High-mobility group box 1 is involved in the initial events of early loss of transplanted islets in mice

Nobuhide Matsuoka, … , Masaru Taniguchi, Yohichi Yasunami


Islet transplantation for the treatment of type 1 diabetes mellitus is limited in its clinical application mainly due to early loss of the transplanted islets, resulting in low transplantation efficiency. NKT cell–dependent IFN-γ production by Gr-1^+CD11b^+ cells is essential for this loss, but the upstream events in the process remain undetermined. Here, we have demonstrated that high-mobility group box 1 (HMGB1) plays a crucial role in the initial events of early loss of transplanted islets in a mouse model of diabetes. Pancreatic islets contained abundant HMGB1, which was released into the circulation soon after islet transplantation into the liver. Treatment with an HMGB1-specific antibody prevented the early islet graft loss and inhibited IFN-γ production by NKT cells and Gr-1^+CD11b^+ cells. Moreover, mice lacking either of the known HMGB1 receptors TLR2 or receptor for advanced glycation end products (RAGE), but not the known HMGB1 receptor TLR4, failed to exhibit early islet graft loss. Mechanistically, HMGB1 stimulated hepatic mononuclear cells (MNCs) in vivo and in vitro; in particular, it upregulated CD40 expression and enhanced IL-12 production by DCs, leading to NKT cell activation and subsequent NKT cell–dependent augmented IFN-γ production by Gr-1^+CD11b^+ cells. Thus, treatment with either IL-12– or CD40L-specific antibody prevented the early islet graft loss. These findings indicate that the HMGB1-mediated pathway eliciting early islet loss is a potential target […]
High-mobility group box 1 is involved in the initial events of early loss of transplanted islets in mice

Nobuhide Matsuoka,¹,² Takeshi Itoh,¹ Hiroshi Watarai,³ Etsuko Sekine-Kondo,³ Naoki Nagata,⁴ Kohji Okamoto,⁴ Toshiyuki Mera,¹,² Hiroshi Yamamoto,⁵ Shingo Yamada,⁶ Ikuro Maruyama,⁷ Masaru Taniguchi,⁸ and Yohichi Yasunami¹

¹Department of Regenerative Medicine and Transplantation and ²Department of Gastrointestinal Surgery, Faculty of Medicine, Fukuoka University, Japan. ³Laboratory for Immune Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan. ⁴Department of Surgery 1, University of Occupational and Environmental Health, School of Medicine, Kitakyushu, Japan. ⁵Department of Biochemistry and Molecular Vascular Biology, Kanazawa University Graduate School of Medical Science, Japan. ⁶Shino-Test Co., Sagamihara, Japan. ⁷Department of Laboratory and Vascular Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Japan.

Islet transplantation for the treatment of type 1 diabetes mellitus is limited in its clinical application mainly due to early loss of the transplanted islets, resulting in low transplantation efficiency. NKT cell–dependent IFN-γ production by Gr-1⁺CD11b⁺ cells is essential for this loss, but the upstream events in the process remain undetermined. Here, we have demonstrated that high-mobility group box 1 (HMGB1) plays a crucial role in the initial events of early loss of transplanted islets in a mouse model of diabetes. Pancreatic islets contained abundant HMGB1, which was released into the circulation soon after islet transplantation into the liver. Treatment with an HMGB1-specific antibody prevented the early islet graft loss and inhibited IFN-γ production by NKT cells and Gr-1⁺CD11b⁺ cells. Moreover, mice lacking either of the known HMGB1 receptors TLR2 or receptor for advanced glycation end products (RAGE), but not the known HMGB1 receptor TLR4, failed to exhibit early islet graft loss. Mechanistically, HMGB1 stimulated hepatic mononuclear cells (MNCs) in vivo and in vitro; in particular, it upregulated CD40 expression and enhanced IL-12 production by DCs, leading to NKT cell activation and subsequent NKT cell–dependent augmented IFN-γ production by Gr-1⁺CD11b⁺ cells. Thus, treatment with either IL-12- or CD40L-specific antibody prevented the early islet graft loss. These findings indicate that the HMGB1-mediated pathway eliciting early islet loss is a potential target for intervention to improve the efficiency of islet transplantation.

Introduction
Pancreatic islet transplantation, although an attractive procedure for the treatment of type 1 diabetes mellitus, usually fails to achieve insulin independence of a diabetic recipient from a single donor due to early loss of transplanted islets and therefore requires sequential transplantations of islets with the use of 2–3 donors (1). Thus, the low efficiency of islet transplantation has been a major obstacle facing islet transplantation and hampers its clinical application.

We have previously shown in mice that loss of transplanted islets soon after transplantation is caused by NKT cell–dependent IFN-γ production by Gr-1⁺CD11b⁺ cells and is successfully prevented by treatment of NKT cells with repeated stimulation with their synthetic ligand, α-galactosylceramide (α-GalCer), to downregulate IFN-γ production of NKT cells, or by depletion of Gr-1⁺CD11b⁺ cells with anti–Gr-1 antibody (2). However, precisely how it is involved in the upstream events in the activation of NKT cells and Gr-1⁺CD11b⁺ cells in the early loss of transplanted islets remains to be solved.

High-mobility group box 1 (HMGB1) protein was initially found to be a DNA-binding protein present in almost all eukaryotic cells, where it stabilizes nucleosome formation and acts as a nuclear factor that enhances transcription (3, 4). Recently, HMGB1 has been demonstrated to play crucial roles in response to tissue damage, indicating that HMGB1 is a prototype of the emerging damage-associated molecular pattern molecule (4, 5). HMGB1 is also known to be secreted by activated immune cells, including macrophages (6, 7), DCs (8), and NK cells (9) in response to infection and inflammatory stimuli. Once secreted, HMGB1 induces inflammatory responses by transduction of cellular signals through its receptors, such as TLR2, TLR4 (10–12), and receptor for advanced glycation end products (RAGE) (8, 13, 14). Moreover, HMGB1 levels are markedly increased during severe sepsis in humans and animals, and administration of neutralizing HMGB1-specific antibodies prevents lethality from sepsis (6). Recent accumulating evidence now suggests that HMGB1 acquires or augments proinflammatory activity by binding to proinflammatory mediators such as LPS, IL-1 (14), and DNA (15–17). These observations indicate that HMGB1 is an essential mediator of organ damage; however, its precise role and mechanism remain unknown. Here, we investigate the mechanisms of action of HMGB1 in the early loss of transplanted islets.

Results
Involvement of HMGB1 in early loss of transplanted islets. It has previously been shown that hyperglycemia of streptozotocin-induced (STZ-induced) diabetic recipient mice was ameliorated after transplantation of 400 syngenic islets in the liver but not of 200 islets (Figure 1A, no treatment), the number of islets isolated from a

Authorship note: Nobuhide Matsuoka, Takeshi Itoh, and Hiroshi Watarai contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2010;120(3):735–743. doi:10.1172/JCI41360.
single mouse pancreas (2). By using the diabetes model mice, we first investigated the effects of anti-HMGB1 antibody to examine whether HMGB1 is directly involved in early loss of transplanted islets. STZ-induced diabetic mice that received 200 syngeneic islets (Islet Tx), and islet transplanted mice treated with anti-HMGB1 antibody or with chicken IgG. NKT cells (top 2 rows) and Gr-1$^+$CD11b$^+$ cells (bottom 2 rows) were analyzed for IFN-γ (second and fourth rows). The numbers in the figures represent the percentage of cells in the corresponding square areas. Representative data from 4 experiments are shown.
production by NKT cells and Gr-1+CD11b+ cells in the liver of mice receiving islets, which are essential components of early loss of transplanted islets, as shown previously (2). For those purposes, mononuclear cells (MNCs) in the liver of recipient mice were isolated at 6 hours after islet transplantation of 200 syngeneic islets into the liver and examined by FACS as to IFN-γ production by NKT cells and Gr-1+CD11b+ cells in the liver. The results are in agreement with the previous findings (2) that, within 6 hours after transplantation of syngeneic islets into the liver, NKT cells and Gr-1+CD11b+ cells accumulated in the liver with upregulated production of IFN-γ (Figure 1B). This upregulated production of IFN-γ after islet transplantation was inhibited by anti-HMGB1. Since the treatment with anti-HMGB1 antibody did not affect the number of infiltrated Gr-1+CD11b+ cells (Figure 1B), the recruitment of Gr-1+CD11b+ cells was due not to HMGB1, but rather probably to the events of transplantation itself. These findings suggest that HMGB1 is essentially involved in the activation of NKT cells and/or Gr-1+CD11b+ cells in the liver after islet transplantation.

Next, we examined the amounts of HMGB1 in isolated islets in comparison with those in other organs, including the thymus, lung, spleen, liver, and pancreas, as well as of FACS-sorted liver MNCs, including NK, NKT, T, B, Gr-1+CD11b+, and Gr-1+CD11b- cells. Currently, there are no data available with respect to the HMGB1 content in the different cell types, although tissue dis-

Involvement of TLR2 and RAGE but not TLR4 in HMGB1-dependent early loss of transplanted islets. We further investigated whether HMGB1-dependent early loss of transplanted islets is dependent on TLR2, TLR4, and/or RAGE, which is known to be a potential receptor of HMGB1 (10–14). Isolated liver MNCs from wild-type mice induced augmented production of IL-12 and IFN-γ in response to HMGB1 in vitro (Figure 2A), which were greatly reduced in Tlr2−/− and Tlr4−/− mice but not in Tlr4−/− liver MNCs, whose cytokine production levels were equivalent to those of wild-type mice in response to HMGB1.

To elucidate which receptor(s) for HMGB1 are actually involved in early loss of transplanted islets, STZ-induced diabetic Tlr2−/−, Tlr4−/−, or Rage−/− mice that received 200 syngeneic islets were investigated for glucose levels in the serum. Interestingly, all of Tlr2−/− or Rage−/− mice (5 of 5) became normoglycemic, while Tlr4−/− mice remained hyperglycemic after transplantation, indicating that TLR2 and RAGE, but not TLR4, play an essential role in the early loss of transplanted islets (Figure 2B).

Pancreatic islet cells are a major source of HMGB1, which mediates IFN-γ production by NKT cells and Gr-1+CD11b+ cells. To validate the involvement of HMGB1 in early loss of transplanted islets, we carried out histological examination on islets before and after transplantation. HMGB1 was detected at a high level in cytoplasm as well as of nucleus of transplanted islets at 3 hours after transplantation, while HMGB1 was stained only in the nucleus of islets in the naive pancreas and of isolated islets (Figure 3A). The results suggest that HMGB1 is localized in the nucleus of pancreatic islets, shuttled to cytoplasm, and possibly secreted into the circulation soon after transplantation.

Next, we examined the amounts of HMGB1 in isolated islets in comparison with those in other organs, including the thymus, lung, spleen, liver, and pancreas, as well as of FACS-sorted liver MNCs, including NK, NKT, T, B, Gr-1+CD11b+, and Gr-1+CD11b- cells. Currently, there are no data available with respect to the HMGB1 content in the different cell types, although tissue dis-

Figure 2
HMGB1 receptors involved in early loss of transplanted islets. (A) In vitro cytokine production by liver MNCs. Liver MNCs (2 × 10⁶/well) isolated from wild-type, Tlr2−/−, Tlr4−/−, or Rage−/− mice were cultured with indicated doses of HMGB1 in vitro for 48 hours, and IL-12 or IFN-γ levels in the culture supernatant were measured. Representative data from 2 experiments are shown. (B) Nonfasting plasma glucose levels of STZ-induced diabetic wild-type, Tlr2−/−, Tlr4−/−, or Rage−/− mice that received 200 syngeneic islets. Individual lines represent the glucose level of each animal.
distribution of HMGB1 has been reported previously (18). To our surprise, isolated islets contained high levels of HMGB1, which were 20 times more greater than in other organs or FACS-sorted cells tested (Figure 3B). The physiological roles of high concentrations of HMGB1 in islet cells as well as their etiology are a matter of interest and need to be clarified in future studies.

To investigate a direct relationship between HMGB1 and islet cell damage, we cultured isolated mouse islets in the absence or presence of cytotoxic proinflammatory cytokines, including IFN-γ, TNF-α, and IL-1β, which are known to induce islet cell death in vitro (19) with elevated concentrations of HMGB1 in the culture medium (20). IL-10 was used as a control. Islet cell death was assessed by fluorescence microscopy with the use of the DNA-binding dye propidium iodide (PI) and Hoechst 33342 (HO 342) (19). PI, a highly polar dye that is impermeable to cells with preserved membranes, stains DNA red when membranes are damaged. HO 342 freely passes the plasma membrane, readily enters cells with intact membranes, and stains DNA blue. Thus, the nuclei of dead cells stained red by PI, while those of intact cells stained blue without fragmentation and condensation by HO 342. PI-positive islet cells were increased in number with time in the presence of cytotoxic cytokines, while, in contrast, that in the absence of cytotoxic cytokines or the presence of cytokine mixtures or of control cytokine (IL-10). The values are expressed as the mean ± SD in each group (n = 5). (D) Serum HMGB1 levels were measured after STZ injection and also after transplantation of 400 syngeneic islets, which had been performed 72 hours after STZ injection (n = 5–6). The values are expressed as the mean ± SD. *P < 0.05; **P < 0.01.
IL-12 is essential for IFN-γ production. Importantly, the amounts of IL-12 and IFN-γ are elevated due to islet cell damage caused by STZ injection (Figure 3D). The findings suggest that the first peak of the serum HMGB1 elevation is due to islet cell damage caused by STZ injection, which is a toxic agent to β cells of islets, while the second peak is due to the early loss of transplanted islets.

Cell types responsible for HMGB1-mediated cytokine production. We investigated the mechanisms of action of HMGB1 by measuring in vitro production of IFN-γ and IL-12 in the culture of isolated liver MNCs from wild-type and Jst18−/− mice in response to HMGB1, since IFN-γ is critical in the early islet graft loss (2) and also because IL-12 is essential for IFN-γ production by NKT cells (21). Isolated liver MNCs from wild-type mice induced augmented production of IL-12 and IFN-γ in response to HMGB1 in vitro (Figure 4A). Importantly, the amount of IL-12 and IFN-γ produced by liver MNCs in NKT cell–deficient Jst18−/− mice treated with HMGB1 was greatly reduced (Figure 4A), indicating that NKT cells augment HMGB1-dependent IL-12 and IFN-γ production.

We then investigated expression of Thr2 and Rage by quantitative real-time PCR in each FACS-sorted cellular population from the liver, including NK, NKT, T, and B cells (Figure 4B). Thr2 and Rage were detected at high levels on Neu (Figure 4B). DCs also expressed Rage receptors on high levels on Neu (Figure 4B). However, Mo/Mφ expressed modest levels of Thr2 but low levels of Rage, while expression of either Thr2 or Rage was barely detected in other cell populations (NK, NKT, T, and B cells) (Figure 4B).

In order to dissect further the mechanisms of action of HMGB1, we investigated IL-12 and IFN-γ production in the culture of FACS-sorted individual cellular populations from liver MNCs in response to HMGB1. IL-12 was mainly produced from DCs rather than Neu or Mo/Mφ (Figure 4C). However, IFN-γ production was not detected in any individual cell population among all liver MNC subpopulations tested (Figure 4C).
Since IL-12 was produced in vitro from DCs in response to HMGB1 (Figure 4C) and since NKT cell–dependent IFN-γ production by Neu is an essential component of early loss of transplanted islets as shown previously (2), IL-12 and IFN-γ production of FACS-sorted DCs, Mo/Mϕ, or Neu cocultured in the presence of NKT cells with addition of HMGB1 was examined. The production of IL-12 was greatly augmented in response to HMGB1, especially when DCs were cocultured with NKT cells (Figure 4D). The production of IFN-γ became evident in the culture medium of DCs cocultured with NKT cells in the presence of HMGB1 (Figure 4D). The cell types responsible for the production of IFN-γ in response to HMGB1 in Figure 4D were NKT cells but not DCs, because intracellular cytokine staining revealed that NKT cells, but not DCs, produced IFN-γ (Figure 4E). It was also shown that Neu production of IFN-γ was augmented in the presence of NKT cells (Figure 4D).

It is known that IFN-γ production by NKT cells is largely dependent on the interaction between CD40L expression on activated NKT cells and CD40 expression on DCs (22). Thus, we measured CD40 expression on DCs and Neu stimulated with HMGB1. CD40 surface expression was detected in both cell types in resting conditions, while upregulation of CD40 was observed in DCs rather than Neu in HMGB1-treated conditions (Figure 5A). Furthermore, production of both IL-12 and IFN-γ mounted in vitro by HMGB1 stimulation was blocked by anti-CD40L antibody (Figure 5B), indicating that augmented IL-12 production from DCs and Neu and also IFN-γ production by NKT cells and Neu are triggered by CD40/CD40L interaction.

To confirm the data shown in Figure 5, A and B, we determined in vivo requirement of IL-12 and CD40-CD40L interaction in early loss of transplanted islets. Hyperglycemia of STZ diabetic mice receiving 200 syngenic islets in the liver was ameliorated by treatment with either anti–IL-12 or anti-CD40L antibody once at the time of islet transplantation, while that of mice treated with control antibody was not (Figure 5C). Together with the previous studies showing that the anti–IFN-γ treatment normalizes hyperglycemia (22), the results indicate that IL-12 and CD40-CD40L interaction together with IFN-γ actually play a crucial role in vivo in early loss of transplanted islets.

Discussion
Among the most important findings of the present study is that pancreatic islets contain abundant HMGB1 compared with other organs and individual cell populations in the liver, the site of islet transplantation. Immunohistochemical staining of the pancreas revealed that HMGB1 is mainly stained in the nucleus of islet cells but not in other cell types, while HMGB1 is detected in the circulation after islet cell damage. In fact, the plasma concentration of HMGB1 in wild-type mice was elevated and peaked at 24 hours after i.v. injection of STZ and returned to the preinjection level 72 hours after STZ injection. The plasma levels of HMGB1 in diabetic recipient mice were elevated after islet transplantation with a peak at 6 hours and returned to pretreatment levels by 24 hours. These findings suggest that the first peak of the elevated HMGB1 levels is caused by destruction of islet cells by a toxic agent of STZ specific to β cells of islets and that the second peak in recipient...
mice after islet transplantation is related to the damage of islet grafts soon after transplantation. Thus, combined with in vitro findings of elevated concentrations of HMGB1 in the culture medium of isolated islets in the presence of cytotoxic cytokines, the plasma levels of HMGB1 may reflect the degree of islet damage in the liver after transplantation. Furthermore, the treatment with anti-HMGB1 antibody delayed the onset of diabetes in NOD mice, suggesting that HMGB1 plays a significant role in disease progression (23).

The above findings prompted us to determine whether HMGB1 is involved in the early loss of transplanted islets, which occurs within 6 hours after islet transplantation and is an event caused by inflammatory cytokines, as we previously reported (2, 23). In fact, the hyperglycemia of islet-transplanted diabetic mice was ameliorated by treatment with anti-HMGB1 antibody, indicating that HMGB1 is essentially involved in the early loss of transplanted islets.

Concerning the mechanisms of HMGB1-mediated early islet graft loss, 3 cell types, NKT cells, Gr-1 CD11b+CD11c− F4/80− DCs, and Gr-1 CD11b+ Neu, were found to be involved in the initial phase of early loss of transplanted islets. Among these cell types, the primary cellular targets of HMGB1 does not seem to be NK T cells, since the receptors for HMGB1 (10–14) TLR2 and RAGE but not TLR4 (Figure 2B) are expressed on DCs, Mo/Mϕ, and Neu, but not on NK T cells (Figure 4B). IL-12, which is essential for NK T cell–dependent production of IFN-γ, was mainly produced by DCs after HMGB1 stimulation (Figure 4C). Thus, it is likely that the first target for HMGB1 is DCs, which in turn activate NK T cells. Then, activated NK T cells themselves produce IFN-γ and also stimulate Neu to produce IFN-γ (Figure 4D), which is an essential component of HMGB1-mediated early loss of transplanted islets.

Thus, the present study unveils a role of DCs in HMGB1-dependent IFN-γ production by NK T cells. DCs stimulated with HMGB1 in vitro upregulate their CD40 expression and produce IL-12, which is markedly augmented in the presence of NK T cells, facilitating IFN-γ production by NK T cells and subsequently that of Neu. The requirement of CD40-CD40L interaction and IL-12 is confirmed by the fact that anti-CD40L and anti–IL-12 antibodies prevented early loss of transplanted islets, leading to amelioration of hyperglycemia of islet-transplanted diabetic recipient mice, while the corresponding control antibody did not. Thus, the uncovered pathways involved in the early loss of transplanted islets in the present study afford further new targets for intervention to improve the efficiency of islet transplantation.

TLR2, TLR4, and RAGE as potential receptors for HMGB1 (10–14) are expressed mainly on DCs, Mo/Mϕ, and Neu (Figure 4B). However, in vitro and in vivo experiments revealed that TLR2 and RAGE but not TLR4 are involved in the early loss of transplanted islets (Figure 2B). It has been reported that HMGB1-mediated biological effects and usage of their receptors are different in the experimental models. For example, TLR4, but not TLR2 or RAGE, has been shown to be an HMGB1 receptor in hepatic reperfusion injury (24). Similarly, HMGB1 signaling through TLR2 and TLR4 but not RAGE contributes to LPS-induced inflammation (11). In the case of SLE, HMGB1 present in DNA-containing immune complexes triggers activation of autoreactive B cells and plasmacytoid DCs through RAGE (17). These differences in HMGB1-mediated effects might be due to the presence, in different systems, of cell types with distinctly different HMGB1 receptor expression profiles, and also due to the formation of complexes of HMGB1 with different molecules under varying disease conditions.

Concerning the form of HMGB1, HMGB1 acquires and/or augments inflammatory effects when it binds to other inflammatory molecules, such as IL-1β, the TLR4 ligand LPS, the TLR9 ligand CpG-ODN, or the TLR1-TLR2 ligand Pam3CSK (14–17). Recent studies on HMGB1-deficient mice also showed that HMGB proteins function as universal sentinels for nucleic acids (25). However, in the present studies, it still remains unsolved what types of molecules interact with HMGB1 protein to mediate its function.

Chen et al. (26) have reported that the direct effects of RAGE on conventional T cell functions resulted in the prolongation of syngeneic and allogeneic islet graft transplanted in the subcapsular space of kidney, in that anti–CD3/CD28–induced T cell proliferation, mixed lymphocyte reaction, and T cell production of IL-10, IL-5, and TNF-α but not IFN-γ were inhibited in RAGE-deficient mice and mice receiving RAGE inhibitor. Since no conventional T cells were involved in the early loss of islet transplanted in the liver, and also because IFN-γ, but not IL-10 nor IL-5, is a major player in the early islet loss, the mechanisms observed in the present studies are different from those described by Chen et al. (26).

Concerning the potential sites for islet transplantation—including the liver, renal subcapsular space, omental pouch, abdominal cavity, intramuscular site, subcutaneum—the liver is currently the only site where insulin independence in patients with type 1 diabetes mellitus can be achieved with clinical islet transplantation, as reported by Shapiro et al. (1). Although we do not have any data on islet transplantation at non–NKT cell–dense sites, the NKT cell–mediated early loss of islets can occur at any tissue, as it has been demonstrated in the allogeneic heart transplantation model that NKT cells migrate immediately into non–NKT cell–dense transplantation sites, where CXCL16, the ligand for chemokine receptor CXCR6 selectively expressed on NKT cells, is expressed (27).

Taken collectively, the findings in the present study shed light on the mechanisms involved in the early loss of transplanted islets as follows. First, islet cells themselves are a major source of HMGB1, which is released from transplanted islets. Since the plasma levels of HMGB1 reflect the degree of islet damage, HMGB1 could be a marker to predict rejection of transplanted islets. Second, HMGB1 stimulates production of inflammatory cytokines including IL-12 and IFN-γ in concert with DCs, NK T cells, and Neu in the liver receiving islets. Third, these inflammatory cytokines accelerated the injuries of transplanted islets. Thus, a vicious cycle harmful to transplanted islets is now unveiled. Therefore, each pathway involved in the early loss of transplanted islets revealed by the present study is a potential target for intervention to improve efficiency of islet transplantation.

Methods

Mice. C57BL/6 mice were purchased from Charles River Japan Inc. or CLEA Japan Inc. Fox18-deficient mice were generated previously (28) and backcrossed more than 10 times to C57BL/6 mice. RAGE−/− mice (29) were described previously. Thβ2−/− and Thβ4−/− mice were provided by Shizuo Akira (Osaka University, Osaka, Japan). Mice were kept under specific pathogen–free conditions and used at 8–16 weeks of age. All experiments were in accordance with protocols approved by the Animal Care and Use Committee of Fukuoka University and RIKEN.

Islet isolation and transplantation. Islets were isolated (30, 31) and transplanted into the liver via the portal vein (32) of STZ-induced (180 mg/kg, Sigma-Aldrich) diabetic mice at 3 days after the injection of STZ. The nonfasting plasma glucose levels of mice were measured using a Beckman glucose analyzer (Beckman Japan).
Immunohistochemistry. The pancreas of naive mice, isolated islets, and the liver of transplant recipients were fixed in 10% formaldehyde solution, processed, and embedded in paraffin. The sections were stained immunohistochemically with anti-mouse insulin antibody (Novocastra) and rabbit anti-bovine HMG1 antibody (Shino-Test Co.) by a streptavidin-biotin-peroxidase complex method (33).

HMG1 and cytokine measurement. HMG1 levels in mouse serum and in the culture medium of isolated islets were determined with an ELISA kit (Shino-Test Co.) (34). IFN-γ concentrations in the culture supernatant of liver MNCs were determined by FACS with cytometric beads assay (CBA) (BD Biosciences). IL-12 concentration in the medium was measured by ELISA (Endogen).

For measurement of tissue concentration of HMG1, individual tissues (1–2 mg wet weight/organ), isolated islets (200 total), and FACS-sorted cells (2 × 10^6 to 6 × 10^6) of each population in the liver were sonicated in PBS. Then, the resulting tissues were treated as reported by Sanders (35) in which perchloric acid (HClO4) was added to the homogenates with a concentration of 0.75 M. The content of HMG1 in the solution was measured with ELISA after the adjustment of pH to 7.0 as well as the appropriate dilution with PBS containing 1% bovine calf serum. The sonicated tissues were also used for measuring DNA content with a Wako assay kit.

Reagents. Bovine HMG1 was purchased from Shino-Test Co. Bovine HMG1 was extracted from the bovine thymus and further purified by CM-Sephadex C25 ion column chromatography according to the method described by Sanders (35). The biological activity of purified HMG1 was reported elsewhere (36). Anti-HMG1 antibody was purchased from Shino-Test Co. This is a polyclonal antibody made by immunizing chicken with purified bovine HMG1, and the neutralizing effect of the anti-HMG1 antibody was reported previously (37, 38). Goat anti-mouse IL-12 antibodies and rabbit anti-mouse CD40L antibody were purchased from BD Biosciences and Sigma-Aldrich, respectively. Recombinant mouse IFN-γ, TNF-α, IL-1β, and IL-10 were purchased from Sigma-Aldrich.

Flow cytometry. Antibodies used for flow cytometric analysis were as follows: anti-mouse FcRγII/III (2.4G2), FITC- or Pacific blue–conjugated anti-CD3 (145-2C11), FITC- or PerCP-Cy5.5-conjugated anti-CD11b (M1/70), allophycocyanin-conjugated (APC-conjugated) anti-IFN-γ (XM12.1), peridinin–chlorophyll protein– (PerCP-) or FITC–conjugated anti-Gr-1 (RB6-8C5), PE-conjugated NK1.1 (PK136), PE-Cy7–conjugated CD19 (1D3), biotinylated anti-CD11c (N418), APC-Cy7–conjugated avidin, APC-conjugated anti-F4/80 (BM8) (BD Biosciences or eBioscience). PE- or APC-labeled α-GaCer–loaded CD1d dimer was prepared as described (39). Intracellular cytokine staining was performed as previously described (39). Flow cytometry was performed using a FACSCalibur and FACSArray (BD) with FlowJo software (Tree Star). The purity of sorted cells was usually greater than 99%.

Cell preparation and culture. Liver MNCs were prepared as described previously (40). For in vitro culture, liver MNCs and those of FACS-sorted cells were cultured in RPMI medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum (Biosource) and 100 μg/ml kanamycin (Meiji Seika) and isolated islets in DMEM medium (Nissui) supplemented with 2% BSA (Sigma-Aldrich) and 100 μg/ml kanamycin in a CO2 incubator (95% air plus 5% CO2) at 24°C or 37°C.

Quantitative real-time PCR. Total RNA was isolated from FACS-purified cell populations using TRIZOL reagent (Invitrogen). cDNA was prepared by Superscript III RTnase H Reverse Transcriptase with random hexamers (Invitrogen). Quantitative real-time PCR was performed with SYBR GreenER qPCR SuperMix (Invitrogen) for ABI PRISM 7900HT (Applied Biosystems). Total mRNA from cells (2 × 10^6) was used as templates to analyze expression levels of Th2, Rage, or Hprt. Gene-specific primer sequences were as follows: Th2-fw, GGGGCTTCACTTCTCTGCTT, Th2-rv, AGCATCCTCTGAGATTGACG; Rage-fw, 5′-GTGTGGCGGGAAC- TAAAGG-3′, Rage-rv, 5′-CTGGCTTCCAGGAACTG-3′; Hprt-fw, 5′- TCTCCCTCAGCCGTTT-3′, Hprt-rv, 5′-CTGGTGTATCATGCTGTA- ATC-3′. Quantitative analysis was performed by the ΔΔCt method by using Hprt as an internal control.

Statistics. The statistical significance of differences was determined by 1-tailed Student’s t test. Values were expressed as mean ± SD from independent experiments. Any difference with a P value less than 0.05 was considered significant.

Acknowledgments

We are grateful to Peter Burrows for helpful comments and constructive criticisms in the preparation of the manuscript. Technical support by Yuko Ueda (Department of Surgery, University of Occupational and Environmental Health, School of Medicine) is greatly appreciated. This work was supported by a grant from the Global FU Program of Fukuoka University (Y. Yasunami), a Health Science Research Grant from the Ministry of Health, Labor and Welfare, Japan (Y. Yasunami), and the Shimura Memorial Foundation (Y. Yasunami).

Received for publication October 6, 2009, and accepted in revised form December 9, 2009.

Address correspondence to: Masaru Taniguchi, Laboratory for Immune Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Kanagawa 230-0045, Japan. Phone: 81.45.503.7001; Fax: 81.45.503.7003; E-mail: taniguti@rcai.riken.or.jp. Or to: Yohichi Yasunami, Department of Regenerative Medicine and Transplantation, Faculty of Medicine Fukuoka University, Fukuoka, Fukuoka 814-0180, Japan. Phone: 81.92.801.1011 ext. 3630; Fax: 81.92.801.1019; E-mail: yasunami@fukuoka-u.ac.jp.


