Immunomodulatory Effect of Nuclear Factor-κB Inhibition by Dehydroxymethylepoxyquinomicin in Combination With Donor-Specific Blood Transfusion

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Background. Nuclear factor-κB (NF-κB) is a key molecule in alloimmune responses, however, its role in tolerance induction is not clear. We have previously reported that dehydroxymethylepoxyquinomycin (DHMEQ), a novel NF-κB inhibitor, prolongs cardiac allograft survival. In this study, we evaluated the immunomodulatory effects of DHMEQ when combined with a donor-specific blood transfusion (DST), and assessed whether the treatment induces tolerance in a mouse heart transplantation model.

Methods. DST (20×10^6 splenocytes) was given intravenously at day −7. DHMEQ (30 mg/kg/day) was administered intraperitoneally for 14 days after DST. Graft survival and histology were evaluated. The underlying mechanisms of immunomodulation by DST and DHMEQ treatments were investigated by assessing alloimmune responses after transplantation.

Results. In fully mismatched $H2^d$-to-$H2^b$ heart transplants, DST alone prolonged allograft median survival time to 15 days, whereas when DST was combined with DHMEQ treatment, the graft median survival time was prolonged to 39.5 days. When the donor-recipient strain combination was reversed, that is, $H2^b$-to-$H2^d$, heart transplants were accepted (>150 days survival) in more than 60% of recipients treated with a DST and DHMEQ, whereas control allografts were all rejected within 8 days. The combined therapy markedly inhibited immune responses by both the direct and indirect allorecognition pathways mainly attributed to promotion of activation-induced cell death and Treg generation.

Conclusions. Our results demonstrate the distinctive ability of NF-κB inhibition in combination with donor alloantigen to promote transplantation tolerance through multiple cellular mechanisms.

Keywords: Donor-specific blood transfusion, Nuclear factor-kappa B, Heart transplantation, Activation-induced cell death, Regulatory T cells.

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The transcription factor, nuclear factor-κB (NF-κB), consists of hetero- or homodimeric complexes of p65 (Rel-A), p50, p52, Rel-B, and c-Rel, and is a key regulator of function in T cells, B cells, dendritic cells (DCs), and macrophages (*1*). Signals through the interleukin (IL)-1-receptor, tumor necrosis factor-receptor, CD40, and through toll-like receptors and antigen (Ag) receptors activate NF-κB, resulting in cellular activation, proliferation, and maturation (*2*, *3*). In transplantation, NF-κB plays a crucial role both in ischemia-reperfusion injury and allograft rejection. NF-κB family genes are upregulated in rejecting cardiac allografts without any immunosuppression, while treatment with anti-CD40L monoclonal antibody (mAb) downregulates the same genes (*4*). Furthermore, a NF-κB decoy that blocks nuclear transcription of the κB site inhibits acute rejection (*5*), and IκB (δN)-Tg that express a transdominant inhibitor of NF-κB in T cells and c-Rel-deficient mice accept heart allografts long term (*6*). In addition, the proteasome inhib-

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itor bortezomib, which inhibits NF-κB activation by preventing the degradation of cellular proteins through the ubiquitin-proteasome pathway, has been shown to suppress both cellular and antibody mediated rejection in clinical kidney transplantation (*7*).

Dehydroxymethylepoxyquinomycin (DHMEQ), a derivative of epoxyquinomicin C, is a newly developed NF-κB inhibitor (*8*). This low molecular weight drug is unique in its mechanism of action: it binds directly to the cysteine-38 residue of p65 therefore inhibiting DNA binding of NF-κB (*9*). We have previously demonstrated the ability of DHMEQ to suppress the activation and proliferation of T cells, resulting in the prolongation of a fully major histocompatibility complex-mismatched cardiac allograft survival (*10*). DHMEQ has also been shown to be beneficial in experimental models of ischemia-reperfusion injury (*2*), inflammatory diseases (*11*, *12*), and neoplasia (*13*).

Donor-specific blood transfusions (DSTs) have been shown to promote tolerance both clinically and experimentally (*14*, *15*). Mechanisms that have been considered to be responsible for this effect include T-cell deletion by means of activation-induced cell death (AICD), the promotion of T-cell anergy, and the generation of regulatory T cells (Tregs) (*16*, *17*). DST in conjunction with conventional immunosuppressants (*18*), costimulation blockers (*19*, *20*), or other new agents such as induction of HO-1 expression (*21*) has been shown to be a successful method for promoting allograft survival experimentally. Many of the immunosuppressive agents used in these studies target molecules with signaling pathways that result in the downstream activation of NF-κB. Given this association, we aimed to determine the immunomodulatory effects of the direct inhibition of NF-κB using DHMEQ in combination with a DST. We show that this strategy results in long-term graft survival and the induction of immunological unresponsiveness in a fully allogeneic mouse heart transplantation model through the inhibition of alloimmune responses by the promotion of alloreactive T-cell deletion and Treg generation.

RESULTS

Impact of Combined Treatment With DHMEQ on DST on Cardiac Allograft Survival

We initially examined the effects of the NF-κB inhibitor DHMEQ in conjunction with a DST in a stringent BALB/c ($H2^d$) to C57BL/6 ($H2^b$) mouse heart transplantation model. Vehicle-treated control mice rejected allografts within 7 days. DST or DHMEQ treatment alone modestly prolonged graft survival (median survival time [MST]) to 15 and 20 days, respectively. In contrast, DHMEQ together with a DST prolonged graft survival significantly (MST=39 days) compared with DST or DHMEQ monotherapy (Fig. 1A). Histopathology of the vehicle-treated control grafts on day 7 revealed typical features of acute rejection including a heavy cellular infiltrate with tissue necrosis (Fig. 1B), whereas DHMEQ in combination with DST treatment reduced intragraft cellular infiltrates in particular $CD4^+$ and $CD8^+$ cells significantly (Fig. 1B,C). By using splenocytes from cardiac recipient mice as responder cells, we examined alloimmune responses against donor Ags by assessing mixed lymphocyte reaction (MLR), interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) and Ag-specific $CD4^+CD154^+$ T-cell population (*22*). At 4 days after transplantation, MLR was significantly suppressed with both treatments, DST and DST plus DHMEQ, as compared with the vehicle-treated control (Fig. 1D). Although the strength of MLR inhibition did not differ between DST with or without DHMEQ treatment on day 4, the proliferation of spleen cells from transplant recipients treated with DST plus DHMEQ was significantly lower than that of DST treatment alone, when obtained on day 7 (Fig. 1D). In parallel with the data of MLR, treatment with DST plus DHMEQ significantly reduced the frequency of IFN-γ producing precursors on day 7 as compared with DST or DHMEQ treatment alone but not on day 4 as compared with the DST treatment alone (Fig. 1E). Meanwhile, the population of Ag-specific $CD4^+CD154^+$ T cells in the spleen was significantly reduced by addition of DHMEQ to the DST treatment as assessed on days 4 and 7 (Fig. 1F). Assessed by different facets, these data indicate that combined DHMEQ and DST treatment continue to suppress alloimmune responses effectively after transplantation, whereas DST or DHMEQ treatment alone did not inhibit the alloimmune responses to the same extent in the early posttransplant period.

Next, we assessed whether combined DHMEQ and DST treatment promotes similar effects when the strains of the donor and recipient mice are reversed, that is, $H2^b$-to-$H2^d$ heart transplants. In this model, vehicle-treated control mice rejected allografts within 8 days. DHMEQ monotherapy prolonged graft MST modestly to 16 days, and DST alone prolonged graft MST to 34 days. In sharp contrast, in the DHMEQ- and DST-treated mice, five (62.5%) of eight allografts were accepted for over 100 days (Fig. 2A). Histolopathologic examination revealed that cardiac allografts removed at day 100 from recipients treated with the combination of DHMEQ and DST were well-preserved with a lack of any cellular infiltrates or tissue destruction (Fig. 2B). Direct antidonor cellular responses remained significantly reduced 100 days after transplantation following combined DHMEQ and DST treatment compared with DST alone (Fig. 2C-E). Furthermore, indirect alloresponses as examined by ELISPOT using a donor-cell lysate stimulus (Fig. 2D,F) were also significantly inhibited by the combined treatment. Nevertheless, splenocytes were capable of proliferating against third-party Ag (Fig. 2G). These data suggest that treatment with DHMEQ together with DST induces a donor Ag-specific hyporesponsiveness.

Treatment With DHMEQ Deletes Alloreactive T Cells by Promoting AICD

To further examine how NF-κB inhibition may enhance the immunomodulatory effects of a DST, we first examined the effect at 7 days after DST because this time point is able to address the exact mechanisms of DST plus DHMEQ in the recipient by excluding the response to an allograft. In this case, DST or DHMEQ monotherapy did not inhibit the splenocyte proliferation; however, treatment with DST plus DHMEQ significantly inhibited the lymphocyte proliferation against donor-Ags at the same time point (Fig. 3A; see **SDC 1,** http://links.lww.com/TP/A642). We further looked at the $CD4^+CD154^+$ donor-Ag reactive T cells. Although the percentage of this cell population was comparable between the treatment groups (Fig. 3C), the absolute cell number was sig-

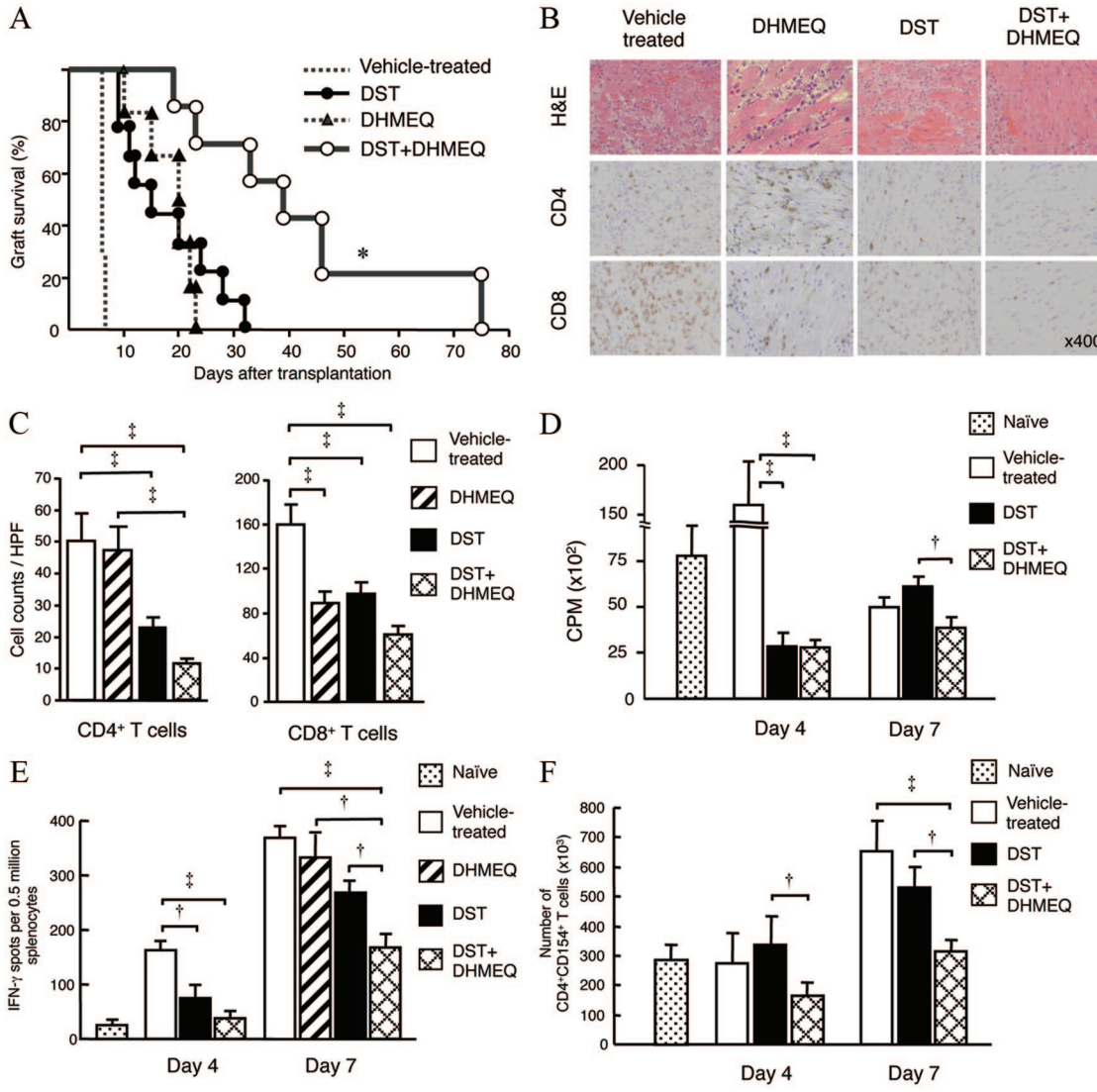

FIGURE 1. Dehydroxymethylepoxyquinomycin (DHMEQ) markedly prolongs cardiac allograft survival time in combination with donor-specific blood transfusion (DST). (A) DHMEQ was administered intraperitoneally at 30 mg/kg daily for 14 days from 7 days before transplantation (day −7). DST was performed by using donor-strain splenocytes (20×10^6) on day −7. In a stringent $H2^d$ to $H2^b$ mouse heart transplantation, DHMEQ in conjunction with DST (DST+DHMEQ) significantly prolonged heart allograft median survival time (MST) to 39.5 days (n=9) (*$P<0.01$ vs. vehicle-treated control [n=8], DST alone [n=9] and DHMEQ alone [n=8] by the log-rank test). (B) Histopathological (hematoxylin-eosin [H&E]) and immunohistochemical (CD4 and CD8) findings of cardiac allografts on day 7 after heart transplantation (original magnification ×400). (C–F) Data were pooled from two independent experiments. (C) The number of graft infiltrating $CD4^+$ and $CD8^+$ T cells on day 7 after heart transplantation was counted in three different high power fields. Data represent mean±SEM for four to seven mice per group (‡$P<0.01$). (D–F) Splenocytes that obtained from recipient $H2^b$ mice on both days 4 and 7 after heart transplantation were restimulated with irradiated donor strain splenocytes. Data are mean±SEM (†$P<0.05$, ‡$P<0.005$). (D) The data shown are the thymidine uptakes of splenocytes on 2 day after restimulation. Data represent mean±SEM for four mice (day 4) and for five to seven mice (day 7)per group, respectively. (E) The frequency of interferon (IFN)-γ producing alloreactive cells at 24 hr after restimulation was assessed by the ELISPOT. Data are mean±SEM for six mice (day 4) and three to four mice (day 7) per group, respectively. (F) The CD154 expressions among $CD4^+$ T cells at 6 hr after restimulation were assessed by intracellular staining for fluorescence-activated cell sorting (FACS). This figure shows the number of $CD154^+CD4^+$ T cells on both days 4 and 7. Data are mean±SEM for seven (days 4) and six mice (days 7) per group.

nificantly decreased by DST plus DHMEQ (Fig. 3B). To assess the alloreactive function of these cells, we performed MLR by using the same number of $CD4^+$ T cells isolated from treated animals as responder cells. In this setting, the suppressive effect of DST+DHMEQ on MLR was lost (Fig. 3D). Taken together, these data indicated that DST+DHMEQ promoted the deletion of alloreactive $CD4^+$ T cells rather than suppressing the function or rendering them anergic. Consistently, DST+DHMEQ significantly reduced the number of $CD4^+CD154^+$ donor Ag-specific alloreactive T cells within recipient spleens when examined at 4 and 7 days after transplantation (Fig. 1F). Based on these results, we hypothesized

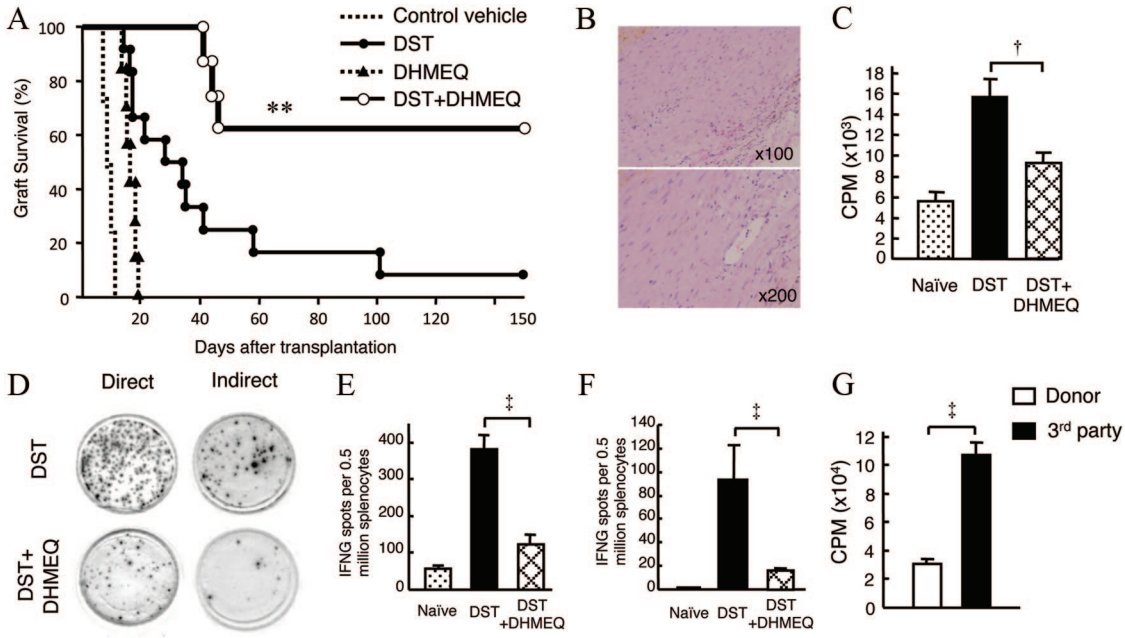

FIGURE 2. Dehydroxymethylepoxyquinomycin (DHMEQ) in conjunction with donor-specific blood transfusion (DST) induces a long-term allograft acceptance in mouse heart transplantation ($H2^b$ to $H2^d$). (A) In $H2^b$ to $H2^d$ mouse heart transplantation, which the strain combination was switched back, five of eight animals accepted the allografts for over 150 days when DHMEQ were given together with DST (**$P<0.01$ vs. DST [n=12], DHMEQ [n=7] monotherapy and vehicle-treated control group [n=6] by the log rank-test). (B) Histopathological (hematoxylin-eosin) findings showed that the cardiac allografts obtained from recipient mice on day 100 after heart transplantation (day 100) that treated with DST+DHMEQ (Original magnification ×100 and 200). (C–G) Responder cells were used with splenocytes obtained from recipient mice on day 100. The data were from three independent experiments. (C) Thymidine uptakes of splenocytes were assessed on 2 days after restimulation with irradiated donor strain splenocytes. Data are mean±SEM for three mice per group (†$P<0.05$). (D–F) The frequency of interferon (IFN)-γ producing cells at 24 hr after restimulation with irradiated donor strain splenocytes (D, E) and cell lysates (D, F) were showed as the immune responses through direct and indirect pathways respectively. Cell lysates were generated from irradiated splenocytes which were frozen and thawed three times after sonication. Cells were transferred onto the ELISPOT plate and incubated for 24 hr before harvesting. Results represent a mean±SEM for three mice per group (‡$P<0.001$). (G) The figure showed the thymidine uptakes of the splenocytes treated with DST+DHMEQ after coculture with donor or third-party strain irradiated splenocytes for 3 days of coculture. Data are mean±SEM for three mice per group (‡$P<0.001$).

that DHMEQ promotes the AICD of alloreactive T cells. To test this, we examined the effect of DHMEQ on T cells in vitro. First, DHMEQ treatment significantly increased the frequency of Annexin V^+ cells among naïve $CD4^+$ T cells after anti-CD3ϵ Ab stimulation (Fig. 3E,F). This was then confirmed in vivo by using carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled lymphocytes (*23*), where DHMEQ treatment significantly increased the percentage of Annexin V^+ cells among dividing $CFSE^+$ $CD4^+$ T cells (Fig. 3G). Taken together, these results suggest that DHMEQ acts on alloreactive T cells in a proapoptotic manner to promote the AICD of alloreactive T cells.

DHMEQ Treatment in Combination With DST Shifts the Balance Between Regulatory and Effector T Cells

To elucidate the mechanisms of long-term graft acceptance further, we examined whether the addition of NF-κB inhibition to DST augments a population of Treg after transplantation. Interestingly, in $H2^d$ recipient's spleens procured from mice 7 days posttransplantation, forkhead box protein (Foxp3) expression among $CD4^+$ T cells was increased by the combined treatment compared with that after DST alone (Fig. 4A). Also, a significant increase in the Treg population by DST plus DHMEQ was seen in both recipient mice strain ($H2^d$ and $H2^b$) as compared with DHMEQ or DST alone treatment group (Fig. 4B,C). Furthermore, DHMEQ plus DST treatment resulted in increased messenger RNA expression levels of the Treg-associated molecules Foxp3, cytotoxic T-Lymphocyte Ag-4 (CTLA-4), TGF-β, and IL-10 compared with vehicle-treated group (Fig. 4D). Considering with the findings that even DST monotherapy significantly increased the $Foxp3^+$ population and its associated molecules as compared with vehicle-treated control (Fig. 4A,C, CTLA-4 and TGF-β in Fig. 4D), possibly DHMEQ enhanced the effect of DST on increasing a Treg population. We then assessed the frequency of allospecific $CD154^+CD4^+$ T cells present after restimulation with donor-Ag. Consistent with data from $H2^b$ transplant recipients (Fig. 1F), the combined treatment of DHMEQ with DST significantly reduced the percentage (Fig. 4E) and absolute number (Fig. 4F) of $CD4^+CD154^+$ T cells. Consequently, the ratio between regulatory and alloreactive $CD4^+$ T cells was significantly increased in combined DHMEQ and DST-treated recipients compared with those

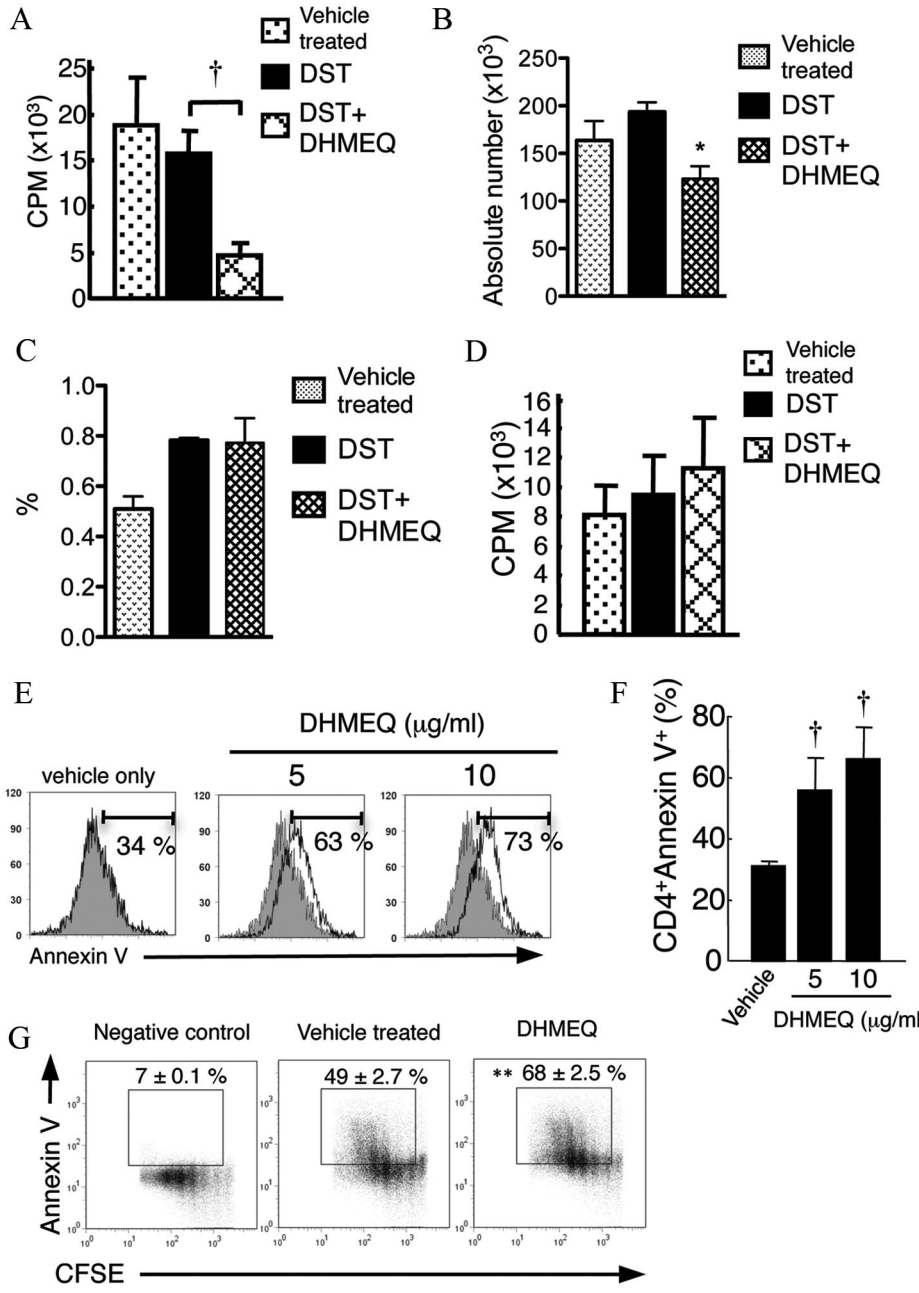

FIGURE 3. Treatment with dehydroxymethylepoxyquinomycin (DHMEQ) deletes the alloreactive T cells as a result of activation-induced cell death (AICD) augmentation. (A–D) Splenocytes (A–C) and the $CD4^+$ T cells (D) isolated from the splenocytes were obtained from treated $H2^b$ mice on 7 day after donor-specific blood transfusion (DST). They stimulated with irradiated donor strain $H2^d$ splenocytes. The data are mean±SEM for five mice per group and were pooled from four independent experiments. (A) The proliferation was examined by counting ^{3}H-thymidine uptake at 48 hr after stimulation. The absolute cell number (B) and percentage (C) of $CD4^+CD154^+$ T cells. (E and F) The figure shows that the percentage of Annexin V^+ among $CD4^+$ T cells, which were treated with DHMEQ at 0, 5, or 10 μg/mL, at 6 hr after stimulation with anti-CD3ε mAb (1 μg/mL). Cells were prepared from $H2^b$ splenocytes. Results are expressed as mean±SD for three mice from two independent experiments (†$P<0.05$ vs. vehicle-treated control). (G) Carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled lymphocytes from $H2^b$ mice were injected into lethally irradiated (9 Gy) $H2^d$ hosts treated with or without DHMEQ at 30 mg/kg/day daily. The lymphocytes from spleens and lymph nodes were harvested at 72 hours after cell transfer. The figure shows the percentage of Annexin V^+ among divided cells after gating CD4. As a negative control, no staining cells are shown because Annexin V does not have isotype control. The three independent experiments are representative and data in image are mean±SEM for five mice per group (‡$P<0.001$ vs. vehicle-treated control).

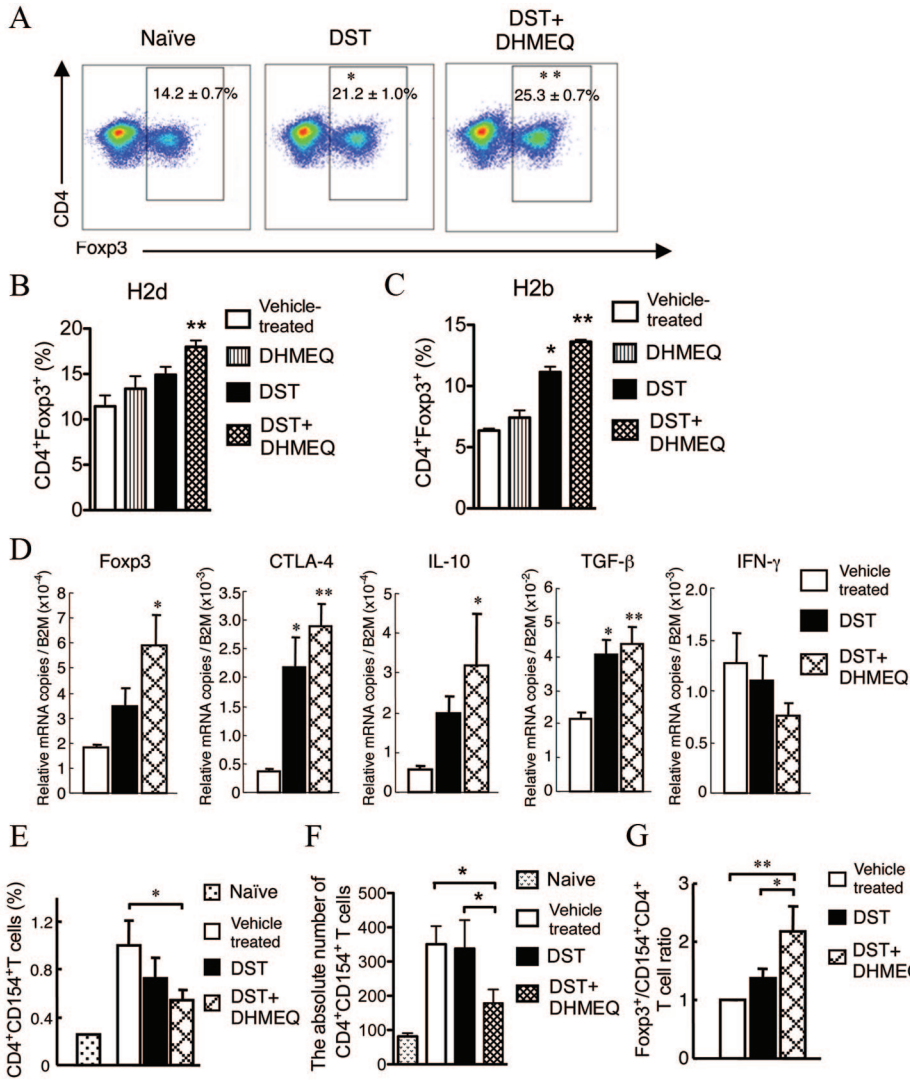

FIGURE 4. Dehydroxymethylepoxyquinomycin (DHMEQ) shifts the balance between Treg and alloreactive T cells to immunosuppressive state. (A, D-G) The lymphocytes were suspended from the spleens obtained from $H2^d$ recipient mice on day 7 after heart transplantation. (A) The population of $Foxp3^+CD4^+$ T cell is shown in the figure. Data are representative of three independent experiments and mean±SEM for four to five mice per group in the figure. (B and C) The figures showed the percentage of Foxp3 in $CD4^+$ T cell in spleen retrieved from $H2^d$ (B) or $H2^b$ (C) mice on day 7 after donor-specific blood transfusion (DST) together with or without DHMEQ treatment (**P<0.05 vs. DST, *P<0.05 vs. vehicle-treated control, Data are mean±SEM for three to six mice in two independent experiments). (D) The expression of Foxp3, CTLA-4, interleukin (IL)-10, TGF-β, and interferon (IFN)-γ messenger RNA were assessed by using a real-time polymerase chain reaction (PCR) method. Data are mean±SEM for three to four mice per group in three independent experiments, *P<0.05, **P<0.005, vs. vehicle-treated control. (E and F) The figures showed the percentage (E) and the absolute cell number (F) of $CD154^+CD4^+$ T cell which was assessed at 6 hr after restimulation with irradiated donor strain splenocytes. Data are mean±SD (E) and SEM (F) for four mice per group in three independent experiments. (G) The figure shows the ratio between $Foxp3^+$ and $CD154^+$ among $CD4^+$ T cells. Data are mean±SEM (n=3 for each group, *P<0.05, **P<0.001).

receiving a DST alone (Fig. 4G), suggesting a shift in the balance from an effector to a regulatory population, a condition which is important for promoting an overall suppressive state (*24*).

Treg Generated In Vivo by Combined DST and DHMEQ Treatment Prevent Allograft Rejection in an Ag-Specific Manner

Finally, we examined the suppressive activity of the Treg that were generated by the DST and DHMEQ treatment. $CD4^+CD25^+$ T cells (purity >90% $Foxp3^+$, data not shown) were isolated from the spleens of mice and subsequently adoptively transferred to $H2^d$ mice that had received an $H2^b$ cardiac allograft. When $CD4^+CD25^+$ T cells were isolated from naïve $H2^d$ mice, or from vehicle-treated $H2^d$ mice that had received an $H2^b$ cardiac allograft 7 days previously, MST was only modestly prolonged to 20 days and 15.5 days, respectively. In contrast, adoptively transferred $CD4^+CD25^+$ T cells isolated from cardiac allograft-recipient mice treated with combined DST and DHMEQ significantly prolonged

the MST of $H2^b$ cardiac allografts in $H2^d$ mice to 31.5 days (Fig. 5A). These same Tregs were unable to prolong survival of third-party ($H2^k$) heart grafts (Fig. 5B). These results indicated that Treg generated by the combined DST with DHMEQ treatment suppress antidonor responses in an Ag-specific manner to prolong allograft survival.

DISCUSSION

We have demonstrated in this study that inhibition of NF-κB activation after a DST augmented the immunomodulatory effects of the DST and promoted the induction of immunological unresponsiveness in $H2^d$ recipients of $H2^b$ cardiac allografts. NF-κB inhibition along with DST reduced the absolute number of alloreactive T cells (Fig. 1F) through the induction of AICD in the alloreactive T-cell population, both in vitro and in vivo (Fig. 3E-G). This is consistent with previous work demonstrating a role for DHMEQ in inducing tumor cell apoptosis through downregulation of the antiapoptotic genes, Bcl-xL and c-FLIP (*25*). Furthermore, the proapoptotic effect of DHMEQ has been shown in AIDS-derived Burkitt lymphoma cells (*26*), Epstein-Barr virus-infected B-lymphocytes (*27*), anaplastic thyroid cancer cells (*28*), Hodgkin/Reed-Sternberg cells (*29*), and phytohemagglutinin (PHA)-stimulated human peripheral blood monocyte cells (*30*). In this study, DST+DHMEQ significantly reduced the number of donor Ag-reactive cells in both $H2^d$ and $H2^b$ transplant recipients (Figs. 1F, 3B, and 4F). Although $H2^b$ mice were used in the experiments for the results demonstrated in the Figure 3, these data support the idea that DHMEQ induces AICD independently of the mouse strain. In addition, we confirmed that DHMEQ does not induce apoptosis on resting lymphocytes (data not shown). Furthermore, when tacrolimus was added to the current DST plus DHMEQ regimen, the in vivo proapoptotic effect of DHMEQ was fully abolished (manuscript in preparation). These data clearly indicate that T-cell activation is crucial for the in vivo effect of DHMEQ to induce an apoptosis. This was in contrast to our previously shown data that in vitro DHMEQ inhibits the T-cell activation and consequently arrests cell cycle progression at G0/G1 (*10*). Although it is not surprising that the mechanisms are not identical in vitro and in vivo, the mechanism of immunomodulation mediated by DHMEQ in vivo in particular, under a huge Ag stimulation such as in vivo MLR and DST treatment, is different from that seen in vitro in previous our published data. Indeed, the deletion of activated T cells is an important mechanism of DST-mediated immunomodulatory effect, which has previously been shown to promote apoptosis of alloreactive T cells when given alongside a costimulation blockade (*31*, *32*). When DST is given to transgenic mice that overexpress Bcl-xL, these mice were resistant to long-term cardiac allograft survival indicating that inhibition of Bcl-xL expression in T cells with resultant apoptosis is critical for tolerance induction (*19*, *33*). Taken together, our data suggest that NF-κB inhibition has an effect on activated lymphocyte survival and on the subsequent induction of tolerance by the DST.

Another beneficial effect of DST+DHMEQ was to promote an increase in the Treg population. We postulate that these Treg are of the peripherally induced variety due to their potent and efficient inhibition of donor-specific responses (Fig. 5). In general, Treg require T-cell receptor stimulation with their cognate Ag for induction of suppressive function, and it has also recently been shown that the transcription factors NFAT, Runx, and Smad3 are necessary for Treg generation and function (*34*, *35*). It follows that a calcineurin inhibitor that strongly inhibits NFAT activation, would inhibit the development and induction of Treg produced by DST treatment (*36*, *37*). Further, given the role of the NF-κB pathway in T-cell receptor/CD28 signaling, it is likely that this inhibition or stimulation of NF-κB also has a direct effect on Treg generation and function (*38*). Indeed, IκB kinase 2-deficient mice have a reduced number of $Foxp3^+CD4^+$ T cells in the thymus and periphery (*39*).

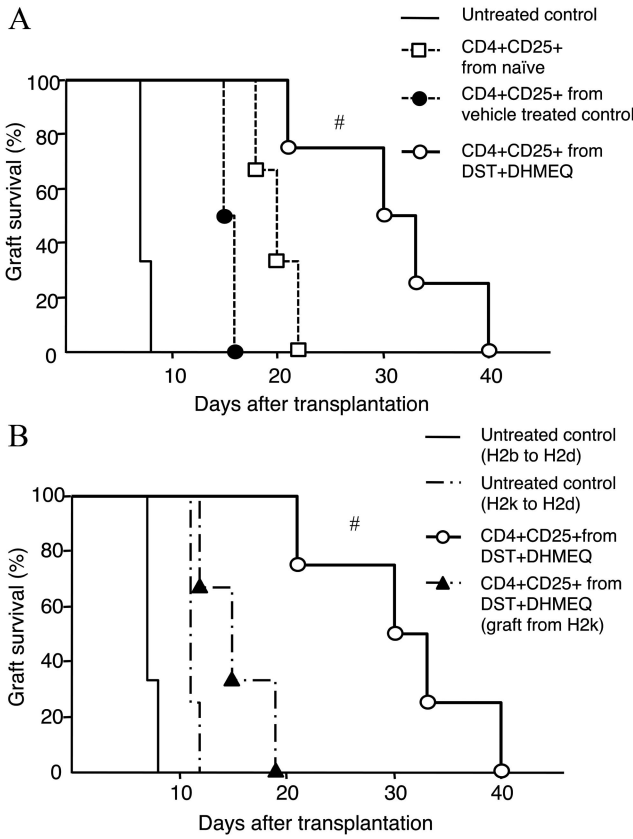

FIGURE 5. The in vivo generated Tregs by the donor-specific blood transfusion (DST)+dehydroxymethylepoxyquinomycin (DHMEQ) treatment are capable of preventing the allograft rejection. (A) Recipient $H2^d$ mice transplanted $H2^b$ heart graft were adoptive transferred with 1×10^6 $CD4^+CD25^+$ T cells, which were isolated from recipient's spleen at day 7 after heart transplantation in each group. The allografts of recipient mice that were adoptive transferred with DST+DHMEQ-treated recipient's $CD4^+CD25^+$ T cells ($CD4^+CD25^+$ from DST+DHMEQ, n=4), significantly prolonged median survival time (MST) to 31.5 days as compared with all other group (#$P<0.05$ by log-rank test, Untreated control [n=6, MST=7 days], $CD4^+CD25^+$ from naïve [n=3, MST=20 days], $CD4^+CD25^+$ from vehicle-treated control [n=4, MST=15.5 days]). (B) When transplanted with the C3H ($H2^k$) cardiac allografts into adoptive transferred recipient mice, the DST+DHMEQ-treated recipient's $CD4^+CD25^+$ T cells were unable to prolong MST (n=4, MST=15 days) as compared with when transplanted with a donor strain graft (#$P<0.05$ by log-rank test).

Meanwhile, it has been demonstrated that TGF-β1-induced Foxp3 expression was enhanced in T cells from p50 knockout mice compared with wild-type mice (*40*). Recently, an enhanceosome, which promotes the transcription of the Foxp3 gene and contains the NF-κB family members c-Rel and p65, but not p50 or Rel-B. c-Rel has been shown important for initiating Treg differentiation (*38*). DHMEQ has been shown to bind to the CEGRSAGSI residues present in p65, c-Rel, and Rel-B but not to p50 or p52 (*9*), and inhibiting the nuclear transport of p65 (*8*). Although the reciprocal effect of DHMEQ on the development of Treg was not important in our in vivo study, the influence of DHMEQ at the single-cell level requires further clarification.

The promotion of Treg development in our study is likely to be secondary to an enhancing effect of NF-κB inhibition on the DST effect. Our data demonstrate that DHMEQ treatment alone does not alter the Treg population, whereas DST treatment alone induces an increase in the percentage of $CD4^+Foxp3^+$ Treg and the Treg function-associated molecules CTLA-4 and TGF-β (Fig. 4A, C, and D). Although further work is necessary to clarify how NF-κB inhibition enhances DST-mediated Treg induction, we consider that there are two possibilities. First, the increase in the Treg population may be due to deletion of alloreactive T cells in association with relative resistance of Treg to apoptosis. Second, DHMEQ may influence Ag-presenting cells to enhance Treg expansion. Indeed, it has previously been reported that Treg are anergic and resistant to apoptosis (*35*), and that DCs play crucial role in the expansion of Treg under DST treatment (*41*, *42*). Tolerogenic DCs are particularly potent in inducing a protolerogenic state through shifting the balance from an effector to a Treg population. Rel-B in the NF-κB pathway is crucial for the maturation of DC (*43*), and NF-κB inhibition causes a reduction in the expression of major histocompatibility complex and costimulatory molecules on DC thereby impairing their ability to activate allospecific effector T cells (*44*, *45*). The inhibition of the upstream segment of the NF-κB signaling pathway by ex vivo treatment of DC with lymphoid follicle 15-0195 promotes the development of tolerogenic DC, which play a pivotal role on generation of Ag-specific Treg (*46*). Indeed, we and others have shown the ability of DHMEQ to inhibit the maturation of both mouse and human DC (*30*). On investigating the effect of DHMEQ on DC further, we recently found these DCs prolong the survival of cardiac grafts (manuscript in preparation). It is possible that the combination of a DST with DHMEQ promotes the development of DC that peripherally induces Treg, which maintain an immunosuppressive state for a long term.

In this study, we demonstrate that DST+DHMEQ markedly prolongs allograft survival in $H2^b$-to-$H2^d$ strain combinations compared with $H2^d$-to-$H2^b$. This may be because a Th2-skewed environment facilitates a better graft survival under the DST+DHMEQ treatment. Others have suggested that the NF-κB pathway is important in the differentiation of $CD4^+$ T-cell subsets. For example, P65 and Rel-B are required for Th1 cell differentiation (*47*, *48*), and Rel-B-deficient cells cannot differentiate into Th1 cells due to a resultant decrease in the expression of T-box and Stat4 (*49*). On the other hand, Bcl-3, a member of the IκB family that binds p50 and p52 is required for the differentiation of Th2 cells (*49*, *50*). As mentioned earlier, it has been shown that DHMEQ target RelA, c-Rel, and RelB, but not in p50 and p52 (*9*). Consistently, Iwata et al. (*51*) have shown that DHMEQ suppresses the Ag-specific T-cell response in addition to downregulating the production of Th1- but not Th2-type cytokines in vitro. Indeed, DHMEQ markedly inhibited IFN-γ production in our study. Because it has been shown that the Th2 skewed condition is associated with Treg generation (*52*), treatment with DHMEQ plus DST in $H2^d$ cardiac recipients under a Th2-skewed environment may have resulted in a better graft survival prolongation.

In conclusion, NF-κB inhibition seems to augment the tolerogenic effects of DST through a mechanism involving AICD and an increase in Treg development and suppressive activity. These findings provide a novel method for tolerance induction, which may be potentially translated for use in clinical transplantation. We envisage DHMEQ to be a useful agent to add to the clinical armament of immunosuppressive agents.

MATERIALS AND METHODS

Animals

Male C57BL/6 ($H2^b$), BALB/c ($H2^d$), and C3H/HeJ ($H2^k$) mice were purchased from SLC Inc. (Shizuoka, Japan) and were used at 8 to 12 weeks of age. All experiments were conducted under protocols approved by the institutional Committee of Ethical Animal Research. Animal care was performed in accordance with the guidelines provided by the Office of Animal Care and Use.

Cells and Cell Isolation

Lymphocytes were isolated from mouse spleens after red blood cell lysis. When necessary, $CD4^+$ and $CD4^+CD25^+$ T cells were purified (purity >93%) by negative selection with an AutoMACS system (Miltenyi Biotec, Tokyo, Japan).

Transplantation

Heterotopic abdominal heart transplantation was performed as previously described (*53*). Graft function was monitored by daily palpation. Rejection was defined as complete cessation of cardiac contractility as determined by direct visualization.

Treatment Protocol

DHMEQ was dissolved in 4% dimethyl sulfoxide and 0.5% carboxymethyl cellulose solution, and was administered intraperitoneally at 10 mg/kg or 15 mg/kg twice or three times daily (total dose: 30 mg/kg/day) for 2 weeks, from 7 days before transplantation (day −7). For DSTs, donor strain splenocytes (20×10^6 cells) were suspended in phosphate-buffered saline and injected intravenously on day −7.

Mixed Lymphocyte Reaction

Recipient splenocytes (5×10^5 cells/well) were cocultured with irradiated (30 Gy, ^{137}Cs) donor or third-party splenocytes (5×10^5 cells/well) in a 96-well round-bottom plate and Rosewell Park Memorial Institute 1640 medium (supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin) in a 5% CO_2-humidified atmosphere. ^3H-thymidine (MP Biomedical Inc., Irvine, CA) (1 μCi/well) was added 8 hr before cell harvesting, and thymidine incorporation was assessed using a liquid scintillation counter.

Monitoring CFSE Labeling Lymphocyte In Vivo

In vivo MLR using CFSE labeling technique was performed as previously described (*23*). Briefly, CFSE (5 μM; Molecular Probes, Eugene, OR)-labeled lymphocytes from $H2^b$ mice were injected intravenously to sublethally irradiated (9 Gy) $H2^d$ mice. Seventy-two hours later, lymphocytes were

harvested and stained with anti-Annexin V Ab (BD Pharmingen, San Diego, CA).

Flow Cytometric Analysis

Cells were stained with mAbs against PerCP-Cy-5.5-CD4 (RM4-5), PerCP-Cy-5.5-CD8 (53-6,7), FITC-CD25 (7D4), PE-CD154 (MR-1), (BD Pharmingen), and PE-FoxP3 (FJK16s) (eBioscience, San Diego, CA). Fluorescence-activated cell sorting (FACS) analysis was carried out with a FACS Calibur (Becton Dickinson, Palo Alto, CA). To investigate the frequency of donor-Ag specific alloreactive T cells, recipient splenocytes were restimulated with donor Ag in the presence of 1 μg/mL CD28 mAb (BD Pharmingen) for 6 hr. Brefeldin A (Sigma-Aldrich, Inc., St. Louis, MO) at 5 μg/mL was added 4 hr before harvesting. Intracellular CD154 expression in $CD4^+$ T cells was detected by intracellular staining as previously described (*22*). Foxp3 staining was performed according to manufacturer's guidelines (eBioscience).

ELISPOT Assay

Recipient splenocytes were cocultured with irradiated donor splenocytes in an IFN-γ capture antibody precoated ELISPOT plate (BD Biosciences, San Diego, CA). IFN-γ spots were detected by a biotinylated antimouse IFN-γ Ab and visualized using HRP-streptavidin followed by AEC staining (BD Biosciences). Spots were counted using the KS ELISPOT system (Carl Zeiss, Jena, Germany).

RNA Extraction and Real-Time Polymerase Chain Reaction Analysis

RNA extraction was performed using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Five micrograms of DNAse (Invitrogen) pretreated RNA was reverse transcribed to 60 μL of complementary DNA (cDNA). Each quantitative polymerase chain reaction reaction consisted of 20 μL of cDNA (containing 250 ng of cDNA), 10 μL of SYBR Green master mix (Bio-Rad, Hercules, CA) and 250 nmol of sense and antisense primers. Primers were designed using Primer3 software (Primer3 Web site). The primers used were Foxp3 (ACAATATGCGACCCCCTTTC; CGGGGTGGTTTCTGAAGTAG), CTLA-4 (GTCTGTGCCACGACATTCAC; CATGAGTTCCACCTTGCAGA), IL-10 (TGAATTCCCTGGGTGAGAAG; TGGCCTTGTAGACACCTTGG), and TGF-β (GTCAGACATTCGGGAAGCAG; TGCCGTACAACTCCAGTGAC). Quantitative polymerase chain reaction was performed at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min. Fluorescence was measured during the annealing/extension phase. Expression was measured as copies of any given gene divided by copies of the housekeeping gene, B2M (ACGCCTGCAGAGTTAAGCAT; GCTATTTCTTTCTGCGTGCAT).

Histopathologic Analysis of Cardiac Grafts

Grafts samples fixed in 10% phosphate-buffered formalin before being embedded in paraffin, sectioned using a microtome, and stained with H&E. Graft samples were also embedded in optimal cutting temperature compound (Sakura Finetek Inc, CA) and snap frozen in liquid nitrogen. Frozen sections were cryosectioned stained with anti-CD4 mAb (GK1.5: Santa Cruz Biotech, Santa Cruz, CA) and anti-CD8 mAb (KT15: Chemicon International, Temecula, CA) using an avidin-biotin complex method. Positive cells were quantified in each tissue section using a light microscope (magnification ×400).

Statistical Analysis

Allograft survival was analyzed using a Kaplan-Meier method, and data compared by applying a log-rank test. For comparison of other data, a one-way ANOVA (post hoc test: Fisher's protected least significant difference) was used and values expressed as mean±SD or SEM. Differences were considered significant when P values were less than 0.05.

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