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ORIGINAL ARTICLE

Combination therapy of an iNKT cell ligand and CD40–CD154 blockade establishes islet allograft acceptance in nonmyeloablative bone marrow transplant recipients

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Abstract

Aims Islet transplantation is an effective therapeutic option for type 1 diabetes. Although maintenance immunosuppression therapy is required to prevent allogeneic rejection and recurrence of autoimmunity, long-term allograft survival has not yet been achieved partly because of its adverse effects. The induction of donor-specific immunotolerance is a promising approach for long-term allograft survival without maintenance immunosuppression therapy. We previously reported that combination therapy using a liposomal ligand for invariant natural killer T cells, RGI-2001, and anti-CD154 antibody established mixed hematopoietic chimerism for the induction of donor-specific immunotolerance. This study investigated whether the protocol could promote islet allograft acceptance in experimental diabetes.

Methods Streptozotocin-induced diabetic BALB/c mice were transplanted with bone marrow cells from C57BL/6 donors and received combination therapy of RGI-2001 and anti-CD154 antibody after 3-Gy total body irradiation. 3 Weeks after bone marrow transplantation, islets isolated from C57BL/6 donors were transplanted under the kidney capsule.

Results Mixed chimerism was established in diabetic mice receiving the tolerance induction protocol. After islet transplantation, blood glucose levels improved and normoglycemia persisted for over 100 days. Hyperglycemia recurred after islet grafts were removed. Histopathological examinations showed insulin-positive staining and absence of cellular infiltration in the islet grafts. T cells of recipients showed donor-specific hyporesponsiveness, and anti-donor antibodies were not detected.

Conclusions The tolerance induction protocol with combination therapy of RGI-2001 and anti-CD154 antibody promoted islet allograft acceptance in a mouse diabetic model. This protocol may be clinically applied to islet transplantation for type 1 diabetes mellitus.

Keywords Islet transplantation · Immunotolerance · Streptozotocin · Type 1 diabetes · Mice

Introduction

Type 1 diabetes is caused by an autoimmune-mediated destruction of insulin-producing β-cells within pancreatic islets. Islet transplantation is an effective therapeutic option for type 1 diabetes [1, 2]. In the vast majority of islet transplantations, isolated islets from the donor pancreas are infused into the recipient's portal vein by percutaneous cannulation. Recipients are treated with immunosuppression therapy and other medications designed to enhance islet engraftment and survival [3]. However, long-term graft survival and insulin independence have not yet been achieved [1, 4, 5]. The rate of 3-year graft survival is reportedly 44–70% [4, 5], and the majority of recipients experience graft loss within 5 years of islet transplantation [1].

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The hyperglycemic status of diabetic recipients is an obstacle to islet graft survival. Prolonged exposure to high glucose concentrations has deleterious effects on islet β-cell viability [6–9]. Islet apoptosis was augmented by increasing the glucose concentration both in vitro and in vivo [10]. Hyperglycemia increases oxygen consumption [11, 12], suggesting that hyperglycemia-exacerbated hypoxia may cause islet apoptosis. Immunological issues preventing graft survival also involve early destruction of infused islets mediated by innate immune responses, and later, graft rejection mediated by alloimmune and autoimmune responses. Immediately after islet infusion through the portal vein, an instant blood-mediated inflammatory reaction is triggered by the activated coagulation cascade and the complement system, which subsequently induce innate immune responses. Thereafter, alloimmune and autoimmune reactions continuously impinge on islet graft survival in autoimmune recipients. To address these issues, several medication protocols have been designed to protect islet allografts from immune reactions. Although maintenance immunosuppression therapy is highly effective for preventing islet injuries mediated by alloimmune and autoimmune responses, long-term immunosuppression therapy is associated with increased rates of infections, malignancies, and hypertension [13, 14]. Some of the commonly used immunosuppressants are toxic to the islets themselves, which limits graft survival [3, 15]. As a novel approach that protects islet allografts with less toxic effect, mesenchymal stem cell therapies, which are still in the research and development phase, are expected [16].

The induction of donor-specific immunotolerance is also a promising approach for long-term allograft survival without maintenance immunosuppression therapy. This approach preserves the immune system against third-party antigens, reducing infection risk. Additionally, avoiding chronic immunosuppression therapy reduces malignancy risk and adverse events, including posttransplant diabetes. However, induction of donor-specific immunotolerance has not yet been achieved in human islet transplantation.

In our series of reports, a novel immunotolerance protocol has been studied. Sublethal irradiation, bone marrow transplantation (BMT), and a combination therapy consisting of a liposomal α-galactosylceramide (αGC-liposome), which is a ligand for invariant natural killer T (iNKT) cells, and an anti-CD154 antibody, MR1, could induce donor-specific immunotolerance by establishing mixed hematopoietic chimerism in mouse recipients [17]. In this protocol, we showed that the migration of donor-derived dendritic cells into recipient thymus and in vivo expansion of regulatory T cells (Tregs) could play a role in the clonal deletion of alloreactive $CD8^+$T cells and the establishment of mixed chimerism [18]. We also found that the myeloid-derived suppressor cells in donor BM cells were indispensable for in vivo Treg expansion and chimerism establishment [19]. For clinical application, we have suggested that the number of BM cells might be reduced by adding peripheral blood mononuclear cells with veto activity, which is involved in the depletion of graft-reactive $CD8^+$T cells and establishment of complete chimerism [20].

A new investigational drug of αGC-liposomes, RGI-2001, has a significant prophylactic effect on acute graft-versus-host disease through Treg expansion in mice and humans [21, 22]. The combination therapy consisting of RGI-2001 and MR1 induces Treg expansion effectively and leads to mixed hematopoietic chimerism [17–20]. In the present study, to investigate whether our protocol can be applied in islet transplantation, we performed allogeneic islet transplantation in diabetic mice induced by streptozotocin (STZ).

Research design and methods

Mice

Male BALB/c, C57BL/6, and C3H/He mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All mice were bred and maintained under specific pathogen-free conditions in the animal facility of Tokyo Women's Medical University, Japan. The Tokyo Women's Medical University internal committee on the use and care of laboratory animals approved all experiments (Reference ID: AE16-105-2).

Diabetes induction

Diabetes was induced by single intraperitoneal injection of 190 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, USA) in 8-week-old BALB/c mice. Blood glucose levels were measured by One Touch Ultra glucometer (Johnson & Johnson K.K., Tokyo, Japan). Diabetes was diagnosed when blood glucose levels were ≥ 300 mg/dL.

Chimerism induction

1 Week after STZ injection, the diabetic BALB/c ($H2^d$) mice received nonmyeloablative total body irradiation (3 Gy) prior to the intravenous injection of 20×10^6 whole bone marrow cells derived from fully allogeneic C57BL/6 ($H2^b$) mice [8]. Following BMT, the recipient mice were treated with a combination therapy consisting of intravenous injection of 0.2 μg RGI-2001 (REGiMMUNE Corp.) and intraperitoneal injection of 0.5 mg MR1 (BioXcell, West Lebanon, NH, USA). We evaluated the percentage of $H2Kb^+$ donor-derived cells in peripheral blood by flow cytometry using the FACSCanto™ II (BD Biosciences, San Jose, CA, USA) and analyzed the data using the FlowJo software (Tree Star, Ashland, OR, USA) every 2 weeks after BMT.

Islet transplantation

Islets were isolated from 10-week-old C57BL/6 pancreases by in situ collagenase P (Roche, Indianapolis, IN, USA) digestion. In brief, 1 mg/dL collagenase P dissolved in Hanks' balanced salt solution (HBSS) was injected into pancreases via the common bile duct using a 27-G needle. The enzyme-infused pancreases were removed and digested in 2 mL of collagenase P solution at 37 °C for 16 min. The digested pancreases were washed twice with HBSS and filtered through a stainless-steel sieve to collect the islets in the flow-through. The islets were washed twice, isolated by density-gradient centrifugation using Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA), and picked up using a micropipette under a microscope. Two hundred of the purified islets were implanted in each mouse under the left kidney capsule under general anesthesia with sevoflurane.

Evaluation of graft function

Blood glucose levels after islet transplantation were monitored weekly. Blood glucose levels ≤ 250 mg/dL were defined as successful graft function. Mice without a decrease of less than 250 mg/dL until 63 days after islet transplantation were diagnosed as primary non-function. Graft loss was diagnosed when blood glucose levels were > 250 mg/dL on two consecutive measurements. To confirm graft function, islet grafts were removed by nephrectomy 100 days after islet transplantation. The removed grafts were fixed with 10% paraformaldehyde and embedded with paraffin. Periodic acid–Schiff (PAS) staining, anti-CD3 staining, and insulin staining were performed. For anti-CD3 staining, we used rabbit anti-CD3 antibody (Abcam). For insulin staining, we used guinea pig anti-insulin antibody (Abcam).

Glucose tolerance test

To evaluate graft function, glucose tolerance test was performed 60 days after islet transplantation. As a control group, naïve BALB/c mice were used. The mice were fasted for 2 h and glucose was injected into the peritoneal cavity (0.04 mg/mouse). Blood glucose levels were measured at 0, 15, 30, 60, and 120 min after glucose injection.

Mixed lymphocyte reaction assay

T cells (i.e., the responders) were negatively isolated from recipient splenocytes 100 days after islet transplantation using a pan T cell isolation kit (Milteny Biotec, Auburn, CA, USA), and then labeled with carboxyfluorescein succinimidyl ester (CFSE). Antigen-presenting cells (i.e., the stimulators) were prepared from splenocytes of naïve BALB/c, C57BL/6, or C3H/He mice by T-cell depletion using mouse pan T beads (Life Technologies, Oslo, Norway), and then irradiated (30 Gy). The responders were incubated with each stimulator at a ratio of 1:1 in RPMI 1640 medium containing 10% FBS, 0.1 mM HEPES (pH 7.2–7.5), 1 × MEM non-essential amino acid solution, 1 mM sodium pyruvate, 1 × penicillin–streptomycin, and 100 μM 2-mercaptoethanol. 4 Days later, the proportion of CFSE-positive cells was calculated using flow cytometry.

Flow cytometry crossmatch assay

Recipients' sera were incubated with naïve C57BL/6 splenocytes 100 days after islet transplantation and with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (eBioscience, San Diego, CA, USA) and phycoerythrin (PE)-conjugated anti-mouse CD3ε antibodies (145-2C11, eBioscience). Mean fluorescence intensity of recipient IgG reactivity against donor T cells was measured by flow cytometry, and values were standardized to molecules of equivalent soluble fluorochrome (MESF).

Statistical analysis

Data were analyzed using Mann–Whitney *U* test in the Prism 7 software (GraphPad Software, La Jolla, CA, USA). Islet allograft survival was plotted using Kaplan–Meier cumulative survival curves and differences in survival between groups determined using the log-rank (Mantel–Cox) test in the Prism 7 software. Differences with P values < 0.05 were considered significant.

Results

BMT with RGI-2001 and MR1 combination therapy established mixed hematopoietic chimerism in STZ-induced diabetic mice

1 Week after STZ injection, diabetic mice received BMT with a combination therapy consisting of RGI-2001 and MR1 (combination therapy group) or without the therapy (control group). 2 Weeks after BMT, $H2Kb^+$ donor-derived cells were detected in 21 out of 24 mice (88%) from the combination therapy group, but not in mice from the control group ($n = 4$; Fig. 1a, b). These results show that our protocol can effectively establish mixed chimerism in diabetic mice induced by STZ.

Islet allografts transplanted into chimeric mice can be effective

3 Weeks after BMT, 200 islets from C57BL/6 mice were implanted under the kidney capsule in 18 chimeric mice

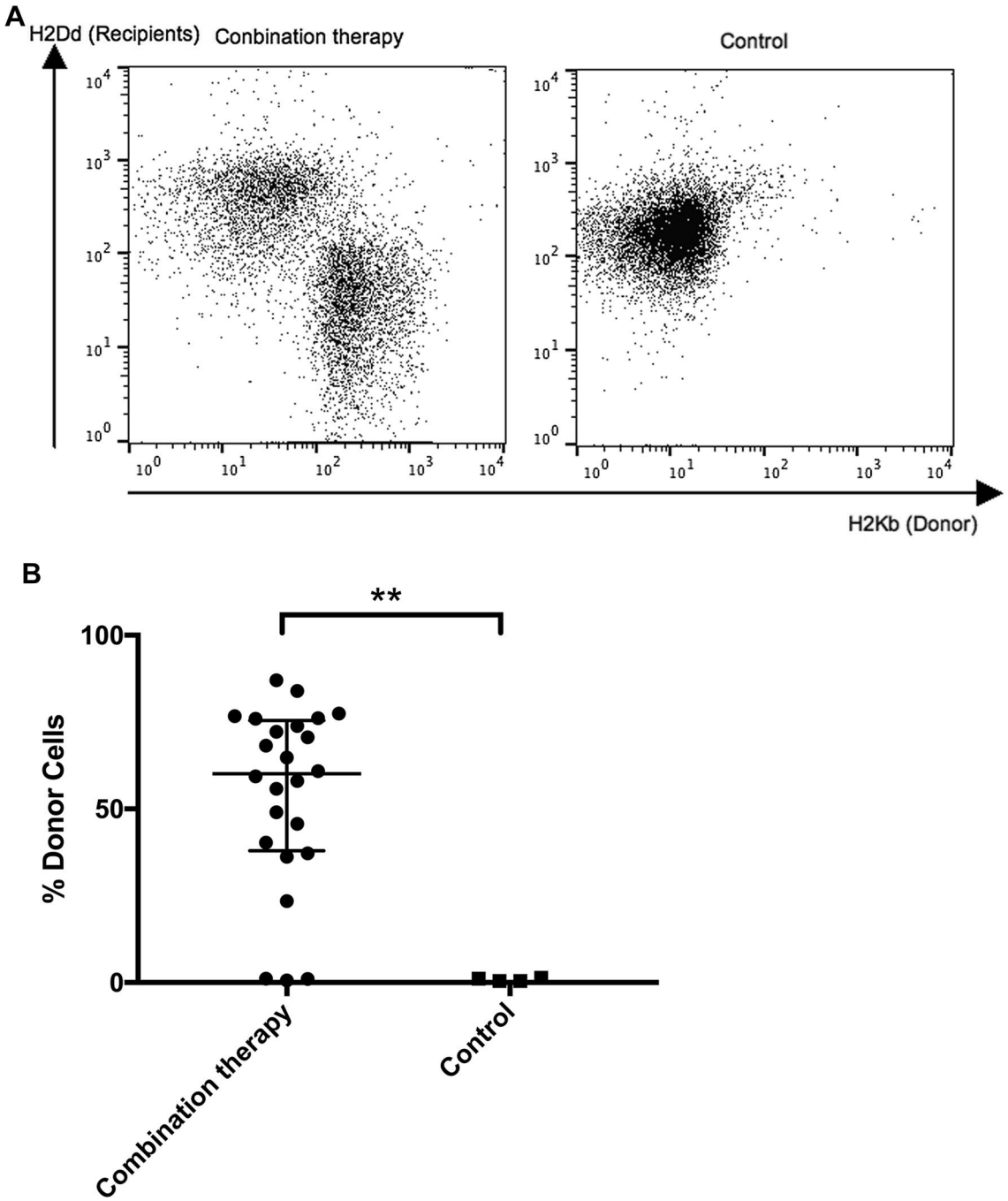

Fig. 1 The percentages of donor-derived cells in peripheral blood. BMT with a combination therapy consisting of RGI-2001 and MR1 established mixed hematopoietic chimerism in diabetic mice. Following BMT, the recipient mice were treated with a combination therapy consisting of RGI-2001 and MR1 (combination therapy group) or received no therapy (control group). The percentages of $H2K^{b+}$ donor-derived cells in peripheral blood were evaluated by flow cytometry 2 weeks after BMT. **a** Representative $H2K^{b+}/H2D^{d+}$ dot plots of the combination therapy and control groups. **b** The proportion of $H2K^{b+}$ donor cells in peripheral blood samples from the combination therapy group ($n=24$) and the control group ($n=4$). Data are presented as median with interquartile range. $^{**}P<0.01$ (Mann–Whitney *U* test). Data are summarized from four independent experiments

from the combination therapy group. Blood glucose levels were decreased in 14 mice (78%) and normoglycemia was maintained until 100 days after islet transplantation (Fig. 2a, solid lines). In the remaining four mice (22%), hyperglycemia was not improved (Fig. 2a, arrowheads). Hyperglycemia was also not improved in the three chimeric mice that did not receive islet transplantation in the combination therapy group (data not shown). All chimeric mice

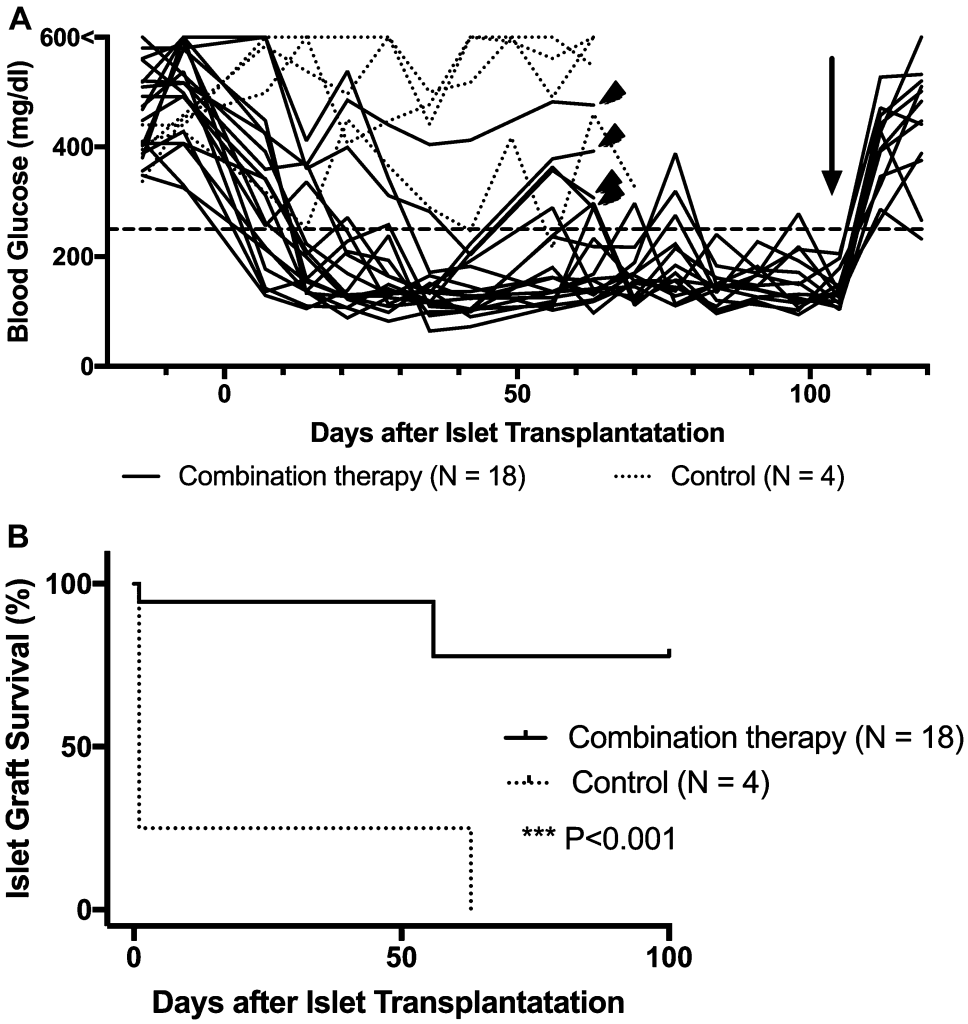

Fig. 2 Islet allografts in chimeric mice established by bone marrow transplantation (BMT) and the combination therapy were effective. 3 Weeks after BMT with administration of a combination therapy consisting of RGI-2001 and MR1 (combination therapy group, $n=18$) or without the therapy (control group, $n=4$), 200 islets from C57BL/6 mice were implanted under the kidney capsule. Blood glucose levels in the combination therapy group (**a**, solid line) and in the control group (**a**, dotted line) were monitored weekly. Hyperglycemia was not improved in four mice from the combination therapy group (**a**, arrowheads). The islet grafts that survived after 100 days of islet transplantation were removed by nephrectomy to confirm graft function (**a**, downward arrow). The difference in the survival rate between the combination therapy group and the control group was statistically significant (**b**, $P<0.0001$, log-rank test). Data are summarized from four independent experiments

were alive until nephrectomy. All mice in the control group ($n=4$) exhibited severe hyperglycemia despite receiving islet transplantation (Fig. 2a, dotted lines). The survival curve is shown in Fig. 2b. The difference in the survival rate between the combination therapy group and the control group was statistically significant ($P<0.001$, log-rank test, Fig. 2b).

These results suggest that islet allografts were effective longitudinally in most mixed chimeric mice receiving BMT and a combination therapy consisting of RGI-2001 and MR1.

Transplanted islet allografts secreted sufficient insulin to improve hyperglycemia

To examine the islet graft function, an intraperitoneal glucose tolerance test was performed. There were no statistically significant differences between the combination therapy group ($n=11$) and naïve BALB/c mice ($n=10$) at all time points (Fig. 3).

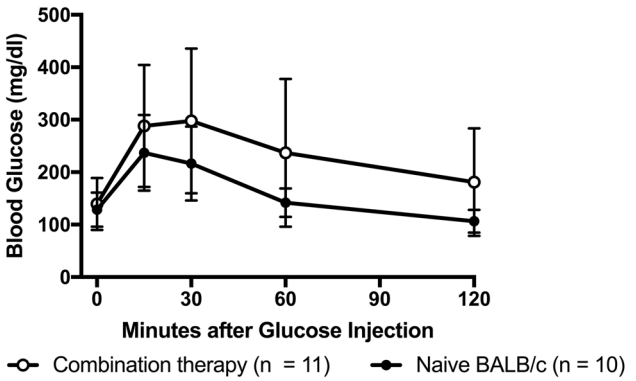

Fig. 3 Glucose tolerance test at 60 days after islet transplantation. The recipient mice treated with a combination therapy consisting of RGI-2001 and MR1 (combination therapy group, open circle, $n=11$) and naïve BALB/c mice (closed circle, $n=10$) were fasted for 2 h and glucose was injected into the peritoneal cavity (0.04 mg/mouse). Blood glucose levels were measured at 0, 15, 30, 60 and 120 min after glucose injection. Data were summarized from two independent experiments. Data are presented as mean ± SD. *$P<0.05$, (Mann–Whitney U test)

To confirm insulin production by islet grafts, we removed them from the recipient mice by nephrectomy 100 days after islet transplantation. Following graft removal, hyperglycemia recurred, suggesting that the islet grafts had been functional (Fig. 2b, downward arrow). In the histological evaluation of the islet grafts from the combination therapy group, PAS staining showed no cellular infiltration, insulin staining was positive, and anti-CD3 staining was negative (Fig. 4, left). In contrast, the islet grafts of the control group disappeared and could not be observed by day 100 after islet transplantation (Fig. 4, right). These results demonstrate that islet graft function

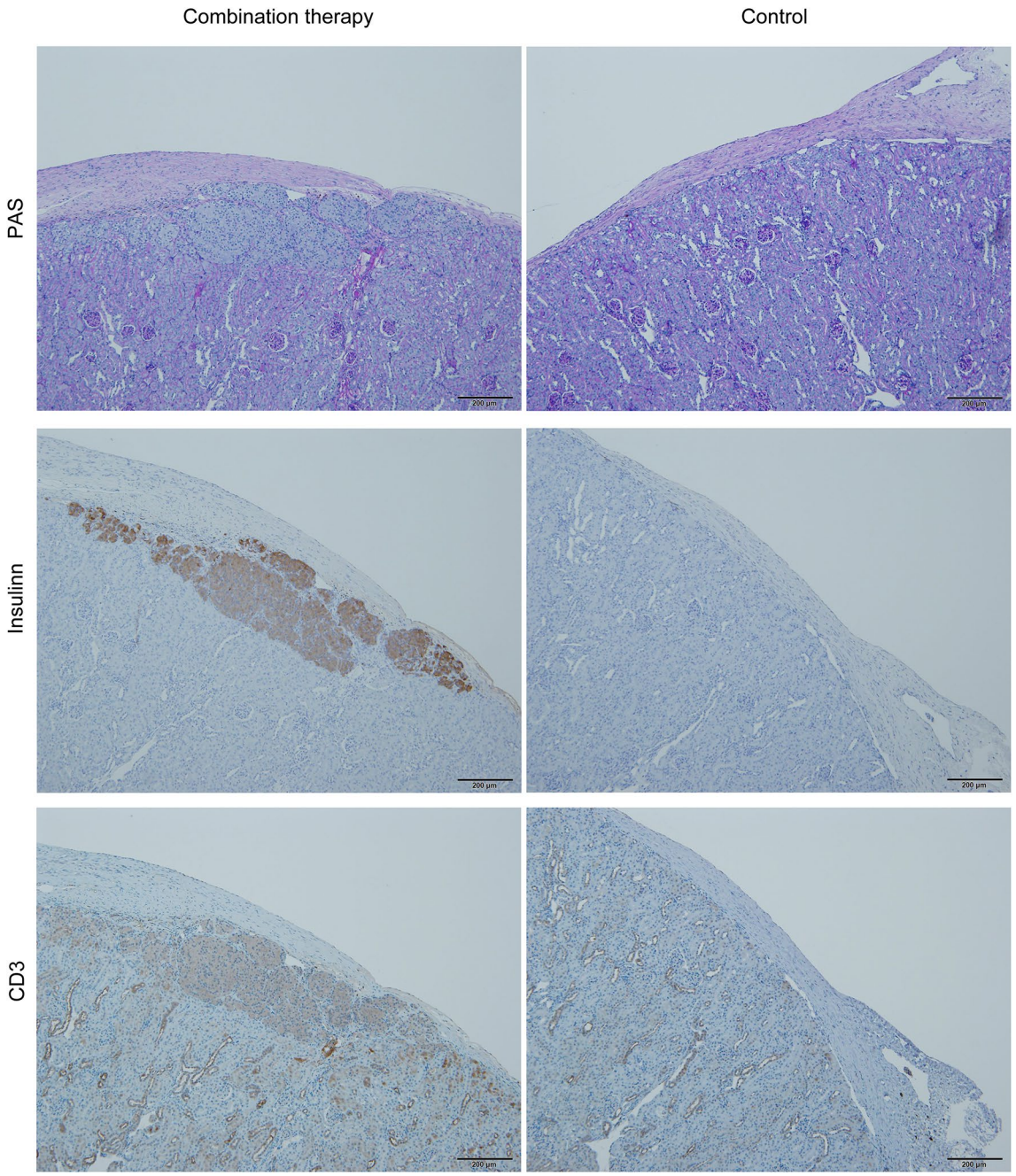

Fig. 4 Histological evaluation of the islet grafts. Islet grafts were removed from the recipients 100 days after islet transplantation by nephrectomy. The upper rows were the representative microscopic images of Periodic acid–Schiff (PAS) staining in the combination therapy group (left, $n=15$) and the control group (right, $n=5$). The middle rows were the representative microscopic images of insulin staining in the combination therapy group (left, $n=5$) and the control group (right, $n=5$). The lower rows were the representative microscopic images of anti CD3 staining in the combination therapy group (left, $n=5$) and the control group (right, $n=5$)

was maintained and improved hyperglycemia in the combination therapy group.

T cells of chimeric mice show hyporesponsiveness to donor antigens

To examine the recipient T-cell response to donor (C57BL/6) and third-party (C3H/He) antigens, we performed mixed lymphocyte reaction assays 100 days after islet transplantation. The combination therapy group ($n=5$) showed T-cell hyporesponsiveness to donor antigens as well as to recipient antigens (Fig. 5a). On the other hand, the control group ($n=4$) showed T-cell hyperresponsiveness to the donor antigens (Fig. 5a; vs. the combination therapy group, $P<0.05$, Mann–Whitney U test). T-cell responsiveness to the third-party antigens did not differ between the two

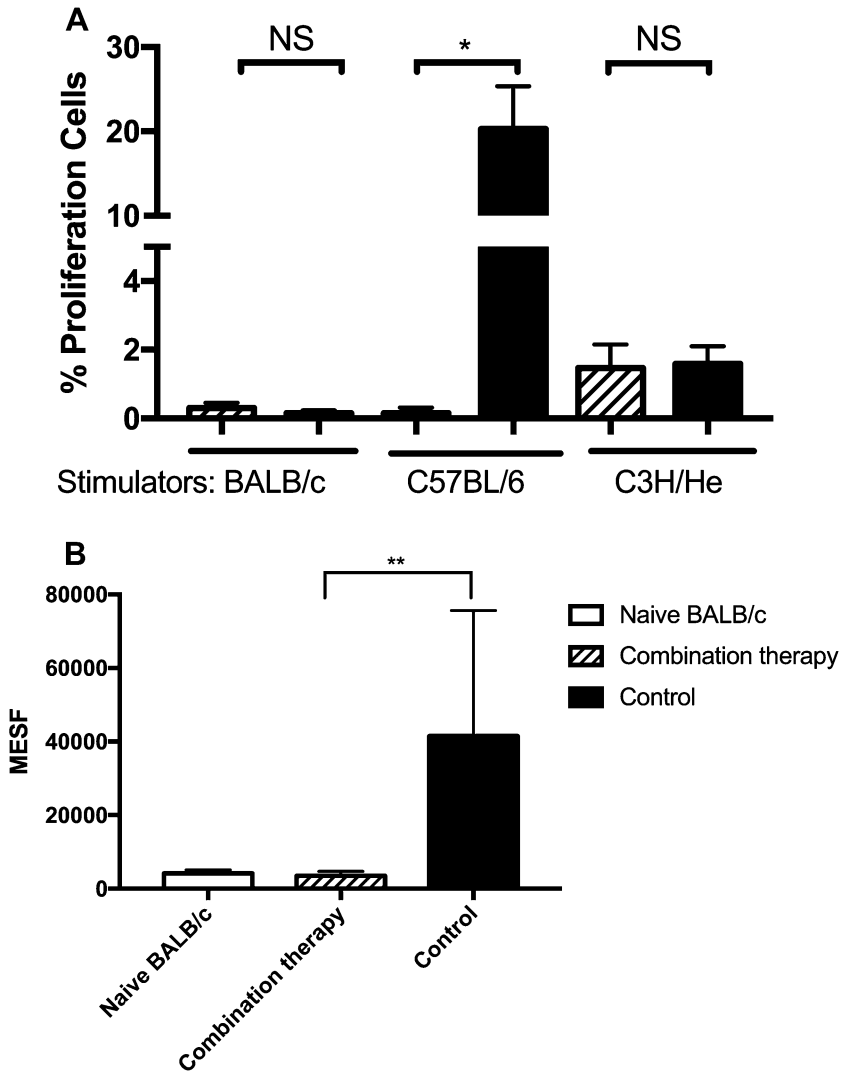

Fig. 5 Chimeric mouse T cells showed hyporesponsiveness to donor antigens while keeping normal responsiveness to third-party antigens and were not sensitized to donor antigens. **a** Mixed lymphocyte reaction assay for evaluation of recipient T-cell responsiveness to donor or third-party antigens. T cells (responders) were isolated from BALB/c recipient splenocytes from the combination therapy group ($n=5$) or the control group ($n=4$) 100 days after islet transplantation, and then labeled with carboxyfluorescein succinimidyl ester (CFSE). Antigen-presenting cells (stimulators) were prepared from the splenocytes of naïve BALB/c (recipient), C57BL/6 (donor), or C3H/He mice (third party), and then irradiated (30 Gy). The responders were incubated with each of the stimulators for 4 days, and the proportion of CFSE-positive cells was calculated using flow cytometry. Data are presented as mean ± SD. $^{*}P<0.05$, *NS* not significant (Mann–Whitney U test). **b** Flow cytometric crossmatch assay for anti-donor antibody detection in the recipients. Recipients' sera from the combination therapy group ($n=16$) or the control group ($n=10$) were incubated 100 days after islet transplantation with naïve C57BL/6 splenocytes. The mean fluorescence intensity of recipient IgG reactive to donor T cells was measured by flow cytometry. The values were standardized to molecules of equivalent soluble fluorochrome (MESF) values. Data were summarized from four independent experiments. Data are presented as mean ± SD. $^{**}P<0.01$ (Mann–Whitney U test)

groups (Fig. 5a). These results indicate that donor-specific immunotolerance was induced in mice from the combination therapy group.

Chimeric mice are not sensitized to donor antigens

Next, we examined the production of anti-donor antibody in the recipients 100 days after islet transplantation to evaluate their humoral immune response. MESFs of anti-donor IgG were comparable between the combination therapy group ($n = 16$) and naïve BALB/c mice ($n = 10$; Fig. 5b). This result shows that mice in the combination therapy group were not sensitized to donor antigens.

On the other hand, the control group exhibited higher MESFs than the control-naïve mice ($P < 0.01$, Mann–Whitney U test, Fig. 5b), suggesting that donor bone marrow cells had been rapidly rejected, and anti-donor IgG was produced in the control group. Taken together, these data demonstrate that allogeneic islet transplant recipients receiving BMT with the combination therapy were not sensitized to donor antigens, even without immunosuppression therapy.

Discussion

In this study, we examined whether our immunotolerance protocol using BMT with a combination therapy consisting of an iNKT cell ligand and CD40-CD154 blockade can be applied to islet transplantation for diabetes mellitus. We performed allogeneic islet transplantation for diabetic mice after BMT and a combination therapy consisting of RGI-2001 and MR1, which successfully established mixed hematopoietic chimerism (Fig. 1a, b) and long-term islet graft survival without immunosuppression therapy (Fig. 2a, b). Our previous study showed that the chimera formation rate was 94% in normal BALB/c mice treated with this protocol [17]. The present study showed that the chimera formation rate in diabetic BALB/c mice was 88% (Fig. 1b), which was similar to that shown in non-diabetic mice in our previous study. These data suggest that our protocol can be applied to diabetic mice as well as non-diabetic mice. In chimeric mice, the percentages of donor cells before islet transplantation were 23–97% (Fig. 1b). This variation had no effects on graft acceptance and function because there were no statistically significant relationships between the percentage of donor cells and the rate of graft survival or the blood glucose level after islet transplantation (data not shown).

We here show that the combination therapy group receiving 200 islets under the kidney capsule had substantially improved blood glucose levels (Fig. 2b). In a previous study of islet transplantation under the kidney capsule in mice, 50 islets were the suboptimal amount combined with insulin therapy; however, 200 islets achieved normoglycemia without insulin treatment [10]. Our study also showed that hyperglycemia was improved by transplanting 200 islets because there were no significant differences between the combination therapy group and naïve BALB/c mice during the glucose tolerance test (Fig. 3).

Moreover, we confirmed donor-specific tolerance induction in the combination therapy group by mixed lymphocyte reaction assay and flow cytometry crossmatch assay. The combination therapy group showed T-cell hyporesponsiveness to donor antigens as well as to recipient antigens (Fig. 5a), and no antibodies to the donor antigens were detected in the recipients' peripheral blood (Fig. 5b). In human islet transplantation, the de novo expression of anti-donor antibodies correlates with lower graft survival [23, 24]. Lorenzo et al. showed that increased expression of post-transplant antibodies including donor-specific alloantibodies and autoantibodies is related to lower rates of graft survival [23]. Brooks et al. showed that de novo expression of donor-specific antibodies was absolutely associated with loss of graft function despite maintained immunosuppression [24]. Increase in posttransplant antibodies after islet transplantation is an important prognostic marker and its identification can potentially lead to improved outcomes [23]. The advantage of preventing this de novo anti-donor antibody expression with our protocol has the potential to achieve long-term graft survival without maintenance immunosuppression therapy.

Thus far, chimerism induction for STZ-induced diabetic mice has been attempted in islet transplantation models. Li et al. reported that stable allogeneic chimerism and long-term islet allograft survival can be achieved by BMT with nonmyeloablative conditioning consisting of total body irradiation (TBI), anti-lymphocyte serum (ALS), and cyclophosphamide [25]. Luo et al. showed that fully stable allogeneic chimeras induced with BMT after nonmyeloablative conditioning with TBI, sirolimus, and lymphocyte-depleting antibodies exhibit robust donor-specific tolerance to islet grafts [26]. The mechanism of our protocol differs from that of these regimens; in our protocol, the combination treatment of iNKT cell activation and CD40–CD154 blockade induces expansion and activation of regulatory T cells associated with chimera establishment [17, 18].

Several tolerance induction approaches have been reported in the spontaneous autoimmune diabetic mice, NOD mice [27–32]. Some protocols achieved chimerism and donor-specific tolerance without radiation conditioning prior to BMT. Liang et al. showed that allogeneic BMT with transfer of donor $CD8^+$ T cells and administration of anti-CD3 monoclonal antibody induced donor-specific tolerance in nonirradiated NOD mice [27]. Racine and Wang et al. have succeeded in establishing a protocol of tolerance induction using major histocompatibility complex (MHC)-mismatched BMT under radiation-free conditions

with anti-CD3 and anti-CD8 monoclonal antibodies [28, 29]. In our protocol, TBI is indispensable for tolerance induction in STZ-induced diabetic mice (data not shown). The next challenge will be to investigate further improvements in our tolerance induction protocol in NOD mice.

In clinical practice, maintenance immunosuppression therapy is thought to prevent graft rejection mediated by allogeneic and autoimmune responses [1, 23, 24]. Induction of mixed hematopoietic chimerism with MHC-mismatched, but not with matched, BMT prevented autoimmune diabetes with thymic deletion of host autoreactive T cells [28] and effectively promoted transplantation tolerance in NOD mice [29]. The MHC-mismatched chimerism induced by BMT not only tolerizes the de novo developed, but also the residual pre-existing host-type T cells, which manifest both alloreactivity and autoreactivity, in a mismatched MHC class II-dependent manner [29]. Nikolic et al. reported that mixed hematopoietic chimerism induces tolerance to donor islets and reverses established autoimmunity in diabetic NOD mice [33]. These previous data suggest that our tolerance induction protocol, which establishes mixed hematopoietic chimerism with allogeneic BMT prior to islet transplantation, has the potential to prevent both autoimmunity and alloimmunity without maintenance immunosuppression therapy.

Although here we show that the tolerance induction protocol with combination therapy of RGI-2001 and anti-CD154 antibody promoted islet allograft acceptance in an STZ-induced diabetic mouse model, further studies are necessary to clarify the application of the protocol to islet transplantation. We have to evaluate the efficacy of the tolerance induction protocol in different diabetic models, such as autoimmune and other animal models. We should also investigate the effect of the protocol on engraftment of donor islets after infusion into the recipient's portal vein, which is commonly used in clinical islet transplantation. These further studies could also contribute to the establishment of a more appropriate transplantation tolerance procedure for islet transplantation based on the combination therapy of RGI-2001 and anti-CD154 antibody. Additionally, important issues still need to be addressed before applying this protocol in clinical practice. One is that clinical application of anti-human CD154 antibody has been hampered by severe thromboembolic complications [34]. However, Kim et al. showed that a novel anti-CD154 antibody lacking Fc-binding activity (Fc-silent anti-CD154 antibody) was as equally potent as the conventional anti-CD154 antibody at prolonging renal allograft survival, without evidence of thromboembolism in a nonhuman primate preclinical model [35]. Our protocol could, therefore, be modified using the Fc-silent anti-CD154 antibody to induce immunotolerance without the risk of thromboembolism.

In conclusion, our immunotolerance protocol consisting of BMT and the combination therapy of an iNKT cell ligand and CD40–CD154 blockade promoted islet allograft acceptance in a mouse diabetic model. This suggests that this protocol may have the potential to prevent alloimmunity without the need for immunosuppression therapy, and thus can be clinically applied to islet transplantation for type 1 diabetes mellitus.

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Compliance with ethical standards

Conflict of interest Y. Ishii is the executive chairman of REGiMMUNE Corp. The other authors declare that they have no conflict of interest.

Human and animal right disclosure Animal studies were approved by the Tokyo Women's Medical University internal committee on the use and care of laboratory animals approved all experiments (Reference ID: AE16-105-2). All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985) as well as the current version of the Japanese law "Act on Welfare and Management of Animals" (revised 2013).

Informed consent For this type of study formal informed consent is not required.

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