

## Small bowel transplantation tolerance achieved by costimulatory blockade leading to mixed chimerism

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### 1. ABSTRACT

We evaluated mixed chimerism with costimulatory blockade for the achievement murine allogeneic small bowel transplantation (SBTx) tolerance. B6 mice received various combinations of anti-CD8 (day -2) and anti-CD154 mAbs with or without 3Gy total body irradiation (TBI) (day -1), and  $20 \times 10^6$  fully MHC-mismatched B10.A bone marrow cells (BMC, day -1). Heterotopic SBTx was performed on day 0. Chimerism in peripheral blood was followed by flow cytometric (FCM) analysis and the frequency of TCR V $\beta$  usage was determined by FCM to assess deletion of donor-reactive T cells. All animals without any treatment (n=6) showed acute rejection within 18 days after transplantation. Mice treated with anti-CD8 and anti-CD154 mAbs alone rejected their grafts within 100 days after transplantation (n=10). Mice treated with anti-CD8 and anti-CD154 mAbs, TBI, and BMT achieved long-term multilineage mixed chimerism and accepted small bowel allografts permanently (>350 days) without any evidence of graft-versus-host disease(n=11). There was specific deletion of donor-reactive cells and skin was accepted as allografts from B10.A donors, but 3rd party B10.BR skin was rejected. Donor-specific tolerance was achieved by inducing mixed chimerism with costimulatory blockade in murine SBTx recipients. This approach which provides a reliable method to induce SBTx tolerance, has potential clinical applications.

### 2. INTRODUCTION

Owing to the development of new immunosuppressive agents (1-4), small bowel transplantation (SBTx) has recently been developed as a therapeutic option for patients with short bowel syndrome or gastroschisis. Although short-term graft survival has been remarkably successful, long-term outcomes have not been satisfactory. Two causes of death, infection and acute rejection, are still severe problems after SBTx. The immune response evoked by an intestinal allograft is more difficult to control than that with transplantation of other solid organs because the intestine contains a large amount of lymphoid tissue. In fact, the patients require the use of high dose non-specific immunosuppressive agents, and face opportunistic infections. To resolve these problems, induction of transplantation tolerance is essential.

Orloff et al. have reported the permanent engraftment of intestinal allograft in rat model using 10Gy lethal irradiation to induce mixed chimerism (5). Sharabi et al. have demonstrated that a relatively non-toxic conditioning regimen consisting of host treatment with depleting anti-CD4 and anti-CD8 mAbs, 3Gy total body irradiation (TBI), and 7Gy local irradiation to the thymus can reliably permit the engraftment of fully allogeneic marrow, which leads to the induction of mixed hematopoietic chimerism and skin graft transplantation tolerance(6).

More recently, many investigators have reported the importance of costimulatory blockade in preventing rejection. According to these effects of costimulatory blocking, Guo *et al.* and Fishbein *et al.* have demonstrated the blockade of CD40-CD154 interaction can prolong the intestinal allograft survival(7, 8). However, these studies demonstrated that allografts had finally rejected. It is suggested that further treatments are necessary to induce small bowel transplantation tolerance.

We have recently demonstrated that a minimally toxic regimen (3GyTBI,  $20 \times 10^6$  donor bone marrow cells, and single injection of anti-CD8 and anti-CD154 mAbs) could induce permanent multilineage mixed chimerism and tolerance(9). This regimen is completely reliable to allow the skin graft transplantation tolerance, even in murine fully MHC-mismatched strain combination model. This reliable regimen have not been investigated the effectiveness in SBTx model. In this study, we evaluated the possibilities of mixed chimerism to induce murine small bowel transplantation.

### 3. MATERIALS AND METHODS

#### 3.1. Mice

Eight-to-twelve-week-old male C57BL/6 (B6: H-2<sup>b</sup>), B10.A (H-2<sup>a</sup>), and B10.BR (H-2<sup>k</sup>) were purchased from Japan SLC Inc. (Hamamatsu, Japan). These animals were housed under specific pathogen-free conditions with free access to food and water in temperature-controlled environment under a 12-hour light-dark cycle at the institute of Laboratory Animals, Yamaguchi University Graduate School of Medicine. B6 mice were used as recipients, B10.A mice as donors, and B10.BR mice as third-party skin graft transplantation.

#### 3.2. Small bowel transplantation

Heterotopic small bowel transplantation (SBTx) was performed by modified technique of Monchik and Russell using standard micro vascular techniques (10). Donor and recipient mice were anesthetized with an intraperitoneal injection of 40mg/kg pentobarbital. About 5cm ileum was removed on a vascular pedicle consisting of the superior mesenteric artery with an abdominal aorta and the portal vein from donor mice. The excised intestinal graft was stored for about 30min in cold saline while the recipient operation was started. The donor abdominal aorta was anastomosed end-to-side to the recipient infrarenal aorta and the donor portal vein to the recipient inferior vena cava. The proximal and distal ends of the intestinal graft were exteriorised stomas (Thiry-Vella loop). The grafts were observed every other day and rejection was defined as the first day on which the mucosa of the stoma was necrotic.

#### 3.3. Conditioning and BMT

Age-matched (8- to 12-week-old) male mice received 3 Gy TBI and were injected intravenously on the same day (day 0) with  $20 \times 10^6$  unseparated bone marrow cells (BMCs) harvested from MHC-full mismatched B10.A donors (8–12 week old). Mice were injected intraperitoneally with a single dose (1.4mg) of rat IgG2b

anti-mouse CD8 mAb 2.43 (11) on day -1. Hamster anti-mouse CD154 mAb, MR1 (12) was injected intraperitoneally on day 0 (2 mg).

#### 3.4. Flow cytometric analysis (FCM) of multilineage mixed chimerism in peripheral blood

FCM of multilineage chimerism was performed as described previously (9). Briefly, forward angle and 90° light scatter properties were used to distinguish lymphocytes, monocytes, and granulocytes in the peripheral WBCs. Two-color FCM was used to distinguish donor and host cells of particular lineages. Lineages of leukocytes were analyzed with FITC-conjugated mAbs including anti-CD4, anti-CD8, anti-B220, and anti-MAC1 mAb (all purchased from BD Biosciences Pharmingen, CA). Donor cells were determined with Biotinylated anti-H-2D<sup>d</sup> mAb 34-2-12 (BD Biosciences Pharmingen). Percentage of donor cells was calculated as described previously (9) by subtracting control staining from quadrants containing donor and host cells expressing a particular lineage marker, and by dividing the net percentage of donor cells by the total net percentage of donor plus host cells of that lineage. For assessing the usage of certain V $\beta$  subunits within the TCR repertoires, peripheral blood was stained with FITC-conjugated anti-V $\beta$ 5, anti-V $\beta$ 8, anti-V $\beta$ 11 and Biotinylated anti-CD4 mAb (all purchased from BD Biosciences Pharmingen). All Biotinylated mAb was developed with PE-streptavidin (Caltag, CA). Dead cells were excluded using propidium iodide staining. All the analysis was done in a FACS Caliber flow cytometer (Becton Dickinson, San Jose, CA).

#### 3.5. Skin grafting

Full thickness tail skin ( $\sim 1.0\text{cm}^2$ ) from B10.A and B10.BR were grafted on the recipient dorsal thoracic wall, sutured with 6-0 nylon and bandaged. Bandages were removed 1 week after transplantation, and the graft was observed every other day. Rejection was defined as the first day on which the entire epidermal surface of the graft was necrotic.

#### 3.6. Histology

Tissues were harvested from animals at the time of sacrifice and immediately embedded in OCT compound and snap-frozen in liquid nitrogen. 5- $\mu\text{m}$ -thick tissues slices were obtained for Haematoxylin-Eosin staining.

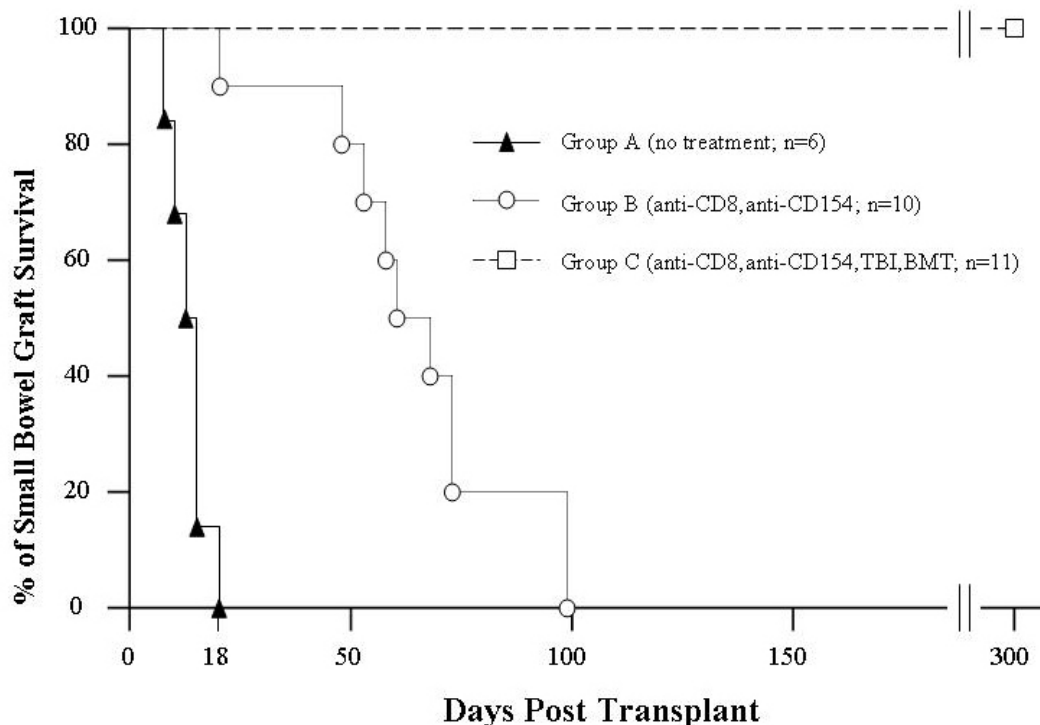
#### 3.7. Statistical analysis

Statistical significance was determined with a two-tailed Student's *t* test for comparison of means with unequal variances.

### 4. RESULTS

#### 4.1. Treatment with anti-CD8 plus anti-CD154 mAbs leads to prolonged survival of MHC-full mismatched allogeneic small bowel grafts

Untreated B6 recipients (Group A, n=6) rejected B10.A small bowel grafts within 18 days after transplantation (median survival time [MST] 10 days; Figure 1). In B6 mice that received anti-CD8 mAb on day -1 and anti-CD154 mAb on day 0 (Group B, n=10), grafted



**Figure 1.** Graft survival of small bowel allograft. Naïve mice rejected transplanted small bowel grafts within 18 days after transplantation (n=6, MST=10days). Anti-CD8 plus anti-CD154 mAbs treatment prolonged small bowel graft acceptance (n=10, MST=63days), however, all the grafts were rejected before 100 days after transplantation. In contrast, mice treated with anti-CD8, anti-CD154 mAbs plus TBI/BMT demonstrated permanent acceptance of small bowel grafts (n=11).

B10.A small bowel survived for an extended period (MST=63 days), but were rejected by 98 days after transplantation (Figure 1). These results demonstrate that anti-CD8 plus anti-CD154 mAbs treatment dramatically prolonged allogeneic small bowel graft survival. However, those treatments alone could not induce permanent acceptance of allogeneic small bowel grafts.

#### 4.2. Additional treatment of allogeneic bone marrow transplantation demonstrated permanent acceptance of allogeneic small bowel grafts

To investigate the effect of allogeneic BMT, B6 mice received  $20 \times 10^6$  B10.A BMCs one day before SBTx. When mice treated with anti-CD8 mAb on day -1, followed by anti-CD154 mAb and 3 Gy TBI plus BMT on day 0, all the B6 mice accepted B10.A small bowel grafts over 350 days (Group C, n=11). Those animals did not demonstrate evidence of graft-versus-host disease (GVHD) during the follow-up period.

#### 4.3. Acute graft rejection was prevented by costimulatory blockade with or without BMT

Transplanted small bowels were removed and histological examination was performed 14 days after transplantation (Figure 2). At this time point, the allogeneic stomas of untreated mice were rejected. Histological findings demonstrated destruction of the villous epithelium and infiltration of inflammatory cells (Figure 2-A). In contrast, the allogeneic stoma of mice

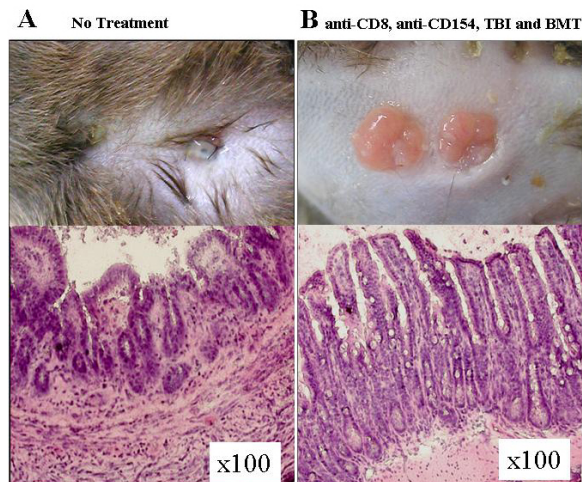
treated with anti-CD8 and anti-CD154 mAbs (data not shown) and the stoma of mice treated with anti-CD8 and anti-CD154 mAbs plus TBI/BMT (Figure 2-B) demonstrated good complexion, and histological findings demonstrated viable and well-preserved structure of mucosa and villus 14 days after transplantation in these mice.

#### 4.4. No rejection was observed in small bowel grafts 350 days after transplantation in mixed chimeras

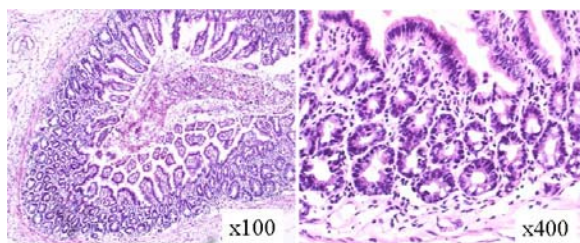
Transplanted small bowels were removed 350 days after SBTx, and the grade of rejection was evaluated (n=11). There were no infiltrating cells, and well-preserved villi were seen. Villous atrophy is considered to reflect non-functioning small bowel after transplantation. These findings suggested that transplanted small bowel grafts were permanently accepted without rejection in the mixed chimeras (Figure 3).

#### 4.5. Mixed chimerism is essential for induction of small bowel transplantation tolerance

To investigate whether or not recipients developed mixed chimerism in the peripheral blood, flow cytometric analysis was performed. Mice treated with anti-CD8 mAb and anti-CD154 mAb demonstrated multilineage mixed chimerism 14 days after BMT. Although CD8 positive cells derived from both donor and recipient were depleted by the effect of anti-CD8 mAb 14 days after BMT, CD8 cells were recovered over time and



**Figure 2.** Histological findings (H-E stain) at 14 days post SBTx : Naïve mice rejected small bowel grafts (n=5) (A). Mice received anti-CD8, anti-CD154 mAbs, TBI, and BMT accepted small bowel grafts (n=5) (B).



**Figure 3.** Histological findings (H-E stain) at 350 days post SBTx : Mice treated with anti-CD8, anti-CD154 mAbs, TBI, and BMT accepted small bowel grafts permanently (n=11). There was no cell infiltration, and well preserved villi were seen in the transplanted small bowel grafts.

demonstrated mixed chimerism (40.0-88.7% of CD8 cells were donor-derived) in mice treated with anti-CD8 and anti-CD154 mAbs plus TBI/BMT. This mixed chimerism persists permanently (Figure 4). In contrast, mice that received anti-CD8 mAb (day-1) and anti-CD154 mAb (day 0) without BMT did not show mixed chimerism (data not shown).

#### 4.6. Donor-reactive T cell deletion in mixed chimeras that accepted small bowel grafts

Central deletion is one of the major mechanisms maintaining tolerance in mixed chimeras prepared by a variety of regimens. We therefore examined whether or not donor-reactive T cells in PBL were deleted by assessing the usage of certain V $\beta$  subunits within the TCR repertoires, as described previously (9, 13-15). The donor strain B10.A expresses I-E, which is required to present superantigens derived from mammary tumor virus 8 and 9 endogenous retroviruses encoded in the B6/B10 background genome (16-18). Developing thymocytes expressing TCR containing V $\beta$ 5 and V $\beta$ 11 subunits, which bind to these superantigens, are deleted in I-E-positive B10.A mice, but not in B6 mice, because they do not express I-E (18, 19).

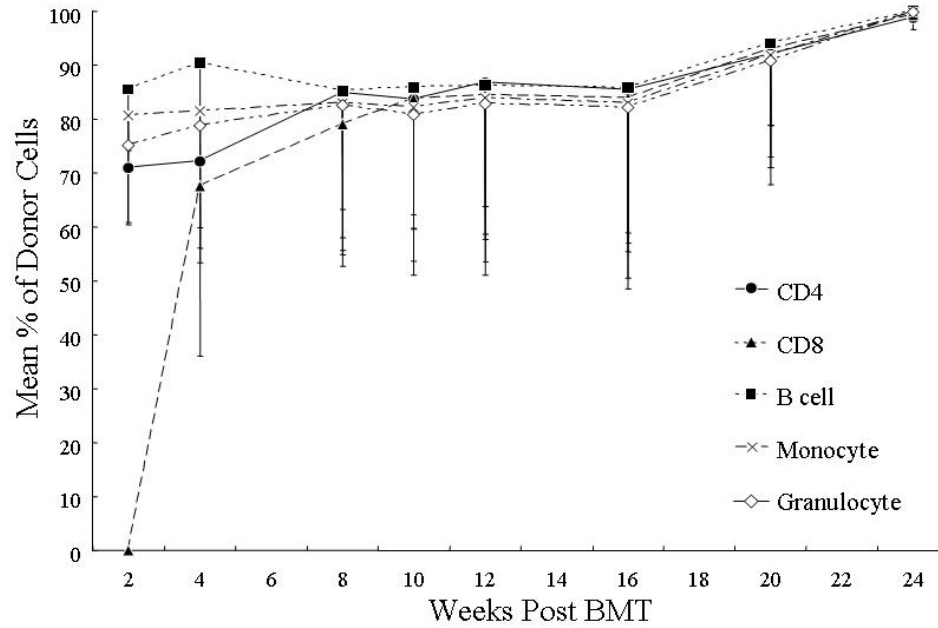
We examined whether or not donor-reactive T cells in peripheral blood were depleted by assessing the usage of certain V $\beta$  subunits within the TCR repertoires (Figure 5). In the B10.A to B.6 strain combination, V $\beta$ 5<sup>+</sup> and V $\beta$ 11<sup>+</sup> CD4<sup>+</sup> T cells recognize endogenous superantigens presented by donor (B10.A) MHC, and therefore are markers for donor-reactive T cells. Additionally, V $\beta$ 8<sup>+</sup> CD4<sup>+</sup> T cells do not recognize these superantigens and serve as controls to assure specificity of the deletion. By 6 weeks post-BMT, mice treated with anti-CD8 and anti-CD154 mAbs plus TBI and BMT showed profound reductions in the percentage of V $\beta$ 5<sup>+</sup> CD4<sup>+</sup> T cells (normal B6,  $2.32 \pm 0.18\%$ ; normal B10.A,  $0.00\%$ ) to  $0.3 \pm 0.43\%$ , and V $\beta$ 11<sup>+</sup> CD4<sup>+</sup> T cells (normal B6,  $5.16 \pm 0.14\%$ ; normal B10.A,  $0.10 \pm 0.10\%$ ) to  $0.35 \pm 0.43\%$ . In addition, these reductions in the percentage of V $\beta$ 5<sup>+</sup> and V $\beta$ 11<sup>+</sup> CD4<sup>+</sup> T cells persisted for at least 26 weeks post-BMT. In contrast, mice treated with anti-CD8 and anti-CD154 mAbs alone did not show deletion of V $\beta$ 5<sup>+</sup> CD4<sup>+</sup> T cells ( $1.91 \pm 0.46\%$ ) or V $\beta$ 11<sup>+</sup> CD4<sup>+</sup> T cells ( $4.44 \pm 0.76\%$ ). These data suggested that central deletion was a major mechanism maintaining mixed chimerism.

#### 4.7. Donor-specific skin graft acceptance in mixed chimeras receiving small bowel grafts following treatment with anti-CD8/CD154 mAbs plus 3 Gy TBI/BMT

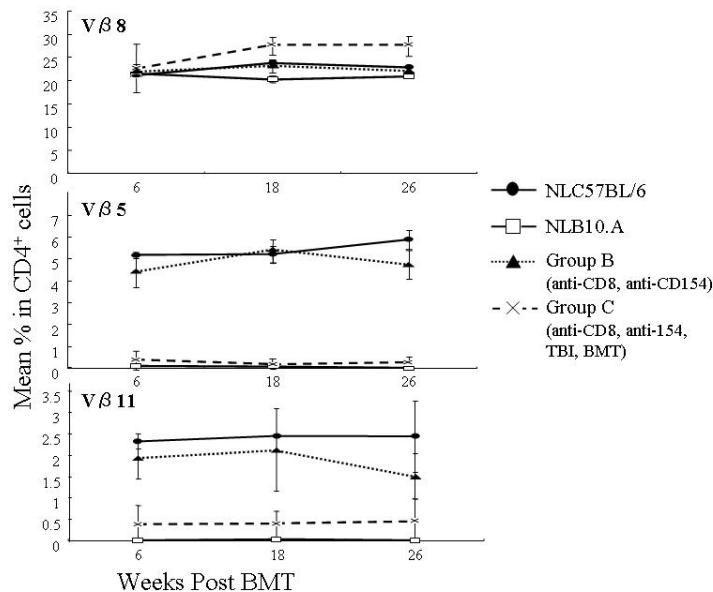
To determine whether or not donor-specific tolerance was induced in mice that accepted small bowel grafts permanently under treatment of anti-CD8 and anti-CD154 mAbs plus TBI/BMT, skin grafting was performed 300 days after SBTx. Mice treated with anti-CD8 and anti-CD154 mAbs that did not accept small bowel grafts rejected both B10.A and B10.BR skin graft within 14 days after skin grafting (Figure 6A, B). On the other hand, mice treated with anti-CD8 and anti-CD154 mAbs plus TBI/BMT, that accepted small bowel grafts permanently, also accepted donor B10.A skin grafts permanently (Figure 6A). However, these mice rejected third party B10.BR skin within 14 days (Figure 6B). These data demonstrated that mice that accepted small bowel grafts achieved donor-specific transplantation tolerance.

## DISCUSSION

Despite the development of immunosuppressive agents, high levels of immunosuppression are required to maintain acceptance of small bowel grafts (1-3, 20). This non-specific immunosuppression causes bacterial, viral and fungal infections, as well as lymphoproliferative disorders, and may lead to other cancers, and organ failure due to chronic rejection and sepsis. To avoid these side effects of an immunosuppressive environment, tolerance induction is essential. However, current immunosuppressive drugs or monoclonal antibodies are insufficient to induce tolerance in clinical transplantation. Blocking the CD40-CD154 pathway has achieved prolongation of donor heart and islet graft survival in rodents (21, 22). These grafts contain little lymphoid tissue and considered to be a relatively easy organ to be accepted. Small bowel grafts, in contrast, contain enormous amounts of lymphoid tissue, and their



**Figure 4.** Long-term multilineage mixed chimerism in mice treated with anti-CD8 and anti-CD154 mAbs, TBI, and BMT.

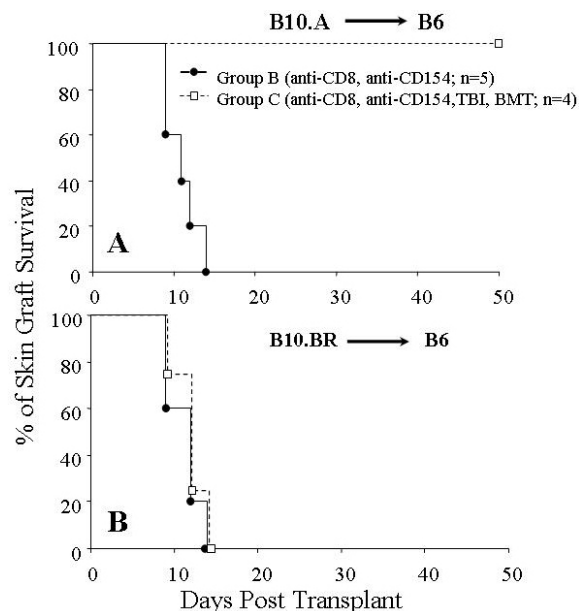


**Figure 5.** Deletion of donor-reactive peripheral blood CD4 T cells in chimeras prepared with anti-CD8 and anti-CD154 mAbs, TBI, and BMT: Every mice contained same level of Vβ8<sup>+</sup> cells. Mice received anti-CD8 and 154 mAbs showed the same mean percentage of CD4<sup>+</sup> Vβ5<sup>+</sup> and Vβ11<sup>+</sup> peripheral T cells as normal C57BL/6 mice. Mice treated with anti-CD8/CD154 mAbs, TBI, and BMT showed the deletion of CD4<sup>+</sup> Vβ5<sup>+</sup> and Vβ11<sup>+</sup> peripheral T cells.

effector cells exist in the lamina propria are activated soon after transplantation, cause severe rejection.

Mixed chimerism has been developed as a promising method to induce reliable tolerance (6, 9). Blocking the CD40-CD154 pathway avoids the need for

thymic irradiation (23) or repeated administration of monoclonal antibodies, especially anti-CD4 mAb (9). Mixed chimeras also accept long-term xenogeneic grafts (24, 25), suggesting that mixed chimerism is one of the most reliable methods to induce transplantation tolerance. Small bowel grafts are one of the most difficult organ grafts



**Figure 6.** Graft survival of skin allograft transplanted 300 days after small bowel transplantation. (A) Donor (B10.A) skin grafts were accepted in mice that accepted small bowel grafts (anti-CD8/CD154 mAbs, TBI/BMT treatment), but were rejected in mice that rejected small bowel grafts (anti-CD8/CD154 mAbs treatment alone). (B) Third party B10.BR skin was rejected within 14 days in all the mice.

to be accepted, and are shown here to be accepted in mixed chimeras. Mice treated with anti-CD8 and anti-CD154 mAbs alone demonstrated prolonged acceptance of allogeneic small bowel grafts, but eventually rejected them. Additional transplantation of donor bone marrow cells led to permanent multilineage mixed chimerism as previously described (9). These mice accepted donor small bowel grafts permanently. Histological findings revealed no infiltrating cells and well-preserved villi with slight atrophy that may have been caused by disuse in the heterotopically transplanted small bowels.

To assess whether robust donor-specific transplantation tolerance was induced or not, skin grafting was performed 300 days after small bowel transplantation. Permanent acceptance of donor skin grafts was achieved in mice that accepted first small bowel grafts, which nevertheless rejected third party skin grafts rapidly. On the other hand, skin grafts were rejected rapidly in mice treated with anti-CD8 and anti-CD154 mAbs without TBI/BMT. These data indicated that donor-specific tolerance was achieved in mixed chimeras.

Mixed chimerism is known as one of the most powerful methods to induce tolerance, and the blockade of costimulation in this study plays an important role in the induction of mixed chimerism. Anti-CD154 mAb, which blocks CD40-CD154 interaction, is not a depleting monoclonal antibody, and CD4 T cells are preserved (26). Preserving CD4 cells is important to prevent bacterial

infection because small bowel grafts are exposed to a bacterial rich environment.

No mice in this study developed GVHD. One of the reasons is that the anti-CD8 depleting mAb and anti-CD154 blocking mAb probably affect not only the recipient but also the infused donor marrow. Moreover, blocking CD40-CD154 interaction may prevent recipient-reactive cells that exist in the transplanted small bowels from attacking the host. To address this hypothesis, further investigations are necessary.

In summary, we demonstrated in this study that the regimen including anti-CD8 and anti-CD154 mAb, TBI/BMT was a reliable method to induce donor-specific small bowel transplantation tolerance. Induction of tolerance based on mixed chimerism is very reliable, so induction of small bowel transplantation tolerance in humans may benefit from chimerism induction.

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