do not achieve systemic tolerance as assessed by *in vitro* MLR and CML assays.

Additional discrepancies between costimulation-blockade protocols with and without HCT include a more prominent role for calcineurin-dependent signaling (201;196), IL-2 and AICD (198;202;200;203), IFN-γ (195;204), and regulatory T cells (195) in the latter. Moreover, linked suppression and “infectious tolerance” can be observed in mice receiving minor antigen-mismatched (MHC-matched) skin or islet allografts (205;206). These mechanisms do not appear to be involved in long-term tolerance induced by costimulation blockade with HCT when pre-existing and newly developing donor-reactive T cells are completely deleted.

Overall, the literature on tolerance induction with costimulation blockade and other immunosuppressive agents, with or without HCT, can be synthesized in the following manner: When donor-reactive T cells are fully deleted, regulatory cells specific for donor reactivity are not expanded. In the absence of strong regulatory mechanisms, central deletion is essential for maintenance of long-term tolerance since even the senescent thymus continually generates new alloreactive T cells (166). Strong regulatory mechanisms are only induced in the presence of persistent anti-donor reactivity, so the challenge is to reliably create conditions that control such alloreactivity while permitting expansion of these regulatory cells. Vascularized allografts in combination with immunosuppressive agents achieve these conditions more reliably in rodents than in large animal models.

B cell and NK cell tolerance

NAb-producing B cells and NK cells do not normally pose a major barrier to allogeneic marrow engraftment, but they pose major barriers to xenogeneic hematopoietic cell engraftment. Establishment of mixed chimerism assures that newly developing anti-donor B cells are tolerized by either deletion or by receptor editing through encounter with membrane-bound donor antigens expressed on donor hematopoietic cells, similar to autoreactive B cells in normal individuals (207;208). Furthermore, generation of T cell-dependent alloantibodies by recipient B cells is prevented in mixed chimeras by T cell tolerance, which results in a lack of help for alloantibody responses. In another model, the absence of T cell help has been associated with deletion of donor-reactive mature B cells (209). However, even when anti-donor Abs (e.g., NAbs against carbohydrates expressed by xenogeneic donors) pre-exist in the host, these cells are tolerized by establishment of mixed chimerism (153;154;155). The pre-existing B cells producing these IgM NAbs, which are mainly splenic B-1b B cells (210), are tolerized within two weeks of BMT by a mechanism that appears to involve anergy followed by deletion (211). B-1b cell tolerance induction by mixed chimerism is dependent on a population of

recipient stromal cells expressing CR1/CR2 complement receptors, which may be follicular dendritic cells (212).

When recipient T cell alloreactivity is fully blocked, NK cells pose a minimal barrier to alloengraftment (213) unless other conditions are limiting. For example, recipient NK cell depletion can enhance alloengraftment in non-myelosuppressed recipients or in recipients of limiting marrow doses (213;88). Donor and recipient NK cells develop mutual tolerance in mixed allogeneic chimeras (214). Such tolerance was reported to be associated with down-regulation of activating Ly49 receptors recognizing the alloantigens (88), but this finding has not been universal (215).

NK cells pose a much stronger barrier to xenogeneic than to allogeneic marrow engraftment. Their depletion is necessary to allow engraftment of high doses of T cell-depleted marrow even from a closely related species (148;216). Once mixed xenogeneic chimerism is established, however, the recovering recipient NK cells are unable to reject donor marrow cells. In contrast to the donor-specific NK cell unresponsiveness observed in mixed allogeneic chimeras (214), a more global NK cell unresponsiveness is induced by mixed xenogeneic chimerism, such that rejection of class I-deficient marrow is also impaired (153). Murine NK cells acquire cytolytic activity through interactions of inhibitory Ly49 receptors with “self” MHC molecules. Some NK cells lack an inhibitory receptor for “self” and are non-functional. Hence their “self-tolerance” reflects a failure to be “licensed” through such inhibitory interactions (217). However, this “licensing” model does not explain the dominant NK cell tolerance induced in the presence of two completely MHC-mismatched marrow types in mixed allogeneic chimeras (214). Since each marrow type should be capable of “licensing” different sets of NK cells expressing inhibitory ligands for their differing class I molecules, the presence of two sets of cells with different MHCs would thereby result in increased overall NK cell reactivity rather than unresponsiveness to both the donor and recipient. Based on the NK cell tolerance observed in mixed allogeneic and xenogeniec chimeras, we favor a model in which developing NK cells receive activating signals from surrounding cells, and only become fully functional if these activating signals are attenuated by interactions with inhibitory ligands. Thus, in mixed allogeneic chimeras, individual NK cells that become fully functional will have encountered both donor and recipient cells during development and have receptors for inhibitory ligands on both donor and recipient hematopoietic cells. In the case of mixed xenogeneic chimeras, the NK cells receive unopposed activating signals from xenogeneic donor hematopoietic cells since inhibitory NK cell receptors are usually unable to interact with xenogeneic MHC molecules (218;219;220), while activating NK receptors are often able to receive functional signal in response to xenogeneic MHC ligation (221;222;223;224). The high level of stimulation without inhibitory signals from xenogeneic cells may result in “anergy” of developing NK cells, resulting in failure to kill any target. This hypothesis is in keeping with the “disarming” model of NK cell tolerance (225), which explains the dominant tolerance induced by the presence of class I-deficient cells in mixed wild-type plus class I-deficient chimeras (226). Consistent with this, tolerant NK cells in fully MHC-mismatched mixed allogeneic chimeras retained the ability to reject class I-deficient marrow (214), while the presence of low levels of xenogeneic chimerism causes a similar, global functional NK cell defect to that described in the presence of chimerism from class I-deficient marrow, suggesting that xenogeneic class I does not interact significantly with NK cell inhibitory ligands.

Infection-related Issues in Mixed Chimeras

The presence of viral infection during conditioning for allogeneic BMT can lead to death due to failure to clear the virus (227), highlighting the importance of avoiding such exposures during the period of immunosuppression, even with non-myeloablative conditioning. Once established, mixed hematopoietic chimeras are markedly more immunocompetent than full

donor chimeras due to the continued presence of recipient APCs expressing the same recipient MHC molecules as those present on thymic epithelial cells during positive selection (228; 229;114;230;231;232). This promotes superior T cell responsiveness to foreign peptides because T cells positively selected on recipient-type MHC react better with those same MHC molecules than with allogeneic MHC. In contrast to full allogeneic chimeras, mixed chimeras generated across full MHC barriers with a lethal TBI regimen demonstrated superior anti-viral responses after challenge. These CTL responses, though mediated by both donor and recipient-derived T cells, were restricted exclusively by recipient MHC molecules (233), which mediated positive selection in the host thymus. Although anti-viral memory responses are generally intact and can effectively clear viruses (234), the exquisite host restriction of the CTL response may preclude killing of infected donor cells, which thereby provide a persistent viral reservoir (235). This problem can be overcome by partial class I sharing between donor and recipient, a situation that always prevails in haploidentical related donor transplantation (235).

Induction of mixed allogeneic chimerism with non-myeloablative regimens involving costimulation blockade without T cell depletion can be blocked by concurrent acute, chronic, or latent viral infections (236;237;227). The ability of acute or chronic viral infection to inhibit mixed chimerism induction may involve activation of APCs in a manner that inhibits tolerance induction (237), likely through the involvement of TLRs. The ability of latent or prior infection to block chimerism induction may reflect cross-reactivity of virus-induced alloreactive memory cells (238) (particularly $CD8^+$ central memory cells) with donor antigens (239). This effect could be overcome by impairing NF-κB activity while costimulation was blocked (239). These studies may help to explain why chimerism is not durable in non-human primates or humans (see below) since exposure to pathogens that may induce heterologous immunity is much more significant in these species. Indeed, memory CD8 cells have been shown to pose a major barrier to achievement of mixed allogeneic chimerism in monkeys. This barrier can be overcome with CD8-depleting antibody (240).

Moving From Animal Models to Clinical Trials

Organ allotransplantation as currently practiced with non-specific immunosuppressive therapy is associated with high early success rates, often exceeding 90%. Thus, any attempt at tolerance induction, which would involve removal of chronic immunosuppressive therapy, currently the mainstay of transplantation strategies, must be expected to achieve at least equally good early graft survival rates. A major limitation to the success of clinical organ transplantation has been late graft loss, which is largely due to chronic rejection. The rates of late graft loss have not improved substantially in recent years, despite the development of new immunosuppressive drugs. Tolerance would prevent chronic rejection, as has been described in animal models (241) and thereby largely prevent late graft loss. Unfortunately, no markers have been identified to reliably indicate whether or not immunological tolerance has been achieved in patients, resulting in an absence of laboratory parameters upon which to base immunosuppression withdrawal.

Because equal or better efficacy compared to current transplant protocols must be expected from tolerance trials, there are stringent prerequisites for animal data. Fortunately, organ transplant studies in large animals mimic clinical transplantation quite well from an immunological standpoint, allowing both safety and efficacy of new therapies to be evaluated. Several strategies reported to induce tolerance in rodent models have either failed to achieve tolerance in large animals (242;198;243;244) or have not been evaluated. The combination of anti-CD154, rapamycin, and DST led to “operational tolerance” in three of five cynomolgus monkeys, suggesting a possible efficacious approach. “Operational tolerance” denotes graft acceptance without chronic immunosuppressive therapy. This state has been achieved in numerous rodent models involving primarily vascularized heart, kidney, and liver allografts

with short courses of immunosuppression (245;246;247;248) and, in some cases, no immunosuppression at all (249;250). "Operational tolerance" is often not systemic, so that anti-donor immune responses can be detected *in vitro* or even *in vivo*. In some instances a second graft from the same donor is accepted without immunosuppression, whereas in others it is not. Acceptance of both the first and the second graft may depend on the type of tissue or organ that is transplanted, as some are clearly more immunogenic than others (251). In general, primarily vascularized organs are more readily accepted than non-vascularized tissue. It is a common error to attribute graft acceptance to the short-term treatment given to the rodent recipient, when in fact the short-term immunosuppressive treatment has merely permitted the inherent tolerogenicity of the allograft to prevail over the destructive immune response. In many instances, this organ tolerogenicity in rodents can be attributed to the ability of such grafts to induce regulatory T cell responses (184). Unfortunately, these regulatory responses tend not to prevail so readily following vascularized allografting in large animals. Thus, to move from rodent models to clinical trials of tolerance induction, efficacy in stringent rodent models such as MHC-mismatched skin grafts, which are among the most immunogenic and least tolerogenic grafts, must first be demonstrated in rodents. Next, both efficacy and safety must be demonstrated in large animal models.

HCT is the only tolerance approach thus far that has met these criteria and is being evaluated in clinical trials. Rodent studies discussed above showed clearly that non-myeloablative regimens can permit achievement of durable multilineage mixed allogeneic chimerism and donor-specific tolerance that is maintained by a central deletional mechanism. Durable chimerism is associated with tolerance to the most highly immunogenic grafts, including skin (96) and small intestine (252). This approach was translated successfully to large animal models, including the pig (253;136), dog (140), and non-human primates (142;145). In the case of non-human primates, the chimerism achieved was only transient, but the combination of simultaneous kidney and bone marrow transplantation from the same MHC-mismatched donor was, in most cases, associated with tolerance induction. The bone marrow graft was essential to the achievement of tolerance and could not be replaced by donor spleen cell infusion (254;255;145). Although central deletional tolerance is unlikely to explain the long-term tolerance in these animals lacking durable chimerism, donor APCs can be detected in the thymi of these recipients early post-transplant (145), suggesting a possible role in the achievement of tolerance. The kidney graft itself is essential for tolerance induction, as failure to implant the donor kidney within the first two to three months after BMT results in failure of tolerance induction (256).

The successful protocols in cynomolgus monkeys described above involved the use of low-dose TBI, thymic irradiation, equine ATG, splenectomy, and post-transplant cyclosporine (142). More recent protocols have employed anti-CD154 mAb to avoid the requirement for splenectomy, which is needed to avoid humoral responses to the donor (145). While all of these reagents, with the exception of anti-CD154 (which is associated with significant thromboembolic complications), have been used in humans with acceptable safety profiles, it would still be a radical departure to use such a protocol in place of standard immunosuppressive therapy in a solid organ transplant recipient. Even if successful in achieving tolerance, this protocol might be associated with significant early toxicity in humans.

The opportunity to evaluate non-myeloablative protocols for achieving mixed chimerism and organ allograft tolerance in humans was provided, in fact, by studies in patients with hematologic malignancies that demonstrated safety and potential efficacy. As discussed above, we have developed regimens aiming to achieve mixed allogeneic chimerism without GvHD using *in vivo* T cell depletion with non-myeloablative conditioning, with the goal of later giving DLI to "quiescent" mixed chimeras and thereby confining the GvH response to the lymphohematopoietic system. Non-myeloablative regimens that successfully achieved mixed

chimerism in these clinical trials allowed two trials of combined kidney and bone marrow transplantation to be initiated. The first involved patients with end-stage renal disease due to multiple myeloma (257). This patient group is usually not considered for HCT because of the expected high morbidity associated with renal failure and is not considered for renal transplantation because of their otherwise (except by allogeneic HCT) incurable malignancy. However, once the excellent safety data and tumor responses were observed in patients with advanced lymphoid malignancies, including myeloma, using this strategy (77;258), pilot studies supported in part by the Immune Tolerance Network (ITN) were performed using combined HLA-identical, related donor kidney and bone marrow transplantation in myeloma patients. It was hoped that concomitant BMT might allow establishment of immunological tolerance of the kidney allograft, while curing the myeloma. Conditioning included myelosuppressive treatment with cyclophosphamide, T cell depletion with equine ATG, and 7 Gy thymic irradiation, followed by combined kidney and BMT from the same donor, with a very short course (approximately 60 days) of post-transplant cyclosporine followed by DLI. Two out of six patients developed full donor chimerism and the other four patients lost their BM grafts within three months but retained their donor kidney allografts without immunosuppression. Follow-up ranges from two and a half to nine years in this group of patients, who provide the first demonstration that organ allograft tolerance can be intentionally achieved in humans (257).

Safety data in a related trial involving HLA-mismatched BMT (with no kidney transplant) in patients with hematologic malignancies provided the impetus to extend this approach of combined renal and BMT to the HLA-mismatched setting. (63). Four patients with hematologic malignancies received haploidentical, related bone marrow grafts with a conditioning regimen that included cyclophosphamide, thymic irradiation, and peri-transplant treatment with a humanized anti-CD2 mAb, a more potent T cell-depleting agent than the equine ATG used in the above HLA-identical studies in myeloma patients. Only transient chimerism was achieved, and loss of donor hematopoiesis was associated with robust recipient hematopoiesis, demonstrating the non-myeloablative nature of the regimen. Although loss of chimerism was undesirable from the standpoint of anti-tumor effects, none of these patients developed GvHD. Achievement of this critical safety parameter in the setting of HLA-mismatched BMT, together with the non-human primate data indicating that transient chimerism promoted tolerance in recipients of combined kidney and BMT, provided the basis for a second clinical tolerance trial sponsored by the ITN, this time using haploidentical (instead of HLA-identical), related donors in recipients without malignant disease (259). Five patients were transplanted, with follow-up currently ranging from about two to greater than five years. Four out of five patients were successfully taken off their initial immunosuppressive monotherapy with calcineurin inhibitor and have had stable graft function off immunosuppression for greater than one to almost five years. One patient lost his graft early due to acute humoral rejection. This trial is the first to intentionally achieve tolerance to an organ allograft across HLA barriers.

Mechanisms of tolerance in patients receiving combined kidney and bone marrow transplants

In vitro studies performed on the myeloma patients who received combined HLA-identical bone marrow and kidney transplantation suggested that donor kidney-specific tolerance may develop. When tested, recipient T cells did not respond to donor renal tubular epithelial cells, but, in some instances, demonstrated sensitized responses to minor histocompatibility antigens expressed on donor hematopoietic cells in association with loss of chimerism (257). Persistent anti-donor CML responses were detected almost two years post-transplant in a patient accepting her renal allograft without immunosuppression (257). A state of “split tolerance” (tolerance to antigens expressed by the kidney but not to those uniquely expressed by donor hematopoietic cells) is consistent with a role for the renal allograft itself in the

maintenance of operational tolerance. Moreover, studies in the non-human primate model described above strongly implicate a role for the kidney itself in maintaining tolerance (256).

In contrast to HLA-identical transplant recipients, who do not show robust bulk *in vitro* responses to their donor prior to transplant, patients receiving HLA-mismatched transplants normally do have such responses prior to tranplantation. *In vitro* analyses of the patients without malignant disease who received HLA-mismatched combined kidney and bone marrow transplantation revealed the progressive development of donor-specific unresponsiveness in both MLR and CML assays following the transplant in all four patients who accepted their grafts without immunosuppression (259). These data indicate that a systemic state of tolerance eventually developed in recipients of HLA-mismatched transplants. These observations contrast with those in recipients of HLA-identical transplants, suggesting that the mechanisms of tolerance may differ in the HLA-identical versus the mismatched setting. However, it is unsatisfying to envision completely different mechanisms resulting in similar outcomes in both groups of patients. In keeping with the hypothesis proposed for the HLA-identical group that tolerance is restricted to antigens expressed by the kidney, we favor the possibility that, in recipients of HLA-mismatched transplants, the pre-existing bulk anti-donor response disappears following the transplant because the majority of allogeneic MHC/peptide complexes that induce the strong alloresponse *in vitro* are shared by both the kidney and the hematopoietic cells. The immunodominance of the response to allogeneic MHC/peptide complexes shared by the kidney and the hematopoietic cells may preclude the sensitization of T cells recognizing antigens expressed only on hematopoietic cells. If this is the case, it is difficult to envision how the marrow could be rejected without the kidney also being rejected. However, the loss of chimerism in these patients occurs very early, when T cells are markedly depleted by the conditioning, and it is not clear that the loss of chimerism is due to an immune response. The marrow grafts in these patients may have been lost due to inadequate HSC engraftment and competition from surviving host hematopoietic cells. Comparison of the *in vitro* results in combined haploidentical kidney and BMT patients with those from patients with hematologic malignancies who received a similar haploidentical BMT regimen without a kidney transplant suggests a role for the kidney in achieving tolerance. In contrast to the combined transplant recipients, the “BMT only” patients showed generally weak alloresponses but tended to have stronger anti-donor than anti-third party responses following the loss of chimerism (260).

While much remains to be learned about the mechanisms of tolerance, intragraft levels of FoxP3 relative to Granzyme B mRNA were increased in tolerant patients compared to patients on immunosuppression, consistent with the possibility that regulatory T cells might play a role in tolerance (259). *In vitro* assays to assess the possible role of regulatory cells, which are enriched among the low T cell numbers initially present in recipients of this regimen without a kidney transplant (260), may suggest a possible role for these cells in tolerance induction. The difficulty in demonstrating this phenomenon in PBMCs suggests that the relevant suppression may occur in the graft itself.

Role of HCT in Treatment of Autoimmune Disease

Many clinical trials have begun to evaluate the utility of autologous HCT for the treatment of a variety of severe autoimmune diseases, including systemic sclerosis, systemic lupus erythematosis, other vasculitides, multiple sclerosis (reviewed in (261;262)), and, most recently, type I diabetes (263). These studies are still in progress, and neither the best approach to conditioning (myeloablative, non-myeloablative but lymphoablative, or non-lymphoablative) nor the best HCT product (unmodified peripheral blood stem cells (PBSCs) versus selected HSCs) nor the curative potential at various disease stages has been determined. Two potential mechanisms might lead to therapeutic benefit: 1) Ablation of existing T and B

cells, including autoreactive ones. Such ablation would require strong lymphoablative conditioning and a purified HSC preparation. However, it may not be possible to fully deplete autoreactive memory T cells or plasma cells, even with the most aggressive lymphoablative regimens. 2) “Resetting” the immune system by reducing the number of destructive autoreactive lymphocytes might tip the immune balance in favor of regulatory cells. Autologous HCT was associated with deviation of autoreactive T cell responses to an IL-10-producing phenotype in children with juvenile idiopathic arthritis in association with restoration of normal Treg levels, which was attributed in part to preferential homeostatic expansion of this population and to renewed thymopoiesis (264).

Animal models have shown that allogeneic HCT can reverse autoimmunity (265), and several potential mechanistic pathways may explain this phenomenon. When compared, allogeneic HCT appears to have greater long-term curative potential than autologous HCT, apparently due, at least in part, to a graft-versus-autoimmunity (GvA) effect, which probably reflects elimination of recipient lymphocytes by GvH-reactive donor T cells (266;267). However, in some models, mixed allogeneic T cell chimerism has been associated with reversal of autoimmunity (268), suggesting that other mechanisms come into play. These can be broadly divided into two categories: 1) negative selection of newly developing autoreactive thymocytes or B cells due to the presentation of cross-reactive antigens by allogeneic donor MHC molecules; and 2) promotion by donor T cells or other hematopoietic cells of normal regulatory mechanisms that may be deficient in the autoimmune recipient. These mechanisms have recently been reviewed in detail (269). Allogeneic HCT has long been validated as the treatment of choice for acquired aplastic anemia, which is considered to be an autoimmune disease. Recommendations are being developed for initiation of trials of allogeneic HCT in this setting (270). However, the risks of allogeneic HCT are high and its morbidity is increased in patients with end organ damage due to their autoimmune disease. It has been suggested that initial trials of allogeneic HCT should involve reduced intensity conditioning in patients who could not tolerate the high intensity regimens often used with autologous HCT and who have aggressive disease with a poor prognosis (260). An added benefit of HCT is that these transplanted cells can induce tolerance to antigens from the same donor, so that the recipient might easily be able to accept needed donor replacement tissues (such as islets in type 1 diabetes) without requiring chronic immunosuppressive therapy.

Conclusions

The therapeutic potential of allogeneic HCT is vast, with its ability to cure hematologic malignancies, autoimmune diseases and inherited hematopoietic and metabolic abnormalities and to induce immune tolerance to allografts and xenografts. The development of reduced intensity regimens for achieving allogeneic hematopoietic engraftment across major histocompatibility barriers and its recent application in clinical trials of kidney transplantation is highly encouraging. A deeper understanding of the mechanisms by which HCT overcomes pre-existing allo- and autoimmunity will permit the development of even more successful and less potentially toxic approaches to exploiting this capacity.

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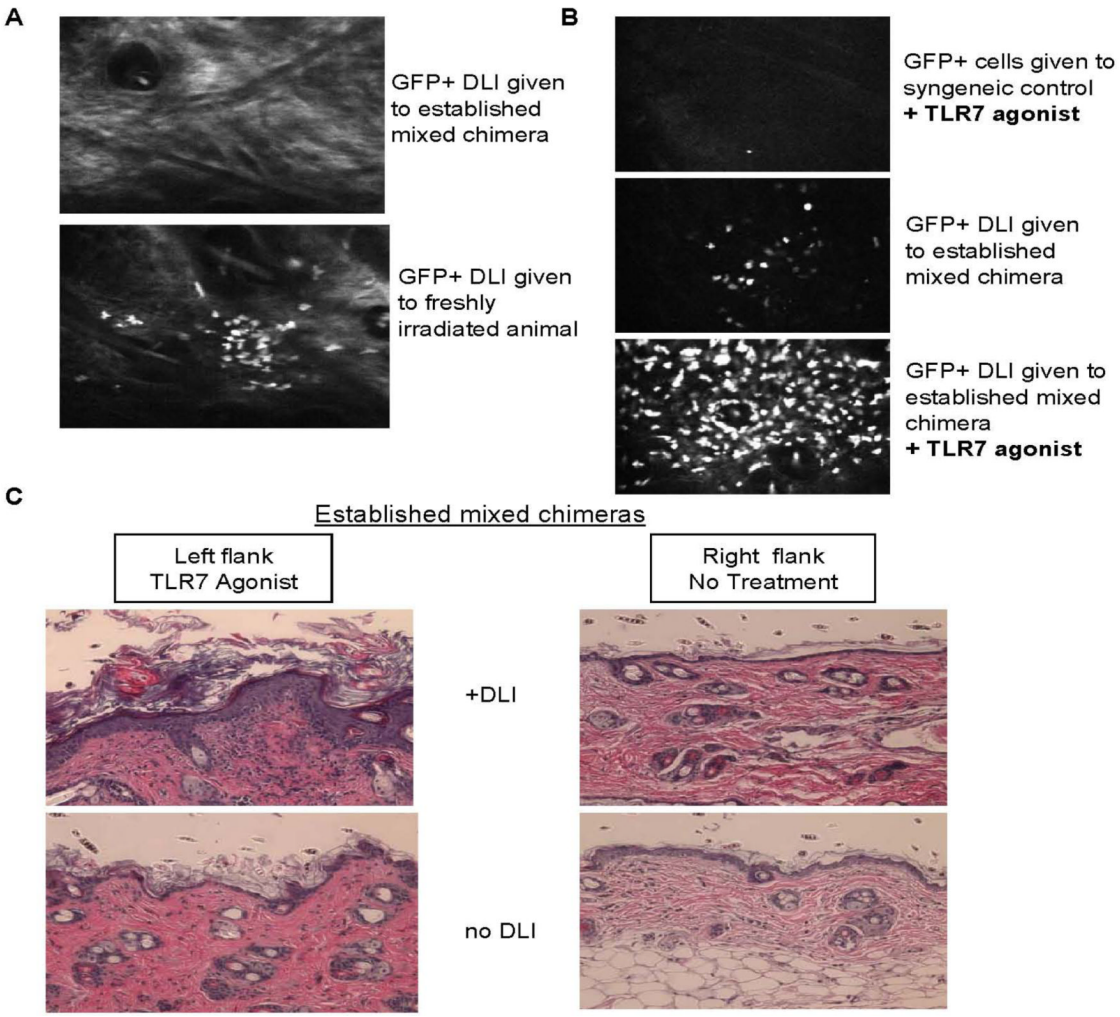

Figure 1.

GvL but not GvHD is observed in established mixed chimeras receiving DLI because tissue inflammation has subsided, preventing donor T cells from trafficking to target organs and restricting the GvH response to the lymphohematopoietic system. (**A**) Intravital two-photon microscopy of established mixed chimeras 5 days after administering GFP^+ B6 splenic T cells as DLI indicates that DLI T cells do not accumulate in skin of established mixed chimeras ($B6^+BDF1 \rightarrow BDF1$), but do so in skin of freshly irradiated animals receiving the same inoculum. Not shown are dynamic imaging studies indicating that the lack of T cell accumulation in skin of mixed chimeras reflects a marked reduction in rolling and tight adhesion of T cells in mixed chimera skin vessels compared to freshly irradiated mice. (**B**) Shows that the addition of local inflammation induced by a TLR agonist (the TLR7 agonist imiquimod painted on the skin) permits DLI to induce local GVHD in skin of mixed chimeras (shown12 days post-DLI with GFP^+ B6 (donor) splenocytes). Histology (**C**) of the left (imiquimod-treated) and right (untreated) flank of established mixed chimeras receiving (top) or not receiving (bottom) DLI shows that the skin GVHD is confined to the imiquimod-painted skin area and requires DLI.

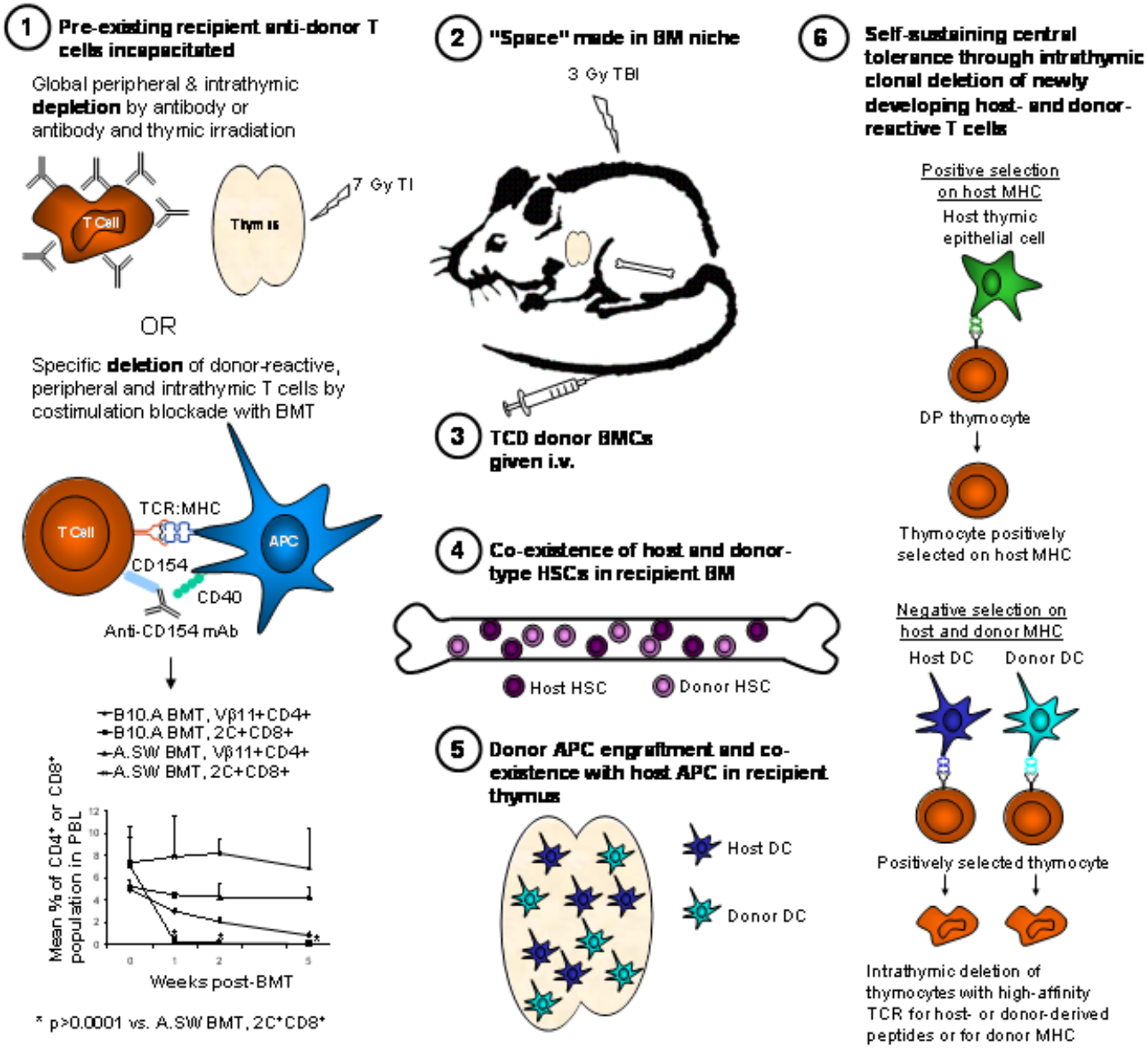

Figure 2.

Schematic representation of the mechanisms of tolerance in mixed hematopoietic chimeras. In order to prevent rejection of donor HCT, pre-existing anti-donor T cells in the host must be removed from the periphery and the thymus (1). This can be done by global T cell depletion (left) or by specific deletion induced by costimulation blockade (right), demonstrated by deletion of donor-reactive $Vb11^{+}CD4^{+}$ and $2C^{+}CD8^{+}$ over time when relevant B10.A BMT is given. Once niches in the BM are freed via non-myeloablative conditioning (2), TCD donor BMCs injected i.v. (3) engraft in the recipient BM and coexist with host cells (4). HSCs from host and donor give rise to host and donor APC, respectively, that engraft in the recipient thymus (5) and mediate central deletion of newly developing host- and donor-reactive T cells (6). This mechanism of intrathymic clonal deletion of developing alloreactive T cells is the sole mechanism maintaining lifelong tolerance in mixed chimeras.