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Mechanisms of tolerance induced via mixed chimerism

Megan Sykes

Transplantation Biology Research Center, Department of Surgery, Massachusetts General Hospital/Harvard Medical School, MGH East, Bldg 149-5102 13th Street, Boston, MA 02129

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1. ABSTRACT

Mixed hematopoietic chimerism provides a powerful means of inducing robust, donor-specific tolerance. In this article, the minimal requirements for achieving mixed chimerism, the development of new reagents that promote its achievement, and the mechanisms by which peripheral and intrathymic tolerance are achieved via mixed chimerism are discussed. An emerging understanding of these mechanisms, along with the development of new immunosuppressive reagents, is allowing advancement toward clinical application of this approach.

2. INTRODUCTION

The induction of specific transplantation tolerance would overcome all of the major obstacles limiting the advancement of transplantation. Although numerous regimens leading to the indefinite acceptance of vascularized allografts have been reported in rodent models, the efficacy of these approaches in humans cannot be predicted, as most of these strategies fail to achieve similar results when attempted in large animals. Tolerance in rodents that is geared toward ultimate clinical application, therefore, must have high reliability and

reproducibility, must not be perturbed by intercurrent infections or other immunological events, and must be evident by the most stringent tests (e.g. the permanent acceptance of fully MHC-mismatched skin grafts in euthymic recipients).

3. TOLERANCE INDUCTION THROUGH HEMATOPOIETIC CHIMERISM

It has been known since the early studies of Owen and Medawar (1-4), that hematopoietic chimerism induced in immunologically immature fetal or neonatal animals leads to transplantation tolerance. Subsequently, it was shown that tolerance could be achieved by bone marrow transplantation (BMT) to adult rodents whose immune and hematopoietic systems were first ablated with lethal total body irradiation (TBI)(5). However, the clinical application of BMT for tolerance induction has largely been prohibited by the toxicity of the host conditioning thought to be necessary to allow bone marrow (BM) engraftment, and by the formidable problems of graft-versus-host disease (GVHD) and failure of engraftment, especially when major HLA barriers are transgressed (6-8). To develop a less toxic approach to using BMT for tolerance induction, it is first necessary to identify the host factors resisting alloengraftment that can be specifically targeted to permit donor marrow engraftment without GVHD.

Mixed chimerism describes a state wherein hematopoietic populations of both the recipient and the donor co-exist. This state can be achieved in immunologically mature recipients by milder treatments that do not ablate host hematopoietic cells (9). Mixed chimerism is associated with improved immunocompetence compared to MHC-mismatched full allogeneic chimerism (10-14), because mixed but not full chimeras contain a life-long source of host-type antigen-presenting cells (APC) that can most effectively present antigens to T cells that develop in the recipient thymus. These T cells are positively selected by host MHC on thymic epithelial cells, and therefore recognize peptide antigens in the periphery most efficiently in the context of host MHC.

4. ACHIEVEMENT OF MIXED CHIMERISM WITH NON-MYELOABLATIVE CONDITIONING

Mixed chimerism achieved with high-dose total lymphoid irradiation or lethal TBI followed by reconstitution with mixed T cell-depleted host-type and allogeneic marrow induces tolerance in rodents (13, 15-19), but these approaches are not clinically applicable because of the complications associated with such strategies in humans. Efforts at achieving mixed chimerism through less toxic approaches include host pretreatment with depleting doses of anti-CD4 and anti-CD8 mAbs along with a sublethal dose of TBI (3 Gy, which is only minimally and transiently myelosuppressive in mice (20)) along with selective irradiation to the thymic area (thymic irradiation, TI) (9). Lasting multilineage mixed chimerism is achieved across full MHC barriers with these treatments, along with robust donor-specific tolerance that is illustrated by permanent acceptance of donor skin grafted any time after BMT, with normal rejection of third party allografts (9). The tolerance is systemic, as is illustrated by donor-specific unresponsiveness in MLR and CML assays (21). TI can be replaced in this model by the repeated administration of anti-CD4 and anti-CD8 mAbs (on Day -1, in addition to a first dose on Day -5 or -6) (22, 23), or by a single treatment with either anti-CD154 mAb or CTLA4Ig (24). A variety of additional protocols using different forms of myelosuppression and T cell elimination for the induction of mixed chimerism in rodents have been reported (25-30) (31).

Since the adult thymus is very slow to regenerate T cells after lymphoablative conditioning (32-34), it would be desirable to avoid recipient T cell depletion in regimens for the induction of mixed chimerism. We demonstrated that the T cell barrier to allogeneic marrow engraftment can be overcome without T cell depletion by using costimulatory blockade, and that the combination of BMT and costimulatory blockade led to donor-specific tolerance (35). This robust, systemic tolerance is maintained long-term by central deletion, but initially involves additional mechanisms required to tolerize pre-existing peripheral T cells. Several groups have now developed additional protocols for mixed chimerism and tolerance induction using costimulatory blockade (36-42). Although tolerance has been achieved in rodent vascularized allograft and islet allograft models with costimulatory blockade alone or with donor-specific transfusion (43-49), or in skin graft models in thymectomized mice (47), it is only in combination with BMT that costimulatory blockade has achieved tolerance that is systemic and measurable by the most stringent criterion, acceptance of fully MHC-mismatched skin grafts, in euthymic mice (35).

5. GENERAL REQUIREMENTS FOR SUCCESSFUL MIXED CHIMERISM AND DURABLE TOLERANCE INDUCTION

The barriers that must be overcome by all protocols that reliably achieve mixed chimerism and tolerance are summarized in Figure 1. Since adult recipients contain mature T cells with anti-donor reactivity in the periphery and thymus, these must be eliminated or inactivated by the initial host conditioning (for example by depletion or using costimulatory blockers), to prevent the rejection of the infused donor BM. Even though a single injection of T cell depleting mAbs leads to near-complete depletion of the peripheral T cell pool, residual donor-reactive thymocytes can cause intrathymic rejection of donor cells entering the thymus, which ultimately leads to failure to tolerize the initially-recovering T cell repertoire (50). Chimerism is not stable, but starts to decline soon after BMT, even if relatively high levels of peripheral non-thymic chimerism are initially achieved (9, 23) A repeat anti-T cell antibody injection (50) or costimulatory blocker (24) eliminates this residual thymic alloreactivity.

While it is certainly important to overcome the barrier imposed by the large number of naïve alloreactive host T cells in order to to achieve durable marrow engraftment, memory T cells derived from responses to

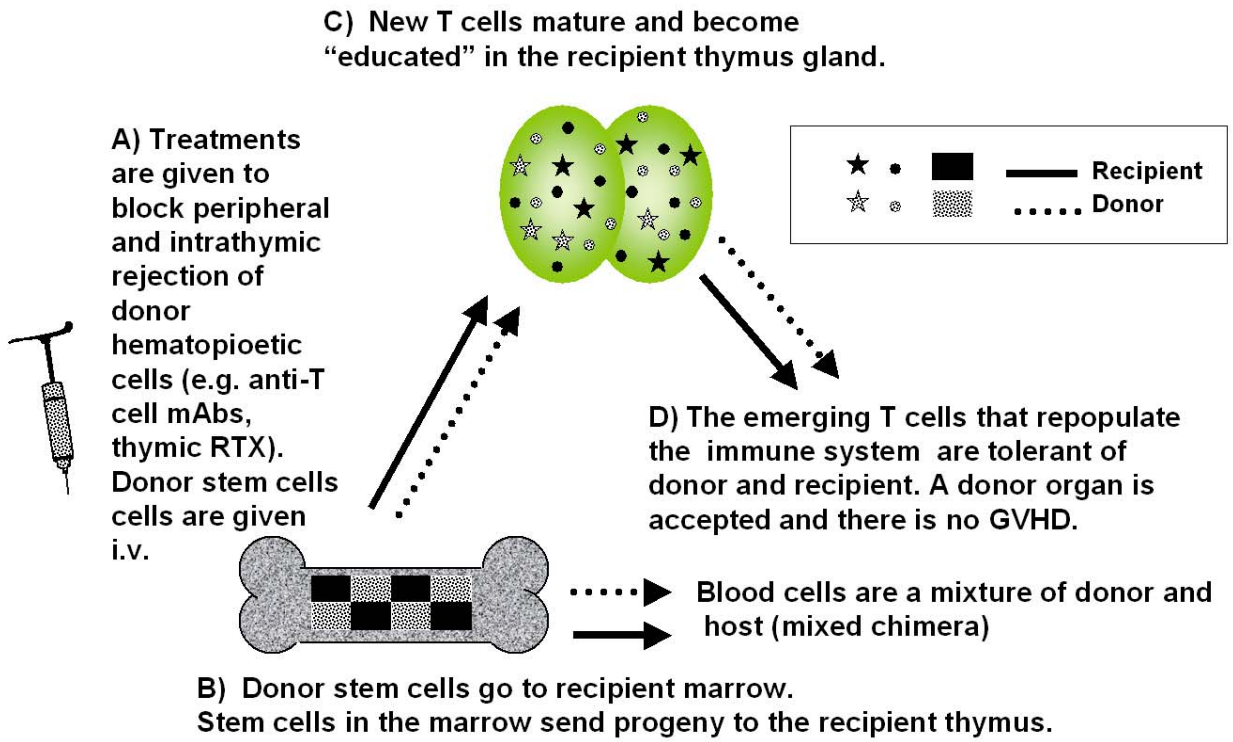

Figure 1. Schematic illustration of the basic requirements for the achievement of durable mixed chimerism and central T cell tolerance via allogeneic BMT. A. Treatments must be given to overcome both peripheral and intrathymic T cell-mediated alloresistance. Such treatments include T cell depleting mAbs, thymic irradiation and/or costimulatory blockade, etc. Donor T cells in the marrow are depleted or tolerized in the mAb-containing environment in the recipient at the time of BMT. B. If anti-donor alloreactivity is blocked and sufficient numbers of donor hematopoietic stem cells are given, they will engraft in the recipient marrow. The number of stem cells required to achieve such engraftment is decreased by the administration of myelotoxic treatments to the recipient, including low-dose TBI or busulfan. Donor and recipient stem cells co-existing in the marrow contribute to multilineage hematopoiesis, resulting in mixed chimerism, and send progeny to the host thymus on an ongoing basis throughout life. C. Some of these cells originating from the donor and host marrow differentiate into dendritic cells and other APC in the thymus, contributing to negative selection of subsequently developing thymocytes of both donor and host origin. D. The emerging T cells that repopulate the peripheral lymphoid tissues are tolerant of donor and recipient. A donor organ is accepted and there is no GVHD.

pathogens may have allogeneic cross-reactivity and present an additional barrier to marrow engraftment (51). Memory cells may be resistant to T cell depleting antibodies (52, 53) and costimulatory blockade (54), and the development of additional treatments to specifically target these cells might be needed for a minimal regimen achieving durable mixed chimerism in humans.

While recipient natural killer (NK) cells also pose a barrier to allogeneic BM engraftment (55, 56),(57) they present only minimal resistance to engraftment of pluripotent hematopoietic stem cells, and this resistance is readily overcome by giving slightly increased marrow doses (58). While *in vitro* studies suggested that NK cells of the two strains composing a mixed chimera (MC) may not be "tolerant" of each other's MHC (59), *in vivo* studies have shown clearly that NK cells develop mutual tolerance in MCs (60).

Natural antibodies can also pose a barrier to hematopoietic cell engraftment both in animal models (61, 62)(e.g. xenoantibodies) and in humans, in which recipient antibodies to donor AB blood group antigens can produce a pure red cell aplasia (63-65). However, studies in animal models have shown clearly that natural or even presensitized antibody responses to donor carbohydrate antigens such as the alphaGal determinant, which is of great importance in xenotransplantation, are also tolerized by the induction of mixed chimerism (62, 66-68).

If the immune barriers are adequately overcome, hematopoietic stem cells contained in the donor BM inoculum will "home" to the BM compartment of the recipient (Figure 1). Stem cell engraftment is facilitated by recipient irradiation or other myelosuppressive therapy, which is critical when conventional marrow doses are given (20). Even local TBI causes a systemic increase in CD45 congenic donor stem cell engraftment, probably by promoting the initial expansion of donor stem cells in irradiated sites (69). Studies in syngeneic and congenic models have shown that this requirement to create "space" can be overcome by the administration of high doses of

donor stem cells (70-74). When T cell-mediated resistance to allogeneic marrow engraftment was overcome with anti-T cell mAbs and TI or with costimulatory blockade, high doses of allogeneic marrow could also engraft without treatment of the hosts with TBI or chemotherapy (75) (76).

6. CENTRAL DELETION AS THE MAIN MECHANISM MAINTAINING TOLERANCE IN MIXED CHIMERAS

Once stem cells have engrafted, they co-exist with recipient stem cells and give rise to cells of all hematopoietic lineages for the life of the recipient. Most importantly, hematopoietic progenitor cells seed the thymus through a separately regulated process (74), giving rise to dendritic cells which mediate clonal deletion (Figure 1). In the normal thymus, self-reactive T cells are clonally deleted during their maturation through the physiologic process of negative selection. Antigens expressed on cells of hematopoietic origin within the thymus are the most effective mediators of negative selection (77-80). In MCs, hematopoietic cells from both the recipient and the donor locate to the thymus, leading to deletion of both host-reactive and donor-reactive T cells (23, 81) (82). The result is a T cell repertoire that is tolerant towards the donor and the host. Donor dendritic cells, which are potent mediators of negative selection, can be found intrathymically in MCs throughout their lives, beginning as early as 8-10 days after BMT (23, 81, 82)(T. Daskivich and M.Sykes, unpublished data). Evidence indicates that central deletion is the only significant mechanism maintaining tolerance in mixed chimeras prepared with an extensively T cell-depleting regimen. When euthymic chimeras established this way received a mAb that depleted donor antigen, tolerance was lost and non-deleted T cells bearing donor-reactive Vbeta appeared in the peripheral repertoire. If the chimeras were thymectomized before donor antigen was depleted, however, tolerance persisted despite the absence of donor antigen, indicating that peripheral antigen was not required to maintain tolerance. T cells with donor-reactive Vbeta did not appear in the repertoire, indicating that these cells had developed in the thymus of control mice after antigen depletion (83). Since persistent antigen would be required for maintenance of peripheral tolerance through anergy (84, 85), this is clearly not a significant mechanism in this model. Active suppression does not play a major role either, as chimerism and tolerance were easily broken in established chimeras by the infusion of naive host-type spleen cells or by the removal of antigen when the host thymus was left intact so that non-tolerant T cells could be subsequently generated in the thymus (83).

7. MECHANISMS OF TOLERANCE ACHIEVED WITH COSTIMULATORY BLOCKADE

Various mechanisms have been implicated in the prolongation of solid organ allograft survival using costimulatory blockade, including anergy, suppression, and deletion (reviewed in (86)). Regulatory $CD25^+$ T cells (Treg), and roles for IFN-gamma, IL-2, activation-induced cell death (AICD), as well as inhibition by calcineurin inhibitors have figured prominently in these models (48, 87-96). However, costimulatory blockade with or without BMT leads to mechanistically different forms of tolerance. The successful models that exclude BMT have generally involved less difficult graft types (e.g. hearts, MHC-matched skin) and susceptible strain combinations. Tolerance in mice receiving costimulatory blockade combined with BMT differs from that in regimens not involving BMT in several respects, including their lack of dependence on IFN-gamma (97) or IL-2 (98), and the less prominent role of regulatory cells (see below).

8. MECHANISMS OF TOLERANCE ACHIEVED WITH COSTIMULATORY BLOCKADE COMBINED WITH BMT

The tolerance achieved with BMT plus costimulatory blockade is effective in "resistant" strain combinations, and leads to the acceptance of the most immunogenic MHC-disparate grafts, including skin and small intestine (35, 99, 100). Since these protocols do not involve global depletion of peripheral T cells, additional mechanisms must explain the acceptance of donor marrow and tolerization of pre-existing recipient T cells. Donor skin grafted one day post-BMT is specifically accepted, demonstrating that the tolerance to donor antigen develops rapidly (99). It should, however, be noted that donor skin is neither required nor sufficient for tolerance induction: engraftment of donor marrow ensures the specific acceptance of donor skin grafted at any time, whereas 3rd party skin grafts are readily rejected, regardless of time of placement. This rapid induction of systemic tolerance is also evident *in vitro* (101).

We have found it useful to analyze the mechanisms of tolerance of peripheral CD4 cells and CD8 cells separately, as there are differences in the pathway to tolerization of each subset using BMT and costimulatory blockade.

8.1. Mechanisms of CD4 T cell tolerance

Depletion of recipient CD8 cells permits the reliable achievement of high levels of mixed chimerism and lasting donor-specific tolerance in mice receiving anti-CD154 on Day 0 and 3 Gy TBI (99). CTLA4Ig was required only to tolerize CD8 cells in the original protocol that excluded CD8 depletion (35). Thus, CD4 cell tolerance could be examined in isolation in CD8-depleted mice receiving low dose TBI and anti-CD154 mAb as the sole conditioning.

To determine the mechanism of the effect of anti-CD154 mAb on CD4 cells, we used $CD154^{-/-}$ and WT mice that were depleted of $CD8^+$ T cells as recipients of fully MHC-mismatched allogeneic BMT (101). Donor marrow was T cell-depleted (TCD) to exclude a role for donor T cells expressing CD40L in promoting engraftment. All $CD154^{-/-}$ mice that received only CD8-depleting mAb and 3 Gy TBI developed lasting multilineage chimerism, similar to WT control mice receiving anti-CD154 mAb plus CD8-depleting mAb and 3 Gy TBI. In contrast, wild-type mice receiving BMT and anti-CD8 mAb without anti-CD154 mAb rejected donor marrow. These results

demonstrate that intact CD40/CD154 interactions are essential for $CD4^+$ T cell-mediated rejection of allogeneic marrow. Deletion of peripheral donor superantigen-reactive CD4 T cells was observed in CD8-depleted mice that receive BMT with anti-CD154, suggesting peripheral deletion as a mechanism of tolerance (99). Similar peripheral deletion of donor superantigen-reactive CD4 cells was seen in $CD154^{-/-}$ recipients of BMT with anti-CD8 without anti-CD154 (101). Clearly, neither a signal to the activated CD4 cell through CD154 nor antibody-mediated depletion of activated, $CD154^+$ CD4 cells is required for this deletion and tolerance. These results argue against speculations made about anti-CD154-mediated T cell clearance in BMT models on the basis of studies not involving BMT and emphasize differences between mechanisms involved when costimulatory blockade is given with or without BMT (102, 103).

A TCR transgenic (Tg) model was used to examine peripheral deletion of a truly alloreactive CD4 cell population. We utilized the AND TCR Tg, which recognizes a pigeon cytochrome c peptide in the context of IE^k, is positively selected on IA^b (104, 105), and cross-reacts with an IA^s alloantigen (106, 107). To establish a physiologically relevant proportion of alloreactive (IA^s – reactive) CD4 T cells that can be tracked, $5x10^6$ C57BL/10 AND^+ $CD4^+$ splenocytes were injected i.v. into C57BL/10 mice. Five days later, these mice (referred to henceforth as B10-AND) received anti-CD8 mAb, 3 Gy TBI, anti-CD154 mAb, and BMT from either B10.A ($H2^a$; not recognized by the AND TCR) or A.SW mice ($H2^s$; IA^s recognized by the AND TCR) to induce mixed chimerism. BMT recipients developed long-term multilineage mixed chimerism. At 1 week post-BMT, the percentage of AND $CD4^+$ T cells in the blood was significantly decreased in mice that received A.SW BMC compared to those receiving B10.A BMC or no BMT. AND cells were undetectable in recipients of A.SW BMC by 4 weeks post-BMT. In contrast, B10-AND mice that received non-ligand-bearing B10.A BMT had similar percentages of AND CD4 T cells as non-BMT control mice. These results demonstrate, in an alloreactive Tg system, that peripheral deletion of donor-reactive CD4 cells occurs over about 4 weeks following BMT (97).

To examine the mechanisms of peripheral CD4 deletion in recipients of this regimen, Tg B6 recipients whose T cells constitutively express Bcl-x_L, which blocks the intrinsic, or mitochondrial pathway of cell death, were used. In contrast to wild-type mice, these animals did not demonstrate early deletion of donor-reactive CD4 T cells and failed to develop long-term mixed chimerism or tolerance (108). Notably, when pathways that play a role in activation-induced cell death (AICD) were blocked (through either Fas deficiency or a 2-week course of treatment with the calcineurin inhibitor CyA), the induction of chimerism and CD4 cell tolerance were not impaired (108, 109). Tolerance develops normally in IFN-gamma-deficient mice (97), also arguing against a role for AICD, in which IFN-gamma plays a critical role (110). Together, our results demonstrate that simply blocking CD40 stimulation in combination with allogeneic BMT is sufficient to completely overcome CD4 cell-mediated anti-donor alloreactivity and induce peripheral deletional tolerance.

Prior to deletion, donor-specific CD4 T cells demonstrate specific MLR non-responsiveness to donor antigens within one week post-BMT with anti-CD154 (101). ELISPOT assays performed following stimulation with donor, host and third party alloantigens revealed rapid, donor-specific tolerance of IL-2, IFN-γ, IL-4, and IL-5-producing cells (97, 108). There was no evidence for increased Th2 cytokine production in response to the donor in tolerant mice (97). The lack of any anti-donor IL-2 response at early time points is not consistent with a role for IL-2-dependent Treg (111-115).

We sought evidence for a role for Tregs in tolerizing peripheral CD4 cells in our model. The data suggest that Tregs play a minor, if any, role in the establishment of mixed chimerism or in the maintenance of tolerance in this model. In stable MCs prepared with anti-CD154 with anti-CD8 or with CTLA4Ig, the infusion of naïve recipient-type splenocytes at 6 or 17 weeks post-BMT (in numbers that are insufficient to break tolerance in models where regulatory cells play a role (116-118)) led to rejection of both the donor bone marrow and skin allografts, arguing against a strong suppressive mechanism (97). Also inconsistent with strong regulatory mechanisms in induction or maintenance of tolerance, MCs prepared with this regimen did not mediate "linked suppression" to either MHC class I or minor antigens co-expressed with donor antigens on skin grafted at the time of BMT or several months later (97). Moreover, when various numbers of tolerant cells obtained 2 weeks post-transplant were transferred into syngeneic, immunodeficient recipients, donor skin grafted to secondary recipients was accepted for at least 60 days, then rejected (97). This rejection coincided with an inability to detect donor cells in the secondary recipients. These chimeric cells may have been destroyed by recipient NK cells. Thus, in the absence of continued chimerism, tolerance does not persist indefinitely, consistent with anergy prior to the observed deletion by 4 weeks. Importantly, co-transfer of 2-week tolerant CD4 cells did not significantly delay rejection of donor skin grafts compared to naïve CD4 cells alone, even when the ratio of tolerant to naïve CD4 T cells given was 3:1 (97) or as high as 25:1 (119). Thus, powerful suppressive mechanisms are not demonstrable in this model, either early or late. A lack of evidence for regulation was also described in mixed chimeras produced with anti-CD154, CTLA4Ig, and busulfan following adoptive transfer of lymphocytes from chimeras several months post-BMT (36).

Given that intrathymic deletion of donor-reactive cells is quite complete (35, 86), the lack of detectable suppression in long-term MCs might not be surprising. However, there is no robust suppressive mechanism maintaining tolerance even before complete deletion of peripheral donor-reactive CD4 cells is achieved by 4 weeks post-BMT (97). In fact, regulatory mechanisms require several months to mature following solid organ transplantation (120), so the ability of BMT with anti-CD154 to delete donor-reactive cells relatively quickly might preclude the maturation of regulatory responses. Our

data are consistent with the interpretation that the presence of T cells with a particular specificity is necessary for the activation and expansion of regulatory cells with similar specificity. If T cells recognizing a given set of alloantigens are deleted, Treg may not have the opportunity to be activated and expand.

Thus, we have established that deletion of alloreactive CD4 T cells occurs over the first 4 weeks post-BMT. Since tolerance is complete (by ELISPOT, MLR, and skin graft studies) by 1 week post-transplant, these results implicate anergy and/or other mechanisms in the initial tolerization of these cells. Exogenous IL-2 did not overcome the early MLR tolerance observed at 1 or 2 weeks post-BMT (97), consistent with a "non-classical" type of anergy described in other models (121, 122).

8.2. Mechanisms of peripheral CD8 T cell tolerance

CD4-independent $CD8^+$ T cells can reject donor marrow in mice receiving 3 Gy TBI and one injection of anti-CD154 as the sole conditioning (99). We identified two ways of tolerizing these CD8 cells without adding any additional host conditioning. One involved the use of donor-specific transfusion (DST). Studies not involving BMT have shown that donor-specific transfusion (DST) in combination with anti-CD154 mAb leads to deletional tolerance of pre-existing peripheral CD8 cells (47, 123). In those studies, the subsequent export from the thymus of non-tolerant T cells led to eventual rejection of donor skin grafts (124). We hypothesized that the combination of BMT, anti-CD154, DST and 3 Gy TBI would lead to permanent chimerism and donor-specific tolerance, since cells arising in the thymus subsequent to the BMT would be intrathymically deleted by donor APC. DST (donor splenocytes) given 7 days prior to BMT led to reliable tolerization of CD8 cells in addition to CD4 cells in mice receiving anti-CD154, BMT and 3 Gy TBI, permitting achievement of high levels of lasting, multilineage mixed chimerism (125). Administration of 3 Gy TBI and DST alone, without anti-CD154 mAb, did not allow induction of mixed chimerism. The mixed chimeras (MCs) prepared with the DST regimen accepted donor B10.A skin grafts indefinitely (>200 days), whereas third party skin was rejected by Day 40. The rejection of third-party skin demonstrates the importance of donor bone marrow in inducing tolerance, and shows that antigen provided by skin grafts is insufficient to induce tolerance in mice receiving DST, anti-CD154 and 3 Gy TBI.

We also found that moving the 3 Gy TBI dose to Day -1 or -2 instead of Day 0 permitted quite reliable tolerization of peripheral CD8 cells (in addition to CD4 cells) in recipients of anti-CD154 without DST. Lasting, high levels of donor chimerism, specific skin graft tolerance and MLR and CML tolerance were achieved with these regimens (125). The mechanisms of tolerance in mice receiving BMT with anti-CD154 and 3 Gy TBI on Day -1 appear to be similar to those in animals receiving BMT with anti-CD154 and Day 0 TBI, with DST to tolerize peripheral CD8 cells. In contrast to tolerance induction with DST plus skin grafting (without BMT) in thymectomized mice (48), IFN-gamma was not required for tolerance in the models involving BMT (Y.Takeuchi and M.Sykes, unpublished data)(98). CTLA4 appears to be critical in achieving tolerance in the BMT (98)(and Y.Takeuchi and M.Sykes, unpublished data) and non-BMT (126) models.

Depletion of CD4 cells abrogates the achievement of CD8 cell tolerance and hence mixed chimerism with both the DST and Day -1 TBI BMT regimens (98, 125). CD4 cells were only required for a short period, as depletion of this cell population before Day 10 post-BMT led to a significant reduction in the incidence of mixed chimerism, whereas CD4 depletion on Day 10 or later had little or no effect (98).

Since "natural" CD4 Treg have been shown to be mainly $CD25^+$ (127), we evaluated the effect of depleting anti-CD25 mAb on chimerism and tolerance in mice receiving BMT following 3 Gy TBI on Day -1 and anti-CD154 mAb on Day 0. Depleting anti-CD25 mAb had no effect on the induction of mixed chimerism in this model, despite successful depletion of $CD4^+CD25^+$ cells for >2 weeks (98). Likewise, neutralizing anti-IL-2 mAb failed to block chimerism and tolerance induction (98). In contrast, these same treatments prevented chimerism and tolerance induction in the CTLA4Ig/anti-CD40L /3 Gy TBI regimen (128), suggesting different tolerance mechanisms in the two models.

To investigate the fate of donor-reactive CD8 T cells in our model, we constructed B6 recipient mice with $2C^+$ T cell chimerism using 3 Gy TBI and BMT from B6 2C TCR Tg mice. The 2C TCR is from a B6 CD8 cell clone recognizing the L^d alloantigen (129), and we mixed these cells into syngeneic wild-type mice to provide a trackable T cell population with known allospecificity. Eight weeks later, when T cell development from the Tg BMC had reconstituted approximately 7% of the peripheral CD8 T cell pool, these 2C/B6 mice received 3 Gy TBI on Day -1 and anti-CD154 and BMT on Day 0 followed by BMT from L^d ligand-bearing B10.A mice or from control A.SW donors, which do not express L^d. In recipients of L^{d+} B10.A BMT, $2C^+CD8^+$ cells disappeared almost completely from PBL within one week post-BMT. In contrast, percentages of $2C^+CD8^+$ cells were constant in PBL of 2C/B6 mice that received irrelevant A.SW BMT. Thus, donor-reactive CD8 cells in the periphery are specifically and rapidly deleted following BMT with Day-1 TBI and anti-CD154 mAb (98). The deletion of donor-reactive CD8 cells is much more rapid than that of CD4 cells recognizing donor antigens, which required about 4 weeks (97).

We examined the effect of CD4 depletion on deletion of peripheral donor-reactive CD8 cells. Surprisingly, treatment with anti-CD4 mAb on Day -1 did not markedly impair the initial deletion of 2C CD8 cells. However, after the first week, the proportion of 2C Tg CD8 cells began to increase in CD4-depleted mice, while those in control animals continued to decline (98). By 5 and 7 weeks post-BMT, when 2C cells were undectable in MCs, the CD4-depleted animals, which again failed to achieve mixed chimerism, showed marked expansion of donor-

reactive 2C CD8 cells (98). Thus, CD4 cells play a role in permitting the complete deletion of donor-reactive CD8 cells, but their role is complex and may not be direct.

Thus, the CD4 cells that contribute to peripheral CD8 cell tolerance in this model act early, are not $CD25^+$, and are not dependent on IL-2, in marked contrast to "natural" regulatory cells (127). We hypothesize that these are "ordinary" alloreactive CD4 cells whose activity becomes suppressive for CD8 cells when the CD4 cell CD154-APC CD40 interaction is disrupted. The APC may be an important intermediary in this tolerization of CD8 cells. Typical "natural" regulatory cells may play a greater role in chimerism models in which peripheral deletion of donor-reactive T cells does not occur completely and/or quickly. It is tempting to speculate that incomplete peripheral deletion of donor-reactive CD8 cells may explain the implied role for $CD25^+$ regulatory cells in animals receiving 3 Gy TBI, anti-CD154 mAb and CTLA4Ig (128), which contrasts to the model discussed above, in which rapid peripheral deletion of donor-reactive CD8 cells occurs (98).

8.3. The relationship between deletional and "regulatory" tolerance

In the models involving low-dose TBI, high levels of both donor and host hematopoiesis exist in the mixed chimeras, making it likely that both donor and host stem cells contribute to hematopoiesis at all times, ensuring the continuous contribution of donor and host cells to intrathymic APC that induce deletion of newly developing T cells. Some models, however, achieve only low levels of donor chimerism, such as those involving high doses of marrow given without TBI (76, 119). In such models, regulatory cells appear to play a significant role in maintaining long-term tolerance, and the deletion of donor-reactive T cells appears to be less complete (119, 130). These results are consistent with the interpretation that regulatory cells suppressing an alloresponse are only activated when alloreactive T cells are present, which is not the case when complete deletional tolerance exists. A threshold level of donor hematopoietic stem cell engraftment may be required to ensure the constant contribution of donor-derived thymic dendritic cells to thymic negative selection. This hypothesis is suggested by data showing that relatively few hematopoietic stem cells contribute to hematopoiesis at any given time (131). When only low levels of donor chimerism are present, there will be times when central deletion of donor-reactive T cells does not occur because donor stem cells are not contributing sufficiently to the thymic dendritic cell pool at that particular time. The emergence of small numbers of donor-reactive T cells from the thymus may then promote peripheral expansion of regulatory cells specific for the donor. Regulatory mechanisms may also be important in large animal (132) and clinical tolerance protocols involving BMT for renal allograft tolerance (133)(Kawai et al, manuscript in preparation), in which long-term donor hematopoiesis is not maintained.

9. ABSENCE OF GVHD IN MIXED CHIMERAS

GVHD does not occur in the rodent mixed chimerism models discussed above, nor in a related primate model (132), despite the use of unseparated donor bone marrow cells. This is most readily explained by the continued presence of the T cell-depleting antibodies (23, 81) or costimulatory blockers in the recipients' serum at the time of BMT, which deplete or inactivate the much smaller number of donor T cells by the same mechanisms that affect the entire recipient T cell pool. For clinical application of the mixed chimerism approach, it will be important to reliably avoid GVHD with *ex vivo* or *in vivo* donor T cell depletion or tolerization.

10. CONCLUSIONS

Mixed chimerism induces a reliable and robust form of tolerance. If regimens for achieving mixed chimerism in patients could be developed that are associated with acceptably low toxicity, the problems of chronic rejection (38, 134) and the complications of chronic immunosuppressive therapy would be completely eliminated. The recent extension of approaches from rodents to large animal models (described elsewhere in this issue) and humans (135-137) is highly encouraging. Further progress in applying the most minimally toxic approaches involving costimulatory blockade from rodents to humans will require an in-depth understanding of the mechanisms of tolerance achieved with this approach in rodents. Since the most promising agent, anti-CD154mAb, is associated with significant thromboembolic complications in large animals and humans (138-142), it will be important to identify the molecular pathways involved in its tolerogenic capacity in conjunction with BMT so that other reagents can be used to replace anti-CD154. Additionally, methods of effectively tolerizing memory T cells, which are relevant to the presensitized recipient and heterologous immunity that poses additional barriers (51), must be developed. Understanding the mechanisms of tolerance of both naïve and memory T cells will ultimately facilitate the development of less toxic regimens using reagents that target the critical pathways elucidated. The development of such conditioning regimens will lead to new hope for patients in need of organ transplants and those with hematologic disorders such as hemoglobinopathies.

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12. REFERENCES

1. R. D. Owen: Immunogenetic consequences of vascular anastomoses between bovine twins. *Science*, 102, 400-401 (1945)
2. D. Anderson, R. E. Billingham, G. H. Lampkin, and P. B. Medawar: The use of skin grafting to distinguish between monozygotic and dizygotic twins in cattle. *Heredity*, 5, 379-397 (1951)
3. R. E. Billingham, G. H. Lampkin, P. B. Medawar, and H. Ll. Williams: Tolerance to homografts, twin diagnosis, and

the freemartin condition in cattle. *Heredity*, 6, 201-212 (1952)
4. R. E. Billingham, L. Brent, and P. B. Medawar: "Actively acquired tolerance" of foreign cells. *Nature*, 172, 603-606 (1953)
5. J. M. Main and R. T. Prehn: Succesful skin homografts after the administration of high dosage X radiation and homologous bone marrow. *Journal of the National Cancer Institute*, 15, 1023 (1955)
6. R. A. Clift and R. Storb: Histoincompatible bone marrow transplants in humans. *Annual Reviews of Immunology*, 5, 43-64 (1987)
7. C. Anasetti, D. Amos, P. G. Beatty, F. R. Appelbaum, W. Bensinger, C. D. Buckner, R. Clift, C. Doney, P. J. Martin, E. Mickelson, B. Nisperos, J. O'Quigley, R. Ramberg, J. E. Sanders, P. Stewart, R. Storb, K. M. Sullivan, R. P. Witherspoon, E. D. Thomas, and J. A. Hansen: Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *New England Journal of Medicine*, 320, 197-204 (1989)
8. N. A. Kernan, N. Flomenberg, B. Dupont, and R. J. O'Reilly: Graft rejection in recipients of T-cell-depleted HLA-nonidentical marrow transplants for leukemia. *Transplantation*, 43, 842-847 (1987)
9. Y. Sharabi and D. H. Sachs: Mixed chimerism and permanent specific transplantation tolerance induced by a non-lethal preparative regimen. *Journal of Experimental Medicine*, 169, 493-502 (1989)
10. A. Singer, K. S. Hathcock, and R. J. Hodes: Self recognition in allogeneic radiation chimeras. A radiation resistant host element dictates the self specificity and immune response gene phenotype of T-helper cells. *Journal of Experimental Medicine*, 153, 1286-1301 (1981)
11. R. M. Zinkernagel, G. N. Callahan, A. Althage, S. Cooper, P. A. Klein, and J. Klein: On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *Journal of Experimental Medicine*, 147, 882-896 (1978)
12. R. M. Zinkernagel, A. Althage, G. Callahan, and R. M. Welsh Jr.: On the immunocompetence of H-2 incompatible irradiation bone marrow chimeras. *Journal of Immunology*, 124, 2356-2365 (1980)
13. S. T. Ildstad, S. M. Wren, J. A. Bluestone, S. A. Barbieri, and D. H. Sachs: Characterization of mixed allogeneic chimeras. Immunocompetence, in vitro reactivity, and genetic specificity of tolerance. *Journal of Experimental Medicine*, 162, 231-244 (1985)
14. E. Ruedi, M. Sykes, S. T. Ildstad, C. H. Chester, A. Althage, H. Hengartner, D. H. Sachs, and R. M. Zinkernagel: Antiviral T cell competence and restriction specificty of mixed allogeneic (P1+P2-->P1) irradiation chimeras. *Cellular Immunology*, 121, 185-195 (1989)
15. S. Slavin, S. Strober, Z. Fuks, and H. S. Kaplan: Induction of specific tissue transplantation tolerance using fractionated total lymphoid irradiation in adult mice: long-term survival of allogeneic bone marrow and skin grafts. *Journal of Experimental Medicine*, 146, 34 (1977)
16. S. Slavin, Z. Fuks, H. S. Kaplan, and S. Strober: Transplantation of allogeneic bone marrow without graft-versus-host disease using total lymphoid irradiation. *Journal of Experimental Medicine*, 147, 963-972 (1978)
17. S. Slavin, B. Reitz, C. P. Bieber, H. S. Kaplan, and S. Strober: Transplantation tolerance in adult rats using total lymphoid irradiation: permanent survival of skin, heart, and marrow allografts. *Journal of Experimental Medicine*, 147, 700-707 (1978)
18. D. A. Vallera, C. C. B. Soderling, G. J. Carlson, and J. H. Kersey: Bone marrow transplantation across major histocompatibility barriers in mice. II. T cell requirement for engraftment in toltal lymphoid irradiation-conditioned recipients. *Transplantation*, 33, 243-248 (1982)
19. S. T. Ildstad and D. H. Sachs: Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature*, 307(5947), 168-170 (1984)
20. Y. Tomita, D. H. Sachs, and M. Sykes: Myelosuppressive conditioning is required to achieve engraftment of pluripotent stem cells contained in moderate doses of syngeneic bone marrow. *Blood*, 83, 939-948 (1994)
21. Y. Sharabi, V. S. Abraham, M. Sykes, and D. H. Sachs: Mixed allogeneic chimeras prepared by a non-myeloablative regimen: requirement for chimerism to maintain tolerance. *Bone Marrow Transplantation*, 9, 191-197 (1992)
22. Y. Tomita, D. H. Sachs, A. Khan, and M. Sykes: Additional mAb injections can replace thymic irradiation to allow induction of mixed chimerism and tolerance in mice receiving bone marrow transplantation after conditioning with anti-T cell mAbs and 3 Gy whole body irradiation. *Transplantation*, 61, 469-477 (1996)
23. Y. Tomita, A. Khan, and M. Sykes: Mechanism by which additional monoclonal antibody injections overcome the requirement for thymic irradiation to achieve mixed chimerism in mice receiving bone marrow transplantation after conditioning with anti-T cell mAbs and 3 Gy whole body irradiation. *Transplantation*, 61, 477-485 (1996)
24. T. Wekerle, M. H. Sayegh, H. Ito, J. Hill, A. Chandraker, D. A. Pearson, K. G. Swenson, G. Zhao, and M. Sykes: Anti-CD154 or CTLA4Ig obviates the need for thymic irradiation in a non-myeloablative conditioning regimen for the induction of mixed hematopoietic chimerism and tolerance. *Transplantation*, 68, 1348-1355 (1999)
25. H. Mayumi and R. A. Good: Long-lasting skin allograft tolerance in adult mice induced across fully allogeneic (multimajor H-2 plus multiminor histocompatibility) antigen barriers by a tolerance-inducing method using cyclophosphamide. *Journal of Experimental Medicine*, 169, 213-238 (1989)
26. K. Nomoto, K. Yung-Yun, K. Omoto, M. Umesue, Y. Murakami, and G. Matsuzaki: Tolerance induction in a fully allogeneic combination using anti-T cell receptor-ab monoclonal antibody, low dose irradiation, and donor bone marrow transfusion. *Transplantation*, 59, 395-401 (1995)
27. Y. L. Colson, S. M. Wren, M. J. Schuchert, K. D. Patrene, P. C. Johnson, S. S. Boggs, and S. T. Ildstad: A nonlethal conditioning approach to achieve durable multilineage mixed chimerism and tolerance across major, minor, and hematopoietic histocompatibility barriers. *Journal of Immunology*, 155, 4179-4188 (1995)
28. A. De Vries-van der Zwan, A. C. Besseling, F. Kievits, E. Van Twuyver, and L. P. De Waal: Anti-CD3 treatment

facilitates engraftment of full H-2-disparate donor bone marrow cells and subsequent skin allograft tolerance. Transplantation, 58, 610-617 (1994)
29. A. De Vries-van der Zwan, A. C. Besseling, L. P. De Waal, and C. J. P. Boog: Specific tolerance induction and transplantation: a single-day protocol. Blood, 89, 2596-2601 (1997)
30. U. Koch and R. Korngold: A synthetic CD4-CDR3 peptide analog enhances bone marrow engraftment across major histocompatibility barriers. Blood, 89, 2880-2890 (1997)
31. A. De Vries-van der Zwan, M. A. van der Pol, A. C. Besseling, L. P. De Waal, and C. J. P. Boog: Haematopoietic stem cells can induce specific skin graft acceptance across a full MHC barrier. Bone Marrow Transplantation, 22, 91-98 (1998)
32. C. L. Mackall, T. A. Fleisher, M. R. Brown, M. P. Andrich, C. C. Chen, I. M. Feuerstein, M. E. Horowitz, I. T. Magrath, A. T. Shad, S. M. Steinberg, L. H. Wexler, and R. E. Gress: Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. *New England Journal of Medicine*, 332, 143-149 (1995)
33. B. F. Haynes, M. L. Markert, G. D. Sempowski, D. D. Patel, and L. P. Hale: The role of the thymus in immune reconstiutution in aging, bone marrow transplantion, and HIV-1 infection. *Annual Reviews of Immunology*, 18, 529-560 (2000)
34. F. T. Hakim, S. A. Memon, R. Cepeda, E. C. Jones, C. K. Chow, C. Kasten-Sportes, J. Odom, B. A. Vance, B. L. Christensen, C. L. Mackall, and R. E. Gress: Age-dependent incidence, time course, and consequences of thymic renewal in adults. *J Clin Invest*, 115, 930-939 (2005)
35. T. Wekerle, M. H. Sayegh, J. Hill, Y. Zhao, A. Chandraker, K. G. Swenson, G. Zhao, and M. Sykes: Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. *Journal of Experimental Medicine*, 187, 2037-2044 (1998)
36. A. B. Adams, M. M. Durham, L. Kean, N. Shirasugi, J. Ha, M. A. Williams, P. A. Rees, M. C. Cheung, S. Mittelstaedt, A. W. Bingaman, D. R. Archer, T. C. Pearson, E. K. Waller, and C. P. Larsen: Costimulation blockade, busulfan, and bone marrow promote titratable macrochimerism, induce transplantation tolerance, and correct genetic hemoglobinopathies with minimal myelosuppression. *Journal of Immunology*, 167, 1103-1111 (2001)
37. M. M. Durham, A. W. Bingaman, A. B. Adams, J. Ha, S. Y. Waitze, T. C. Pearson, and C. P. Larsen: Cutting edge: administration of anti-CD40 ligand and donor bone marrow leads to hemopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. *Journal of Immunology*, 165, 1-4 (2000)
38. N. Shirasugi, A. B. Adams, M. M. Durham, A. E. Lukacher, H. Xu, P. Rees, S. R. Cowan, M. A. Williams, T. C. Pearson, and C. P. Larsen: Prevention of chronic rejection in murine cardiac allografts: a comparison of chimerism- and nonchimerism-inducing costimulation blockade-based tolerance induction regimens. *J Immunol*, 169, 2677-2684 (2002)
39. P. A. Taylor, C. J. Lees, J. M. Wilson, M. J. Erhardt, M. T. Campbell, R. J. Noelle, and B. R. Blazar: Combined effects of calcineurin inhibitors or sirolimus with anti-CD40L mAb on alloengraftment under nonmyeloablative conditions. *Blood*, 100, 3400-3407 (2002)
40. P. A. Taylor, C. J. Lees, H. Waldmann, R. J. Noelle, and B. R. Blazar: Requirements for the promotion of allogeneic engraftment by anti-CD154 (anti-CD40L) monoclonal antibody under nonmyeloablative conditions. *Blood*, 98, 467-474 (2001)
41. D. A. Hale, R. Gottschalk, A. Umemura, T. Maki, and A. P. Monaco: Establishment of stable multilineage hematopoietic chimerism and donor-specific tolerance without irradiation. Transplantation, 69, 1242-1251 (2000)
42. J. F. Lambert, G. A. Colvin, S. Zhong, H. Wang, L. D'Hondt, M. Abedi, A. E. Frimberger, F. M. Stewart, and P. J. Quesenberry: H2-mismatched transplantation with repetitive cell infusions and CD40 ligand antibody infusions without myeloablation. *Br.J Haematol.*, 119, 155-163 (2002)
43. L. A. Turka, P. S. Linsley, H. Lin, W. Brady, J. M. Leiden, R. Wei, M. L. Gibson, X. Zhen, S. Myrdal, D. Gordon, T. Bailey, S. F. Bolling, and C. B. Thompson: T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. *Proceedings of the National Academy of Science USA*, 89, 11102 (1992)
44. M. H. Sayegh, E. Akalin, W. W. Hancock, M. E. Russell, C. B. Carpenter, P. S. Linsley, and L. A. Turka: CD28-B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. *Journal of Experimental Medicine*, 181, 1869-1874 (1995)
45. D. C. Parker, D. L. Greiner, N. E. Phillips, M. C. Appel, A. W. Steele, F. H. Durie, R. J. Noelle, J. P. Mordes, and A. A. Rossini: Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proceedings of the National Academy of Science USA*, 92, 9560-9564 (1995)
46. M. H. Sayegh, X.-G. Zheng, C. Magee, W. W. Hancock, and L. A. Turka: Donor antigen is necessary for the prevention of chronic rejection in CTLA4Ig-treated murine cardiac allograft recipients. *Transplantation*, 64, 1646-1650 (1997)
47. T. G. Markees, N. E. Phillips, R. J. Noelle, L. D. Shultz, J. P. Mordes, D. L. Greiner, and A. A. Rossini: Prolonged survival of mouse skin allografts in recipients treated with donor splenocytes and antibody to CD40 ligand. *Transplantation*, 64, 329-335 (1997)
48. T. G. Markees, N. E. Phillips, E. J. Gordon, R. J. Noelle, L. D. Shultz, J. P. Mordes, D. L. Greiner, and A. A. Rossini: Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires $CD4^+$ T cells, Interferon-g, and CTLA4. *Journal of Clinical Investigation*, 101, 2446-2455 (1998)
49. T. A. Judge, Z. Wu, X.-G. Zheng, A. H. Sharpe, M. H. Sayegh, and L. A. Turka: The role of CD80, CD86 and CTLA4 in alloimmune responses and the induction of long-term allograft survival. *Journal of Immunology*, 162, 1947-1951 (1999)
50. B. Nikolic, A. Khan, and M. Sykes: Induction of tolerance by mixed chimerism with nonmyeloblative host conditioning: the importance of overcoming intrathymic alloresistance. *Biol.Blood Marrow Transplant.*, 7, 144-153 (2001)

51. A. B. Adams, M. A. Williams, T. R. Jones, N. Shirasugi, M. M. Durham, S. M. Kaech, E. J. Wherry, T. Onami, J. G. Lanier, K. E. Kokko, T. C. Pearson, R. Ahmed, and C. P. Larsen: Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin.Invest*, 111, 1887-1895 (2003)
52. J. H. Chace, J. S. Cowdery, and E. H. Field: Effect of anti-CD4 on CD4 subsets. I. Anti-CD4 preferentially deletes resting, naive CD4 cells and spares activated CD4 cells. *Journal of Immunology*, 152, 405-412 (1994)
53. J. P. Pearl, J. Parris, D. A. Hale, S. C. Hoffmann, W. B. Bernstein, K. L. McCoy, S. J. Swanson, R. B. Mannon, M. Roederer, and A. D. Kirk: Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. *Am J Transplant*, 5, 465-474 (2005)
54. Y. Chen, P. S. Heeger, and A. Valujskikh: In vivo helper functions of alloreactive memory CD4+ T cells remain intact despite donor-specific transfusion and anti-CD40 ligand therapy. *J Immunol*, 172, 5456-5466 (2004)
55. R. Kiessling, P. S. Hochman, O. Haller, G. M. Shearer, H. Wigzell, and G. Cudkowicz: Evidence for a similar or common mechanism for natural killer activity and resistance to hemopoietic grafts. *Eur.J.Immunol.*, 7, 655-663 (1977)
56. W. J. Murphy, V. Kumar, and M. Bennett: Rejection of bone marrow allografts by mice with severe combined immune deficiency (scid). Evidence that natural killer cells can mediate the specificity of marrow graft rejection. *Journal of Experimental Medicine*, 165, 1212-1217 (1987)
57. L. S. Kean, K. Hamby, B. Koehn, E. Lee, S. Coley, L. Stempora, A. B. Adams, E. Heiss, T. C. Pearson, and C. P. Larsen: NK cells mediate costimulation blockade-resistant rejection of allogeneic stem cells during nonmyeloablative transplantation. *Am J Transplant*, 6, 292-304 (2006)
58. L. A. Lee, J. J. Sergio, and M. Sykes: Natural killer cells weakly resist engraftment of allogeneic long-term multilineage-repopulating hematopoietic stem cells. *Transplantation*, 61, 125-132 (1996)
59. J. O. Manilay, G. L. Waneck, and M. Sykes: Altered expression of Ly-49 receptors on natural killer cells developing in mixed allogeneic bone marrow chimeras . *Int.Immunol.*, 10, 1943-1955 (1998)
60. Y. Zhao, H. Ohdan, J. O. Manilay, and M. Sykes: NK cell tolerance in mixed allogeneic chimeras. *Journal of Immunology*, 170, 5398-5405 (2003)
61. I. Aksentijevich, D. H. Sachs, and M. Sykes: Natural antibodies can inhibit bone marrow engraftment in the rat-->mouse species combination. *Journal of Immunology*, 147, 4140-4146 (1991)
62. H. Ohdan, K. G. Swenson, H. Kitamura, Y.-G. Yang, and M. Sykes: Tolerization of Gala1,3Gal-reactive B cells in presensitized a1,3-galactosyltransferase-deficient mice by nonmyeloablative induction of mixed chimerism. *Xenotransplantation*, 8, 227-238 (2002)
63. R. J. Benjamin, J. M. Connors, S. McGurk, W. H. Churchill, and J. H. Antin: Prolonged erythroid aplasia after major ABO-mismatched transplantation for chronic myelogenous leukemia. *Biol.Blood Marrow Transplant.*, 4, 151-156 (1998)
64. J. P. Gmür, J. Burger, A. Schaffner, K. Neftel, O. Oelz, D. Frey, and M. Metaxas: Pure red cell aplasia of long duration complicating major ABO-incompatible bone marrow transplantation. *Blood*, 75, 290-295 (1990)
65. J. D. Seebach, G. Stussi, J. R. Passweg, F. R. Loberiza, Jr., J. L. Gajewski, A. Keating, M. Goerner, P. A. Rowlings, P. Tiberghien, G. J. Elfenbein, R. P. Gale, J. J. Van Rood, V. Reddy, E. Gluckman, B. J. Bolwell, T. R. Klumpp, M. M. Horowitz, O. Ringden, and A. J. Barrett: ABO Blood Group Barrier in Allogeneic Bone Marrow Transplantation Revisited. *Biol Blood Marrow Transplant*, 11, 1006-1013 (2005)
66. I. Aksentijevich, D. H. Sachs, Y. Sharabi, T. M. I. Sundt, and M. Sykes: Humoral tolerance in mixed xenogeneic chimeras prepared by a non-myeloablative conditioning regimen. *Transplant.Proc.*, 23, 880-882 (1991)
67. H. Ohdan, Y.-G. Yang, A. Shimizu, K. G. Swenson, and M. Sykes: Mixed bone marrow chimerism induced without lethal conditioning prevents T cell and anti-Gala1,3Gal antibody-mediated heart graft rejection. *Journal of Clinical Investigation*, 104, 281-290 (1999)
68. H. Ohdan, Y.-G. Yang, K. G. Swenson, H. Kitamura, and M. Sykes: T cell and B cell tolerance to gala1,3gal-expressing heart xenografts is achieved in a1,3-galactosyltransferase-deficient mice by non-myeloablative induction of mixed chimerism. *Transplantation*, 71, 1532-1542 (2001)
69. H. Ito, Y. Takeuchi, J. Shaffer, and M. Sykes: Local irradiation enhances congenic donor pluripotent hematopoietic stem cell engraftment similarly in irradiated and non-irradiated sites. *Blood*, 103, 1949-1954 (2003)
70. F. M. Stewart, R. B. Crittenden, P. A. Lowry, S. Pearson-White, and P. J. Quesenberry: Long-term engraftment of normal and post-5-fluorouracil murine marrow into normal nonmyeloablated mice. *Blood*, 81, 2566-2571 (1993)
71. H. S. Ramshaw, S. S. Rao, R. B. Crittenden, S. O. Peters, H.-U. Weier, and P. J. Quesenberry: Engraftment of bone marrow cells into normal unprepared hosts: effects of 5-fluorouracil and cell cycle status. *Blood*, 86, 924-929 (1995)
72. H. S. Ramshaw, R. B. Crittenden, M. Dooner, S. O. Peters, S. S. Rao, and P. J. Quesenberry: High levels of engraftment with a single infusion of bone marrow cells into normal unprepared mice. *Biol.Blood Marrow Transplant.*, 1, 74-80 (1995)
73. S. S. Rao, S. O. Peters, R. B. Crittenden, F. M. Stewart, H. S. Ramshaw, and P. J. Quesenberry: Stem cell transplantation in the normal nonmyeloablated host: Relationship between cell dose, schedule, and engraftment. *Experimental Hematology*, 25, 114-121 (1997)
74. M. Sykes, G. L. Szot, K. Swenson, D. A. Pearson, and T. Wekerle: Separate regulation of peripheral hematopoietic and thymic engraftment. *Experimental Hematology*, 26, 457-465 (1998)
75. M. Sykes, G. L. Szot, K. Swenson, and D. A. Pearson: Induction of high levels of allogeneic hematopoietic reconstitution and donor-specific tolerance without myelosuppressive conditioning. *Nature Medicine*, 3, 783-787 (1997)
76. T. Wekerle, J. Kurtz, H. Ito, J. V. Ronquillo, V. Dong, G. Zhao, J. Shaffer, M. H. Sayegh, and M. Sykes: Allogeneic bone marrow translantation with costimulatory

blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nature Medicine*, 6, 464-469 (2000)
77. J. W. Kappler, N. Roehm, and P. Marrack: T cell tolerance by clonal elimination in the thymus. *Cell*, 49, 273-280 (1987)
78. F. Ramsdell, T. Lantz, and B. J. Fowlkes: A nondeletional mechanism of thymic self tolerance. *Science*, 246, 1038-1041 (1989)
79. T. Brocker, M. Riedinger, and K. Karjalainen: Targeted expression of major histocompatibiity complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. *Journal of Experimental Medicine*, 185, 541-550 (1997)
80. M. Inaba, K. Inaba, M. Hosono, T. Kumamoto, T. Ishida, S. Muramatsu, T. Masuda, and S. Ikehara: Distinct mechanisms of neonatal tolerance induced by dendritic cells and thymic B cells. *Journal of Experimental Medicine*, 173, 549-559 (1991)
81. Y. Tomita, A. Khan, and M. Sykes: Role of intrathymic clonal deletion and peripheral anergy in transplantation tolerance induced by bone marrow transplantion in mice conditioned with a non-myeloablative regimen. *Journal of Immunology*, 153, 1087-1098 (1994)
82. J. O. Manilay, D. A. Pearson, J. J. Sergio, K. G. Swenson, and M. Sykes: Intrathymic deletion of alloreactive T cells in mixed bone marrow chimeras prepared with a nonmyeloablative conditioning regimen. *Transplantation*, 66, 96-102 (1998)
83. A. Khan, Y. Tomita, and M. Sykes: Thymic dependence of loss of tolerance in mixed allogeneic bone marrow chimeras after depletion of donor antigen. Peripheral mechanisms do not contribute to maintenance of tolerance. *Transplantation*, 62, 380-387 (1996)
84. F. Ramsdell and B. J. Fowlkes: Maintenance of in vivo tolerance by persistence of antigen. *Science*, 257, 1130-1134 (1992)
85. B. Rocha, C. Tanchot, and H. Von Boehmer: Clonal anergy blocks in vivo growth of mature T cells and can be reversed in the absence of antigen. *Journal of Experimental Medicine*, 177, 1517-1521 (1993)
86. T. Wekerle, J. Kurtz, S. Bigenzahn, Y. Takeuchi, and M. Sykes: Mechanisms of transplant tolerance induction with costimulatory blockade. *Curr.Opin.Immunol.*, 14, 592-600 (2002)
87. K. Honey, S. P. Cobbold, and H. Waldmann: CD40 ligand blockade induces CD4+ T cell tolerance and linked suppression. *Journal of Immunology*, 163, 4805-4810 (1999)
88. L. Graca, K. Honey, E. Adams, S. P. Cobbold, and H. Waldmann: Cutting edge: anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *Journal of Immunology*, 165, 4783-4786 (2000)
89. B. T. Konieczny, Z. Dai, E. T. Elwood, S. Saleem, P. S. Linsley, F. K. Baddoura, C. P. Larsen, T. C. Pearson, and F. G. Lakkis: IFN-g is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. *Journal of Immunology*, 160, 2059-2064 (1998)
90. Z. Dai, B. T. Konieczny, F. K. Baddoura, and F. G. Lakkis: Impaired alloantigen-mediated T cell apoptosis and failure to induce long-term allograft survival in IL-2-deficient mice. *Journal of Immunology*, 161, 1659-1663 (1998)
91. A. Chandraker, M. E. Russell, T. Glysing-Jensen, T. A. Willett, and M. H. Sayegh: T-cell costimulatory blockade in experimental chronic cardiac allograft rejection: effects of cyclosporine and donor antigen. *Transplantation*, 63, 1053-1058 (1997)
92. Y. Li, X. X. Zheng, X. C. Li, M. S. Zand, and T. B. Strom: Combined costimulation blockade plus rapamycin but not cyclosporine produces permanent engraftment. *Transplantation*, 66, 1387-1388 (1998)
93. M. Sho, H. Harada, D. M. Rothstein, and M. H. Sayegh: CD45RB-targeting strategies for promoting long-term allograft survival and preventingchronic allograft vasculopathy. *Transplantation*, 75, 1142-1146 (2003)
94. K. Kishimoto, S. Sandner, J. Imitola, M. Sho, Y. Li, P. B. Langmuir, D. M. Rothstein, T. B. Strom, L. A. Turka, and M. H. Sayegh: Th1 cytokines, programmed cell death, and alloreactive T cell clone size in transplant tolerance. *J Clin Invest*, 109, 1471-1479 (2002)
95. X. C. Li, A. D. Wells, T. B. Strom, and L. A. Turka: The role of T cell apoptosis in transplantation tolerance. *Curr.Opin.Immunol.*, 12, 522-527 (2000)
96. A. D. Wells, X. C. Li, Y. Li, M. Walsh, X. XC. Zheng, Z. Wu, G. Nunez, A. Tang, M. Sayegh, W. W. Hancock, T. B. Strom, and L. A. Turka: Requirement for T cell apoptosis in the induction of peripheral transplantation tolerance. *Nature Medicine*, 5, 1303-1312 (1999)
97. J. Kurtz, J. Shaffer, N. Anosova, G. Benichou, and M. Sykes: Mechanisms of early peripheral CD4 T cell tolerance induction by anti-CD154 monoclonal antibody and allogeneic bone marrow transplantation: Evidence for anergy and deletion, but not regulatory cells. *Blood*, 103, 4336-4343 (2004)
98. T. Fehr, Y. Takeuchi, J. Kurtz, and M. Sykes: Rapid peripheral deletion of donor-reactive CD8 T cells permits mixed allogeneic chimerism and tolerance induction in recipients of anti-CD40L and allogeneic BMT: Short-term regulation by CD4 cells. *Eur J Immunol*, 35, 2679-2690 (2005)
99. H. Ito, J. Kurtz, J. Shaffer, and M. Sykes: CD4 T cell-mediated alloresistance to fully MHC-mismatched allogeneic bone marrow engraftment is dependent on CD40-CD40L interactions, and lasting T cell tolerance is induced by bone marrow transplantation with initial blockade of this pathway. *Journal of Immunology*, 166, 2981 (2001)
100. Z. Guo, J. Wang, Y. Dong, A. B. Adams, N. Shirasugi, O. Kim, J. Hart, M. Newton-West, T. C. Pearson, C. P. Larsen, and K. A. Newell: Long-term survival of intestinal allografts induced by costimulation blockade, busulfan and donor bone marrow infusion. *Am J Transplant*, 3, 1091-1098 (2003)
101. J. Kurtz, H. Ito, T. Wekerle, J. Shaffer, and M. Sykes: Mechanisms involved in the establishment of tolerance through costimulatory blockade and BMT: Lack of requirement for CD40L-mediated signaling for tolerance or deletion of donor-reactive CD4+ cells. *Am.J.Transplant.*, 1, 339-349 (2001)
102. N. J. Monk, R. E. Hargreaves, J. E. Marsh, C. A. Farrar, S. H. Sacks, M. Millrain, E. Simpson, J. Dyson, and

S. Jurcevic: Fc-dependent depletion of activated T cells occurs through CD40L-specific antibody rather than costimulation blockade. *Nat Med*, 9, 1275-1280 (2003)
103. H. Waldmann: The new immunosuppression: just kill the T cell. *Nat Med*, 9, 1259-1260 (2003)
104. J. Kaye, M.-L. Hsu, M.-E. Sauron, S. C. Jameson, N. R. J. Gasgiogne, and S. M. Hedrick: Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature*, 341, 746 (1989)
105. J. Kaye, N. J. Vasquez, and S. M. Hedrick: Involvement of the same region of the T cell antigen receptor in thymic selection and foreign peptide recognition. *Journal of Immunology*, 148, 3342-3353 (1992)
106. P. Fink, L. Matis, D. McElligot, M. Bookman, and S. Hedrick: Correlations between T-cell specificity and the structure of the antigen receptor. *Nature*, 321, 219 (1986)
107. N. J. Vasquez, J. Kaye, and S. M. Hedrick: In vivo and in vitro clonal deletion of double-positive thymocytes. *Journal of Experimental Medicine*, 175, 1307-1316 (1992)
108. J. Kurtz, A. Lie, M. Griffith, S. Eysaman, J. Shaffer, N. Anosova, L. Turka, G. Benichou, and M. Sykes: Lack of role for CsA-sensitive or Fas pathways in the tolerization of CD4+ T cells via BMT and anti-CD40L. *Am.J.Transplant.*, 3, 804-816 (2003)
109. T. Wekerle, J. Kurtz, M. H. Sayegh, H. Ito, A. D. Wells, S. Bensinger, J. Shaffer, L. A. Turka, and M. Sykes: Peripheral deletion after bone marrow transplantation with costimulatory blockade has features of both activation-induced cell death and passive cell death. *Journal of Immunology*, 166, 2311-2316 (2001)
110. Y. Rafaeli, L. Van Parijs, S. I. Alexander, and A. K. Abbas: Interferon gamma Is required for activation-induced death of T lymphocytes. *J Exp Med*, 196, 999-1005 (2002)
111. M. A. Curotto de Lafaille and J. J. Lafaille: CD4(+) regulatory T cells in autoimmunity and allergy. *Curr Opin Immunol*, 14, 771-778 (2002)
112. M. Murakami, A. Sakamoto, J. Bender, J. Kappler, and P. Marrack: CD25+CD4+ T cells contribute to the control of memory CD8+ T cells. *Proc Natl Acad Sci U S A*, 99, 8832-8837 (2002)
113. Q. Tang, K. J. Henriksen, E. K. Boden, A. J. Tooley, J. Ye, S. K. Subudhi, X. X. Zheng, T. B. Strom, and J. A. Bluestone: Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol*, 171, 3348-3352 (2003)
114. S. J. Bensinger, P. T. Walsh, J. Zhang, M. Carroll, R. Parsons, J. C. Rathmell, C. B. Thompson, M. A. Burchill, M. A. Farrar, and L. A. Turka: Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. *J Immunol*, 172, 5287-5296 (2004)
115. A. M. Thornton, E. E. Donovan, C. A. Piccirillo, and E. M. Shevach: Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. *J Immunol*, 172, 6519-6523 (2004)
116. R. S. Gruchalla, P. G. Strome, and J. W. Streilein: Analysis of neonatally induced tolerance of H-2 alloantigens. III. Ease of abolition of tolerance of class I, but not class II, antigens with infusions of syngeneic, immunocompetent cells. *Transplantation*, 36, 318-323 (1983)
117. H. Ramseier: Immunization against abolition of transplantation tolerance. *Eur.J.Immunol.*, 3, 156-164 (1973)
118. R. E. Billingham, L. Brent, and P. B. Medawar: Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. *Philos.Trans.Royal Society*, 239(Ser.B), 44 (1955)
119. C. Domenig, A. Sanchez-Fueyo, J. Kurtz, S. P. Alexopoulos, C. Mariat, M. Sykes, T. B. Strom, and X. X. Zheng: Roles of deletion and regulation in creating mixed chimerism and allograft tolerance using a nonlymphoablative irradiation-free protocol. *J Immunol*, 175, 51-60 (2005)
120. R. Scully, S. Qin, S. Cobbold, and H. Waldmann: Mechanisms in CD4 antibody-mediated transplantation tolerance: kinetics of induction, antigen dependency and role of regulatory T cells. *Eur.J.Immunol.*, 24, 2383-2392 (1994)
121. H. Quill: Anergy as a mechanism of peripheral T cell tolerance. *Journal of Immunology*, 156, 1325-1327 (1996)
122. A. D. Wells, M. C. Walsh, J. A. Bluestone, and L. A. Turka: Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. *J Clin Invest*, 108, 895-903 (2001)
123. N. N. Iwakoshi, J. P. Mordes, T. G. Markees, N. E. Phillips, A. A. Rossini, and D. L. Greiner: Treatment of allograft recipients with donor-specific transfusion and anti-CD154 antibody leads to deletion of alloreactive CD8+ T cells and prolonged graft survival in a CTLA4-dependent manner. *Journal of Immunology*, 164, 512-521 (2000)
124. N. N. Iwakoshi, T. G. Markees, N. Turgeon, T. Thornley, A. Cuthbert, J. Leif, N. E. Phillips, J. P. Mordes, D. L. Greiner, and A. A. Rossini: Skin allograft maintenance in a new synchimeric model system of tolerance. *Journal of Immunology*, 167, 6623-6630 (2001)
125. Y. Takeuchi, H. Ito, J. Kurtz, T. Wekerle, L. Ho, and M. Sykes: Earlier low-dose TBI or DST overcomes CD8+ T-cell-mediated alloresistance to allogeneic marrow in recipients of anti-CD40L. *Am J Transplant*, 4, 31-40 (2004)
126. X.-X. Zheng, T. G. Markees, W. W. Hancock, Y. Li, D. L. Greiner, X. C. Li, J. P. Mordes, M. H. Sayegh, A. A. Rossini, and T. B. Strom: CTLA4 signals are required to optimally induce allograft tolerance with combined donor-specific transfusion and anti-CD154 monoclonal antibody treatment. *Journal of Immunology*, 162, 4983-4990 (1999)
127. A. O'Garra and P. Vieira: Regulatory T cells and mechanisms of immune system control. *Nat Med*, 10, 801-805 (2004)
128. S. Bigenzahn, P. Blaha, Z. Koporc, I. Pree, E. Selzer, H. Bergmeister, F. Wrba, C. Heusser, K. Wagner, F. Muehlbacher, and T. Wekerle: The role of non-deletional tolerance mechanisms in a murine model of mixed chimerism with costimulation blockade. *Am J Transplant*, 5, 1237-1247 (2005)
129. G. Schonrich, G. Strauss, K.-P. Muller, L. Dustin, D. Y. Loh, N. Auphan, A.-M. Schmitt-Verhulst, B. Arnold, and G. J. Hammerling: Distinct requirements of positive and negative selection for selecting cell type and CD8 interaction. *Journal of Immunology*, 151, 4098-4105 (1993)
130. F. Bemelman, K. Honey, E. Adams, S. Cobbold, and H. Waldmann: Bone marrow transplantation induces either

clonal deletion or infectious tolerance depending on the dose. *Journal of Immunology*, 160, 2645-2648 (1998)
131. I. R. Lemischka, D. H. Raulet, and R. C. Mulligan: Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell*, 45, 917-927 (1986)
132. T. Kawai, A. B. Cosimi, R. B. COLVIN, J. Powelson, J. Eason, T. Kozlowski, M. Sykes, R. Monroy, M. Tanaka, and D. H. Sachs: Mixed allogeneic chimerism and renal allograft tolerance in cynomologous monkeys. *Transplantation*, 59, 256-262 (1995)
133. Fudaba, Y., T. R. Spitzer, J. Shaffer, T. Kawai, T. Fehr, F. Delmonico, F. Preffer, N. Tolkoff-Rubin, B. Dey, S. Saidman, A. Kraus, T. Bonnefoix, S. MCafee, K. Power, K. Kattleman, R. B. COLVIN, D. H. Sachs, A. B. Cosimi, & M. Sykes: Myeloma responses and tolerance following combined kidney and non-myeloablative marrow transplantation: in vivo and in vitro analyses. (2006)
Ref Type: In Press
134. K. Hayamizu, F. Lan, P. Huie, R. K. Sibley, and S. Strober: Comparison of chimeric and non-chimeric tolerance using posttransplant total lymphiod irradiation. Cytokine expression and chronic rejection. *Transplantation*, 68, 1036-1044 (1999)
135. T. R. Spitzer, F. Delmonico, N. Tolkoff-Rubin, S. MCafee, R. Sackstein, S. Saidman, C. Colby, M. Sykes, D. H. Sachs, and A. B. Cosimi: Combined HLA-matched donor bone marrow and renal transplantation for multiple myeloma with end stage renal disease: The induction of allograft tolerance through mixed lymphohematopoietic chimerism. *Transplantation*, 68, 480-484 (1999)
136. L. H. Buhler, T. R. Spitzer, M. Sykes, D. H. Sachs, F. L. Delmonico, N. Rubin-Tolkoff, S. L. Saidman, R. Sackstein, S. MCafee, B. Dey, C. Colby, and A. B. Cosimi: Induction of kidney allograft tolerance after transient lymphohematopoietic chimerism in patients with multiple myeloma and end-stage renal disease. *Transplantation*, 74, 1405-1409 (2002)
137. T. Fehr and M. Sykes: Tolerance induction in clinical transplantation. *Transpl Immunol*, 13, 117-130 (2004)
138. T. Kawai, D. Andrews, R. B. COLVIN, D. H. Sachs, and A. B. Cosimi: Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand [letter]. *Nat.Med.*, 6, 114 (2000)
139. I. Koyama, T. Kawai, D. Andrews, S. Boskovic, O. Nadazdin, S. L. Wee, H. Sogawa, D. L. Wu, R. N. Smith, R. B. COLVIN, D. H. Sachs, and A. B. Cosimi: Thrombophilia associated with anti-CD154 monoclonal antibody treatment and its prophylaxis in nonhuman primates. *Transplantation*, 77, 460-462 (2004)
140. J. L. Ferrant, C. D. Benjamin, A. H. Cutler, S. L. Kalled, Y. M. Hsu, E. A. Garber, D. M. Hess, R. I. Shapiro, N. S. Kenyon, D. M. Harlan, A. D. Kirk, L. C. Burkly, and F. R. Taylor: The contribution of Fc effector mechanisms in the efficacy of anti-CD154 immunotherapy depends on the nature of the immune challenge. *Int.Immunol*, 16, 1583-1594 (2004)
141. T. Kanmaz, J. J. Fechner, Jr., J. Torrealba, H. T. Kim, Y. Dong, T. D. Oberley, J. M. Schultz, D. D. Bloom, M. Katayama, W. Dar, J. Markovits, W. Schuler, H. Hu, M. M. Hamawy, and S. J. Knechtle: Monotherapy with the novel human anti-CD154 monoclonal antibody ABI793 in rhesus monkey renal transplantation model. *Transplantation*, 77, 914-920 (2004)
142. W. Schuler, M. Bigaud, V. Brinkmann, F. Di Padova, S. Geisse, H. Gram, V. Hungerford, B. Kleuser, C. Kristofic, K. Menninger, R. Tees, G. Wieczorek, C. Wilt, C. Wioland, and M. Zurini: Efficacy and safety of ABI793, a novel human anti-human CD154 monoclonal antibody, in cynomolgus monkey renal allotransplantation. *Transplantation,* 77,717-726 (2004)

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Send correspondence to: Dr Megan Sykes, Transplantation Biology Research Center, Department of Surgery, Massachusetts General Hospital/Harvard Medical School, MGH East, Bldg 149-5102 13th Street, Boston, MA 02129, 617-726-4070, Fax:: 617-724-9892, E-mail: Megan.Sykes@tbrc.mgh.harvard.edu

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