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ORIGINAL ARTICLE

Suppressing memory T cell activation induces islet allograft tolerance in alloantigen-primed mice

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Summary

Memory T cells are known to play a key role in prevention of allograft tolerance in alloantigen-primed mice. Here, we used an adoptively transferred memory T cell model and an alloantigen-primed model to evaluate the abilities of different combinations of monoclonal antibodies (mAb) to block key signaling pathways involved in activation of effector and memory T cells. In the adoptively transferred model, the use of anti-CD134L mAb effectively prevented activation of $CD4^+$ memory T cells and significantly prolonged islet survival, similar to the action of anti-CD122 mAb to $CD8^+$ memory T cells. In the alloantigen-primed model, use of anti-CD134L and anti-CD122 mAbs in addition to co-stimulatory blockade with anti-CD154 and anti-LFA-1 prolonged secondary allograft survival and significantly reduced the proportion of memory T cells; meanwhile, this combination therapy increased the proportion of regulatory T cells (Tregs) in the spleen, inhibited lymphocyte infiltration in the graft, and suppressed alloresponse of recipient splenic T cells. However, we also detected high levels of alloantibodies in the serum which caused high levels of damage to the allogeneic spleen cells. Our results suggest that combination of four mAbs can significantly suppress the function of memory T cells and prolong allograft survival in alloantigen primed animals.

Introduction

Islet transplantation has become a common surgical procedure since the Edmonton protocol was established in 1999 [1] for treatment of insulin-dependent diabetics. While the success of the procedure is initially high at around 90%, the proportion of patients achieving insulin independence with this procedure is reduced to 10% by the fifth year after transplantation [2,3], at which time the patients would require a second transplant. Although little has been reported concerning changes in islet graft survival between the first and second transplantations, a significant reduction in renal graft survival is observed between the secondary and tertiary transplantations (82.0% survival following primary transplantation versus 55.5% survival following secondary transplantation versus 27.0% survival following tertiary transplantation) [4].

Memory cells that cause graft rejection mainly develop through previous transplantation, but they can also develop from blood transfusions, pregnancies, or continuous exposure to bacterial and viral pathogens. Nearly half of adult T cells have a memory phenotype [5–7] capable of responding to allogeneic cells and mediating allograft rejection by cross-reactivity [8]. Among the many strategies being investigated to prevent rejection, use of agents to block co-stimulatory molecules to induce tolerance are promising; however, it was suggested that the failure of these strategies in older animals may be because of the

increased proportion of memory T cells in these hosts compared with young rodents housed in specific pathogen-free conditions [9].

Two main subsets of memory T cells are $CD4^+$ and $CD8^+$ memory T cells with the phenotype of $CD44^{high}$ in mice. The memory T cells are more easily activated than naïve T cells, and recent studies have shown potential ways to suppress them. For instance, the activation of $CD4^+$ memory T cells relies on the CD134/CD134L (OX40/OX40L) pathway [10,11]. Vu *et al.* found that memory T cells, generated by either homeostatic proliferation or donor antigen priming, induced prompt skin allograft rejection with CD28/CD154 blockade alone, but blocking the CD134/CD134L pathway in combination with CD28/CD154 blockade induced long-term skin allograft survival [12]. $CD122^+CD8^+$ memory T cells were also found to play an important role in secondary skin transplantation, and additional use of depleting CD122 mAb can significantly prolong survival of the engrafted skin [13].

Since graft rejection in primary transplantation is mainly mediated by T cells, in this study we attempted to block both effector and memory T cells following secondary transplantation in order to induce allo-specific tolerance. Use of appropriate models for investigating mechanisms of rejection and approaches for preventing it is important for successful translation into clinical therapies. Adoptive transfer models are classical models for studying memory cells *in vivo* [14–19]. As there are few reports on secondary islet allograft transplantations, the alloantigen-primed model may also provide us with an additional experimental framework which would be more similar to the clinical situation and give a meaningful evaluation of immunosuppressive protocols. Thus, we endeavored to induce islet graft tolerance in an antigen-primed transplantation model by the use of non-depleting anti-CD134L mAb and depleting anti-CD122 mAb to suppress the function of $CD4^+$ and $CD8^+$ memory T cells, respectively. In addition, we used anti-CD154 and anti-LFA-1 mAb to block activation of naïve T cells, which displayed promising effects in our preliminary experiments. Our results demonstrated that blockade of the CD134/CD134L pathway and depletion of $CD122^+$ cells can provide a highly tolerant environment for islet allografts and prolong their survival in the alloantigen-primed model.

Materials and methods

Animals

All animals were purchased from Slac Laboratory Animal Co. Ltd (Shanghai, China). Female B6 ($H\text{-}2K^b$) and BALB/c ($H\text{-}2K^d$) mice (8–12 weeks old) were used as graft recipients and donors respectively. Female C3H ($H\text{-}2K^k$) mice (8–12 weeks old) was used as source of the third party stimulator cells in mixed lymphocyte reactions (MLR) test. All animals were maintained and bred in the specific pathogen-free facility, and procedures followed NIH publication 'Principles of Laboratory Animal Care'.

Antibodies

All administered antibodies were produced by Bioexpress (West Lebanon, NH, USA), including antibodies to CD154 (MR-1, M), LFA-1 (M17/4, L), CD134L (RM134L, R), CD122 (TM-β1, T), and their respective isotype controls. Antibodies for flow cytometric analysis, including FITC anti-CD4 (GK1.5), FITC anti-CD8 (53-6.7), PE anti-CD44 (IM7), PECy5-CD62L (MEL14), and their isotype controls were purchased from BioLegend (San Diego, CA, USA). The mouse regulatory T cell staining kit was purchased from eBioscience (San Diego, CA, USA).

Chemical induction of diabetes

A 180–220 mg/kg dose of streptozocin (STZ, Sigma-Aldrich, St Louis, MO, USA) in 0.1 M citrate buffer (pH 4.4) was injected intraperitoneally (i.p.) to induce diabetes in recipients, as previously described [20]. Blood glucose was measured using a One Touch glucose analyzer (FreeStyle, Abbott, IL, USA). Diabetes onset was defined as two consecutive daily blood glucose measurements of >16.7 mM.

Islet isolation, purification, and transplantation

Donor islets were isolated and transplanted by kidney subcapsular injection as previously described [21]. Briefly, the pancreas was perfused via bile duct cannulation with 3 ml 1.5 mg/ml collagenase V (Sigma-Aldrich) and excised. The pancreas was digested at 37 °C for 20 min then shaken vigorously in cold Hank's balanced salt solution (HBSS). The suspension was allowed to settle, and the sediment was washed three times in HBSS. Islets were sorted manually under a microscope, collected in vials of 600 islets, centrifuged at 92 *g* for 2 min, and transplanted using a 1.2-mm diameter venflon (Becton Dickinson, Stockholm, Sweden) under the kidney capsule of diabetic B6 mice. Islet transplants were considered functional with two consecutive blood glucose measurements <8 mM. The time of islet rejection was defined as the first day of two consecutive blood glucose measurements >11.1 mM.

Adoptively transferred memory T cell model and alloantigen-primed model

Memory $CD4^+$or $CD8^+$ T cells were purified by auto-MACS flow cytometry from BALB/c mice 4 weeks after

B6 lateral thoracic skin transplantation (alloantigen-primed model), at which time all animals had rejected their grafts. $CD4^+CD62L^-CD44^{high}$ memory T cells were isolated using the MagCellect Mouse Memory $CD4^+$ T Cell Isolation Kit (Cat. No. MAGM206, R&D, Minneapolis, MN, USA). $CD8^+$ T cells were first isolated from spleen cells by using the MagCellect Mouse $CD8^+$ T Cell Isolation Kit (Cat. No. MAGM 203, R&D). These isolated cells were then incubated with PECy5 anti-CD62L and PE anti-CD44 and sorted by flow cytometry after gating on the $CD44^{high}CD62L^-$ population. The memory $CD4^+$ or $CD8^+$ T cells were further confirmed by staining with FITC anti-CD4 or FITC anti-CD8, PE anti-CD44 and PECy5 anti-CD62L. The purities of these cells were typically greater than 85% for memory $CD4^+$ and 90% for memory $CD8^+$ T cells detected by flow cytometry (Fig. S1). The viability of these cells was greater than 90% detected by trypan blue. Both $CD4^+$ and $CD8^+$ memory T cells were transferred to diabetic B6 mice by a single intravenous injection (2×10^6 cells/recipient). On the day following the adoptive transfer, 600 BALB/c islets were transplanted under the kidney capsule of the recipient.

In the alloantigen-primed model, diabetes was induced in alloantigen-primed mice, and 600 BALB/c islets were transplanted under the kidney capsule. Treatment consisted of antibodies to CD154, CD134L, CD122 (0.25 mg respectively) and 0.1 mg anti-LFA-1 in various combinations on days 0, 2, 4, and 6 after transplantation (Table 1). Control mice were treated with isotype antibodies.

Pathology studies

Grafts were dissociated from recipient mice at day 5 after transplantation. Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) and examined by a transplant pathologist who was blinded to treatment modality. Representative specimens ($n = 27$) from all treatment modalities were ranked from 1 to 27 (from least to most) for overall rejection/inflammation, with the median in each group presented.

Table 1. Various treatment combinations used in this study.

Recipient mice	Treatment combinations*
Naïve	M+L
$CD4^+$ T cells transferred	M+L or M+L+R
$CD8^+$ T cells transferred	M+L or M+L+T
Alloantigen-primed	M+L or M+L+R or M+L+R+T

*Treatments consisted of 0.25 mg anti-CD154 (M), 0.1 mg anti-LFA-1(L), 0.25 mg anti-CD134L (R), and 0.25 mg anti-CD122 (T) in various combinations as indicated and were administered i.p. on days 0, 2, 4, and 6 after transplantation.

Mixed lymphocyte reactions

T lymphocytes were isolated from spleens of B6 mice using nylon wool columns (Wako, Osaka, Japan) and used as responder cells. Donor BALB/c or third party C3H spleen cells were used as stimulator cells and treated with mitomycin (40 μg/ml, Amresco, Cleveland, OH, USA) before use in the MLR assay. After being mixed (responders:stimulators = 1:10), the cells were cultured at 37 °C for 3 days, and proliferation was quantified using a BrdU ELISA kit (Chemicon, Temecula, CA, USA). Assays were performed in triplicate.

Enzyme-linked immunosorbent assay

Sera from recipient mice ($n = 3$ mice/group) were isolated on day 5 post-transplantation. Enzyme-linked immunosorbent assays (ELISAs) were performed using commercially available kits (Shanghai Yikesai Bioproduct Limited Company, China) to detect IL-2, IFN-γ, IL-10, and TGF-β according to the manufacturer's instructions. Briefly, flat-bottomed 96-well ELISA plates were coated with antibodies against mouse IL-2, IFN-γ, IL-10, or TGF-β antibody in PBS and incubated overnight at 4 °C. After washing and blocking, diluted samples and a biotin conjugate were applied to the wells and incubated for 2 h. After washing, the plates were incubated with Streptavidin-HRP for 1 h at room temperature. Samples were developed using a 3,3′,5,5′-tetramethylbenzidine substrate solution, and the reaction was stopped with 2 M sulfuric acid. ELISA plates were read using a microplate reader at 450 nm.

Quantitative real-time PCR

Grafts were removed from recipients at day 5 after transplantation, and the RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription and quantitative real-time PCR (qRT-PCR) were performed using commercially available reagents (TOYOBO, Osaka, Japan). The StepOne Real-Time PCR System (ABI, Carlsbad, CA, USA). Syber Green I was used to detect amplification, and β-actin was used as a normalizing control. Calculation was performed using the $2^{-\Delta\Delta CT}$ method. Each reaction was carried out in triplicate. The primer sequences used for the qRT-PCR are listed in Table 2.

Microlymphocytotoxicity assay

The following reagents were added to each well of a 96-well plate for the microlymphocytotoxicity (MLC) assay: 3 μl mineral oil, 1 μl serum from alloantigen-primed islet

Table 2. Primer sequences used for qRT-PCR.

Primer name	Sequences
β-actin	Forward 5′-CATCCGTAAAGACCTCTATGCCAAC-3′
	Reverse 5′-ATGGAGCCACCGATCCACA-3′
IFN-γ	Forward 5′-CGGCACAGTCATTGAAAGCCTA-3′
	Reverse 5′-GTTGCTGATGGCCTGATTGTC-3′
IL-2	Forward 5′-GGAGCAGCTGTTGATGGACCTAC-3′
	Reverse 5′-AATCCAGAACATGCCGCAGAG-3′
IL-10	Forward 5′-GACCAGCTGGACAACATACTGCTAA-3′
	Reverse 5′-GATAAGGCTTGGCAACCCAAGTAA-3′;
TGF-β	Forward 5′- TGACGTCACTGGAGTTGTACGG -3′
	Reverse 5′- GGTTCATGTCATGGATGGTGC -3′

transplantation mice, 1 μl serum from guinea pig, and 1 μl of a BALB/c spleen cell (2×10^3 cells/μl). After incubation at 22–25 °C for 45 min, cells were stained using a Trypan Blue staining Cell Viability Assay Kit (Beyotime Institute of Biotechnology, Haimen, China), and the cell death rate was calculated by counting random visual fields. In control wells, instead of 1 μl of serum from alloantigen-primed islet transplantation mice, 1 μl of serum from naïve mice was used. Each reaction was carried out in triplicate.

Statistical analysis

Mean survival time (MST) was analyzed using the Kaplan–Meier method. The Mann–Whitney *U*-test was used to compare the ranks of rejection/inflammation changes in each group. Data from the MLR, ELISA, flow cytometry, qRT-PCR, and MLC experiments were analyzed using one-way analysis of variance and expressed as mean values±standard deviation (SD). Because multiple comparisons were made during the analysis, a Bonferroni correction was calculated and applied. A value of $P < 0.05$ was considered to indicate a statistically significant difference, with $P < 0.01$ and $P < 0.001$ indicating highly statistically significant differences. All analyses were performed using the GRAPHPAD PRISM® (GraphPad, Inc., La Jolla, CA, USA) software.

Results

Antibodies to CD134L and CD122 suppress secondary graft rejection mediated by $CD4^+$ and $CD8^+$ memory T cells, respectively

In order to determine the suppressive effect of anti-CD134L on $CD4^+$ memory T cells and anti-CD122 on $CD8^+$ memory T cells, we transferred allogeneic $CD4^+$ or $CD8^+$ memory T cells to diabetic B6 mice prior to transplantation of BALB/c islets ($n = 6$ per group). After transplantation, mice received therapy with antibodies to CD154 (M), LFA-1 (L), CD134L (R), and CD122 (T) in various combinations. After receiving M+L therapy, the results showed that naïve recipients accepted islet allografts, but recipients of adoptively transferred $CD4^+$ memory T cells rejected the allografts with a MST of 14.17 days; meanwhile, the M+L+R group rejected the allografts with a MST of >60 days (Fig. 1a, $P < 0.001$). In recipients of adoptively transferred $CD8^+$ memory T cells, the MST of the allografts with M+L treatment was 12 days, whereas M+L+T therapy resulted in significant prolongation of graft survival, with four of six grafts surviving >60 days (Fig. 1b, $P < 0.001$).

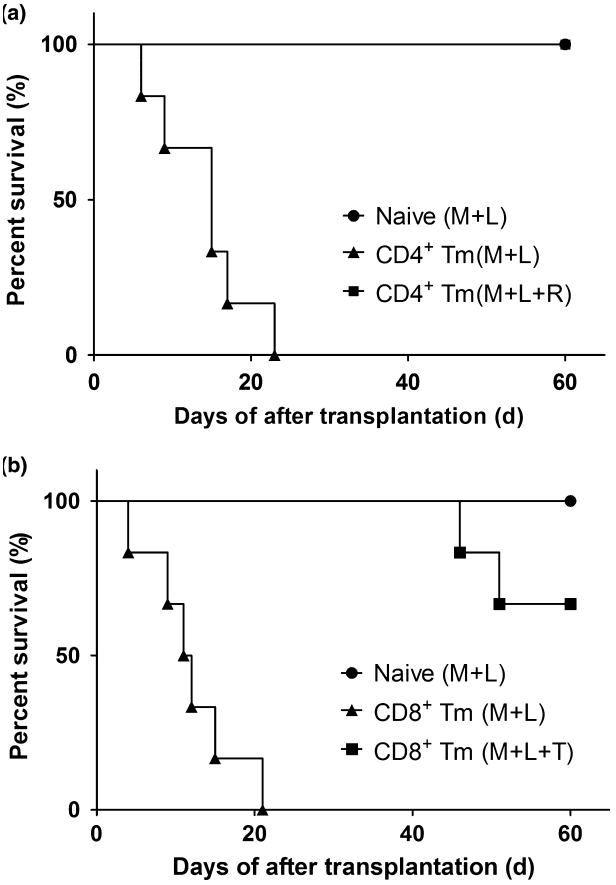

Figure 1 Anti-CD134L mAb suppresses secondary graft rejection mediated by $CD4^+$ memory T cells (a) and anti-CD122 mAb suppresses secondary graft rejection mediated by $CD8^+$ memory T cells (b). Memory T cells are abbreviated as Tm. Allogeneic $CD4^+$ or $CD8^+$ memory T cells were transferred into recipients before islet transplantation ($n = 6$ mice per group). Treatments consisted of various indicated combinations of 0.25 mg anti-CD154 (M), 0.1 mg anti-LFA-1 (L), 0.25 mg anti-CD134L (R), and 0.25 mg anti-CD122 (T) and were administered i.p. on days 0, 2, 4, and 6 after transplantation. The time of islet rejection was defined as the first day of two consecutive blood glucose measurements >11.1 mM. MST was analyzed using the Kaplan–Meier method.

Anti-CD134L with or without anti-CD122 prolongs islet allograft survival in alloantigen-primed mice

After inducing diabetes with STZ treatment, alloantigen-primed B6 mice were transplanted with BALB/c islets and then given various combination therapies ($n = 6$ per group). Compared with the MST of 4.83 days in the M+L group, that of the M+L+R and M+L+R+T groups were both prolonged at 8.33 and 16.2 days, respectively (Fig. 2, $P < 0.001$).

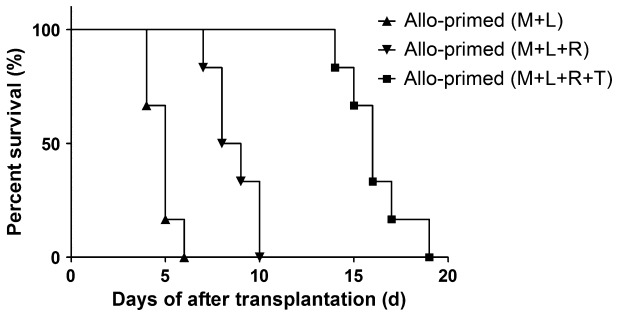

Figure 2 Anti-CD134L with or without anti-CD122 prolongs islet allograft survival in alloantigen-primed mice. Treatments consisted of various indicated combinations of 0.25 mg anti-CD154 (M), 0.1 mg anti-LFA-1 (L), 0.25 mg anti-CD134L (R), and 0.25 mg anti-CD122 (T) and were administered i.p. on days 0, 2, 4, and 6 after transplantation. The time of islet rejection was defined as the first day of two consecutive blood glucose measurements >11.1 mM. MST was analyzed using the Kaplan–Meier method.

Pathological examination of islet allografts

Grafts were dissociated from recipient mice at day 5 after transplantation and routinely processed for histology. Grafts from the M+L group showed thorough damage of islets and were filled with infiltrating lymphocytes (Fig. 3a), whereas grafts in the M+L+R group showed less islet damage and much fewer infiltrating lymphocytes (Fig. 3b). Grafts in the M+L+R+T group showed well-preserved islets without significant evidence of degeneration or destruction and with much less lymphocyte infiltration (Fig. 3c). The overall ranking of rejection/inflammation is shown in Fig. 3d. The rankings given for the M+R+L and M+R+L+T groups were significantly lower than those for M+L groups ($P < 0.05$ in each comparison) and were lowest for the M+L+R+T group.

Production of cytokines related to rejection or tolerance in the grafts

In order to detect the effects of different mAb combinations on the level of rejection or tolerance-associated cytokines in the grafts, we prepared total allograft mRNA for measurement of cytokine transcript expression by using qRT-PCR. Compared with the M+L group, the additional use of anti-CD134L mAb significantly reduced the expression of IL-2 in the allograft, and there was a marked downregulation of the IFN-γ mRNA level in the M+L+R+T group. Compared with the M+L group, the

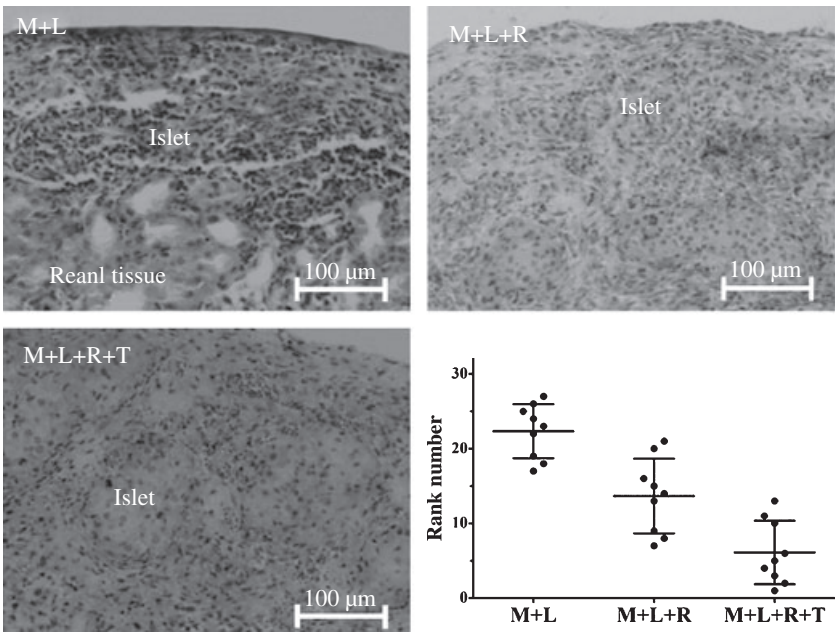

Figure 3 Pathological evaluation of islet allografts from alloantigen-primed mice treated with combination therapies. Islet grafts were dissociated from recipient mice at day 5 after transplantation, and paraffin-embedded tissue sections were stained with H&E. Representative specimens ($n = 27$) from all treatment modalities were ranked from 1 to 27 (from least to most) for overall rejection/inflammation, and the median in each group is shown in the graph (lower right). $^{**}P < 0.01$ when compared between the M+L+R treatment group to the M+L+R+T treatment group or control group, and $^{***}P < 0.001$ when compared between the M+L+R+T treatment group to control group.

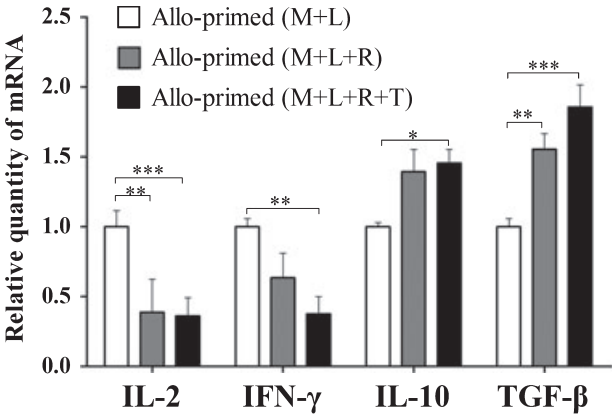

Figure 4 The level of rejection/tolerance associated with cytokines in the grafts of recipients. Grafts were removed from recipient mice at day 5 after transplantation and the mRNA levels of IL-2, IFN-γ, IL-10, and TGF-β were detected by qRT-PCR. Each reaction was carried out in triplicate. Data are shown as the mean±SD and are representative of three separate experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

expressions of IL-10 and TGF-β were significantly increased in the M+L+R and M+L+R+T groups (Fig. 4, $P < 0.05$).

Proportion of memory T cells and $CD4^+Foxp3^+$ Tregs in spleens of recipient mice

To investigate the effects of the combination antibody therapies on changes in memory T cells and $CD4^+Foxp3^+$ Tregs in spleens of recipient mice, splenic T cells from recipient mice were detected 5 days after transplantation by flow cytometry. Compared with the M+L group, the proportions of $CD4^+$ memory T cells/$CD4^+$ T cells in both the M+L+R and M+L+R+T groups were significantly reduced (36.73% in M+L group vs. 20.97% in M+L+R group and 23.33% in M+L+R+T group), and the proportions of $CD8^+$ memory T cells/$CD8^+$ T cells were also significantly reduced (46.93% in M+L group vs. 30.67% in M+L+R group and 18.60% in M+L+R+T group). Of note, the proportion of $CD8^+$ memory T cells/$CD8^+$ T cells in the M+L+R+T group was much lower than that in the M+L+R group (Fig. 5a). By contrast, the proportion of Tregs in the total splenic T cells increased in the M+L+R (1.09%) and M+L+R+T (1.97%) groups as compared with the M+L group (0.72%), and the proportion in the M+L+R+T group was nearly twice that of the M+L+R group (Fig. 5b).

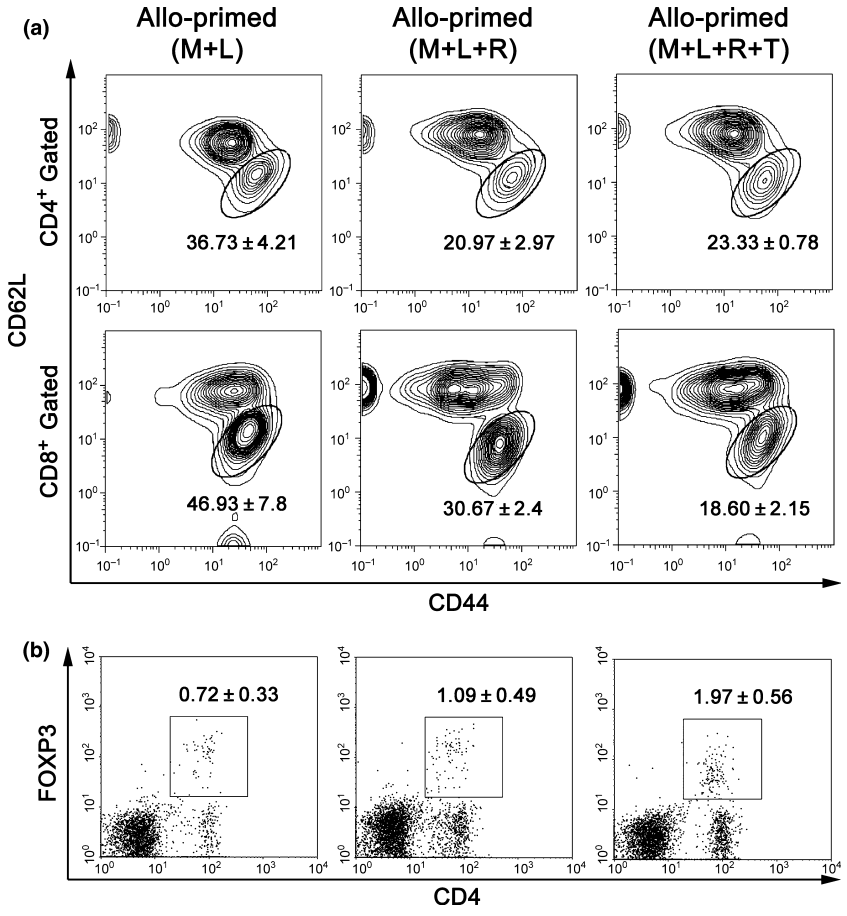

Figure 5 The proportion of memory T cells and $CD4^+Foxp3^+$ Tregs in recipient splenic T cells. (a) Proportion of $CD4^+$ memory T cells/$CD4^+$ T cells and $CD8^+$ memory T cells/$CD8^+$ T cells after therapy. $**P < 0.01$ when comparing the proportion of $CD4^+$ memory T cells between treatment groups (M+L+R and M+L+R+T) to control group, and the proportion of $CD8^+$ memory T cells between the M+L+R+T treatment group to the control group. $*P < 0.05$ when comparing the proportion of $CD8^+$ memory T cells between the M+L+R treatment group to the M+L+R+T treatment group or control group. (b) Proportion of $CD4^+Foxp3^+$ Tregs/splenic T cells of alloantigen-primed recipient mice. $*P < 0.05$ when compared between M+L+R treatment group to control group or M+L+R+T treatment group, and $**P < 0.01$ between M+L+R+T treatment group to control group. Data are all representative of three separate experiments.

Combined antibody therapy induces allo-specific tolerance in recipient mice

Mixed lymphocyte reaction assays were used to test the response of recipient T cells to allogeneic lymphocytes (Fig. 6a and b), and the concentrations of IL-2, IFN-γ, IL-10, and TGF-β in recipient mouse sera were tested by ELISA (Fig. 6c). As shown in Fig. 6a, the proliferation of recipient T cells responding to allogeneic lymphocytes was much lower in the M+L+R and M+L+R+T groups than in the M+L group, with that of the M+L+R+T group being lower than the M+L+R group. By contrast, the response of recipient cells to third party C3H cells was still strong after combination treatments (Fig. 6b). Furthermore, the use of combined therapy significantly reduced IL-2 and IFN-γ concentrations and increased IL-10 and TGF-β concentrations in alloantigen-primed recipient mouse sera (Fig. 6c). As the addition of anti-CD122 greatly enhanced these effects on cytokine production, these results suggest that combined therapy inhibited the responder cells of allogeneic-primed mice, and that these suppressive effects could be significantly enhanced by anti-CD122.

Lethal alloantibodies in alloantigen-primed recipient mice

To determine whether alloantibodies contribute to the failure of long-term tolerance of allografts in alloantigen-primed mice, we performed MLC assays with the sera of alloantigen-primed recipient mice collected at day 5 after transplantation. The M+L, M+L+R, and M+L+R+T groups all had high cell death rates compared with the negative control (69% in M+L group, 48.6% in M+L+R group, and 42% in M+L+R+T group vs. 9.6% in negative control) (Fig. 7).

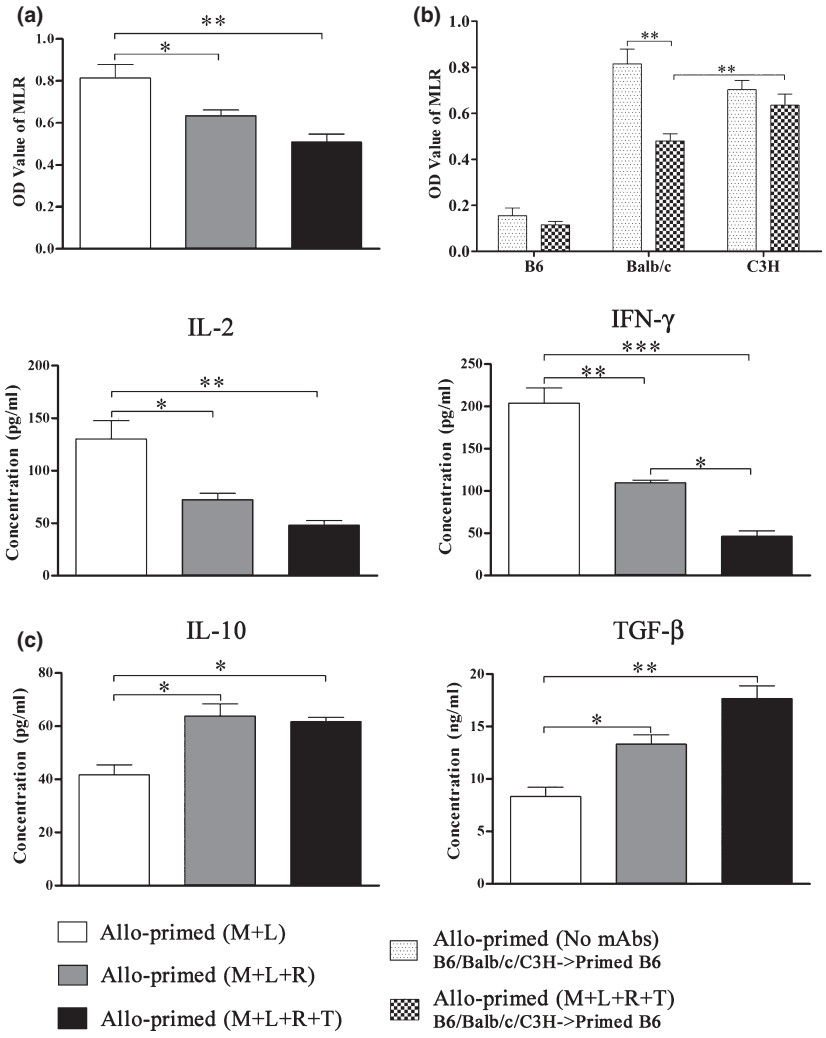

Figure 6 Combined therapies influenced the response of recipient splenic T cells to allogenic lymphocytes and the level of various cytokines in serum of recipients. MLR assays were used to test the proliferative responses of recipient splenic T cells to donor BALB/c (a) or third party C3H lymphocytes (b), and ELISAs were used to test the concentrations of IL-2, IFN-γ, IL-10, and TGF-β in recipient mouse serum (c). Each reaction was carried out in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

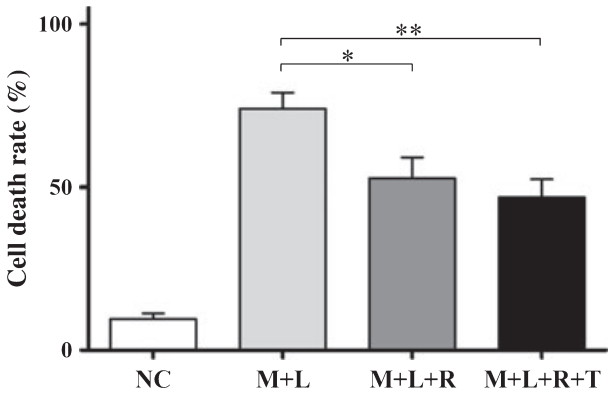

Figure 7 Microlymphocytotoxicity assays of alloantigen-primed recipient sera. The sera of alloantigen-primed recipients were prepared at day 5 after transplantation and were cultured with recipient spleen lymphocytes and sera of guinea pigs for 45 min. The cells were then stained with trypan blue and cell death rate was calculated by counting. Each reaction was carried out in triplicate. NC, negative control. $*P < 0.05$; $**P < 0.01$.

Discussion

In allograft transplantations, reducing activation of memory T cells is critical for survival of the graft and clinical outcome for the recipient. Fortunately, in recent years studies of regulatory pathways have increasingly promoted promising ways to control the allo-memory T cells in an effort to prevent allograft rejection. The CD134/CD134L pathway has been shown to play a key role in the generation, survival, and activation of $CD4^+$ memory T cells [12,22–24], and $CD122^+CD8^+$ memory T cells are an important subset of $CD8^+$ memory T cells that mediate secondary transplantation in alloantigen-primed mice [13]. In this study, we used an anti-CD134L mAb to inhibit the CD134/CD134L pathway and an anti-CD122 mAb to deplete $CD122^+CD8^+$ memory T cells. Both of these treatments have dominant suppressive effects on $CD4^+$ or $CD8^+$ memory T cells in the adoptively transferred models (Fig. 1), respectively. The results are consistent with those of Vu *et al.* [12] and Minamimura *et al.* [13].

What we truly wanted to know, however, was whether these strategies could still be effective in the alloantigen-primed model. In the clinic, a patient who acquires and retains allo-specific memory cells will have short survival of islet transplants and will require a second or even third transplant. Therefore, it becomes necessary to find strategies to prolong the graft survival in these patients. Here, we tested antibodies to CD134L and CD122 together in alloantigen-primed mice, and as our results indicated, the additional use of these two mAbs can promote longer survival of the allograft than with treatment with only anti-CD154 and anti-LFA-1. While this enhanced combination therapy could not induce islet allograft tolerance (Fig. 2), our data now demonstrate that the survival of islet allografts could be prolonged in the alloantigen-primed model mainly by use of the anti-CD134L and anti-CD122 mAbs (Fig. 5). As the CD134/CD134L pathway plays a critical role in the generation and survival of $CD4^+$ memory T cells [22–24], and a considerable proportion of $CD44^{high}CD8^+$ memory T cells that are involved in the memory immune response stably express CD122 [25,26], it is logical that the additional use of anti-CD134L and anti-CD122 could effectively reduce the proportion of effector memory ($CD44^{high}CD62^{low}$) T cells (Fig. 5) in alloantigen-primed recipients. Our data also showed that the proportion of Tregs in the spleen increased when the CD134/CD134L pathway was blocked with anti-CD134L, which was consistent with hypothesis provided by Chen and Xiao *et al.* [27,28].

Effector memory T cells can recirculate in peripheral tissues and will be rapidly recruited to initiate early responses at the graft site [29]. $CD8^+$ memory T cells specific for alloantigens exhibit rapid effector functions, faster proliferation, increased responses to low antigen doses and direct cytolytic activity compared with allospecific naïve CD8 T cells [30,31]. In our study, the anti-CD122 mAb which was used to deplete $CD122^+CD8^+$ memory T cells could effectively prevent lymphocyte infiltration to the allograft and showed well-preserved islets without significant evidence of degeneration or destruction (Fig. 3). This finding demonstrated the effectiveness of $CD8^+$ memory T cell depletion by anti-CD122 mAb in preventing early graft rejection.

Memory T cells produce effector cytokines *in situ* to recruit additional immune cells that mediate early graft tissue damage [29]. Our study also provided evidence that the combination therapies could inhibit production of these effector cytokines. The ELISA results (Fig. 6c) indicated that the use of anti-CD134L mAb can significantly reduce the secretion of Th1 cytokines, such as IL-2 and IFN-γ, which suggested the induction of anergy in the pre-existing $CD4^+$ memory cells. Moreover, the increase of IL-10 correlated with the use of anti-CD134L, which was consistent with the conclusion of Ito *et al.* [32] that the CD134L pathway inhibits IL-10-producing regulatory T cells.

TGF-β also has an important role in maintaining tolerance in the allo-primed model [33]. In our study, the concentration of TGF-β increased in the serum following the increase of Tregs (Fig. 6c), consistent with the TGF-β mRNA expression detected by qRT-PCR (Fig. 4). The response of recipient splenic T cells to donor BALB/c lymphocytes was also attenuated after treatment with the four combined antibodies and was much weaker than the response to third party C3H splenic T cells (Fig. 6b). All

these results indicated that the combined therapy of antibodies to CD134L, CD122, CD154, and LFA-1 have the potential to induce donor-specific tolerance.

We observed a significant difference in the prolongation of islet allografts between memory T cells in the adoptively transferred and alloantigen-primed islet transplantation models (Figs. 1 and 2). This difference may be because of the complex background of the allo-primed model. While less attention has been paid to other memory cells in allo-rejection, memory B cells may well be one important reason for eventual rejection even with inhibition of the memory T cells. Memory B cells can be activated, proliferate, and be converted to alloantibody-secreting plasma cells in the secondary response, and have the potential ability to prevent anti-CD154-mediated graft acceptance [34,35]. Our MLC results detected a persistent level of alloantibodies in the alloantigen-primed recipient mice capable of killing allogeneic lymphocytes immediately in the presence of complement (Fig. 7). Further study will be required to determine whether memory B cells or other types of cells, such as natural killer cells [36] do indeed play important roles in rejection in the allo-primed recipient.

In summary, graft rejection is accelerated, and tolerance is more difficult to induce in allo-primed recipients, but the use of anti-CD134L and anti-CD122 mAbs can significantly prolong the survival of islet allograft in alloantigen-primed mice by inducing anergy in memory T cells, depleting or converting them into Tregs. However, this strategy cannot induce long-term survival of allografts, and one reason for the failure of tolerance induction could be the presence of a large quantity of alloantibodies that can directly damage islet allografts in alloantigen-primed recipients. Therefore, additional studies are needed to develop better tolerance inducing strategies.

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Authorship

JX: performed research and wrote the paper, JC: designed research, WS: performed islet preparations, TL: performed flow cytometry analysis, YW: performed MLR assays, BX: performed skin transplantations, HT: contributed important reagents and modified the paper, FT: modified the paper, RH: contributed supporting funds, ZQ: contributed important reagents and supporting funds.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Memory $CD4^+$ or $CD8^+$ T cells were autoMACS and FLOW CYTOMETRY-purified from BALB/c mice 5 to 6 weeks after B6 full-thickness skin transplantation.

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References

1. Shapiro AM, Ricordi C, Hering B. Edmonton's islet success has indeed been replicated elsewhere. *Lancet* 2003; **362**: 1242.
2. Hering BJ, Kandaswamy R, Ansite JD, *et al.* Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. *JAMA* 2005; **293**: 830.
3. Ryan EA, Paty BW, Senior PA, *et al.* Five-year follow-up after clinical islet transplantation. *Diabetes* 2005; **54**: 2060.
4. Park SC, Moon IS, Koh YB. Second and third kidney transplantations. *Transplant Proc* 2006; **38**: 1995.
5. Hernandez-Fuentes MP, Stevenson S, Barroso-Herrera O, Lechler RI. Establishing the optimal method to estimate the frequencies of alloreactive CD4+ T cells. *Transplant Proc* 2002; **34**: 2855.
6. Heeger PS, Greenspan NS, Kuhlenschmidt S, *et al.* Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol* 1999; **163**: 2267.
7. McFarland RD, Douek DC, Koup RA, Picker LJ. Identification of a human recent thymic emigrant phenotype. *Proc Natl Acad Sci U S A* 2000; **97**: 4215.
8. Taylor DK, Neujahr D, Turka LA. Heterologous immunity and homeostatic proliferation as barriers to tolerance. *Curr Opin Immunol* 2004; **16**: 558.
9. Sachs DH. Tolerance: of mice and men. *J Clin Invest* 2003; **111**: 1819.
10. Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol* 2003; **3**: 609.
11. Rothstein DM, Sayegh MH. T-cell costimulatory pathways in allograft rejection and tolerance. *Immunol Rev* 2003; **196**: 85.
12. Vu MD, Clarkson MR, Yagita H, Turka LA, Sayegh MH, Li XC. Critical, but conditional, role of OX40 in memory T cell-mediated rejection. *J Immunol* 2006; **176**: 1394.

13. Minamimura K, Sato K, Yagita H, Tanaka T, Arii S, Maki T. Strategies to induce marked prolongation of secondary skin allograft survival in alloantigen-primed mice. *Am J Transplant* 2008; **8**: 761.
14. Tang AL, Bingaman AW, Kadavil EA, Leeser DB, Farber DL. Generation and functional capacity of polyclonal alloantigen-specific memory CD4 T cells. *Am J Transplant* 2006; **6**: 1275.
15. Lunsford KE, Jayanshankar K, Eiring AM, *et al.* Alloreactive (CD4-Independent) CD8+ T cells jeopardize long-term survival of intrahepatic islet allografts. *Am J Transplant* 2008; **8**: 1113.
16. Oberbarnscheidt MH, Ng YH, Chalasani G. The roles of CD8 central and effector memory T-cell subsets in allograft rejection. *Am J Transplant* 2008; **8**: 1809.
17. Zhang QW, Rabant M, Schenk A, Valujskikh A. ICOS-Dependent and -independent functions of memory CD4 T cells in allograft rejection. *Am J Transplant* 2008; **8**: 497.
18. Schenk AD, Gorbacheva V, Rabant M, Fairchild RL, Valujskikh A. Effector functions of donor-reactive CD8 memory T cells are dependent on ICOS induced during division in cardiac grafts. *Am J Transplant* 2009; **9**: 64.
19. Chen Y, Heeger PS, Valujskikh A. *In vivo* helper functions of alloreactive memory CD4+ T cells remain intact despite donor-specific transfusion and anti-CD40 ligand therapy. *J Immunol* 2004; **172**: 5456.
20. Lunsford KE, Gao D, Eiring AM, Wang Y, Frankel WL, Bumgardner GL. Evidence for tissue-directed immune responses: analysis of CD4- and CD8-dependent alloimmunity. *Transplantation* 2004; **78**: 1125.
21. Malm H, Pahlman C, Veress B, Corbascio M, Ekberg H. Combined costimulation blockade prevents rejection of allogeneic islets in mice. *Scand J Immunol* 2006; **64**: 398.
22. Gramaglia I, Jember A, Pippig SD, Weinberg AD, Killeen N, Croft M. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *J Immunol* 2000; **165**: 3043.
23. Prell RA, Evans DE, Thalhofer C, Shi T, Funatake C, Weinberg AD. OX40-mediated memory T cell generation is TNF receptor-associated factor 2 dependent. *J Immunol* 2003; **171**: 5997.
24. Sugamura K, Ishii N, Weinberg AD. Therapeutic targeting of the effector T-cell co-stimulatory molecule OX40. *Nat Rev Immunol* 2004; **4**: 420.
25. Murali-Krishna K, Ahmed R. Cutting edge: naive T cells masquerading as memory cells. *J Immunol* 2000; **165**: 1733.
26. Madakamutil LT, Christen U, Lena CJ, *et al.* CD8alphaalpha-mediated survival and differentiation of CD8 memory T cell precursors. *Science* 2004; **304**: 590.
27. Chen M, Xiao X, Demirci G, Li XC. OX40 controls islet allograft tolerance in CD154 deficient mice by regulating FOXP3+ Tregs. *Transplantation* 2008; **85**: 1659.
28. Xiao X, Kroemer A, Gao W, Ishii N, Demirci G, Li XC. OX40/OX40L costimulation affects induction of Foxp3+ regulatory T cells in part by expanding memory T cells *in vivo*. *J Immunol* 2008; **181**: 3193.
29. Bingaman AW, Farber DL. Memory T cells in transplantation: generation, function, and potential role in rejection. *Am J Transplant* 2004; **4**: 846.
30. Kedl RM, Mescher MF. Qualitative differences between naive and memory T cells make a major contribution to the more rapid and efficient memory CD8+ T cell response. *J Immunol* 1998; **161**: 674.
31. Cho BK, Wang C, Sugawa S, Eisen HN, Chen J. Functional differences between memory and naive CD8 T cells. *Proc Natl Acad Sci U S A* 1999; **96**: 2976.
32. Ito T, Wang YH, Duramad O, *et al.* OX40 ligand shuts down IL-10-producing regulatory T cells. *Proc Natl Acad Sci U S A* 2006; **103**: 13138.
33. Muthukumarana PA, Zheng XX, Rosengard BR, Strom TB, Metcalfe SM. In primed allo-tolerance, TIM-3-Ig rapidly suppresses TGFbeta, but has no immediate effect on Foxp3. *Transpl Int* 2008; **21**: 593.
34. Fecteau JF, Roy A, Neron S. Peripheral blood CD27(+) IgG(+) B cells rapidly proliferate and differentiate into immunoglobulin-secreting cells after exposure to low CD154 interaction. *Immunology* 2009; **128**: e353.
35. Sanz I, Wei C, Lee FE, Anolik J. Phenotypic and functional heterogeneity of human memory B cells. *Semin Immunol* 2008; **20**: 67.
36. Pratschke J, Stauch D, Kotsch K. Role of NK and NKT cells in solid organ transplantation. *Transpl Int* 2009; **22**: 859.

