プを含めて，ウイルスベクターを用いない方法も報告されており，今後，ADSCから全能性を持つ細胞を誘導することで，難治性疾患に苦しむ患者への新規治療の可能性がみえてくる[12,13]．

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本論文では，文献6: Kawamoto et al. Disease Markers, 2013[6]の図を使用していますが，同誌の著作権ポリシー(http://www.hindawi.com/journals/dm/guidelines/)によれば適切な引用がなされていれば構わないとされております(Organ Biology編集委員会)．

本稿は，2013年11月9, 10日に東京医科大学病院臨床講堂(東京・新宿)で行われた，第40回日本臓器保存生物医学会学術集会におけるシンポジウム2“再生医療”での発表をもとに書き下ろしたものである．

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HMGB1-Mediated Early Loss of Transplanted Islets Is Prevented by Anti–IL-6R Antibody in Mice

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Objectives: The limited success in achieving insulin independence of patients with type 1 diabetes mellitus after islet transplantation from a single donor, mainly due to early loss of transplanted islets, hampers clinical application of islet transplantation. Previously, we have shown in mice that the early loss of transplanted islets in the liver, the site of islet transplantation, is caused by innate immune rejection triggered by high-mobility group box 1 (HMGB1) protein released from transplanted islets. We herein determined whether the HMGB1-mediated early loss of transplanted mouse islets is prevented by anti–interleukin-6 receptor (IL-6R) antibody.

Methods: The effect of anti–IL-6R antibody on amelioration of hyperglycemia in streptozocin-induced diabetic mice receiving 200 islets into the liver from a single donor was evaluated in association with HMGB1-stimulated interferon-γ production of hepatic mononuclear cells.

Results: Hyperglycemia of diabetic mice receiving 200 syngeneic islets was ameliorated with down-regulation of interferon-γ production of hepatic natural killer T cells and neutrophils when anti–IL-6R was administered at the time of transplantation. This beneficial effect was also seen in allografts when alloimmune rejection was prevented by anti-CD4 antibody.

Conclusions: These findings demonstrate that anti–IL-6R antibody prevented the early loss of intrahepatic islet grafts with inhibiting HMGB1-induced immune activation after islet transplantation.

Key Words: islet transplantation, HMGB1, anti–IL-6R antibody, graft loss

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Pancreatic islet transplantation is an attractive procedure for the treatment of insulin-dependent diabetes mellitus (IDDM).[1,2] Currently, however, islet transplantation has only experienced limited success in achieving insulin independence of patients with IDDM after transplantation from a single donor, and therefore, sequential transplantations of islets with the use of 2 to 3 donor pancreases are required for the treatment of a single IDDM recipient.[1–3] Thus, the low efficiency of islet transplantation has been a major obstacle facing clinical islet transplantation.

Previously, we have shown in mice that a large amount of high-mobility group box 1 (HMGB1) protein is released from islets soon after their transplantation into the liver and induces innate immune rejection in concert with the activation of dendritic cells (DCs), natural killer T (NKT) cells, and Gr-1$^+$CD11b$^+$ cells (neutrophils) to produce interferon γ (IFN-γ) and finally leads to the early loss of transplanted islets.[4,5] The treatments targeting the HMGB1-DC-NKT cells–neutrophils-IFN-γ pathway has been found to prevent early loss of transplanted islets in the liver of recipient mice.[4,5] Importantly, HMGB1 was found to be actually involved in islet graft loss in a clinical setting by the finding that the serum HMGB1 levels of recipients soon after transplantation were inversely correlated to the outcome of autologous islet transplantation.[6] Furthermore, we have also shown that inflammatory cytokines other than IFN-γ, such as tumor necrosis factor α and interleukin 1β, play an important role in the early loss of transplanted islets in the liver,[7] although their precise mechanisms in association with HMGB1-mediated pathway remain unclear.

In the present study, we focused on interleukin 6 (IL-6) and determined whether anti–IL-6 receptor (IL-6R) antibody has any beneficial effect on prevention of early loss of transplanted islets because IL-6 is another proinflammatory cytokine involved in various inflammatory diseases[8–10] and the blockade of IL-6/IL-6R signaling with anti–IL-6R antibody has already been proved efficacious in a clinical setting for the treatment of rheumatoid arthritis[11,12] and Castleman disease.[13–15] Thus, we hypothesize that anti–IL-6R antibody may have a beneficial effect on outcome of islet transplantation by preventing early loss of transplanted islets when applied to islet transplantation.

The present study demonstrates that it is indeed the case; the treatment with anti–IL-6R antibody could prevent early loss of transplanted islets, enabling islet transplantation from one donor to one recipient feasible in mice. Notably, the beneficial effect of anti–IL-6R antibody was found to be mediated by the inhibition of IFN-γ production of hepatic mononuclear cell (HMNC) including NKT cells and neutrophils elicited by HMGB1 released from transplanted islets, which is an essential component of early loss of transplanted islets.[4,5]

MATERIALS AND METHODS

Animals

Male BALB/c (H-2d) and C57BL/6 (H-2b) mice were purchased from Charles River Japan (Kanagawa, Japan). Mice were kept under specific pathogen-free conditions and used at 8 to 16 weeks of age. Diabetes was induced in the recipients by the intravenous (IV) injection of streptozotocin (STZ) (180 mg/kg) (Sigma, St Louis, Mo). The plasma glucose levels of the mice exceeded 400 mg/dL at 2 to 3 days after the STZ injection and the mice remained hyperglycemic at the time of islet transplantation. The experiments were approved by the Institutional Animal Care and Use Committee.

Islet Isolation and Transplantation

Islets were isolated by the static digestion method using collagenase[16] and then separated by centrifugation on Ficoll-Conray gradients.[17] Islets of 150 to 250 μm in diameter were hand-selected using Pasteur pipette with the aid of a dissecting microscope. Hand-picked islets were transplanted into the liver via the

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recipient's portal vein[18] at 3 to 5 days after the induction of diabetes with STZ injection. The nonfasting plasma glucose levels and body weight were monitored 3 times a week in all recipients for 60 days after islet transplantation. The plasma glucose was measured using a GlucoCard DIA meter (Arkray). Normoglycemia after transplantation was defined as 2 consecutive plasma glucose levels reading below 200 mg/dL.

Administration of HMGB1, Anti–IL-6R, and Anti-CD4 Antibody

Bovine HMGB1 was purchased from Shino-test Co (Sagamihara, Japan) and administered IV (100 μg/injection per mouse) to untreated mice. Rat anti–IL-6R mAb (MR16-1) was a generous gift by Chugai Pharmaceutical Co (Tokyo, Japan). Rat anti–IL-6R antibody (500 μg/injection per mouse, MR16-1) and nondepleting anti-CD4 mAb (200 μg/injection per mouse, YTS177, rat IgG1; R&D, Minneapolis, Minn) were administered intraperitoneally (IP) into an appropriate group of mice once at the time of islet transplantation. Rat IgG (Chemicon International, Temecula, Calif) was used as control.

Morphological Study

The livers bearing islet grafts and pancreas were examined morphologically. The liver and pancreas were fixed with Bouin solution, processed, and embedded in paraffin. The sections were prepared for light microscopy and stained with hematoxylin and eosin, and aldehyde and fuchsin.

Flow Cytometry Analysis of HMNCs

Hepatic mononuclear cells were prepared as described previously,[19] and examined by flow cytometry. The following mAbs were used: antimouse Fcγ III/II R (2.4G2), FITC-conjugated anti-CD3ε (145–2C11), FITC- or PE-conjugated anti-CD11b (M1/70), allophycocyanin-conjugated anti–IFN-γ (XMG1.2), PerCP-conjugated anti–Gr-1 (Rb6-8c5), and isotype control (clone R3-34, Rat IgG1) were purchased from BD Biosciences (San Jose, Calif). PE-α-galactosylceramide (α-GalCer)-CD1d tetramers were prepared as previously described.[20] For intracellular staining, cells were incubated with anti-Fcγ III/II R (BD Biosciences), surface stained, fixed, permeabilized, stained with mAbs, and analyzed on a flow cytometer (FACS Calibur; Becton Dickinson, San Jose, Calif). Ten thousand viable cells were analyzed.

Statistical Analysis

The statistical significance of the population of Gr-1$^+$CD11b$^+$ cells was determined by Student t test. The statistical significance with respect to the rate of euglycemia in STZ-induced diabetic mice after islet transplantation was determined by Fisher exact test. The statistical significance of plasma glucose levels of intraperitoneal glucose tolerance test (IPGTT) was determined by 1-way analysis of variance and Tukey/Kramer post hoc test. Differences were considered significant when P values were less than 0.05.

RESULTS

Inhibitory Effect of Anti–IL-6R Antibody on IFN-γ Production of NKT Cells and Neutrophils in the Liver of Mice Treated With IV Injection of HMGB1

As an initial experiment, we determined whether anti–IL-6R antibody has any inhibitory effect on in vivo HMGB1-stimulated IFN-γ production of HMNC of mice. For those purposes, control antibody or rat anti–IL-6R antibody (MR16-1) was administered IP (500 μg/injection per mouse) into mice 5 minutes before the administration of HMGB1 (100 μg/injection per mouse), and HMNCs were isolated at 2 hours after the HMGB1 injection (IV) and examined by FACS. As originally reported,[4] a marked increase in IFN-γ production of hepatic α-GalCer/CD1d-tetramer$^+$ CD3$^+$NKT cells and Gr-1$^+$CD11b$^+$ cells (neutrophils) was seen in mice treated with control antibody compared with untreated mice (Fig. 1, first and second panels). In marked contrast, the IFN-γ production of NKT cells and Gr-1$^+$CD11b$^+$ cells in the liver of mice treated with anti–IL-6R antibody in conjunction with HMGB1 was down-regulated (Fig. 1, third panel). The accumulation of Gr-1$^+$CD11b$^+$ cells in the liver after HMGB1 injection was also observed, however with significant reduction in number when mice were treated with HMGB1 in conjunction with anti–IL-6R antibody compared with control antibody (Fig. 1). The percentage of the Gr-1$^+$CD11b$^+$ cells in HMNC of mice treated with HMGB1 in conjunction with control or anti–IL-6R antibody was 51.1% (7.3%) [mean (SD), n = 3] and 21.2% (8.7%) (n = 3), respectively, with significant difference between the 2 groups ($P < 0.05$ by Student t test).

Beneficial Effects of Anti–IL-6R Antibody on Prevention of Early Loss of Transplanted Islets

The previously mentioned findings suggest that anti–IL-6R antibody (MR16-1) might have a beneficial effect on prevention of early loss of transplanted islets by inactivation of HMGB1-stimulated immune response in the liver receiving islets. To address this question, we determined whether hyperglycemia of STZ-induced diabetic mice is ameliorated after transplantation of a marginal mass of syngeneic islets, namely, 200 islets from a single donor when recipient mice were treated with anti–IL-6R antibody. When diabetic mice received 200 islets and were treated with or without control antibody, all the recipient mice remained

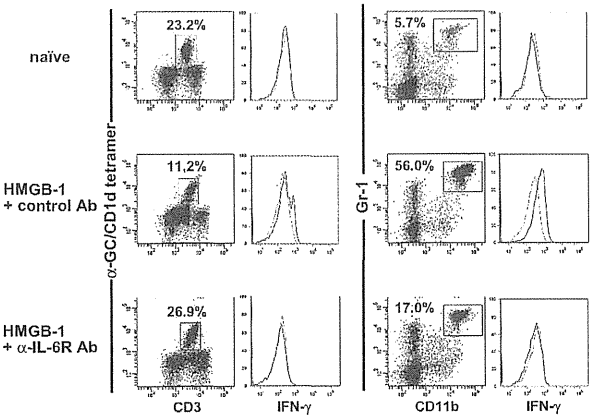

FIGURE 1. Anti–IL-6R antibody prevents IFN-γ production of hepatic NKT cells and neutrophils in mice treated with the IV injection of HMGB1. FACS profiles of liver MNCs from naive mice (first panel) and from mice at 2 hours after the IV injection of HMGB1 (100 μg/injection per mouse) in conjunction with control antibody (second panel) or anti–IL-6R antibody (500 μg/injection per mouse) (third panel), which was administered IP 5 minutes before the injection of HMGB1. NKT (α-GalCer/CD1d-tetramer$^+$ CD3$^+$) cells and Gr-1$^+$CD11b$^+$ cells were gated and analyzed for their IFN-γ production (second and fourth columns). The numbers in each box represent the percentages of cells in the corresponding area. Representative data from 2 to 3 experiments are shown.

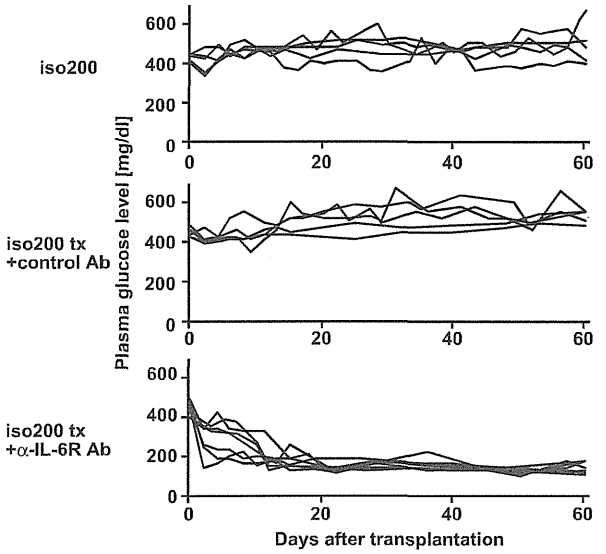

FIGURE 2. Hyperglycemia of STZ-induced diabetic mice treated with anti-IL-6R antibody was ameliorated after transplantation of 200 syngeneic islets into the liver. Nonfasting plasma glucose levels of STZ-induced diabetic mice (C57BL/6) received 200 syngeneic islets without the treatment (upper panel) and treated with control antibody (second panel) or anti-IL-6R antibody (third panel), which was administered IP once at the time of islet transplantation. Individual lines represent nonfasting plasma glucose levels of each animal.

hyperglycemic after the transplantation (Fig. 2, first and second panels). When diabetic mice received the same number of islets and were treated with anti-IL-6R antibody (MR16-1, 500 μg) once at the time of islet transplantation, hyperglycemia of recipient mice was ameliorated and all mice remained normoglycemic for more than 60 days after the transplantation (Fig. 2, third panel), indicating that anti-IL-6R antibody has no deleterious effect on survival of recipient mice and that, importantly, anti-IL-6R antibody improves efficiency of islet transplantation. A histological study revealed that intact or degenerated islets with well or poorly granulated β cells were seen in the liver of the normoglycemic or hyperglycemic recipient mice, respectively, at 60 days after transplantation (histologic examination not shown), as shown in the previous study.[4,5,7] The difference in the euglycemic rates between diabetic mice receiving 200 islets and treated with control antibody and those receiving the same number of islets and treated with anti-IL-6R antibody was statistically significant ($P < 0.05$ by Fisher exact test).

IFN-γ Production of NKT Cells and Neutrophils in the Liver of Mice Receiving Islets Is Down-Regulated by Anti-IL-6R Antibody

To determine whether IFN-γ production of NKT cells and neutrophils in the liver of mice after islet transplantation into the liver was actually inhibited by anti-IL-6R antibody, FACS analysis of mononuclear cells (MNCs) in the liver of mice receiving islets was performed. As a result, the IFN-γ production of both NKT cells and neutrophils in the liver of mice receiving islets and treated with anti-IL-6R antibody was found to be down-regulated at 6 hours after transplantation (Fig. 3, third panel), whereas that of mice treated with control antibody was up-regulated (Fig. 3, second panel), consistent with the previous report.[4] Interestingly, the number of accumulated Gr-1$^+$CD11b$^+$ cells in the liver of mice receiving islets and treated with anti-IL-6R antibody (MR16-1) was reduced compared with mice receiving the similar number of islets and treated with control antibody (Fig. 3, third and second panels).

Improved Glucose Tolerance of Recipient Mice Treated With Anti-IL-6R Antibody

To evaluate the functional mass of islet grafts in the livers of recipient mice, an IPGTT was performed at 60 days after transplantation. The plasma glucose levels of naive untreated C57BL/6 mice (n = 4) were 59.5 (3.1) [mean (SD)], 249.8 (5.6), and 133.3 (4.8) mg/dL at 0, 30, and 120 minutes, respectively, after the IP injection of 1.0 g/kg glucose, and those of diabetic mice (n = 3) without islet transplantation were 459.7 (57.1), 658.3 (16.6), and 561.3 (44.2) mg/dL, respectively (Fig. 4).

The plasma glucose levels of diabetic mice (n = 7) receiving 200 islets and treated with anti-IL-6R antibody (MR16-1) were 75.9 (21.5), 251.9 (36.4), and 164.7 (22.4) mg/dL and those of mice (n = 5) treated with control antibody were 336.4 (76.6), 611.8 (131.8), and 509.6 (140.0) mg/dL at 0, 30, and 120 minutes, respectively, after the injection of glucose (Fig. 4). The difference in the plasma glucose levels at 30 and 120 minutes between the mice with 200 islets and treated with anti-IL-6R antibody and those with 200 islets with control antibody was statistically significant ($P < 0.05$ by 1-way analysis of variance and Tukey/Kramer post hoc test).

Beneficial Effects of Anti-IL-6R Antibody on Prevention of Early Loss of Islet Allografts in the Liver

Finally, we determined whether the beneficial effect of the treatment with anti-IL-6R antibody on prevention of early loss of transplanted syngeneic islets was also seen in islet allografts. Our previous experiments revealed that islet allograft rejection could be prevented by posttransplant short-term administration of nondepleting anti-CD4 antibody after islet transplantation.[7]

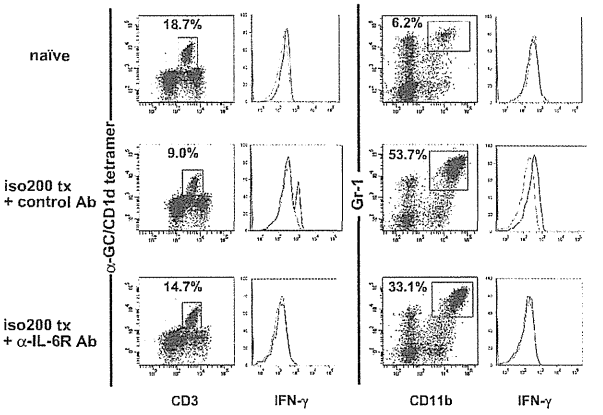

FIGURE 3. Anti-IL-6R antibody prevents IFN-γ production of MNCs in the liver of mice after syngeneic islet transplantation into the liver. Mononuclear cells were isolated from the liver of untreated mice (first panel) and of diabetic mice receiving 200 syngeneic islets and treated with control antibody (second panel) or anti-IL-6R antibody (third panel) at 6 hours after transplantation and examined by flow cytometry. NKT (α-GalCer/CD1d-tetramer$^+$ CD3$^+$) cells and Gr-1$^+$CD11b$^+$ cells were analyzed for their IFN-γ production (second and fourth columns). The figures in individual boxes show the percentage of the cells in the corresponding areas. Representative data from 2 to 3 experiments are shown.

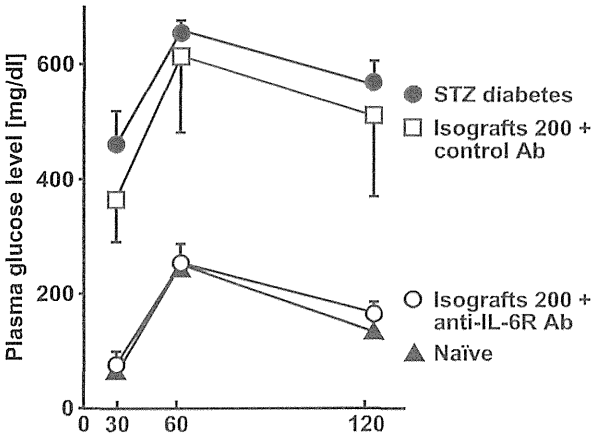

FIGURE 4. Improved glucose tolerance of recipient mice treated with anti–IL-6R antibody at the time of islet transplantation. An IPGTT was performed in STZ-induced diabetic mice before and at 60 days after islet transplantation. Mice were fasted for 15 hours before IPGTT and blood samples were taken from orbital sinuses at 0, 30, and 120 minutes after the IP injection of glucose (1 g/kg). Experimental groups included naive untreated mice (black diamond, n = 4), STZ-induced diabetic mice without islet transplantation (black circle, n = 3), diabetic mice receiving 200 syngeneic islets and treated with control antibody (white square, n = 7), or anti–IL-6R antibody (white circle, n = 5). The difference in the plasma glucose levels at 0, 30, and 120 minutes after the glucose injection between the mice receiving 200 islets treated with control antibody and those receiving 200 islets and treated with anti–IL-6R antibody was statistically significant (*$P < 0.05$ by 1-way analysis of variance and Tukey/Kramer post hoc test).

Therefore, the same protocol to prevent rejection of islet allografts was used to determine whether the beneficial effects of anti–IL-6R antibody on prevention of early loss of transplanted islet allografts under the immunosuppressive treatment with anti-CD4 antibody. Diabetic C57BL/6 mice receiving 200 BALB/c islets and treated with control antibody remained hyperglycemic by 60 days after transplantation (Fig. 5, first panel). On the other hand, diabetic C57BL/6 mice receiving 200 BALB/c islets and treated with anti–IL-6R antibody alone became normoglycemic after islet transplantation and again hyperglycemic at 10.2 (2.3) days [n = 5, mean (SD)] after transplantation due to allograft rejection (Fig. 5, second panel). Morphologically, transplanted islets infiltrated with MNCs were seen at the time of rejection (Fig. 5, right row of second panel). In contrast, diabetic C57BL/6 mice receiving 200 BALB/c islets and treated with anti–IL-6R antibody (MR16-1) (n = 4) remained normoglycemic when allograft rejection is prevented by anti-CD4 antibody treatment (Fig. 5, third panel). Morphologically, intact islet grafts with well-granulated β cells were seen in the liver of mice receiving islet allografts with combined treatment of anti–IL-6R antibody (MR16-1) and anti-CD4 antibody (Fig. 5, right row of third panel). When diabetic C57BL/6 mice received 200 BALB/c islets and treated with anti-CD4 antibody (n = 5), recipient mice remained hyperglycemic by 60 days after transplantation (Fig. 5, fourth panel).

DISCUSSION

These findings clearly demonstrate that the treatment with anti–IL-6R antibody has beneficial effects on prevention of early loss of transplanted syngeneic and allogeneic islets favoring to ameliorate hyperglycemia of STZ-induced diabetic mice receiving a marginal mass of islets from a single donor.

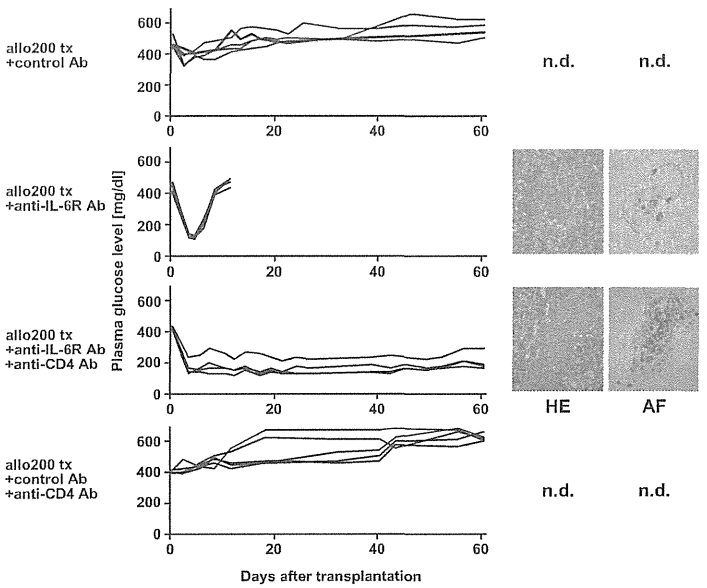

FIGURE 5. Beneficial effects of anti–IL-6R antibody on prevention of early loss of transplanted islet allografts. Two hundred BALB/c islets were grafted into the liver of STZ-induced diabetic C57BL/6 mice. Anti–IL-6R antibody (500 μg/injection per mouse) or control antibody was administered IP once at the time of islet transplantation. Nondepleting anti-CD4 antibody (200 μg/injection per mouse) was administered IP into appropriate groups of recipient mice once at the time of islet transplantation. Experimental groups included STZ-diabetic mice receiving 200 islet allografts and treated with control antibody (first panel), anti–IL-6R antibody (second panel), anti–IL-6R antibody in conjunction with anti-CD4 antibody (third panel), or anti-CD4 antibody alone (fourth panel). Individual lines represent the nonfasting plasma glucose levels of each animal. In the right column, photomicrographs of islet allografts at 12 (second panel) and 60 days (third panel) after transplantation were shown. The sections were stained with hematoxylin and eosin, and aldehyde and fuchsin (original magnification, ×400; n.d. indicates not done).

The early loss of transplanted islets has been a major concern in clinical islet transplantation because it may cause the low efficiency of islet transplantation, in which only the limited number of patients with IDDM becomes insulin-free after islet transplantation from a single donor, and therefore sequential transplantations with the use of 2 to 3 donors are needed for the treatment of a single recipient. In the previous studies, we have shown in mice that the early loss of transplanted islets could be prevented by the treatments targeting the HMGB1-DC-NKT cells–neutrophils-IFN-γ pathway including anti-HMGB1 antibody, anti–IL-12 and anti-CD40L antibody, α-galactosylceramide; a synthetic ligand of NKT cells, anti–Gr-1, anti-CD11b, and anti–IFN-γ antibody.[4,5] Furthermore, we have reported the beneficial effect of adenosine,[21] antithrombin III,[22] and thrombomodulin[23] on prevention of early loss of transplanted islets in the liver of mice. In these previous studies, the common characteristic relevant to acceptance of islet grafts in the liver achieved by individual treatments was the down-regulation of IFN-γ production of NKT cells and neutrophils accumulated in the liver of mice receiving islets within 6 hours after transplantation, while otherwise up-regulated which is thus an essential component of the early loss of transplanted islets. Of note, the treatment with anti–IL-6R antibody of the current study was found to have the similar effect as seen in the previous studies as to the islet graft survival with the down-regulation of IFN-γ production of NKT cells and neutrophils in the liver after transplantation, and however, with reduction in number of neutrophils accumulated in the liver receiving islets. The findings indicate that the treatment with anti–IL-6R antibody (MR16-1) may inhibit not only the HMGB-1-DC-NKT cells-neutrophils-IFN-γ pathway but also locomotion of neutrophils in the liver after transplantation. Recently, Citro et al[24] reported that the inhibition of chemokine signaling through CXCR1/2 and their ligand by CXCR1/2 inhibitor (reparixin) targeting on NKT cells improved the engraftment of intrahepatic islet allografts after transplantation in a clinical setting, suggesting that the locomotion of immune cells induced after islet transplantation is a novel target for intervention to improve the efficiency of islet transplantation. Thus, it is a matter of interest for future investigation to determine whether the beneficial effect of the anti–IL-6R antibody treatment is mediated through the inhibition of neutrophil accumulation as well as production of IFN-γ and how IL-6 itself is involved in the early loss of transplanted islets because it is not clarified in the present study.

Instant blood-mediated immune responses including complement have been reported to play an essential role in the early loss of transplanted islets in the liver.[25,26] However, the mechanistic analysis with respect to simultaneous and/or subsequent in vivo immune responses in the liver, site of islet transplantation, has not been shown. Thus, it might be important to dissect the relationship between the instant blood-mediated immune response and the HMGB1-DC-NKT cells–neutrophils-IFN-γ pathway to elucidate a novel mechanisms involved in the early loss of transplanted islets, facilitating to find new targets for intervention to improve further the efficiency of islet transplantation.

In summary, the present study demonstrates that a single injection of anti–IL-6R antibody to recipient mice at the time of islet transplantation produces prevention of early loss of transplanted islets in the liver, the site of islet transplantation. Because anti–IL-6R antibody has already been introduced in clinics for the treatment of rheumatoid arthritis[11,12] and Castleman disease,[13–15] the safety issue as to its clinical use for islet transplantation has been cleared. Thus, anti–IL-6R antibody may have a great impact to improve the efficiency of islet transplantation when the beneficial effect of anti–IL-6R antibody revealed by the present study in mice holds true in humans.

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