

Treatment with Anti-Fc ϵ RI α (MAR-1) Antibody Prevents Acute Islet Allograft Rejection in a Murine Model

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Objective: Various immunological strategies for tolerance induction against allogeneic tissue grafts (allografts) have been tested in islet transplant recipients; for example, T cell activating co-stimulatory pathway blockade has been shown to prolong islet allograft survival. However, little is known about whether infiltrating inflammatory cells (e.g., basophils) affect islet allograft fates before antigen-specific immune cell development. Herein, we treated mice with a basophil-specific monoclonal antibody (mAb) and examined whether early acute-phase islet allograft rejection could be prevented in recipients.

Methods: Pancreatic islets isolated from C57BL/6 (H-2b) or DBA/2 (H-2d) mice were transplanted under the renal capsules of C57BL/6 recipient mice. Recipients receiving allografts were administered the anti-basophil mAb MAR-1 to examine the antibody-mediated effect on graft survival. At days 4 and 7 post-transplantation, graft-bearing recipient kidneys were harvested for immunohistological analysis and stained with anti-insulin antibody to compare the sizes of grafted islets.

Results: On day 7 post-transplantation, the transplanted pancreatic islet clusters in allograft-recipient kidneys had rapidly decreased in size, whereas those in syngeneic recipients remained larger in both size and number. However, MAR-1-treated recipients had increased the numbers of larger insulin-positive allograft islet cell clusters.

Conclusion: Basophil-specific mAb treatment contributes to enhance and prolong transplanted islet survival in allogeneic recipient mice.

Key words: islet transplantation, graft survival, acute allograft rejection, immunosuppression, basophil

INTRODUCTION

Insulin-dependent (type 1) diabetes mellitus remains a major health concern worldwide. Current treatment options for patients with type 1 diabetes mellitus include insulin injection, whole pancreas transplantation, and pancreatic islet cell transplantation. Pancreatic islet cell transplantation has been shown to be less invasive and associated with fewer surgical complications when compared with whole pancreas transplantation [1] and was found to be a potent alternative exogenous insulin treatment in experimental models and recent clinical trials [2-4]. However, strategies to induce immune tolerance to allografts are required to clinically apply islet transplantation in patients with type 1 diabetes mellitus. Early immunosuppressive protocols used for islet transplantation were similar to those used for kidney transplantation and included azathioprine, cyclosporine, and corticosteroids [5]; later modifications to immunosuppressive treatments included the combination of sirolimus (a mammalian target of rapamycin inhibitor), low-dose tacrolimus (FK506), and glucocorticoids for liver, kidney, and pancreas transplantation [6]. In 2000, a group in Edmonton further modified this regimen by replacing glucocorticoids with daclizumab (an anti-IL-2 receptor mAb) to prevent the diabetogen-

ic side effects of steroids [7]. Based on their methods, several clinical trials of islet transplantation have been conducted and have reported relative improvements in insulin independence [8]; however, despite a 1-year improvement in the insulin independence rate after islet transplantation of up to 80% [9], the improvement at 5 years was less effective than expected [10]. In addition, sirolimus and tacrolimus can cause side effects such as nausea, diarrhea, oral ulceration, anemia, and neutropenia [11].

Given this background, the development of new effective immunosuppressive strategies with better safety profiles that can provide alternatives to sirolimus and tacrolimus is urgently required [12]. One promising strategy for preventing graft rejection is the blockade of co-stimulatory signals and consequent inhibition of T cell activation [13]. T cells become activated upon receiving signals through both the T cell receptor (TCR) and co-stimulatory molecules during antigen stimulation [14]. If T cells recognize antigens (e.g., allo-antigens) via the TCR without proper co-stimulatory molecules, antigen-specific anergy is induced and results in tolerance [15-17]. Therefore, to induce allograft tolerance, CTLA4-Ig and anti-CD154 mAb have been used to block co-stimulatory pathways that promote T cell activation and have been reported to

prolong allograft survival in several experimental transplantation models [18–24]. However, the clinical benefits are not yet well established [25].

It is important to prevent the early loss of transplanted islets, particularly during the stage in which antigen-specific T cells have not yet been induced. Transplanted islets are exposed to allogeneic recipient blood and are therefore subjected to an immediate blood-mediated inflammatory reaction [26–28], which may result in the loss of transplanted islets. Accordingly, very early inflammatory cell invasion should be blocked to avoid this reaction [29].

In this report, we have focused on basophils as a representative of these early inflammatory cells. Basophils comprise approximately 0.5% of all leukocytes and have been generally neglected or considered a minor cell type. Basophils express the high-affinity IgE receptor $Fc\epsilon RI$ on their cell membranes, and the cytoplasm contains basophilic granules that stain blue with Giemsa or toluidine blue stain. Sokol *et al.* found that basophils treated with the cysteine protease papain produce IL-4 and thymic stromal lymphopoeitin (TSLP), both of which promote type 2 T-helper cell (Th2) responses [30]. Accordingly, several recent studies have shown that basophils are involved in the induction of Th2 responses [31–33]; for example, basophils have been reported to play an important role in the production of Th2 cytokines, including IL-4 and IL-13, by T-cells [34–37].

Concurrent indications suggest that the size and number of grafted islets may be determined by a non-immune reaction comprising inflammatory cell infiltration and environmental components in grafted areas [38]. Despite this non-immune nature, such reactions are known to contribute to antigen-specific acquired immunity through innate immune pathways [39]. Recently, increasing evidence suggests that basophils are of interest mainly because they appear at graft sites prior to antigen-specific T cells and are involved in the development of antigen-specific T cells into effector cells [36]. However, as basophils are a very small population among peripheral leukocytes, their involvement in grafted islet rejection is difficult to assess.

Recent studies have described an anti- $Fc\epsilon RI\alpha$ mAb, MAR-1, that specifically binds to $Fc\epsilon RI\alpha$ and competitively blocks the binding of IgE to its receptor [30, 40]. In particular, Sokol *et al.* successfully used this antibody to eliminate basophils *in vivo* and thus examine their immune roles [30]. Accordingly, in this study we examined the efficacy of MAR-1 antibody-mediated immunosuppression in preventing acute islet allograft rejection.

MATERIALS AND METHODS

Mice

Male C57BL/6 (H-2b) and DBA/2 (H-2d) mice (8–10 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan) for use as recipients or donors. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996). The animals were housed under specific pathogen free (SPF) conditions during

the experimental procedures, which were approved according to the guidelines of the Animal Care and Use Committee of Tokai University School of Medicine.

Islet isolation and subcapsular kidney transplantation

Mouse pancreatic islets were isolated and purified using a modification of the method published by Gotoh *et al.* [41]. Collagenase P (Roche, Mannheim, Germany) was dissolved in Hanks' balanced salt solution (HBSS; Sigma, St Louis, MO, USA) to yield a 1-mg/mL collagenase solution, which was placed on ice. C57BL/6 and DBA/2 mice were weighed and anesthetized with isoflurane (Abbott Laboratories, Abbott Park, IL, USA) and fastened to a surgical board. The liver and gallbladder were exposed through an upper midline incision under sterile conditions. The common bile duct was clamped with hemostatic forceps at the opening to the duodenum and cannulated using a PE-10 polyethylene catheter (0.28 mm I.D., 0.61 mm O.D., Becton Dickinson and Company, Sparks, MD, USA) for pancreatic perfusion. Approximately 2.5–3 mL of cold collagenase solution was injected slowly into the common bile duct until the pancreas was fully distended. Following perfusion, the pancreas was separated from the intestines, stomach, and spleen, harvested, and digested with gentle shaking in a water bath at 37°C for 45 min. Digestion was terminated by the addition of cold HBSS supplemented with 5% fetal calf serum (FCS; Thermo Trace, Melbourne, VIC, Australia). The digested tissue was then passed through a stainless steel filter (pore size: 630 μ m; Tokyo Screen Co., Tokyo, Japan) [42]. The filtered tissue was purified over a discontinuous Ficoll density gradient (25%, 23%, 20%, and 11%; GE Healthcare, Buckinghamshire, UK) that was centrifuged at 800 $\times g$ for 10 min at 4°C. Islets were subsequently obtained from the 2 interfaces between the 11%, 20%, and 23% layers.

C57BL/6 recipient mice were weighed and anesthetized with isoflurane. A small incision was made on the left flank to expose the kidney. Based on previous reports [43, 44], approximately 300 isolated pancreatic islets were transplanted under one kidney capsule per C57BL/6 mouse via transrenal injection with a 50- μ L Hamilton syringe and blunt 23-gauge needle.

Antibodies

Hamster IgG isotype control and anti-mouse $Fc\epsilon RI\alpha$ (clone MAR-1) antibodies were purchased from eBioscience (San Diego, CA, USA). Mouse anti-insulin mAb (clone K36AC10) was purchased from Sigma (St. Louis, MO, USA). Anti-mouse IgG F(ab')₂ antibody was obtained from Amersham International plc (Buckinghamshire, UK).

Antibody treatments

C57BL/6 recipient mice were divided into 8 experimental groups as follows: group 1, isografts sacrificed at day 4 after transplantation (n = 7); group 2, isografts sacrificed at day 7 (n = 6); group 3, untreated islet allografts from DBA/2 mice sacrificed at day 4 (n = 8); group 4, untreated allografts sacrificed at day 7 (n = 7); group 5, allografts treated with control IgG and sacrificed at day 7 (n = 7); group 6, allografts treated

with MAR-1 antibody and sacrificed at day 7 (n = 9); group 7, allografts treated with MAR-1 antibody and sacrificed at day 14 (n = 8); and group 8, allografts additionally treated after transplantation with MAR-1 antibody and sacrificed at day 14 (n = 9).

Isotype control IgG or MAR-1 was administered intravenously 3 and 2 days before transplantation at a dose of 0.1 mg/mouse. In the additional treatment group (group 8), MAR-1 was additionally administered 4 days after transplantation at a dose of 0.1 mg/mouse.

Histopathological examination

On day 4, 7, or 14 after islet transplantation, graft-bearing kidneys were removed from recipients to detect transplanted islet cells. Samples were fixed overnight in 10% formalin and prepared for histological analysis. Paraffin-embedded tissues were sectioned at 4- μ m intervals and stained with hematoxylin and eosin (H & E) for morphological evaluation. Five-micrometer-thick sections cut from paraffin-embedded tissues were deparaffinized, and endogenous peroxidase activity was quenched by incubation in 0.3% H₂O₂ in methanol for 30 min. Immunohistochemistry with mouse anti-insulin mAb K36AC10 (1: 2500 dilution) was performed overnight at 4°C according to the indirect method. Section slides were subsequently incubated with anti-mouse IgG F(ab')₂ antibody (1: 100 dilution) for 60 min and visualized following a 3,3'-diaminobenzidine (DAB) reaction. The sections were counterstained with H&E. Immunohistochemical images were captured using a digital microscope camera system (BX50 microscope and DP 70 digital camera; Olympus Corporation, Tokyo, Japan).

Immunohistochemical evaluation

Each insulin-positive cell and cluster was classified and scored using a semi-quantitative scoring system into 5 groups as follows: O, no insulin-stained cells (score 0); C, scattered residual insulin-stained cells (score 1); S, small cell cluster of insulin-stained cells (<10 cells/cluster) (score 2); M, medium-sized cell cluster of insulin-stained cells (<20 cells/cluster) (score 4); and L, large cell cluster of insulin-stained cells (\geq 20 cells/cluster) (score 8). For each group, the number of insulin-stained cell clusters in 10 randomly selected kidney sections per mouse was counted. The total score was calculated as the number \times score (number of C \times 1, number of S \times 2, number of M \times 4, and number of L \times 8). The graft score was calculated for each group by dividing the total score by 10.

Statistical analysis

The non-parametric Mann-Whitney test was used for the statistical analysis of score data between the 2 groups. A P-value of <0.05 was considered statistically significant. All calculations were performed using the SPSS statistical software package version 21 (SPSS, IBM, Armonk, NY, USA).

RESULTS

I. The size and number of grafted islets were reduced on day 7 in allogeneic recipients

To examine how allogeneic graft rejection is initiated at a very early stage (i.e., immediately after

islet transplantation), a murine pancreatic islet cell transplantation model was established, wherein islet cells isolated from allogeneic (DBA/2) and syngeneic (C57BL/6) mice were transplanted under recipient kidney capsules. The average number of injected islet cells into the recipient mice was 307 ± 24 . During the 7-day after islet transplantation period, recipient kidneys were submitted for paraffin sectioning and histological and immunohistological analysis. Sections were stained with an anti-insulin antibody to assess the survival of the transplanted islet cells.

In C57BL/6 recipients transplanted with syngeneic islet cells (i.e., from syngeneic C57BL/6 pancreas), insulin-positive cell clusters comprising several to >30 islet cells were detected near the kidney capsule. The sizes and numbers of the distributed islet clusters appeared almost equal on days 4 and 7 after transplantation (Figs. 1A and B). However, following DBA/2 allogeneic islet cell transplantation, the major insulin-positive clusters in the kidney capsules were much smaller and sparser on day 7 than on day 4, and many infiltrating inflammatory cells had accumulated around the cluster on day 7 (Figs. 1C and D). The major inflammatory cells morphologically appeared to be monocytes and lymphocytes; basophils were almost indistinguishable, presumably because the proportion of basophils in leukocytes is nearly negligible at 0.5%.

To perform a more statistical analysis of differences in the survival statuses of grafted islets in recipients receiving syngeneic vs. allogeneic grafts, insulin-stained cell clusters were arbitrarily classified into 5 groups based on the cluster size: O, C, S, M, and L as described in the Materials and Methods and Fig. 2. Compared with day 4, the graft scores were reduced significantly on day 7 after allogeneic transplantation (Fig. 3B), whereas no significant difference was observed with syngeneic transplantation (Fig. 3A). These results imply that in comparison to syngeneic transplantation, more inflammatory cells gathered around grafted allogeneic islets during the relatively early (7 days) post-transplantation stage.

II. Enhanced survival of allogeneic transplanted islet cells in recipients treated with anti-basophil antibody (MAR-1)

Recent studies indicate the importance of basophils for the early inflammatory reaction [45, 46]. To examine the involvement of basophils in the rejection of allogeneic transplanted grafts, we created a basophil eliminated mouse in the islet transplantation mouse model described above by injecting the anti-basophil antibody MAR-1 because this antibody is reported to deplete basophils *in vivo* [30, 40]. Recipients receiving MAR-1 on days 3 and 2 before transplantation were sacrificed and subject to morphological analysis on day 7 after allo-islet transplantation. Grafted islets in the recipient kidney were detectable in MAR-1 injected mice as relatively large size and number of insulin-positive islets, but they were almost undetectable in control IgG antibody injected mice accompanied by substantial accumulated inflammatory cells, presumably including developed effector T cells (Fig. 4). A graft score analysis also revealed that the reduction in grafted islets was slightly but significantly suppressed in MAR-1 injected

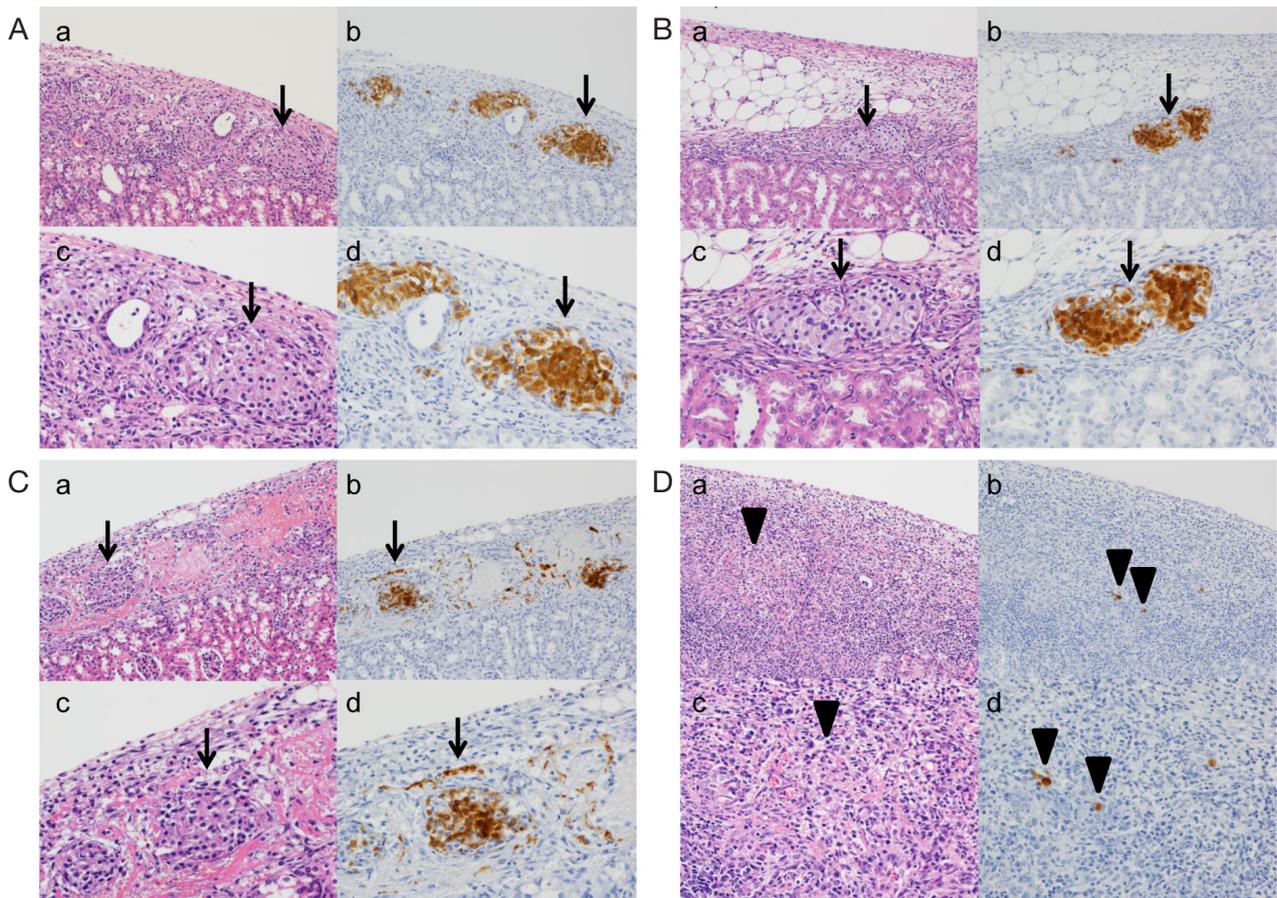


Fig. 1 Morphological detection of grafted islets in the recipient kidney capsule
Representative grafted islets in kidney tissue sections stained with hematoxylin & eosin (a and c) or with anti-insulin (b and d) on day 4 (A and C) and day 7 (B and D) were obtained from recipients transplanted with syngeneic (A and B) or allogeneic (C and D) islet cells. Histological figures shown under lower (a and b, $\times 10$) and higher (c and d, $\times 20$) magnification. Black arrows and arrowheads indicate well- and poorly-organized transplanted islet clusters, respectively.

mice (Fig. 5).

In addition to non-specific inflammatory reaction, basophils are recently known to be involved in the development of antigen-specific T cell effectors by early production of dispensable cytokines such as IL-4. Because antigen-specific T cells are expected to develop around 7 days after stimulation, it may be more effective to attenuate basophil function nearly when the activation of specific T cells is initiated. Thus, using the experimental model described above, another MAR-1 injection was given on day 4 to the recipients who had already received MAR-1 prior to allo-transplantation, followed by cluster survival analysis on day 14. A more prominent effect of basophil deletion was observed in allografted recipients additionally injected with MAR-1 on day 4 after transplantation. The graft scores per section were much higher in the additional treatment group than in the non-additional treatment groups (Fig. 6).

These results indicate that a loss of basophils following MAR-1 administration may attenuate the induction of an acquired allogeneic cell-specific immune reaction as well as non-specific inflammatory cells, resulting in the effective survival of transplanted allo-islet cells.

DISCUSSION

Various strategies for elongating the survival of grafted tissues have been investigated. Regarding

these strategies, current trials have shifted toward the induction of immune tolerance against transplanted allogeneic tissues via mAbs that block or induce positive or negative signaling, respectively, in T cells and have reported some prospective results involving the induction of T cell anergy [13]. In contrast to T cell-specific tolerance in transplantation, however, little attention has been given to the roles of non-specific inflammatory cells in this process, although inflammatory cells may accumulate at recipients' transplantation sites in reaction to surgical injury.

In the present study, we first presented evidence suggesting that among non-specific inflammatory cells, basophils may be involved somehow in allogeneic grafted islet rejection during the early post-transplantation stage. The sizes of transplanted islet cell clusters were compared in recipient C57BL/6 mouse kidneys that had been transplanted with either syngeneic C57BL/6 or allogeneic DBA/2 mouse pancreatic islet cells. During the first 7 days after transplantation, the allogeneic islet cell clusters were highly reduced in the recipients, but were almost unchanged in the syngeneic counterparts. However, following pre-transplantation treatment with the anti-Fc ϵ RI α antibody MAR-1, which had been reported to specifically eliminate basophils *in vivo* [30, 40], the sizes and numbers of grafted allo-islet cell clusters were larger in comparison

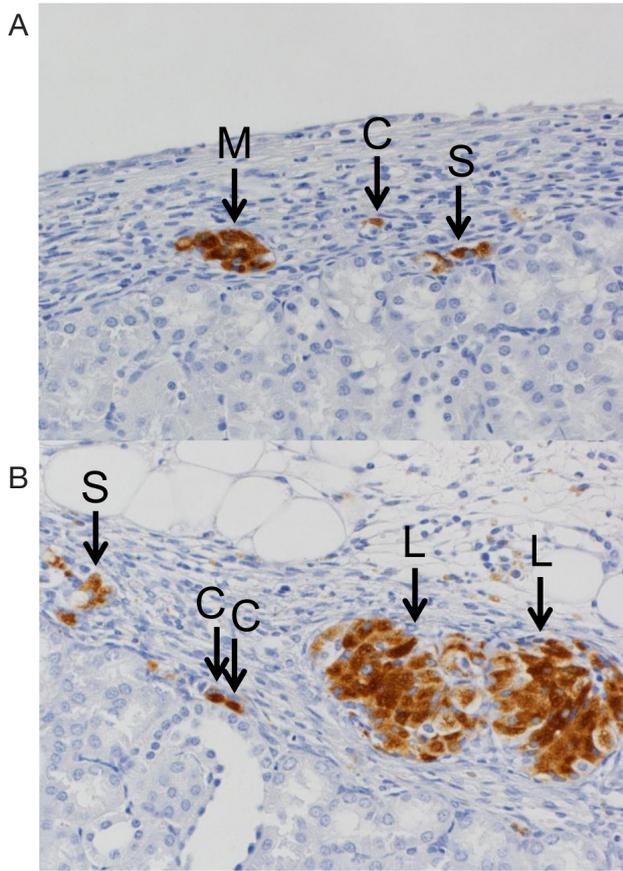


Fig. 2 Scoring of survival grafts
The cell clusters containing insulin-stained cells in kidney tissues were arbitrarily classified into 5 groups according to the cluster size as described in the Materials and Methods: groups L, M, S, C, and O comprised large, intermediate, small, scattered, and no insulin-stained cells, respectively. Furthermore, each of the groups is scored arbitrarily as 8, 4, 2, 1, and 0, respectively.

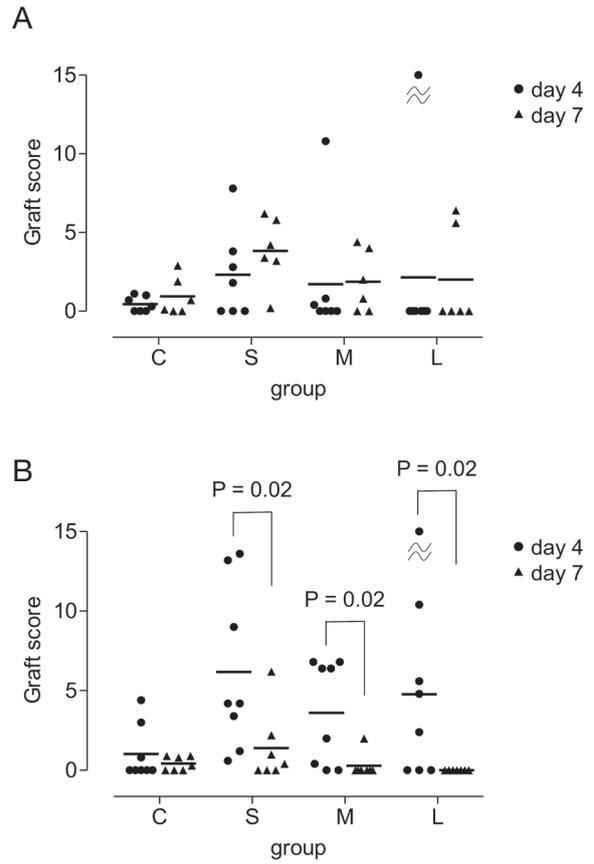


Fig. 3 Lower survival score after transplantation in allogeneic grafts
Survival status of syngeneic (A) and allogeneic graft (B) in the recipient kidneys on days 4 (●) and 7 (▲) after transplantation was scored based on the semi-quantitative scoring method described in Fig. 2. Each plot represents the average score of each group (number of clusters × score of each group/10 counted sections in an individual mouse). Horizontal lines represent the mean values for each group. The change in isograft (A) graft scores was not statistically significant between days 4 (n = 7) and 7 (n = 6). In the allogeneic grafts (B), the graft scores on day 7 (n = 7) were significantly lower than those on day 4 (n = 8) (P = 0.02, Mann-Whitney test).

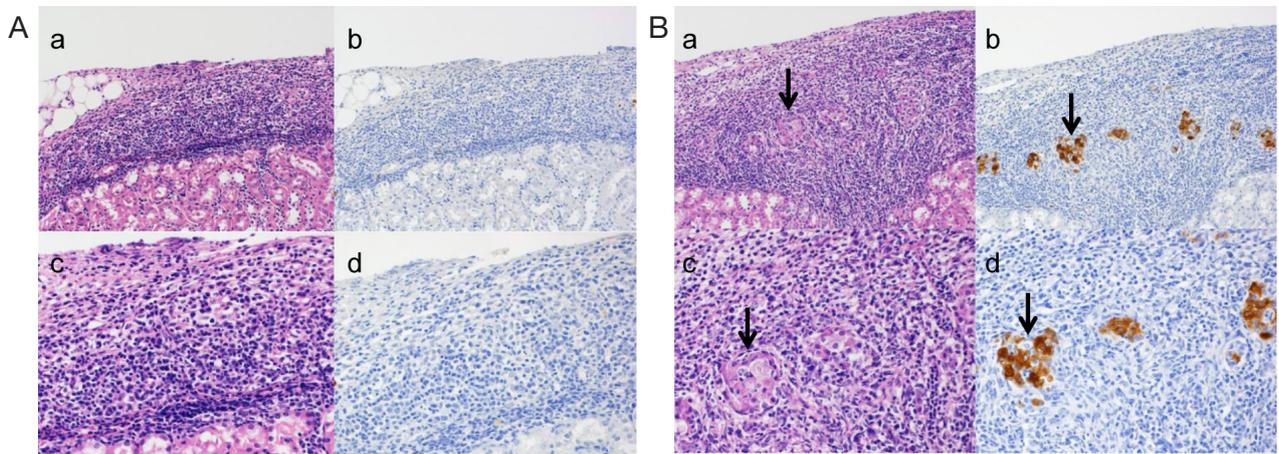


Fig. 4 Histology of allogeneic islet grafts in the recipients treated with MAR-1
Tissue sections of the kidney in the recipients receiving control IgG (A) or MAR-1 (B) were prepared on day 7 after transplantation and were stained with hematoxylin & eosin (a and c) or with anti-insulin (b and d). Histological figures were shown in lower (a and b, ×10) and higher (c and d, ×20) magnification. Black arrows indicate well-organized transplanted islet clusters.

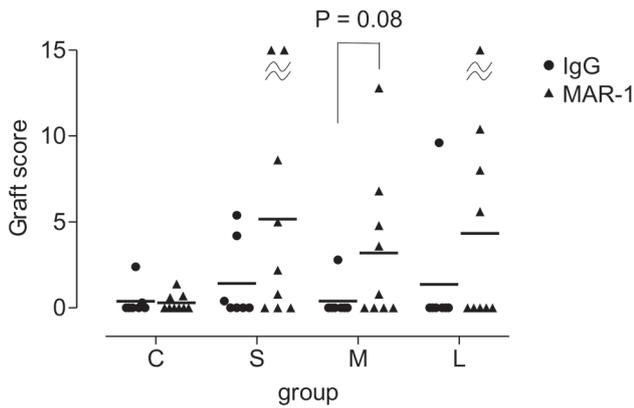


Fig. 5 Effect of MAR-1 treatment on allogeneic grafts
Graft status in the recipient kidney with allogeneic islet grafts on day 7 after transplantation was estimated according to the semi-quantitative score described in Fig. 2. The recipient mice were treated with control IgG (●) (n = 7) or anti-FcεRIα (MAR-1) (▲) (n = 9) on days 3 and 2 before transplantation. Regarding medium-sized insulin-stained cell clusters, the graft scores per section were almost significantly higher in the MAR-1 group than in the IgG group (P = 0.08, Mann–Whitney test).

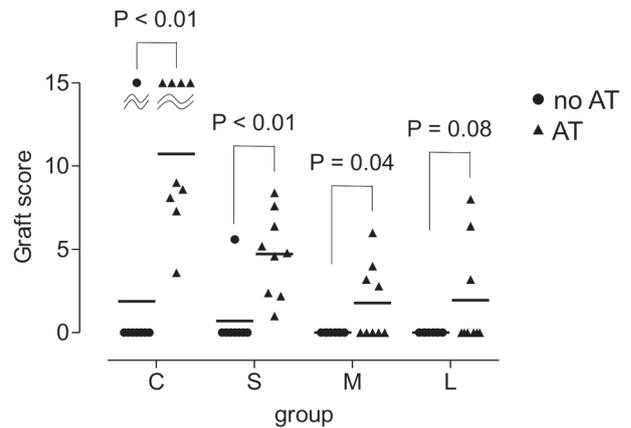


Fig. 6 Effect of additional MAR-1 administration after transplantation
The recipients receiving MAR-1 injection on days 3 and 2 before transplantation were additionally injected with MAR-1 on day 4 after transplantation of allogeneic islet cells, and their kidneys were submitted for examination of graft survival on day 14 after transplantation. Survival status of allogeneic graft was estimated as scores described in Figs. 3 and 5. The graft scores per section were significantly higher in the additional treatment group (AT) (▲) (n = 8) than in the no additional treatment group (no AT) (●) (n = 9) (Mann–Whitney test).

those of the untreated recipients on day 7. This MAR-1 effect on the survival of grafted islet cells was more remarkable when additional MAR-1 was injected on day 4 after transplantation and examined on day 14 (Fig. 6).

Basophils comprise a non-specific inflammatory cell population about which little is known, mainly because of the small size of basophil population in leukocytes and the lack of proper specific markers. However, the recent development of technologies and tools such as specific mAbs for basophil deletion and genetic manipulation of mice have allowed us to investigate basophil functions [30, 40, 46–48]. Accordingly, basophils have received recent attention as non-specific immune cells with a variable inflammatory reaction potential resulting from the production of soluble factors/cytokines [49]. In addition to their role in non-specific inflammation, basophils have been in the spotlight as initiators of acquired immunity, in which CD4+ T cells develop into effector helper T cells in the presence of basophil-produced factors such as IL-4 [50, 51].

In our transplantation experiments, we showed the role of basophils on both non-specific and antigen-specific reaction in allogeneic transplantation by *in vivo* elimination of basophils. In graft transplantation, recipient tissues are exposed to some degree of surgical damage, resulting in some inflammatory reactions. MAR-1 treatment on days 3 and 2 pre-transplantation is targeted to such non-specific inflammatory reactions and showed a slight effect on islet graft survival, although the effect was nearly statistically significant. We injected additional MAR-1 into the recipients on day 4 after islet transplantation, wherein allo-specific T cells were assumed to begin developing into effector cells

and found that grafted islets were highly maintained in the recipient kidney. These results suggest that despite receiving stimulation, allo-specific T cells may less efficiently develop into effector cells under basophil-depleted conditions.

Previous studies have shown prolonged graft survival in transplant recipients that have been treated with CTLA4-Ig and anti-CD154 mAb, presumably because allogeneic cell-specific T cells are rendered anergic due to a defect in co-stimulatory signaling [13]. As effector T cell development is delayed or inefficient in the absence of basophils, we predict that the combination of basophil deletion by anti-basophil mAbs and co-stimulatory signaling blockade using relevant antibodies may provide more effective induction of graft tolerance, although this remains to be tested.

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