Control of Allograft Rejection by Applying a Novel Nuclear Factor-κB Inhibitor, Dehydroxymethylepoxyquinomicin

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Background. Nuclear factor (NF)-κB plays a crucial role in lymphocyte activation, proliferation, and survival. We examined the immunosuppressive effect of a newly developed NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ) in allotransplantation.

Methods. Purified C57BL/6 (H-2^b) T cells were used for in vitro studies examining activation, proliferation, cytokine production and nuclear NF-κB and nuclear factor of activated T cells (NFAT) protein levels. A fully major histocompatibility complex incompatible BALB/c (H-2^d)-to-C57BL/6 mice cardiac transplantation model was utilized for in vivo studies. DHMEQ was given intraperitoneally to transplant recipients at a various dose starting from day 0. In some, DHMEQ was administered concomitantly with tacrolimus.

Results. DHMEQ significantly suppressed αCD3+αCD28 monoclonal antibody–triggered T-cell proliferation, CD25/CD69 expressions, and both interleukin-2 and interferon (IFN)-γ production in a dose-dependent fashion. DHMEQ blocked nuclear translocation of NF-κB but not NFAT in activated T cells. Combined treatment with DHMEQ and tacrolimus significantly suppressed T cell activation as compared to that of mono-therapy with either agent alone. Single DHMEQ treatment moderately prolonged cardiac allograft survival. Further, combination of DHMEQ plus tacrolimus markedly prolonged graft mean survival time (MST) to 59.5 days when compared to either DHMEQ (MST: 10 days) or tacrolimus (MST: 13 days) treatment alone. Such effect was associated with inhibition of mixed lymphocyte reaction against donor antigen, IFN-γ producing splenocytes and graft cellular infiltration as examined at 5 and 12 days posttransplantation.

Conclusion. DHMEQ inhibits nuclear translocation of NF-κB but not NFAT in activated T cells, and prolongs allograft survival. Blocking both NF-κB and NFAT by DHMEQ and tacrolimus induces potent immunosuppression, which may become a new modality in controlling allograft rejection.

Keywords: NF-κB, Dehydroxymethylepoxyquinomicin, Mouse, T cells, Heart transplantation.

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The nuclear factor (NF)-κB is a transcription factor that resides in the cytoplasm of various cell type as a homo- or heterodimer of subunits, p65 (RelA), p50/105, p52/100, RelB, and c-Rel. Upon activation, NF-κB is sequestrated by a family of inhibitors of κB (IκB) proteins following phosphorylation and degradation, and it translocates to the nucleus to induce various gene expression that are critical for cell survival, inflammation and immunity (*1*, *2*). NF-κB plays an important role in activation and proliferation of lymphocytes (*3*), and it has been shown that p65, p50 and p52 are activated within allografts during transplant rejection (*4*). Indeed, in the p50-deficient mice that have a Th2 response defect (*5*), cardiac allograft survivals are modestly prolonged (*6*). Also, long-term cardiac allograft survivals has been demonstrated in the cRel-deficient mice (*7*, *8*), in which those T cells are absent from interleukin (IL)-2 production, IL-2R expression and proliferation (*9*). The I-κB(ΔN) transgenic mice, lacking the phosphorylation site of I-κB also accepted cardiac allografts for over 100 days (*7*). In addition, administration of antisense oligodeoxynucleotide (ODN) against NF-κB has been shown to prolong heart allograft survival in mice (*10*). Although these approaches including genetic engineering can target NF-κB specifically, they are far from clinical applications. On the other hand, conventional immunosuppressants such as glucocorticoids (*11*), tacrolimus, and cyclosporine (*12*) has been reported to inhibit NF-κB to some extent. However, inhibition of NF-κB activation by these agents are nonspecific, and to date, selective NF-κB inhibitors such as proteasome inhibitors (*13*) or others (*14*) are still under study.

Dehydroxymethylepoxyquinomicin (DHMEQ), a newly developed low molecular weight NF-κB inhibitor, is a 5-dehydroxymethyl derivative of the antibiotic epoxyquinomycin C (*15*). DHMEQ has been found to inhibit tumor necrosis factor (TNF)-α induced NF-κB activation by suppressing the nuclear translocation but not I-κB phosphorylation or degradation (*16*). Recently, effect of DHMEQ on breast (*17*), thyroid (*18*), and prostate (*19*) cancers and rheumatoid arthritis (*20*) has been shown. In this study, we examined immunosuppressive effect of DHMEQ using murine T cells and a cardiac transplantation model.

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MATERIALS AND METHODS

Animals

Male C57BL/6 (B6: H-2^b), BALB/c (B/c: H-2^d) and C3H/HeJ (C3H: H-2^k) mice were purchased from SLC Inc. (Shizuoka, Japan). They were maintained in the specific pathogen–free facility, and were used for experiments when six- to eight-weeks old. The experiments were approved by the institutional Animal Care Committee, and were conducted under the guideline of animal care policy.

Cells and Culture Medium

Primary murine leukocytes were isolated from the spleen. T cells (purity: >85%) were further enriched by passing a nylon-wool mesh column (R&D systems, Minneapolis, MN). Roswell Park Memorial Institute (RPMI) 1640 culture media containing 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum, and 50 μM 2-ME was used for cell culture.

Reagents

DHMEQ was synthesized as described previously (*15*), dissolved in dimethylsulfoxide, and adjusted to 50 mg/ml. Tacrolimus (Fujisawa, Osaka, Japan) was adjusted to 1 mg/ml using RPMI 1640. These stock solutions were stored at −80°C until use. For in vivo experiments, DHMEQ stock solution and tacrolimus (20%) powder was dissolved in 0.5% carboxymethyl cellulose (CMC) solution and in distilled water, respectively. They were administered intraperitoneally (i.p.). Monoclonal antibodies (mAbs) against mouse CD3 (145-2C11), CD4 (L3T4), CD25 (7D4), CD28 (37.51) and CD69 (H1.2F3) were purchased from PharMingen (San Diego, CA). Antimouse CD4 (GK1.5: SANTACRUZ, CA) and CD8 (KT15: CHEMICON, CA) antibodies (Abs) were used for immunohistochemistry.

Lymphocyte Stimulation and Proliferation Assay

Purified B6 T cells (5×10^5/well) were stimulated by anti-CD28 (αCD28: 1 μg/ml) and plate precoated anti-CD3 (αCD3: 10 μg/ml) mAbs, and were cultured in 96-well flat-bottom plates. For mixed lymphocyte cultures (MLC), irradiated (30 Gy, ^{137}Cs) B/c-splenocytes (5×10^5/well) were cocultured with B6-splenocytes (5×10^5/well) in 96-well round-bottom plates. Plates were incubated at 37°C under 5% CO_2 plus air. Cells were pulsed with ^{3}H-thymidine (1 μCi/well) 16 hours before culture termination, and ^{3}H-thymidine incorporation was measured using a β-counter (PerkinElmer, Boston, MA).

Flow Cytometric Analysis

Stimulated B6-T cells were stained with isotype control mAbs or specific mAbs against CD4, CD69, and CD25. Cells were analyzed using a FACSCalibur flow cyotmeter and CellQuest software (BD Biosciences, Palo Alto, CA). Ten-thousand $CD4^+$ T cells were acquired for each analysis. For cell cycle analysis, T cells were stained with 7-amino-actinomyain D (7-AAD) and pyronin Y, and were examined using a flow cytometer as described previously (*21*).

Cytokine Measurement

IL-2 and interferon (IFN)-γ levels in culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA) using a cytokine assay kit (R&D Systems). Measurements were performed in duplicate. IFN-γ production of splenocytes obtained from transplant recipients was examined by enzyme-linked immunospot assay (ELISpot). Briefly, responding splenocytes (5×10^5 cells/well) were cocultured with irradiated B/c- or C3H-splenocytes (1×10^6 cells/well) for 24 hr in antimouse IFN-γ mAb precoated BD ELISPOT plate (BD biosciences). IFN-γ spots were detected by a biotinylated antimouse IFN-γ Ab, and were visualized using SAv-HRP and AEC substrate within the kit. Finally, spots were enumerated by KS ELISPOT (Carl Zeiss, Germany). The average number of spots was expressed as spot-forming cells (SFC)/10^6 splenocytes.

Nuclear Protein Extraction and Quantification of Transcription Factor Activity

Following three hr of αCD3+αCD28 mAb stimulation, B6-T cells (10^7 cells/group) were collected, and nuclear protein was extracted according to the kit instruction (Nuclear extract kit: Activemotif, Carlsbad, CA). DNA binding activity of nuclear extracts for p50, p65 and nuclear factor of activated T cells (NFAT) were assayed using the TRANS AM Kit (Activemotif) (*22*).

Cardiac Transplantation and Treatment Protocol

Heterotopic mouse heart transplantation was performed as previously described (*23*). Cardiac recipients were given either DHMEQ (12, 20 or 40 mg/kg), tacrolimus (1.5 mg/kg), or control vehicle (0.5% CMC), starting from day 0 (n=6 each). Graft beating was monitored by daily palpation, and was scored +1 to +4 based on strength of graft contractions. Rejection was defined as cessation of beating, which was confirmed by direct inspection followed by histological examination.

Histology and Immunohistochemistry

Cardiac graft was excised at the time of animal death or sacrifice. Tissues were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin (HE). Graft samples were also embedded in an optimal cutting temperature compound, frozen in liquid nitrogen, and stored at −80°C. Frozen sections were stained with anti-CD4 and CD8 Abs by avidin-biotin complex method (*24*). Positive cells were counted in 3 different high power fields (magnification ×400).

Statistical Analysis

Graft survival time was plotted by a Kaplan-Meier method, and a log-rank test was applied for comparison. Other data are expressed as mean±standard error of the mean (mean±SEM), and statistical analysis was performed by one-way analysis of variance (Fisher's PSLD posthoc test). A P value less than 0.05 was considered statistically significant.

RESULTS

DHMEQ Suppresses T-cell Activation, Proliferation, and Cytokine Production by Inhibiting Nuclear NF-κB Activity

To examine the suppressive effect of DHMEQ on T cell proliferation in vitro, naïve B6 T cells were stimulated with αCD3+αCD28 mAb or irradiated B/c splenocytes, with or

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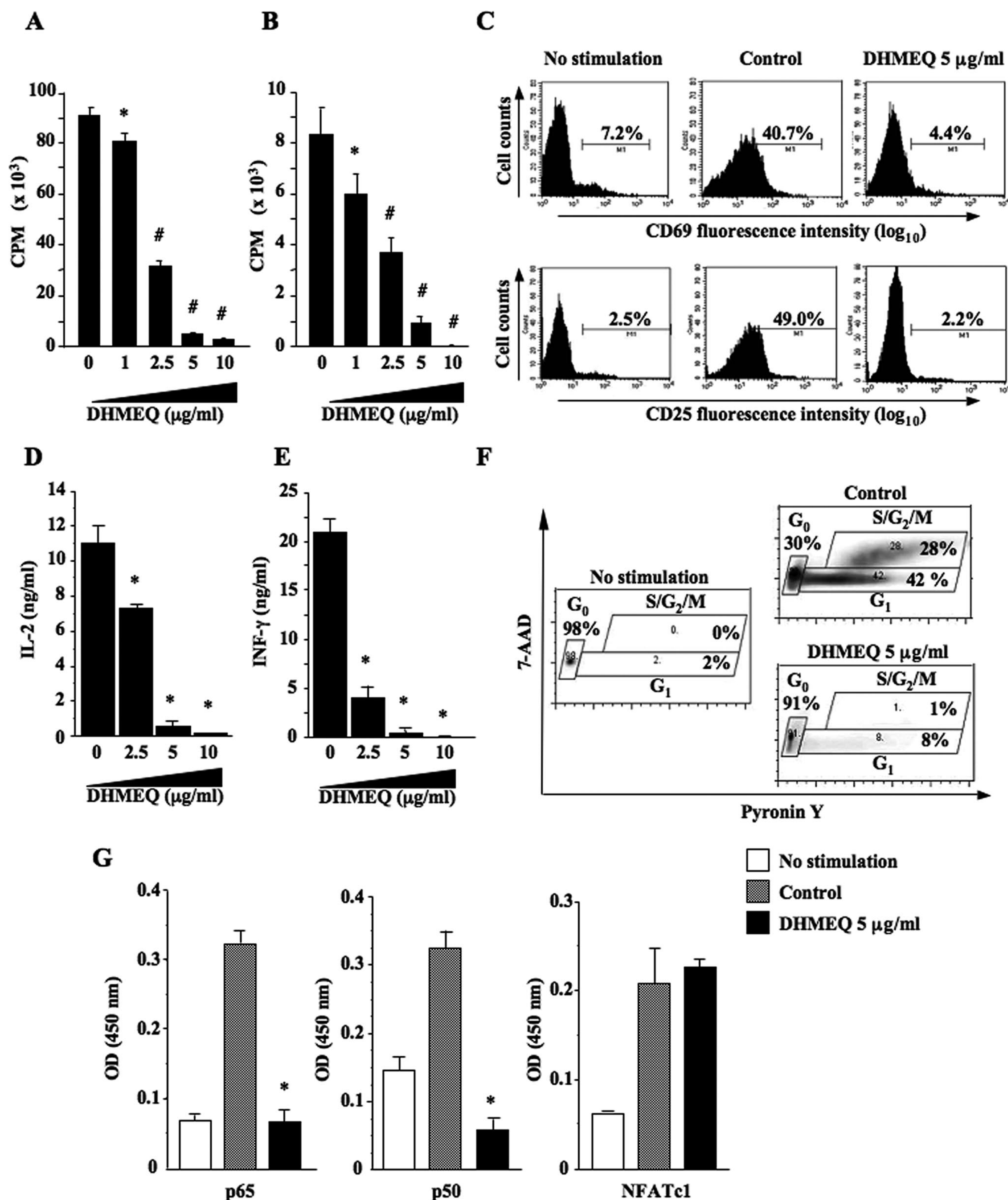

FIGURE 1. DHMEQ suppresses activation, cell cycle progression, proliferation, and cytokine production by inhibiting nuclear NF-κB activity in vitro. (A and B) B6 mouse lymphocyte proliferation following 48-hour stimulation with αCD3+αCD28 (A) or 72-hour stimulation with irradiated B/c splenocytes (B) with or without DHMEQ. Proliferation was examined by counting ^{3}H-thimidine uptake (n=3). DHMEQ suppressed lymphocyte proliferation in a dose-dependent manner ($^{*}P<0.05$ and $^{\#}P<0.001$ vs. control). (C) Representative histograms of surface CD69 and CD25 expression on CD4^{+} T cells following three and nine hours of αCD3+αCD28 stimulation, respectively (n=3). Treatment with DHMEQ at 5 μg/ml suppressed upregulation of CD69 and CD25. (D and E) Cytokine production in αCD3+αCD28 stimulated T cells. IL-2 (D) and IFN-γ levels in culture supernatants following 48 hr-stimulation were measured by ELISA (n=3). DHMEQ treatment

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without DHMEQ. DHMEQ suppressed lymphocyte proliferation in a dose-dependent manner in both settings (Fig. 1A, B). In the control $CD4^+$ T cells, CD69 and CD25 expressions were strongly upregulated at three and nine hours after αCD3+αCD28 mAb stimulation, respectively. In contrast, DHMEQ treatment at 5 mg/ml significantly suppressed upregulation of these activation markers (Fig. 1C). Also, DHMEQ treatment dose-dependently inhibited IL-2 and IFN-γ production in mAb stimulated T cells, as compared to those of control (Fig. 1D and E). Further, the effect of DHMEQ on cell cycle progression was assessed by quantifying DNA and RNA contents. Forty-eight hours after mAb stimulation, T cells entering the $S/G_2/M$ phase (25.1±2.5%) increased when compared to nonstimulated naïve T cells (0.2±0.2%). In contrast, cells in the $S/G_2/M$ phase (1.6±0.4%) were significantly lower in the presence of DHMEQ (Fig. 1F). To examine whether activation of NF-κB and/or NFATc were suppressed by DHMEQ in activated T cells, nuclear NF-κB and NFATc protein levels were quantified. Nuclear p65 and p50 levels were significantly suppressed in mAb stimulated T cells by DHMEQ (5 μg/ml), whereas nuclear NFATc level was unaffected (Fig. 1G).

Combination of DHMEQ and Tacrolimus Efficiently Suppresses T-cell Activation and Proliferation In Vitro

To assess whether the combination of DHMEQ and NFAT inhibitor has an adjunct effect on T cells in vitro, naïve B6 T cells were stimulated in the presence of DHMEQ and/or tacrolimus. DHMEQ plus tacrolimus treatment significantly suppressed proliferation of αCD3+αCD28 mAb driven T cells as compared to that of monotherapy with either agent (Fig. 2A). A similar result was observed in splenocytes stimulated with alloantigens (Fig. 2B). Combination effect of DHMEQ and tacrolimus treatment on activation marker expression was also examined in stimulated $CD4^+$ T cells. Although DHMEQ (2.5 μg/ml) or tacrolimus (0.25 ng/ml) treatment suppressed CD69 and CD25 expressions at a mild-to-moderate degree, combination treatment dramatically suppressed these expression levels (Fig. 2C and 2D). Such an effect was true for cytokine production: both IL-2 and IFN-γ production from stimulated T cells was markedly suppressed by DHMEQ plus tacrolimus treatment (Fig. 2E and 2F).

FIGURE 1. (Continued) suppressed both IL-2 and IFN-γ production in a dose-dependent manner ($^*P<0.001$ vs. control). (F) Cell cycle analysis in cultured B6 mouse T cells by using a flow cytometry. Intracellular 7-AAD and Pyronin Y were stained 48 hr after stimulation with αCD3+αCD28. Representative dot-plots are shown (n=3). Cells in the G_0 phase are shown in the left lower small box, G_1 phase in the lower long box, and $S/G_2/M$ phase in the upper long box. Cell cycle of the stimulated T cells progressed to $S/G_2/M$ phase following αCD3+αCD28 stimulation, however, in the presence of DHMEQ, cell cycle was arrested at G_0/G_1 phase. (G) Nuclear NF-κB and NFAT activity in T cells following three-hour stimulation with αCD3+αCD28. Nuclear NF-κB and NFATc protein levels were quantified using the TransAM kit (n=3). Nuclear p65 and p50 levels were suppressed in activated T cells by DHMEQ treatment at 5 μg/ml ($^*P<0.001$ vs. control), whereas nuclear NFATc level was unaffected.

Inhibition of NF-κB Activation by DHMEQ Prolongs Cardiac Allograft Survival

B/c mouse heart was transplanted into B6 mouse, and recipient was given either DHMEQ or vehicle alone. Vehicle-treated control mice rejected allografts at a median survival time (MST) of six days (Fig. 3A). In contrast, thrice weekly DHMEQ treatment at 12, 20, and 40 mg/kg significantly prolonged graft MST to 9.5 ($P<0.01$), 10 ($P<0.01$), and 13 ($P<0.02$) days, respectively, when compared to control. Also, daily DHMEQ administration at 12 and 20 mg/kg for two weeks significantly prolonged allograft survival to MST of 12.5 ($P<0.01$) and 28.5 ($P<0.001$) days, respectively (Fig. 3A). We further examined the combination effect of DHMEQ and tacrolimus. Thrice weekly DHMEQ treatment (20 mg/kg, 4 weeks) together with daily tacrolimus treatment (1.5 mg/kg, 2 weeks) markedly prolonged graft MST to 59.5 days as compared to either DHMEQ (MST: 10 days; $P<0.001$) or tacrolimus (MST: 13 days; $P<0.001$) treatment alone (Fig. 3B). Microscopic exam revealed that all allografts were lost due to acute cellular rejection (data not shown).

DHMEQ plus Tacrolimus Suppresses Alloimmune Responses In Vivo

To analyze the in vivo immunosuppressive effect of DHMEQ with or without tacrolimus treatment, splenocytes harvested on day five were challenged with donor-antigens in a MLC. Although thrice weekly DHMEQ treatment (20 mg/kg on days 0, 2, and 4; n=4, $P=0.09$) or tacrolimus administration (1.5 mg/kg/day; n=4, $P<0.05$) alone induced a mild-to-moderate suppression (Fig. 4A), combination of both (n=4, $P<0.02$) significantly inhibited splenocyte proliferation as compared to the control (n=3). Alloreactivity of cardiac recipients was also assessed by IFN-γ ELISpot assay. Splenocytes from control recipients on day five showed a high response against B/c but not C3H splenocytes as assessed by the numbers of IFN-γ SFC (Fig. 4B). In contrast, splenocytes from DHMEQ treated recipients had significantly lower response to donor-antigens ($P<0.001$). The number of IFN-γ SFC in DHMEQ plus tacrolimus treated group was significantly lower when compared to that of tacrolimus treatment alone on day 12 ($P=0.003$), while IFN-γ production against third-party antigens was unaffected by treatment with DHMEQ and/or tacrolimus. Finally, we examined histology of cardiac grafts. In the control grafts, severe cellular infiltration, predominantly $CD8^+$ and to a lesser extent $CD4^+$ cells, was noted at five days posttransplantation (Fig. 4C–E). In contrast, DHMEQ and/or tacrolimus treatment suppressed intragraft $CD4^+$ and $CD8^+$ cellular infiltration. Although grafts from tacrolimus treatment group showed severe acute rejection with a heavy infiltration of $CD4^+$ and $CD8^+$ cells on day 12, addition of DHMEQ prevented graft damage and reduced infiltrates of $CD4^+$ and especially, $CD8^+$ cells (Fig. 4C–E).

DISCUSSION

In this study, we have demonstrated that a newly developed agent, DHMEQ, suppressed T-cell proliferation in vitro

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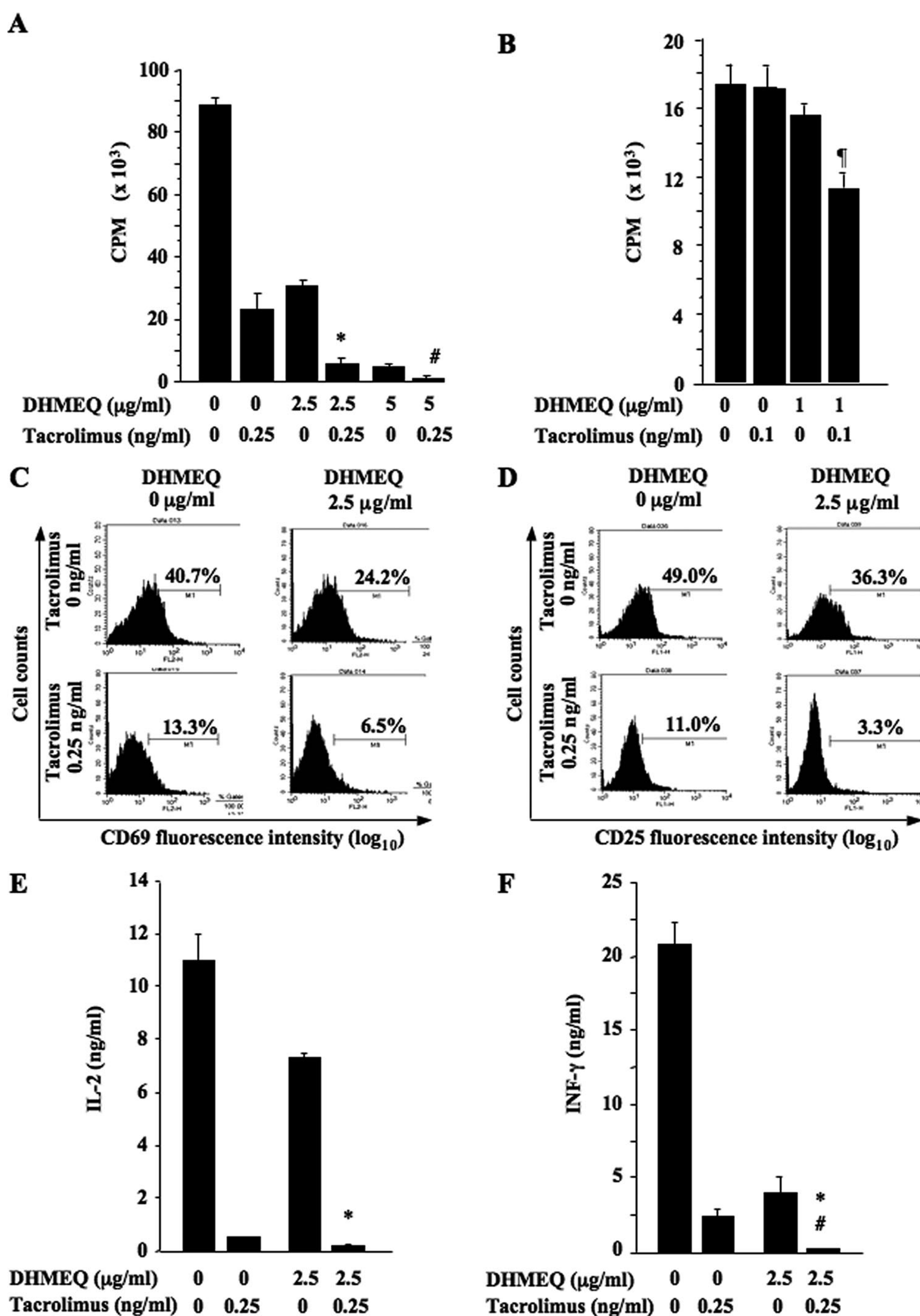

FIGURE 2. Combination of DHMEQ and tacrolimus efficiently suppresses T cell activation, proliferation and cytokine production in vitro. (A and B) B6 mouse lymphocyte proliferation following 48-hour stimulation with αCD3+αCD28 (A) or 72-hour stimulation with irradiated B/c splenocytes (B) in the presence or absence of DHMEQ and/or tacrolimus (n=3). Proliferation was examined by counting ^{3}H-thimidine uptake. Combined treatment with DHMEQ and tacrolimus suppressed proliferation of T cells as compared to that of monotherapy with either agent alone ($^{*}P<0.001$, $^{\#}P<0.001$, and $^{¶}P<0.001$ vs. tacrolimus). (C and D) Representative histograms of surface activation markers following αCD3+αCD28 stimulation (CD69: 3 hr, CD25: 9 hr) on CD4^{+} T cells (n=3). DHMEQ plus tacrolimus treatment markedly suppressed expression of CD69 (C) and CD25 (D) in activated B6 mouse CD4^{+} T cells when compared to either treatment alone. (E and F) Combination effect of DHMEQ and tacrolimus on cytokine production in T cells. IL-2 (E) and IFN-γ (F) concentrations in the culture supernatants of αCD3+αCD28 stimulated T cells was measured by ELISA (n=3). T cells were stimulated for 48 hours. Both IL-2 and IFN-γ production from αCD3+αCD28 stimulated T cells was efficiently suppressed by combined treatment with DHMEQ and tacrolimus when compared to those of treatment with either agent alone ($^{*}P<0.003$ vs. DHMEQ, $^{\#}P<0.05$ vs. tacrolimus).

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FIGURE 3. DHMEQ treatment prolongs a fully MHC incompatible cardiac allograft survival. (A) Graft survival following cardiac transplantation with or without DHMEQ. Cardiac graft from B/c mouse was transplanted into B6 mouse, and recipient was given intraperitoneally either DHMEQ at the indicated doses or control vehicle (n=6). DHMEQ significantly prolonged cardiac allograft survival in a dose-dependent fashion ($P<0.02$ vs. control). (B) Combination effect of DHMEQ plus tacrolimus on allograft survival in a B/c-to-B6 mouse heart transplantation. Recipients were given intraperitoneally DHMEQ with or without tacrolimus at the indicated dose (n=6). DHMEQ treatment in combination with tacrolimus markedly prolonged allograft survival when compared to either DHMEQ or tacrolimus monotherapy ($P<0.001$).

in a dose-dependent fashion. This accompanied inhibition of T-cell activation as assessed by surface CD69/CD25 expressions and IL-2/IFN-γ productions that consequently arrested cell-cycle progression at G0/G1 phase. Further, such T-cell suppression with DHMEQ was induced by selective inhibition of nuclear translocation of p50 and p65 proteins but not NFAT. These correspond to the fact that upon T-cell activation, NF-κB translocates to nucleolus, binds to the DNA site, and upregulates mRNA transcript of the target genes such as IL-2 (*1*). Using Jurkat cells, Ariga et al. (*16*) have demonstrated the specificity of DHMEQ on NF-κB by showing that DHMEQ did not interfere nuclear translocation of other proteins such as Smad2 or Large T antigen.

Reflecting the in vitro effect on T cells, DHMEQ prolonged fully MHC-mismatched cardiac allograft survivals in vivo in a dose-dependent manner. This in vivo effect was associated with suppression of alloreactive precursor cells as assessed by MLC and IFN-γ ELISpot assays, and reduced intragraft cellular infiltration. With DHMEQ monotherapy, however, long-term survival was not achieved as were shown by the others using c-Rel$^{-/-}$ (*8*) or I-κB(ΔN) mice (*7, 8*). Such discrepancy on potency is presumably due to its magnitude of NF-κB inhibition, such as suppression but not abrogation.

In addition to alloreactive T-cell suppression, the mechanisms underlying prolongation of allograft survival with DHMEQ may also be due to its effect on dendritic cells (DCs). It has been shown that activation of NF-κB is required for DC maturation/activation (*25*), and that NF-κB inhibition by either anti-NF-κB ODN (*26*) or 15-deoxyspergualin analogue (*27*) induces "tolerogenic DCs," which allows potent immunosuppression alone or together with other agents. Studies examining the effect of DHMEQ on DCs are currently ongoing in our laboratory.

T-cell activation is dependent on T-cell receptor (TCR) signaling and also costimulatory signaling via receptors such as CD28. Upon antigenic stimulation in T cells, NF-κB and NFAT share a number of properties: rapid nuclear translocation and production of common cytokines through binding to similar DNA binding domains (*28*, *29*). However, the upstream signaling cascades are distinct. Following TCR cross-linking, NFAT is dephosphorylated by the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, and that results in NFAT activation (*30, 31*). However, signaling through TCR and CD28 triggers stimulation of protein kinase Cθ, a member of the Ca^{2+}/calcineurin-independent PKC family, leads to NF-κB activation (*32*). A previous study indicated that the latter pathway is important for calcineurin inhibitor-resistant T-cell activation (*33*). Based on these facts, we tested our hypothesis that simultaneous blockade of NF-κB and NFAT induces a potent immunosuppression. Indeed, our present study revealed that DHMEQ plus tacrolimus treatment robustly suppressed T-cell activation, proliferation, and cytokine production in vitro, and markedly prolonged cardiac allograft survival. These data indicate that the combina-

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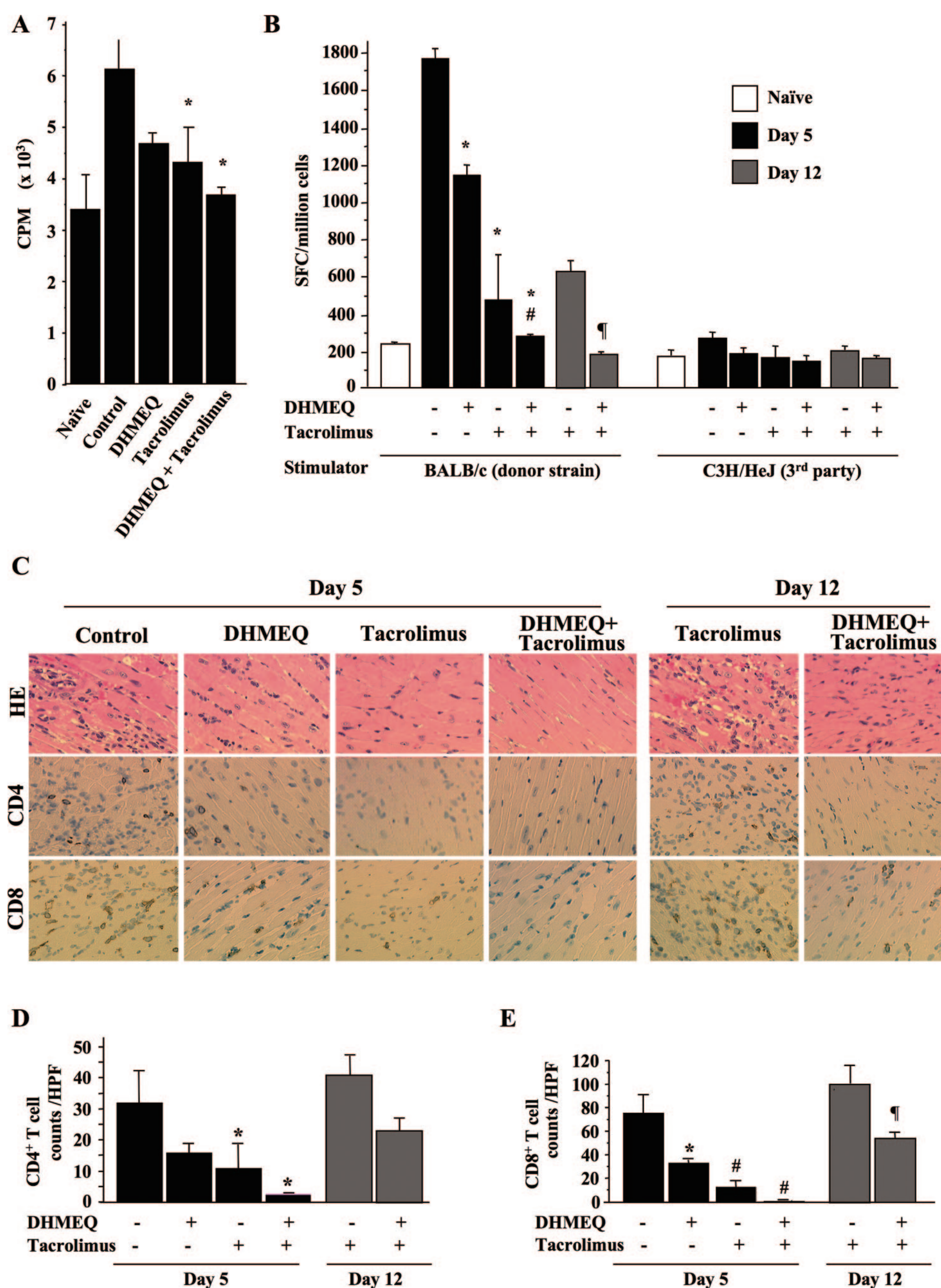

FIGURE 4. Suppression of alloimmune responses and intragraft cellular infiltration by combination treatment with DHMEQ and tacrolimus in vivo. (A) Proliferation of splenocytes, which was obtained from cardiac recipients, against donor antigens. Splenocytes harvested five days after cardiac transplantation were cocultured with irradiated B/c splenocytes for 48 hr, and ^{3}H-thimidine uptake was examined (n=3–4). Combined DHMEQ plus tacrolimus treatment significantly suppressed splenocyte proliferation as compared to the control ($^*P<0.05$ vs. control). (B) The number of alloreactive IFN-γ producing cells within the splenocytes obtained from cardiac recipients on day five and day 12. Splenocytes from recipient animal were cocultured with irradiated donor or third-party splenocytes for 24 hr, and the numbers of IFN-γ spots were examined using ELISpot assay (n=3–4). Splenocytes from DHMEQ treated recipients had significantly lower response to donor ($^*P<0.001$) but not to third-party antigens on day five as compared to control. The number of IFN-γ SFC in DHMEQ plus tacrolimus-treated group was significantly lower when compared to that of DHMEQ treatment alone on day five ($^{\#}P<0.001$) and tacrolimus treatment alone on day 12 ($^{\P}P<0.005$), while IFN-γ production against third-party antigens was unaffected. (C–E) Histology of cardiac grafts following transplantation. Cardiac allografts were stained with HE, anti-CD4, or anti-CD8 mAbs. Representative photographs on day five and day 12 are shown in the panel (original magnification: ×400) (C). The numbers of CD4$^+$ and CD8$^+$ cells in graft sections were counted in three different high power fields and quantified (D, E). Treatment with either tacrolimus alone or DHMEQ plus tacrolimus significantly suppressed intragraft CD4$^+$ and CD8$^+$ cellular infiltration on day five when compared to those of control ($^*P<0.03$ and $^{\#}P<0.001$ vs. control). Combination of DHMEQ and tacrolimus treatment reduced infiltrates of CD4$^+$ and CD8$^+$ cells as compared to those of tacrolimus treatment alone ($^{\P}P<0.01$ vs. tacrolimus).

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tion of NF-κB and calcineurin inhibitors is an effective strategy.

Regarding the toxicity of DHMEQ, no apparent side effects were noted in transplant animals that were given DHMEQ at various doses under the treatment protocol. We further examined toxicity of DHMEQ in naïve mice by administering 20 mg/kg/day for 4 weeks. No difference was seen in weight gain, complete blood cell counts, serum biochemistry, and organ histology (liver, kidney, heart, lung, and spleen) between DHMEQ and vehicle treatment (n=3, data not shown). We also examined adverse effects of DHMEQ when combined with tacrolimus in naïve mice by applying the identical protocol used for transplant experiments. No apparent side effects were noted, including serum alanine transferase, blood urea nitrogen, and creatinine levels (n=3–4, data not shown). Detailed studies confirming safety and efficacy of DHMEQ in higher animal species such as non-human primates are necessary before considering its clinical application.

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