Efficacy of DHMEQ, a NF-κB Inhibitor, in Islet Transplantation: II. Induction DHMEQ Treatment Ameliorates Subsequent Alloimmune Responses and Permits Long-Term Islet Allograft Acceptance

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Background. Long-term graft deterioration remains a major obstacle in the success of pancreatic islet transplantation (PITx). Antigen-independent inflammatory and innate immune responses strengthen subsequent antigen-dependent immunity; further, activation of nuclear factor (NF)-κB plays a key role during these responses. In this study, we tested our hypothesis that, by the inhibition of NF-κB activation, the suppression of these early responses after PITx could facilitate graft acceptance.

Methods. Full major histocompatibility complex (MHC)–mismatched BALB/c (H-2^d) mice islets were transplanted into streptozotocin-induced diabetic C57BL/6 (B6: H-2^b) mice. The NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) was administered for either 3 or 14 days after PITx. To some PITx recipients, tacrolimus was also administered. Islet allograft survival, alloimmune responses, and in vitro effects of DHMEQ on dendritic cells (DCs) were assessed.

Results. With a vehicle treatment, 600 islet allografts were promptly rejected after PITx. In contrast, 3-day treatment with DHMEQ, followed by 2-week treatment with tacrolimus, allowed permanent acceptance of islet allografts. The endogenous danger-signaling molecule high mobility group complex 1 (HMGB1) was elevated in sera shortly after PITx, whereas DHMEQ administration abolished this elevation. DHMEQ suppressed HMGB1-driven cellular activation and proinflammatory cytokine secretion in mouse bone marrow–derived DCs and significantly reduced the capacity of DCs to prime allogeneic T-cell proliferation in vitro. Finally, the DHMEQ plus tacrolimus regimen reverted the diabetic state with only 300 islet allografts.

Conclusions. Inhibition of NF-κB activation by DHMEQ shortly after PITx suppresses HMGB1, which activates DCs and strengthens the magnitude of alloimmune responses; this permits long-term islet allograft acceptance, even in case of fewer islet allografts.

Keywords: Pancreatic islet transplantation, DHMEQ, Nuclear factor-κB.

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Clinical pancreatic islet transplantation (PITx) has become a realistic treatment option for type 1 diabetes mellitus after introduction of the Edmonton protocol (*1*). During the past decade, immunosuppression after PITx has been refined with use of newly developed agents, such as hOKT3-Ala-Ala (*2*), alemtuzumab (*3*), the costimulation blocker belatacept (*4*), and thymoglobulin plus anti-inflammatory antibodies (*5*). Although the primary outcomes of PITx have

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improved steadily, with fewer adverse events, the insulin independence rate has been noted to gradually drop to 44% at 3 years after PITx even in recipients who received PITx recently (*6*). Therefore, a gradual but progressive rejection of islet allografts is inevitable under the current protocols, and a new immunosuppressive strategy is essential for further improvement of PITx.

After PITx, leukocytes, such as macrophages, neutrophils, T cells, and dendritic cells (DCs), are activated, and these activated cells produce proinflammatory mediators, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-8, IL-12, and monocyte chemoattractant protein, which amplify inflammation and immune responses (*7, 8*). Endogenous "danger-signaling" molecules released from damaged islets and other cells or tissues, so-called damage-associated molecular patterns (DAMPs), such as high mobility group complex 1 (HMGB1) (*9*), tissue factor (*10*), heat shock proteins (*11, 12*), and cell fragments elicit innate immune and exaggerated inflammatory responses. Moreover, DAMPs generate immunostimulatory DCs, which play a crucial role in the translation of innate to adaptive immune responses (*13*) and augment allograft rejection and autoimmunity (*14, 15*). As the understanding of innate and adaptive immunology progresses, the importance of preventing early nonspecific immune reactions shortly after PITx is increasingly being recognized, not only for avoiding early islet graft loss but also for controlling subsequent progressive allograft rejection.

The transcription factor nuclear factor (NF)-κB is a key regulator of inflammation and innate- and adaptive-immune responses. It has been shown that NF-κB is involved in the activation, differentiation, proliferation, and survival of various cell types (*16*) and that the transcription of many genes encoding proinflammatory cytokines such as TNF-α, IL-1, IL-2, IL-6, IL-12, and interferon (IFN)-γ are dependent on NF-κB (*17*). In DCs, signaling through DAMP-pattern recognition receptors activates NF-κB, which induces the up-regulation of surface costimulatory molecules and promotes DCs to act as antigen-presenting cells to initiate immune responses (*18*).

Dehydroxymethylepoxyquinomicin (DHMEQ) is a newly developed, low-molecular-weight NF-κB inhibitor that blocks NF-κB activation at the nuclear translocation level by binding to the cysteine-38 residue of p65 (*19, 20*). Our previous findings and those of other studies have demonstrated that DHMEQ exerts both anti-inflammatory (*21, 22*) and immunosuppressive (*23*) effects in rodents. Here, we used DHMEQ to test the hypothesis that the inhibition of inflammatory and innate immune responses associated with PITx, via inhibition of NF-κB activation, would reduce the subsequent alloimmune responses and permit successful long-term allogeneic islet acceptance.

RESULTS

DHMEQ Inhibits HMGB1-Induced DC Activation, Proinflammatory Cytokine Production, and Allospecific Immunostimulatory Capacity

To examine the effect of DHMEQ on the linkage between innate and subsequent adaptive immunity in vitro, we first examined whether DHMEQ suppresses activation and proinflammatory cytokine production of DCs after HMGB1 stimulation. HMGB1 is a DNA-binding protein that is passively released from damaged cells and tissues (*24*) and functions as a mediator of innate immunity and inflammation (*25*). In vitro, HMGB1 significantly stimulated murine bone marrow–derived DCs (BMDCs) maturation and activation, as manifested by increased expression of CD40, CD86, and major histocompatibility complex (MHC) class II. Pretreatment of BMDCs with DHMEQ before HMGB1 stimulation significantly inhibited the up-regulation of these surface markers (Fig. 1A,B). Also, DHMEQ treatment significantly suppressed HMGB1-mediated production of proinflammatory cytokines such as IL-6, IL-12, and TNF-α in the stimulated DCs compared with DHMEQ-untreated controls (Fig. 1C).

To examine the allospecific immunostimulatory capacity of DCs, T cells obtained from C57BL/6 mouse splenocytes was cocultured with HMGB1-triggered BALB/c mouse BMDCs, and T-cell proliferation was assessed. As shown in Figure 1D and E, the DHMEQ-treated DCs were found to significantly suppress allogeneic T-cell proliferation and IFN-γ production from the stimulated T cells compared with control DCs.

Treatment with DHMEQ Shortly After PITx, Followed by Tacrolimus Administration, Facilitates Allograft Acceptance

We then examined the effects of DHMEQ in a PITx model in which fully MHC-mismatched BALB/c (H-2^d) mouse pancreatic islets were transplanted into streptozotocin (STZ)–induced diabetic C57BL/6 (H-2^b) mice. Upon transplant of 600 allogeneic islets, control mice that had been administered carboxymethylcellulose (CMC) rejected the allografts, with a median survival time (MST) of 11 days (Fig. 2A,B). Treatment with DHMEQ prolonged islet allograft survival. However, only a few recipients accepted islet grafts for more than 100 days: 2 of 8 (25.0%) animals treated with DHMEQ for 3 days and 3 of 9 (33.3%) animals treated for 14 days. Tacrolimus administration prolonged allograft survival to a MST of 69 days, and 3 of 8 (37.5%) animals accepted islet grafts for more than 100 days. In contrast, all the recipient mice accepted islet allografts over the longer term (MST, >100 days) when treated first with DHMEQ for 3 days after PITx and with tacrolimus for 14 days thereafter (Fig. 2A,B). Furthermore, to confirm that the prolongation of normoglycemia after PITx was due to acceptance of islet allografts under the current treatment protocol, 600 BALB/c mice islets were transplanted under the kidney capsule of STZ-induced diabetic C57BL/6 mice, and islet-recipients were treated with the combination therapy. Blood glucose level normalized promptly after the PITx, and all animals achieved normoglycemic state thereafter. When transplanted islet allografts were removed together with the kidney 60 days later, blood glucose levels increased to diabetic levels in all animals (Fig. 2C).

Treatment with DHMEQ Suppresses Intragraft Cellular Infiltration and Alloimmune Responses After PITx

The transplanted islet allografts in the recipients' livers were evaluated by histopathology at 9 days after PITx. In the vehicle-treated control animals, severe cellular infiltration

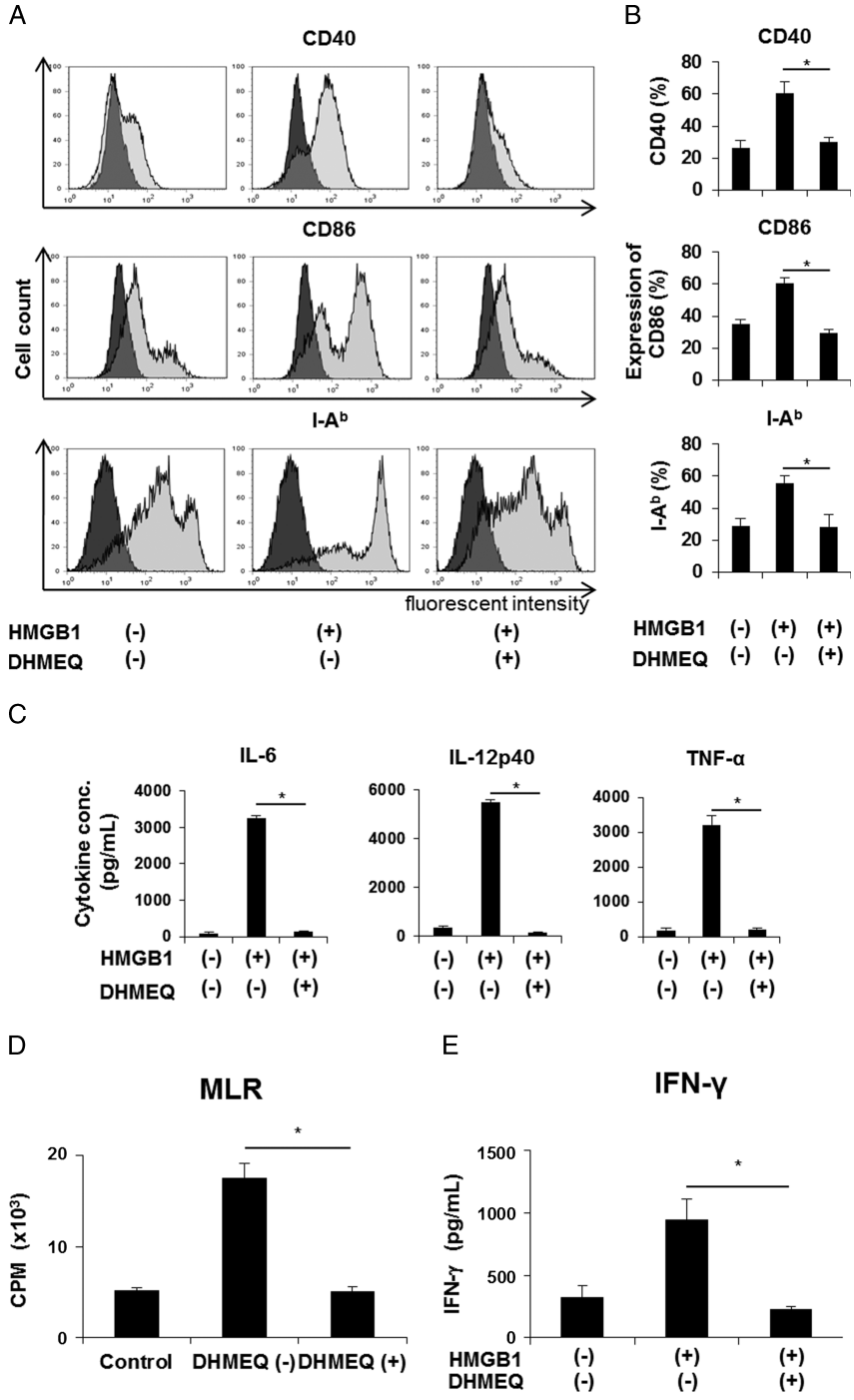

FIGURE 1. DHMEQ inhibits HMGB1-induced DC activation, proinflammatory cytokine production, and allospecific immunostimulatory capacity. A and B, HMGB1 (20 μg/mL) significantly stimulated C57BL/6 mouse BMDC maturation, as manifested by increased expression of surface markers. One hour before HMGB1 stimulation, 5.0 μg/mL DHMEQ was added to the cultures. DHMEQ significantly inhibited HMGB1-induced DC maturation, resulting in significantly lower expression of CD40, CD86, and MHC class II than in cells treated with HMGB1 alone (*P<0.05 vs. HMGB1(+) DHMEQ(–)). C, to evaluate cytokine production, the culture supernatants were subjected to ELISA. DHMEQ significantly inhibited IL-6, IL-12, and TNF-α production in HMGB1-stimulated BMDCs (*P<0.05 vs. HMGB1(+) DHMEQ(–), n=5 each). D and E, MLR was performed using T cells from the spleens of C57BL/6 mice as responder cells. Irradiated control DCs (1×10^4 cells/well) or DCs pretreated with DHMEQ (5.0 μg/mL) were cocultured with the responder cells (1×10^5 cells/well). Plates were incubated at 37°C in 5% CO_2 plus air. Cells were pulsed with ^{3}H-thymidine (1 mCi/well) 8 hr before culture termination, after which ^{3}H-thymidine incorporation was measured. The culture supernatants were collected on day 5 and subjected to ELISA. DHMEQ-treated DCs significantly suppressed allogeneic T-cell proliferation and IFN-γ production by the stimulated T cells compared with that in the control DCs (*P<0.05 vs. HMGB1(+) DHMEQ(–)).

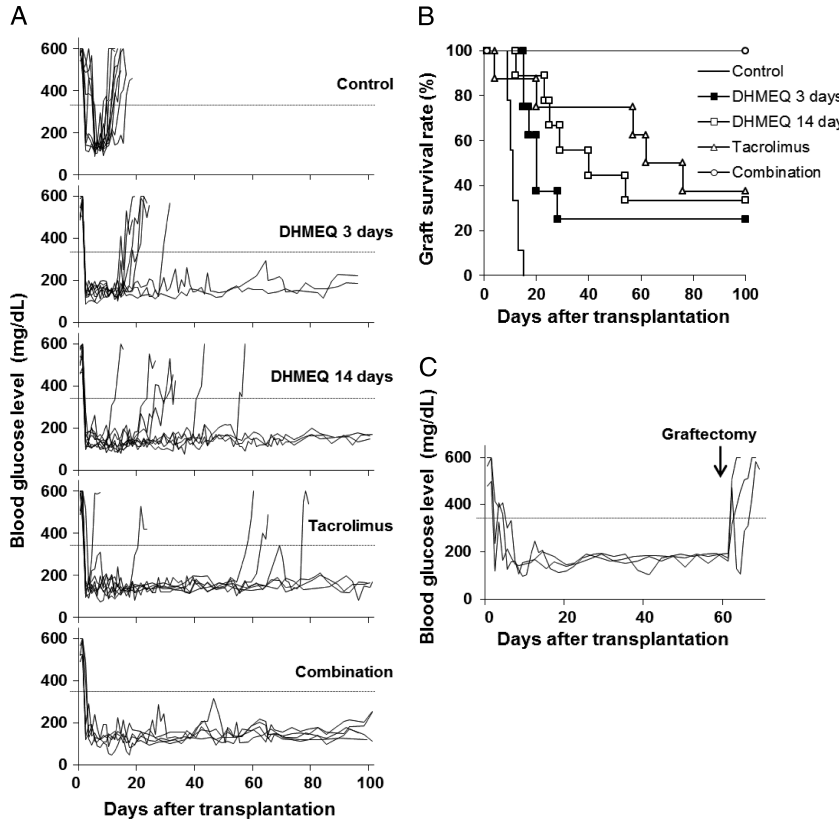

FIGURE 2. Treatment with DHMEQ shortly after PITx followed by tacrolimus administration permits allograft acceptance over a longer term. Changes in blood glucose levels (A) and graft survival time (B) are shown. Six hundred pancreatic islets of BALB/c mice were transplanted into the liver of STZ-induced diabetic C57BL/6 mice. Islet-recipients received 20 mg/kg DHMEQ intraperitoneally once daily for 3 days (DHMEQ 3 days, *n*=8; black squares) or twice daily for 14 days (DHMEQ 14 days, *n*=9; white squares) or vehicle alone (0.5% CMC; Control, *n*=9; bars) for 14 days, starting from day 0. In the tacrolimus monotherapy group, tacrolimus was administered intraperitoneally, at a dose of 1.5 mg/kg/day, for 14 days starting from day 0 (Tacrolimus, *n*=8; white triangles). As a combination therapy, the recipients were given 20 mg/kg DHMEQ daily for 3 days after PITx followed by 1.5 mg/kg tacrolimus daily for 14 days (Combination, *n*=5; white circles). In the control animals, allografts were rejected within 15 days. In the DHMEQ 3 or 14 days or Tacrolimus groups, only a few recipient animals accepted islet allografts for an extended period (MST, 20, 40, and 69 days, respectively). The combination therapy significantly facilitated acceptance of the allografts (MST, >100 days) compared with other groups ($P<0.05$, Kaplan–Meier log-rank test). C, six hundred pancreatic islets of BALB/c mice were transplanted under the kidney capsule of STZ-induced diabetic C57BL/6 mice (n=3). Islet-recipients were treated with the combination therapy. Blood glucose level normalized promptly in all animals after the PITx. When the kidney with islet allografts was removed at 60 days after PITx, blood glucose level increased in all animals.

into and surrounding islet allografts were noted. Immunohistochemistry revealed that those infiltrates mainly consisted of $CD4^+$ and $CD8^+$ cells. On the contrary, treatment with DHMEQ significantly suppressed cellular infiltrates of both $CD4^+$ and $CD8^+$ cells compared with those in the control (Fig. 3A,B).

The in vivo alloimmune response after PITx was evaluated using IFN-γ ELIspot assay. Splenocytes were obtained from recipient animals on day 9 post-PITx and were cocultured with irradiated donor (BALB/c) mouse splenocytes, and the numbers of IFN-γ spots were then examined. Splenocytes from DHMEQ-treated recipients exhibited a significantly lower response to the donor antigen as assessed by the frequency of alloreactive IFN-γ–producing cells when compared with that of control animals (Fig. 3C).

Treatment with DHMEQ Shortly After PITx Inhibits Elevation of HMGB1 and Suppresses Subsequent Alloimmune Responses In Vivo

To investigate whether the induction treatment with DHMEQ inhibits early innate/inflammatory responses after PITx and reduces subsequent alloimmune responses, we firstly examined serum levels of HMGB1 in the current mouse allogeneic PITx model. As reported by Matsuoka et al. (*9*), serum HMGB1 levels were elevated at 6 hr after islet grafting in the control mice. In contrast, administration of DHMEQ significantly suppressed the elevation of HMGB1 in sera (Fig. 4A). We then assessed the impact of DHMEQ given shortly after PITx on subsequent alloimmune responses. Tacrolimus (at a dose of 1.5 mg/kg/day) was administered to PITx recipient animals with or without DHMEQ induction treatment (from days 0 to 3). Splenocytes

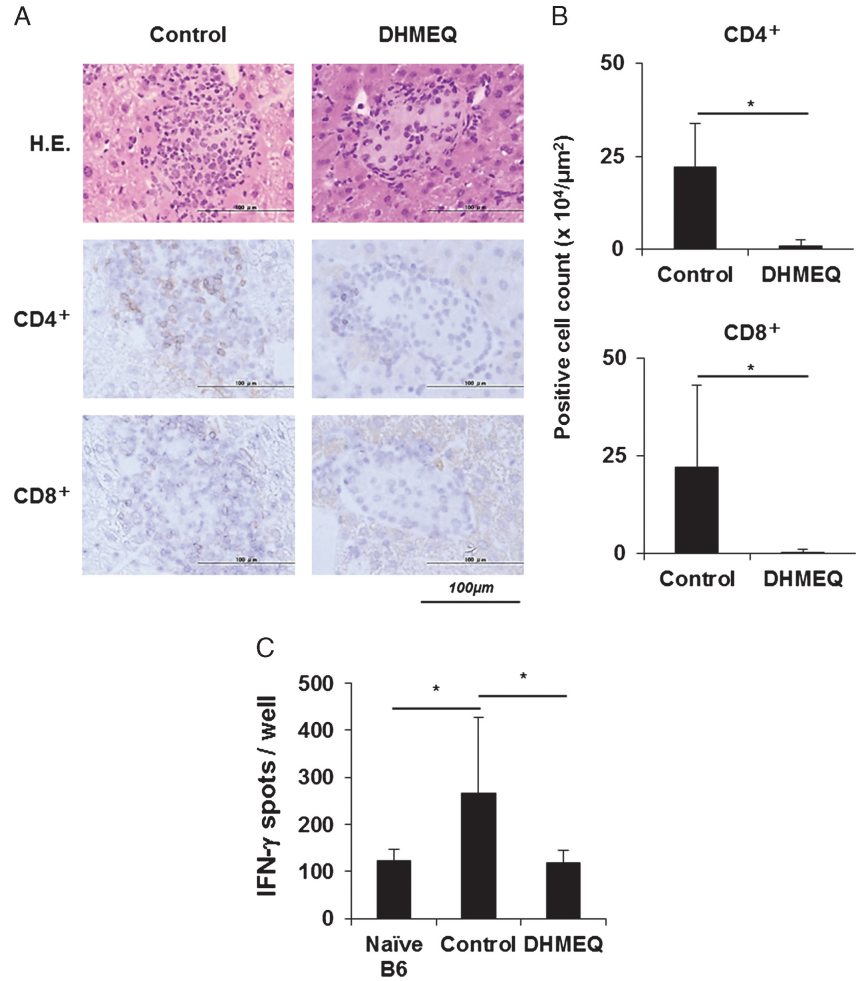

FIGURE 3. Treatment with DHMEQ suppresses intragraft cellular infiltration and alloimmune responses after PITx. A and B, at 9 days after PITx, islet allografts in the recipient liver were stained with hematoxylin-eosin, anti-CD4 mAbs, and anti-CD8 mAbs, and the numbers of $CD4^+$ and $CD8^+$ cells in islet allografts were counted and quantified. In the vehicle-treated control animals, severe cellular infiltrations into the islet allografts, mainly consisted of $CD4^+$ and $CD8^+$ cells, were noted. On the contrary, treatment with DHMEQ significantly suppressed these cellular infiltrations compared with those of control (*P<0.05 vs. Control). C, splenocytes obtained from recipient animals at 9 days after PITx were cocultured with irradiated donor (BALB/c) splenocytes for 24 hr, and the numbers of IFN-γ spots were examined using ELIspot assay. Spots were expressed as spot-forming cells/10^6 splenocytes. Splenocytes from DHMEQ-treated recipients had significantly lower response to the donor antigen compared with control (*P<0.05 vs. Control, n=4).

obtained from these animals were cocultured with irradiated BALB/c donor-strain mouse splenocytes, and the T-cell response of the PITx recipients against the donor antigens was examined by an IFN-γ ELIspot assay. The frequency of alloreactive IFN-γ–producing precursors was significantly lower in the DHMEQ induction treatment group compared with that of the tacrolimus (without DHMEQ induction) treatment group (Fig. 4B).

Induction DHMEQ Treatment Plus Tacrolimus Facilitates Long-term Islet Allograft Survival with 300 Allogeneic Islets

Considering that DHMEQ administered shortly after PITx suppresses serum HMGB1 levels, we hypothesized that DHMEQ treatment would rescue the transplanted islets from the damage caused by HMGB1-mediated reactions that occur after PITx and that treatment with induction by DHMEQ and tacrolimus thereafter would result in successful PITx despite a reduced number of transplanted islets. To test this hypothesis, we transplanted 300 BALB/c islet allografts into each diabetic C57BL/6 mouse. The blood glucose level of CMC-treated control mice did not normalize at all. Treatment with 20 mg/kg DHMEQ once daily for 3 days or daily administration of 1.5 mg/kg tacrolimus for 2 weeks permitted a transient recovery from diabetes; however, the islet allografts were rejected shortly after PITx, with graft MSTs of 10 and 13 days, respectively. In contrast, even with this lower number of transplanted islets, combined treatment with DHMEQ for 3 days and tacrolimus for a further 14 days facilitated successful islet engraftment in all

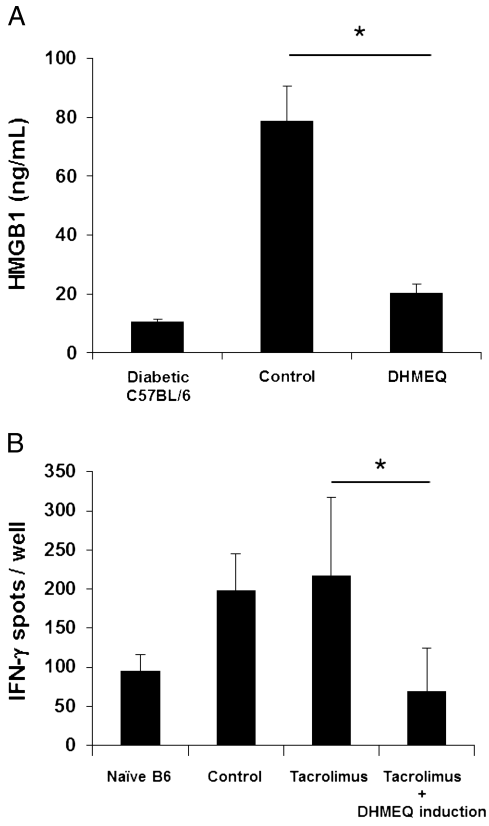

FIGURE 4. Treatment with DHMEQ shortly after PITx suppresses subsequent alloimmune responses in vivo. A, in the transplant model using 600 allogeneic islets, blood samples were collected via the hearts of the recipient mice at 6 hr after PITx, and the serum HMGB1 level was measured by ELISA. Blood samples were similarly collected from STZ-induced diabetic C57BL/6 mice at 6 days after STZ injection. The serum HMGB1 level was elevated in the CMC-treated control animals. In contrast, treatment with DHMEQ significantly suppressed serum HMGB1 levels compared with that in both the control and diabetic nontransplanted C57BL/6 control animals (*P<0.05 vs. control, n=5). B, splenocytes obtained from these animals at 6 days after PITx were cocultured with irradiated BALB/c mouse (donor strain) splenocytes, and the frequency of IFN-γ–producing donor-reactive T cells was assessed by ELIspot assay. In this experiment, tacrolimus (at a dose of 1.5 mg/kg/day from day 4) was administered to PITx recipient animals with or without DHMEQ induction treatment (from days 0 to 3). The frequency of alloreactive IFN-γ–producing precursors in the spleen was significantly lower in the combination treatment (tacrolimus with DHMEQ induction) group compared with the corresponding numbers in the tacrolimus group or control group (P<0.05; n=4, respectively).

the recipient mice and significantly prolonged the graft MST to more than 100 days (Fig. 5A,B).

DISCUSSION

In the present study, we demonstrated that induction treatment with DHMEQ facilitates long-term acceptance of fully MHC-mismatched murine islet allografts when immunosuppression was maintained with tacrolimus for 2 weeks. We have previously shown that NF-κB inhibition by DHMEQ, when administered for 2 weeks, significantly prolonged the survival time of a mouse cardiac graft (*23*). This immunosuppressive effect was mainly attributed to the inhibitory effect of DHMEQ on T cells. The same finding was also made in our current mouse allogeneic PITx model: administration of DHMEQ for 14 days significantly prolonged the median allograft survival time to 40 days. The efficacy of DHMEQ was associated with reduced frequency of alloreactive precursor cells, as assessed by IFN-γ ELIspot assays, and suppression of intragraft cellular infiltration into the transplanted intrahepatic islet allografts. Although 600 islet allografts, a mass volume estimated to be equivalent to islets from three donors, were transplanted, it is noteworthy that reversal of the diabetic state did not persist with a 2-week course of DHMEQ treatment under the current regimen.

In addition, we examined the efficacy of DHMEQ induction therapy on islet allograft survival. This was based on previous findings that DHMEQ exerts a potent anti-inflammatory effect (*21, 22, 26*). Likewise, in an earlier study, we demonstrated that a 3-day treatment with DHMEQ significantly inhibits inflammatory responses associated with PITx and improves the normoglycemic rate in a mouse marginal syngeneic PITx model (*27*). When treatment with DHMEQ was followed by daily, low-dose tacrolimus administration for 2 weeks, islet allograft survival was markedly prolonged to more than 100 days. The IFN-γ ELIspot assay revealed that the DHMEQ induction treatment reduced the frequency of IFN-γ–producing alloreactive cells in the spleen after PITx at a later time point compared with that seen with tacrolimus monotherapy. These results suggested that the inhibition of NF-κB activation by DHMEQ shortly after PITx contributed to a reduction in the magnitude of alloresponses and facilitated subsequent acceptance of islet allografts.

To understand the role of short-term induction treatment with DHMEQ in allograft acceptance in our allogeneic PITx model, we measured serum HMGB1 levels and found that these levels were elevated at 6 hr after allogeneic PITx, whereas DHMEQ treatment significantly inhibited this elevation. HMGB1 is a nuclear protein present in almost all eukaryotic cells; it is passively released by damaged cells and tissues (*24*) and is actively secreted by activated macrophages and mature DCs in response to injury, infection, or other inflammatory stimuli (*28, 29*). Extracellular HMGB1 functions as an "alarmin" and as an innate immune mediator, enhancing the immune response. Matsuoka et al. reported that HMGB1 is released into the circulation soon after PITx and that administration of anti-HMGB1 antibodies prevents early islet graft loss in a syngeneic mouse model (*9*). HMGB1 induces signals via TLR2, TLR4, and RAGE, which initiates the activation of immune cells involved in NF-κB activity (*30, 31*). Previous studies have demonstrated NF-κB activation in islet grafts shortly after intraportal transplantation (*32*); furthermore, TLR4 deficiency has been shown to reduce proinflammatory cytokines and improve the viability of islet grafts (*33*). In addition, in our previous study, we demonstrated that islets isolated from donors who were pretreated with DHMEQ

were resistant to apoptosis, and the use of these islets facilitated normoglycemia in a mouse marginal syngeneic PITx model (*34*).

DCs are the most potent antigen-presenting cells that mediate crosstalk between the innate and subsequent adaptive immunity (*35*). Binding of ligands to TLRs induces DC migration to T-cell areas of draining lymph nodes; these early activated DCs play an important role in the bystander activation of other DCs and the recruitment of NK and NKT cells, which then together support the ensuing adaptive immune response (*36, 37*). In our in vitro study, we demonstrated that the addition of DHMEQ to the culture medium significantly inhibited HMGB1-driven up-regulation of CD40, CD86, and MHC class II cells, as well as the production of proinflammatory cytokines, such as IL-6, IL-12, and TNF-α, in bone marrow–derived murine DCs. These results were concurred with the findings that DHMEQ inhibited IL-6, TNF-α, and IL-12 production upon lipopolysaccharide stimulation in human monocyte-derived DCs (*38*). We also demonstrated that DHMEQ-treated DCs exert a significantly reduced ability to trigger T-cell proliferation and IFN-γ production upon allogeneic stimulation. Although it has been shown that blockade of TLR signals in mice lacking the MyD88 and TLR adaptor molecule 1 was not sufficient to prevent islet allograft rejection (*39*), our results indicate that suppression of the initial innate/inflammatory responses that occur shortly after PITx could potentially reduce the subsequent adaptive immune responses mediated by immune cells such as DCs. Moreover, this suppression would then aid in preventing allograft rejection when used in combination with conventional immunosuppressants.

Our current protocol involved a short treatment with DHMEQ and tacrolimus and enabled reversal of diabetes by islet allograft acceptance, even when the transplant involved only 300 islets. This result also supports our hypothesis that it is important to control early innate/inflammatory responses to ensure allogeneic islet engraftment. It is clear that transplant of allogeneic islets into the liver results in subsequent induction of innate immunity and inflammation. During these early responses, HMGB1—which is released from activated immune cells and damaged islet grafts—triggers DC activation. Those reactions strengthen the adaptive immune responses that are mainly mediated by alloantigen-reactive T cells, subsequently leading to graft rejection. Therefore, it is crucial to block this undesirable negative feedback loop by controlling the initial reactions to ensure successful PITx.

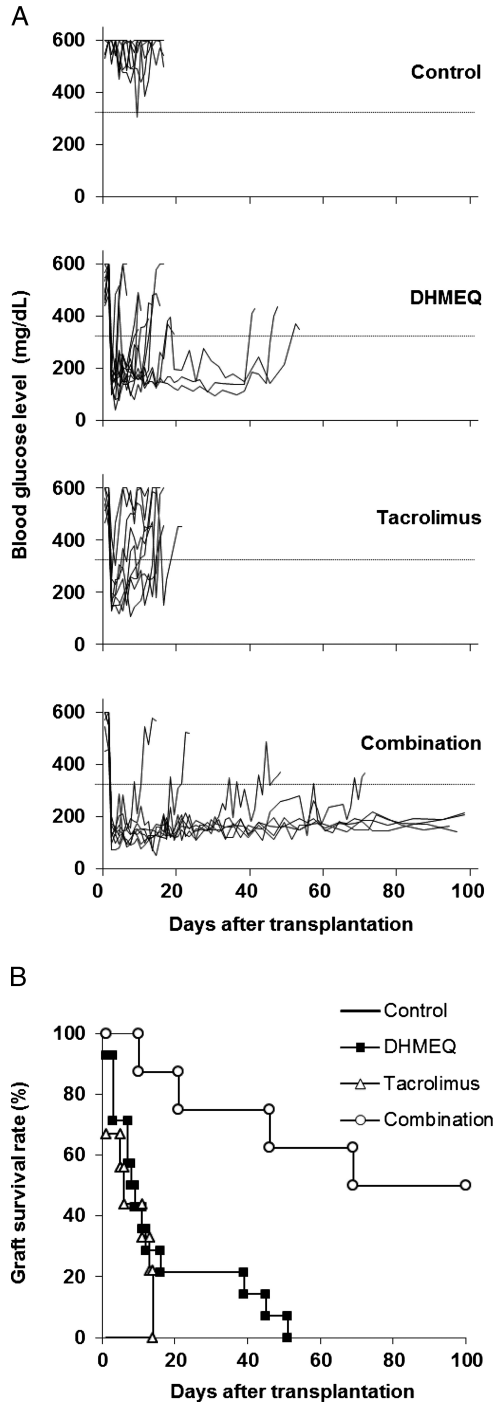

FIGURE 5. DHMEQ induction treatment plus tacrolimus permits long-term islet allograft survival with 300 allogeneic islets. A and B, effect of DHMEQ and tacrolimus on PITx was examined with a model using a reduced islet allograft volume, in which 300 pancreatic islets from BALB/c mice were transplanted into the livers of STZ-induced diabetic C57BL/6 mice. The islet-recipients were administered 20 mg/kg DHMEQ intraperitoneally for 3 days (DHMEQ, n=14; black squares) or 1.5 mg/kg tacrolimus daily (Tacrolimus, n=9; white triangles) or vehicle alone (0.5% CMC; Control, n=8; black bars) for 14 days, starting from day 0. As combination therapy, recipient animals were treated with 20 mg/kg DHMEQ daily for 3 days after PITx followed by 1.5 mg/kg tacrolimus daily for 14 days (Combination, n=8; white circles). Blood glucose levels (A) and graft survival time (B) are shown. In the control animals, blood glucose levels did not normalize at all. Treatment with DHMEQ, or 2-week administration of tacrolimus, facilitated only a transient recovery from diabetes, and islet allografts were rejected shortly after the PITx (MST, 10 and 13 days, respectively). The combination therapy enabled successful islet engraftment in all animals, and significantly prolonged the graft MST, to more than 100 days (P<0.05 vs. DHMEQ and Tacrolimus). *P<0.05 using Kaplan–Meier log-rank test.

We conclude that inhibition of the initial innate/inflammatory reactions by the novel NF-κB inhibitor DHMEQ not only protects islets from early immune responses immediately after grafting, but also reduces alloimmune responses and enables islet allograft acceptance over the longer term, when administered in combination with conventional immunosuppressants. The findings of this study can contribute to establishing a treatment strategy that would enable successful allogeneic PITx in multiple diabetic recipients, even from a single donor.

MATERIALS AND METHODS

Animals

All experiments were approved by the Institutional Animal Care Committee, and the study was conducted according to the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University. Male C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H/HeJ (H-2^k) mice were purchased from SLC (Shizuoka, Japan). The mice were maintained in a specific pathogen-free facility and were used for experiments when they were 10 to 14 weeks old.

Reagents and Antibodies

DHMEQ was synthesized as described previously (*40*). DHMEQ was dissolved in dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO) and stored at –80°C until required. DHMEQ stock solution and tacrolimus powder (Fujisawa Pharmaceutical, Osaka, Japan) were dissolved in 0.5% CMC (Sigma-Aldrich) solution and distilled water, respectively. Purified HMGB1 was purchased from Shino-Test (Kanagawa, Japan). Murine recombinant granulocyte-macrophage colony-stimulating factor and IL-4 were purchased from PeproTech (Rocky Hill, NJ). Anti-mouse fluorescein isothiocyanate–conjugated, phycoerythrin-conjugated, and peridinin chlorophyll protein–conjugated monoclonal antibodies (mAbs), biotinylated mAbs against CD4 (RM4-5), CD86 (GL1), and I-A^b (AF6-120.1), and streptavidin-peridinin chlorophyll protein were obtained from PharMingen (San Diego, CA). Anti-mouse phycoerythrin-conjugated CD40 mAb (3/23) was obtained from BioLegend (San Diego, CA).

DC Preparation, Culture, and Stimulation

Murine BMDCs were generated as described previously (*41*). Cells were cultured in complete medium (CM) of RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% fetal calf serum (FCS), 20 ng/mL granulocyte-macrophage colony-stimulating factor, 20 ng/mL IL-4, 50 μM 2-mercaptoethanol, 100 units/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich). Cells were cultured in the wells of a 24-well plate at a density of 1×10^6 cells/mL/well. On day 2, the medium was replaced with fresh CM. On day 4, nonadherent granulocytes were removed without dislodging clusters of developing DCs, and fresh CM was added. On day 6, free-floating and loosely adherent cells were collected (>90% CD11c) and were used as BMDCs. These cells were then cultured in CM at a density of 1×10^6 cells/mL/well in untreated 24-well plates.

The BMDCs were added at 1×10^6 cells/mL to each well of a 24-well plate and pretreated with or without DHMEQ for 1 hr followed by treatment with HMGB1 (20 μg/mL) for 24 hr. Cells were detached using 3 mM EDTA for 5 min at 37°C and used for analysis.

Mixed Leukocyte Reaction

Splenic T cells from C57BL/6 mice were used as responder cells, and mixed leukocyte reaction (MLR) analysis was performed as described previously (*42*). After HMGB1 stimulation (20 μg/mL), untreated and DHMEQ-pretreated (5 μg/mL) DCs from BALB/c mice (1×10^4 cells/well) were irradiated (30 Gy; 137 Cs) and cocultured with the responder cells (1×10^5 cells/well) in 96-well round-bottomed plates at 37°C in 5% CO_2 with air, for 72 hr, in RPMI 1640 medium containing 10% FCS, 50 μM 2-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were pulsed with ^{3}H-thymidine (1 μCi/well) 8 hr before estimating the thymidine incorporation with a β-counter (Perkin-Elmer, Boston, MA).

Flow Cytometry

Flow cytometry was performed as described previously (*42*). A FACSCalibur flow cytometer and CellQuest software (Becton Dickinson Biosciences, San Jose, CA) were used for flow cytometric analyses. We acquired 10,000 DCs or 30,000 T cells for each analysis.

Measurement of Cytokines and HMGB1

The IL-6, IL-12, and TNF-α protein levels in the DC culture supernatants and the IFN-γ levels in the MLR supernatants were quantified by enzyme-linked immunosorbent assay (ELISA) using OptEIA Sets (PharMingen). All measurements were performed in duplicate. The serum HMGB1 levels in the mice were measured by ELISA (Shino-Test) as described previously (*43*). IFN-γ production of the splenocytes obtained from transplant recipients was examined by ELIspot assay, as described previously (*23*). Splenocytes (5×10^5 cells/well) obtained from the recipients were cocultured with the irradiated BALB/c or C3H splenocytes (1×10^6 cells/well) for 24 hr in an anti-mouse IFN-γ mAb-precoated BD ELIspot plate (Becton Dickinson Biosciences). The IFN-γ spots were detected by biotinylated anti-mouse IFN-γ antibodies, and the spots were enumerated by KS ELIspot software (Carl Zeiss, Munich, Germany) and expressed as spot-forming cells/10^6 splenocytes.

Pancreatic Islet Isolation

The pancreatic islets of BALB/c mice were isolated as described previously (*33*). Islets that were 150 to 200 μm in diameter were used for the analysis. The islets were cultured overnight in RPMI 1640 medium supplemented with 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma-Aldrich) at 37°C in 5% CO_2 and a humidified atmosphere.

Diabetes Mellitus Induction

C57BL/6 islet-recipient mice were rendered diabetic by intraperitoneal administration of 180 mg/kg STZ (Sigma-Aldrich) 5 to 7 days before PITx. Blood glucose levels were monitored with the Accu-Chek blood glucose monitor (Roche Diagnostics, Tokyo, Japan). Diabetes was considered to be established when the blood glucose level of two consecutive measurements exceeded 450 mg/dL.

PITx and Treatment Protocol

The pancreatic islets (600 or 300) of BALB/c mice were transplanted into the C57BL/6 recipient liver via the portal vein. In some, 600 BALB/c mice islets were transplanted under the kidney capsule of C57BC/6 mice. After islet grafting, the blood glucose levels and graft survival time were analyzed. Normoglycemia was defined when the blood glucose level was reduced to below 200 mg/dL for 2 consecutive days. The day of graft rejection was defined as the day when the blood glucose level exceeded 350 mg/dL and remained high for 2 consecutive days.

The islet-recipient animals were given vehicle (0.5% CMC), DHMEQ at 20 mg/kg, or tacrolimus at 1.5 mg/kg per day, intraperitoneally, after PITx. For DHMEQ treatment, one group was administered DHMEQ once daily for 3 days, and the other was given DHMEQ twice daily for 14 days from day 0. In the tacrolimus monotherapy group, tacrolimus was administered for 14 days starting from day 0. As a combination therapy, DHMEQ (20 mg/kg/day) was given daily for 3 days after PITx, and tacrolimus (1.5 mg/kg/day) was administered thereafter for 14 days.

Histologic Study

Islet allografts in the recipient liver were stained with hematoxylin-eosin, anti-CD4, and anti-CD8 mAbs (both from Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis

The graft survival time was plotted using a method based on the Kaplan–Meier estimator. Differences in the duration of graft survival between groups were evaluated using the Kaplan–Meier log-rank test.

Quantitative results were presented as mean±SD and were compared using Student's *t* test. $P<0.05$ was considered statistically significant.

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