Immunosuppressive Effects of DTCM-G, a Novel Inhibitor of the mTOR Downstream Signaling Pathway

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Background. A newly developed compound, 3-[(dodecylthiocarbonyl)methyl]-glutarimide (DTCM-G), has been shown to inhibit nuclear translocation of c-Fos/c-Jun in a murine macrophage cell line. Herein, we studied the immunosuppressive properties and potency of DTCM-G.

Methods. Using purified mouse T cells, the in vitro effects of DTCM-G on activation, cytokine production, proliferation, and cell cycle progression were assessed, and a possible molecular target of DTCM-G was investigated. In a BALB/c (H-2^d) to C57BL/6 (H-2^b) mouse heart transplantation model, transplant recipients were administered DTCM-G, a calcineurin inhibitor (tacrolimus), and a nuclear factor-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ). Treatment drugs were administered daily for 14 days after transplantation. Alloimmune responses were assessed in addition to graft survival time.

Results. After anti-CD3+anti-CD28 monoclonal antibody stimulation, DTCM-G significantly suppressed proliferation, interferon-γ production, and cell cycle progression of activated T cells but not CD25 expression or interleukin-2 production. These effects were accompanied by inhibition of 70-kDa S6 protein kinase phosphorylation, a downstream kinase of the mammalian target of rapamycin. The addition of tacrolimus and DHMEQ to DTCM-G resulted in a robust inhibition of T-cell proliferation. In vivo combination therapy of DTCM-G plus either tacrolimus or DHMEQ significantly suppressed alloreactive interferon-γ–producing precursors and markedly prolonged cardiac allograft survival. Furthermore, combination of all three agents markedly inhibited alloimmune responses and permitted long-term cardiac allograft survival.

Conclusions. DTCM-G inhibits T cells by suppressing the downstream signal of mammalian target of rapamycin. DTCM-G in combination with tacrolimus and DHMEQ induces a strong immunosuppressive effect in vivo.

Keywords: DTCM-G, Cardiac allograft rejection, T-cell activation, Mouse, IL-2.

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After organ transplantation, control of T-cell–mediated immune responses to donor antigens is crucial for preventing acute cellular rejection. Tacrolimus and cyclosporine are calcineurin inhibitors (CNIs) that powerfully inhibit interleukin (IL)-2 production and IL-2 receptor α (IL-2Rα) expression and suppress proliferation of helper T cells by powerfully inhibiting in a nuclear factor of activated T-cell (NFAT)–dependent manner (*1–4*). To date, these agents are among the most common and dependable immunosuppressants for clinical use. However, side effects of CNIs (including but not limited to renal toxicity, neurotoxicity, and hypertension) remain major problems for posttransplantation patients. Recently, application of CNI-sparing or CNI-minimizing immunosuppressive regimens has been given more attention to avoid or reduce these adverse events (*5–9*).

On activation of T cells, transcription factors other than NFAT (such as nuclear factor [NF]-κB) and activator protein (AP)-1] play an important role in the generation and production of IL-2 (*10*). Previous studies suggested that approaches to genetic engineering, which specifically target

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NF-κB (including using knockout and transgenic mice or administration of antisense oligodeoxynucleotide), significantly prolong cardiac allograft survival (*11–14*). In addition, we previously demonstrated that the NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) inhibits IL-2 production and prevents acute cardiac allograft rejection in mice (*15, 16*). Then again, whether inhibition of AP-1 activation protects the allograft from acute rejection is unknown, although administration of oligodeoxynucleotide against AP-1 has been shown to modestly prolong cardiac allograft survival in rats (*17*).

Recently, we reported on a novel chemical compound, 3-[(dodecylthiocarbonyl)methyl]-glutarimide (DTCM-G) (*18, 19*). This agent was derived from 9-methylstreptimidone and has been shown to inhibit lipopolysaccharide (LPS)–induced nitric oxygen production in murine RAW264.7 macrophages by suppressing nuclear translocation of AP-1, including c-Jun and c-Fos. DTCM-G has also been shown to prolong cardiac allograft survival in mice along with inhibition of intragraft cellular infiltration. However, the means by which DTCM-G inhibits T-cell activation and proliferation is uncertain, as is the combination effect with other immunosuppressant agents that work via different mechanisms. Herein, we investigated the mechanism of action of DTCM-G in T cells and examined the immunosuppressive effects of DTCM-G in combination with low-dose tacrolimus and DHMEQ in a major histocompatibility complex–incompatible model of mouse heart transplantation (HTx).

RESULTS

DTCM-G Inhibits T-Cell Proliferation But Not Interleukin-2 Production

In our previous study, we demonstrated that DTCM-G suppresses proliferation and cytotoxicity of C57BL/6 (H-2^b) (B6) mouse lymphocytes and T cells (*19*). To confirm the inhibitory effect of DTCM-G on lymphocytes and T cells, we performed a mixed lymphocyte reaction (MLR) and proliferation assay and found that DTCM-G treatment significantly inhibited proliferation of both MLR and T cells in a dose-dependent manner (Fig. 1A). The calculated 50% inhibitory concentration (IC_{50}) values were 6.28 and 6.02 μg/mL for MLR and T-cell proliferation, respectively. As such, activation, cytokine production, and cell cycle progression were assessed to further investigate the inhibitory properties of DTCM-G on T cells. In the control $CD4^+$ T cells, surface CD69 and CD25 were up-regulated after anti-CD3 (αCD3)+anti-CD28 (αCD28) monoclonal antibody (mAb) stimulation. Under this condition, DTCM-G did not suppress the up-regulation of these surface activation markers (Fig. 1B). The enzyme-linked immunosorbent assay (ELISA) showed that treatment with DTCM-G did not alter IL-2 production compared with that of controls (Fig. 1C). Then again, DTCM-G inhibited interferon (IFN)-γ production in a dose-dependent fashion (Fig. 1C). In the absence of DTCM-G, the cell cycle analysis revealed that 40%±1.4% and 53%±5.3% of activated $CD4^+$ T cells entered into the S phase at 24 and 36 hr, respectively. At 48 hr after αCD3+αCD28 mAb stimulation, 21%±8.4% of $CD4^+$ T cells further progressed into the G_2/M phase (Fig. 1D). In contrast to control cultures, DTCM-G treatment interfered with cell cycle transition of the activated $CD4^+$ T cells from G_1 to S phase at 24 hr. After 36-hr culture with DTCM-G, only a small proportion (13%±4.1%) of $CD4^+$ T cells entered the S phase. Although activated $CD4^+$ T cells further progressed into the G_2/M phase at 48 hr in the control culture, many $CD4^+$ T cells remained in the S phase after DTCM-G treatment (Fig. 1D). These results of cell cycle analysis indicated that DTCM-G delayed the cell cycle entry from G_1 into S phase.

DTCM-G Inhibits Phosphorylation of 70-kDa S6 Protein Kinase Without Affecting Mammalian Target of Rapamycin Signaling Itself

DTCM-G has been shown previously to inhibit nuclear c-Jun and c-Fos activation in a macrophage cell line (*19*). Therefore, we first examined the nuclear binding activity of c-Jun and c-Fos in mAb-stimulated T cells. Compared with T cells cultured with control media, DTCM-G inhibited activation of nuclear c-Jun (20.3%±3.6%) but not c-Fos (9.1%±10.2%; Fig. 2A). Other important transcription factors relating to IL-2 production, such as NF-κB and NFAT, were unaffected by the DTCM-G treatment (Fig. 2A). We then examined the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) expression, which plays an important role in the IL-2R signaling pathway (*20*). DTCM-G inhibited 70-kDa S6 protein kinase (p70s6K) phosphorylation 12 hr after stimulation in the activated T cells, whereas other molecules were unaffected by the DTCM-G treatment, such as Akt, mTOR, and 3-phosphoinositide-dependent protein kinase 1 (PDK1; Fig. 2B).

Combination Therapy With DHMEQ and Tacrolimus Augments the Immunosuppressive Effects of DTCM-G In Vitro

Coadministration of immunosuppressants that work via different mechanisms of action has a great advantage in reducing drug-related side effects while still achieving potent immunosuppression. Therefore, we investigated the combined effects of DTCM-G with a newly developed inhibitor of NF-κB, DHMEQ (*21–23*), and tacrolimus, a widely used immunosuppressant. Before examining the combinatorial effect, MLR and T-cell proliferation were examined using DHMEQ or tacrolimus alone. DHMEQ inhibited both MLR and T-cell proliferation in a dose-dependent manner (Fig. 3A). However, the calculated IC_{50} value was 0.72 μg/mL for MLR and 2.78 μg/mL for T-cell proliferation; approximately four times higher concentration was necessary for DHMEQ to inhibit T-cell proliferation than to inhibit MLR. Conversely, tacrolimus inhibited both MLR and T-cell proliferation in a dose-dependent manner at equivalent concentrations (Fig. 3B). The calculated IC_{50} value was 0.72 μg/mL for MLR and 0.71 μg/mL for T-cell proliferation. Next, to investigate how coadministration inhibits T cells in vitro, we examined T-cell proliferation and Th1-cytokine production, including IL-2 and IFN-γ, when DHMEQ and tacrolimus were combined with DTCM-G. The optimal dose of each drug was determined based on the result of the T-cell proliferation study (Figs. 1A and 3). Monotreatment with DTCM-G (2.5 μg/mL), DHMEQ (1.0 μg/mL), or tacrolimus (0.5 ng/mL) did not suppress T-cell proliferation. In contrast, the combination of DTCM-G with DHMEQ or tacrolimus significantly inhibited T-cell proliferation. In addition, exposure to all three agents completely abolished T-cell proliferation (Fig. 4A).

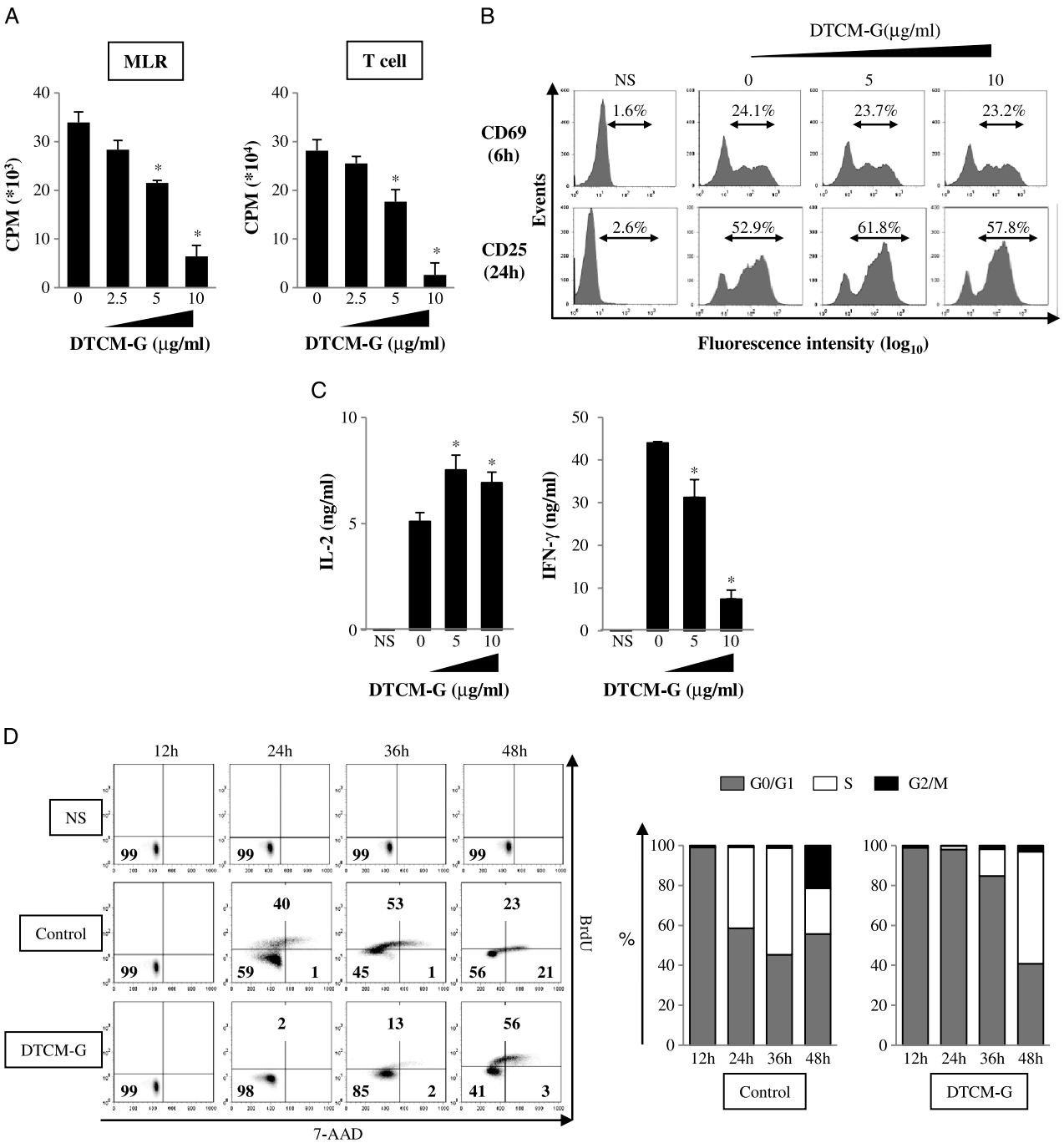

FIGURE 1. DTCM-G inhibits IFN-γ and cell cycle progression, but not activation or IL-2 production, in naïve B6 T cells. A, naïve B6 mouse lymphocyte proliferation after 72-hr stimulation with irradiated BALB/c splenocytes (*left*) or 48-hr stimulation with αCD3+αCD28 mAbs (*right*) in the presence of vehicle-alone (control; 0) or DTCM-G. Cells were pulsed with [^{3}H]thymidine at 8 hr before cell harvest. Each column represents the mean±SEM of six independent experiments. *P<0.05 vs. control. B, surface expression of CD69 (*top*) or CD25 (*bottom*) on T cells after 6- or 24-hr stimulation with αCD3+αCD28 mAbs. T cells were harvested, stained with specific mAbs, and then analyzed by flow cytometry after gating on 30,000 CD4$^+$ events. Results are representative histograms of three independent experiments with similar results. C, IL-2 (*left*) or IFN-γ (*right*) production by T cells after 48-hr mAb stimulation. Protein levels in culture supernatants were measured by ELISA. Each column represents the mean±SEM of three independent experiments. *P<0.05 vs. control. D, cell cycle progression after mAb stimulation with or without 5 μg/mL DTCM-G treatment. Cells were pulsed with bromodeoxyuridine 30 min before cell harvest, stained intracellularly with 7-AAD, and then analyzed by flow cytometry. Results are shown as representative of three independent experiments with similar results (*left*). Each column represents the mean of three independent experiments (*right*). 7-AAD, 7-amino-actinomycin D; B6, C57BL/6; BrdU, bromodeoxyuridine; CPM, counts per minute; DTCM-G, 3-[(dodecylthiocarbonyl)methyl]-glutarimide; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; NS, no stimulation.

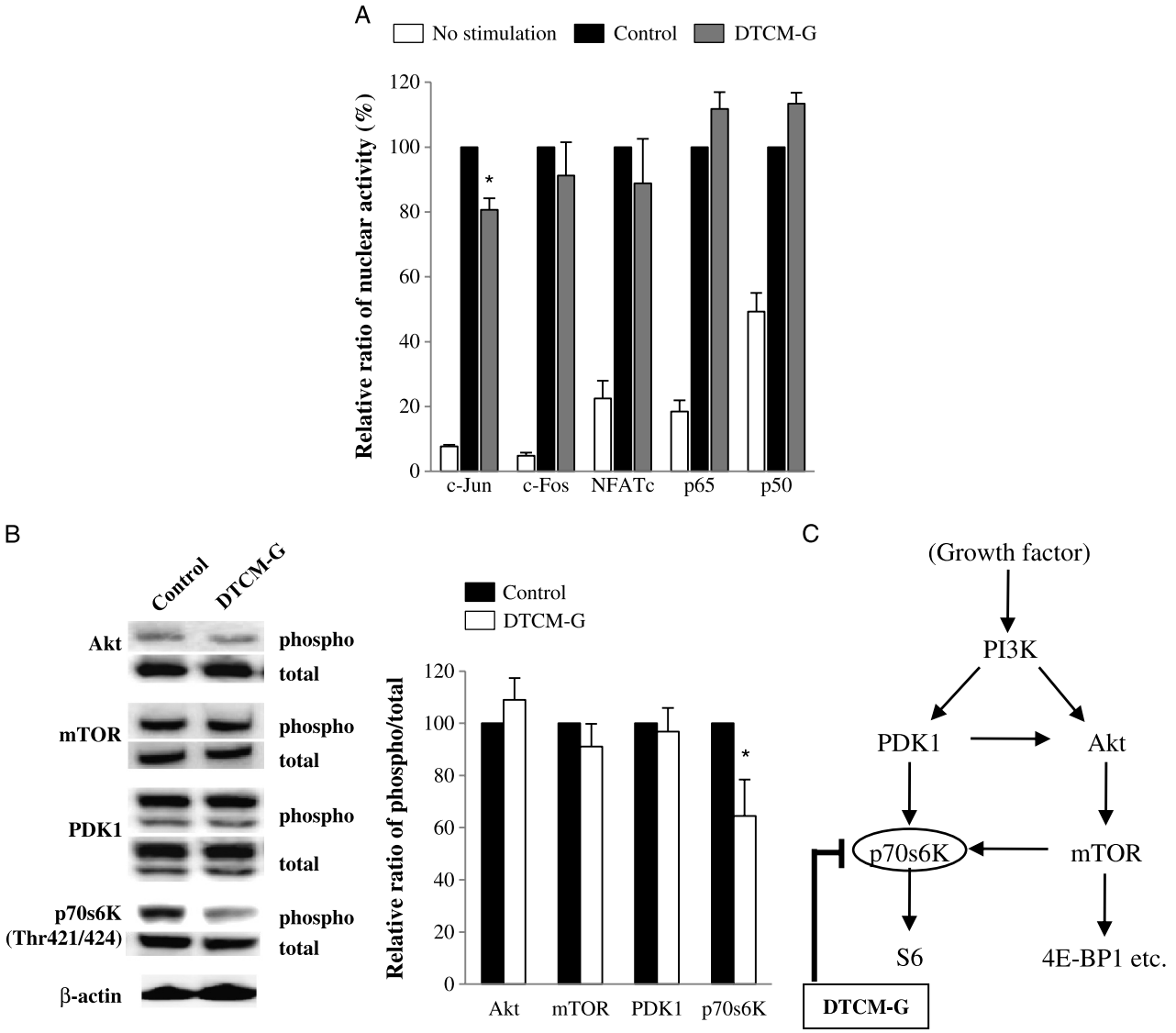

FIGURE 2. DTCM-G mildly suppresses nuclear c-Jun activation and inhibits phosphorylation of p70s6K in activated T cells. naïve B6 T cells were stimulated with αCD3+αCD28 mAbs in the absence (control) or presence of DTCM-G (5 μg/mL). A, Relative activities of nuclear c-Jun, c-Fos, NFATc1, p65, and p50 after 2-hr stimulation. Nuclear extracts were prepared from T cells, and activity was quantified using the TransAM kit. Each column is expressed as mean±SEM of three independent experiments. *P<0.05 vs. control. B, analysis of the PI3K/Akt/mTOR signaling pathway by immunoblot assay. After 12- or 24-hr stimulation, reactions were halted by rapidly cooling on ice and washed with ice-cold phosphate-buffered saline. Whole cell lysates were prepared, transferred to membranes, and immunoblotted with mAbs against phospho/total Akt, mTOR, PDK1, p70s6K, and anti-β-actin mAbs. The bands were visualized by chemiluminescence. Representative immunoblot of three independent experiments is shown (*left*). Densitometry and relative ratio of phospho/total are also shown (*right*). Each column represents the mean±SEM of three independent experiments. *P<0.05 vs. control. C, scheme of the PI3K/Akt/mTOR signaling pathway is shown. DTCM-G, 3-[(dodecylthiocarbonyl)methyl]-glutarimide; mAb, monoclonal antibody; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; p70s6K, 70-kDa S6 protein kinase; PDK1, 3-phosphoinositide-dependent protein kinase 1; PI3K, phosphoinositide 3-kinase (PI3K).

DTCM-G alone (2.5 μg/mL) did not suppress IFN-γ production, but combination of DTCM-G with DHMEQ and tacrolimus significantly inhibited IFN-γ production (Fig. 4B). However, the drugs exhibited dissimilar effects on IL-2 production at a suboptimal concentration. DTCM-G promoted, DHMEQ did not alter, and tacrolimus significantly suppressed IL-2 production (Fig. 4C). Interestingly, DTCM-G did not alter IL-2 production when combined with either DHMEQ or tacrolimus. The triple combination treatment resulted in a potent inhibition of IL-2 (Fig. 4C).

Triple Combination Therapy Exerts a Robust Immunosuppressive Effect In Vivo

Based on the finding that the triple combination treatment strongly suppressed T cells in in vitro (Fig. 4), we further assessed the in vivo effects of DTCM-G in combination

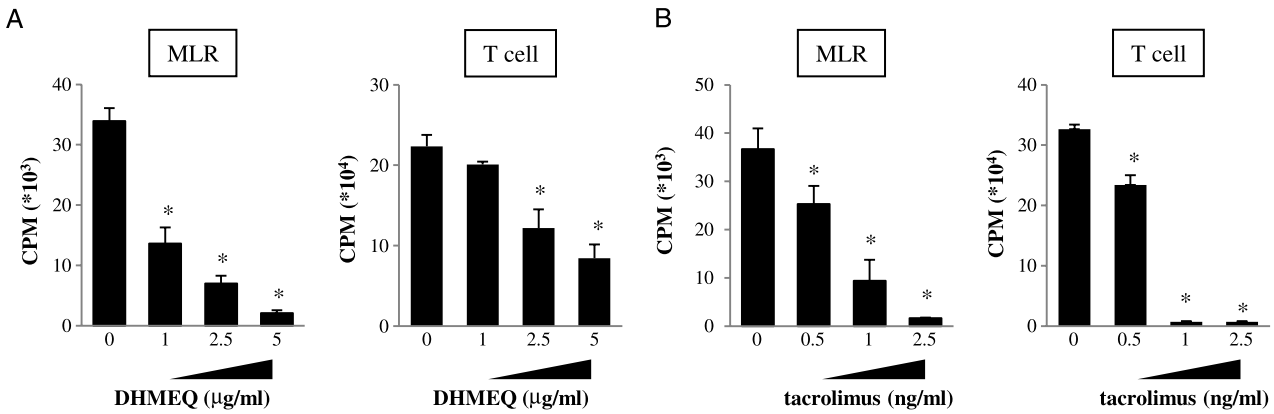

FIGURE 3. Effective concentrations of DHMEQ for MLR and T-cell inhibition are disparate, whereas the IC_{50} values of tacrolimus are equivalent. A, B6 mouse lymphocyte proliferation after 3-day stimulation with irradiated BALB/c mouse splenocytes (*left*) and B6 mouse T-cell proliferation after 2-day stimulation with αCD3/αCD28 mAbs (*right*) with or without DHMEQ. B, B6 mouse lymphocyte proliferation after 3-day stimulation with irradiated BALB/c mouse splenocytes (*left*) and B6 mouse T-cell proliferation after 2-day stimulation with αCD3/αCD28 mAbs (*right*) with or without tacrolimus. Cells were pulsed with [^{3}H]thymidine 8 hr before cell harvest. Each column represents the mean±SEM of three independent experiments. *P<0.05 vs. control. B6, C57BL/6; CPM, counts per minute; DHMEQ, dehydroxymethylepoxyquinomicin; mAb, monoclonal antibody; MLR, mixed lymphocyte reaction.

with DHMEQ and tacrolimus (Table 1). In a BALB/c to B6 mouse HTx model, nontreated heart allografts were rejected within 7 days. Daily treatment with DTCM-G at a dose of 20 mg/kg prolonged allograft survival to a median survival time (MST) of 13 days. At a subtherapeutic dose of DHMEQ (10 mg/kg/day) or tacrolimus (1.5 mg/kg/day), allograft MST reached 12.5 and 13 days, respectively. Addition of DTCM-G (20 mg/kg/day) to subtherapeutic treatment with either DHMEQ or tacrolimus resulted in a significant extension of graft MST to 24 and 32 days compared with that after DTCM-G, DHMEQ, or tacrolimus monotherapy. Furthermore, concomitant in vivo administration of all three drugs for only 2 weeks markedly prolonged allograft MST to 50 days. In a single set of six allografts, 2 (33%) survived for more than 120 days (Table 1). A minor decrease of less than 10% of body weight was noted, although this was chiefly due to the surgical trauma. No obvious toxic events leading to animal death were observed in any of the treatment groups.

The frequency of alloreactive IFN-γ–producing cells was evaluated 10 days after HTx to further assess the in vivo effects of combination therapy (Fig. 5). $CD4^+CD25^+Foxp3^+$ T-cell populations in the spleen were examined at this time point because regulatory T cells play a substantial role in controlling allograft rejection (*24*). DTCM-G, DHMEQ, or tacrolimus monotherapy moderately, but significantly, reduced the frequency of alloreactive IFN-γ–producing cells compared with control mice. In contrast, the combination therapy of all three agents markedly inhibited the frequency of donor-reactive IFN-γ–producing cells compared with each monotherapy (Fig. 5A). The $CD4^+CD25^+Foxp3^+$ T-cell population did not differ irrespective of treatments at 10 days after HTx (Fig. 5B).

DISCUSSION

T-cell activation is a complex process requiring progression through two major phases (*20*). Signals initially act

TABLE 1. Efficacy of DTCM-G in combination with DHMEQ and tacrolimus in BALB/c to B6 mouse HTx

Group	Drug (mg/kg/day)[a]			Graft survival, days	Median, days	P<0.05[b]
	DTCM-G	DHMEQ	Tacrolimus			
1	—	—	—	6, 6, 6, 6, 7, 7	6	
2	20	—	—	12, 12, 13, 14, 14, 15	13.5	vs. group 1
3	—	10	—	9, 10, 11, 14, 15, 18	12.5	vs. group 1
4	—	—	1.5	11, 11, 13, 13, 14, 14	13	vs. group 1
5	20	10	—	20, 21, 23, 25, 27, 28	24	vs. groups 1–3
6	20	—	1.5	16, 24, 26, 38, 41, 41	32	vs. groups 1, 2, 4
7	—	10	1.5	19, 21, 24, 37, 41, 44	30.5	vs. groups 1, 3, 4
8	20	10	1.5	29, 31, 47, 52, 120<, 120<	49.5	vs. groups 1–7

[a] Drug was given orally to cardiac recipient animal for 14 days starting from the day of grafting. As a vehicle control, animals in group 1 were administered 0.5% CMC only.

[b] P was calculated by a log-rank test.

B6, C57BL/6; CMC, methylcellulose; DHMEQ, dehydroxymethylepoxyquinomicin; DTCM-G, 3-[(dodecylthiocarbonyl)methyl]-glutarimide; HTx, heart transplantation.

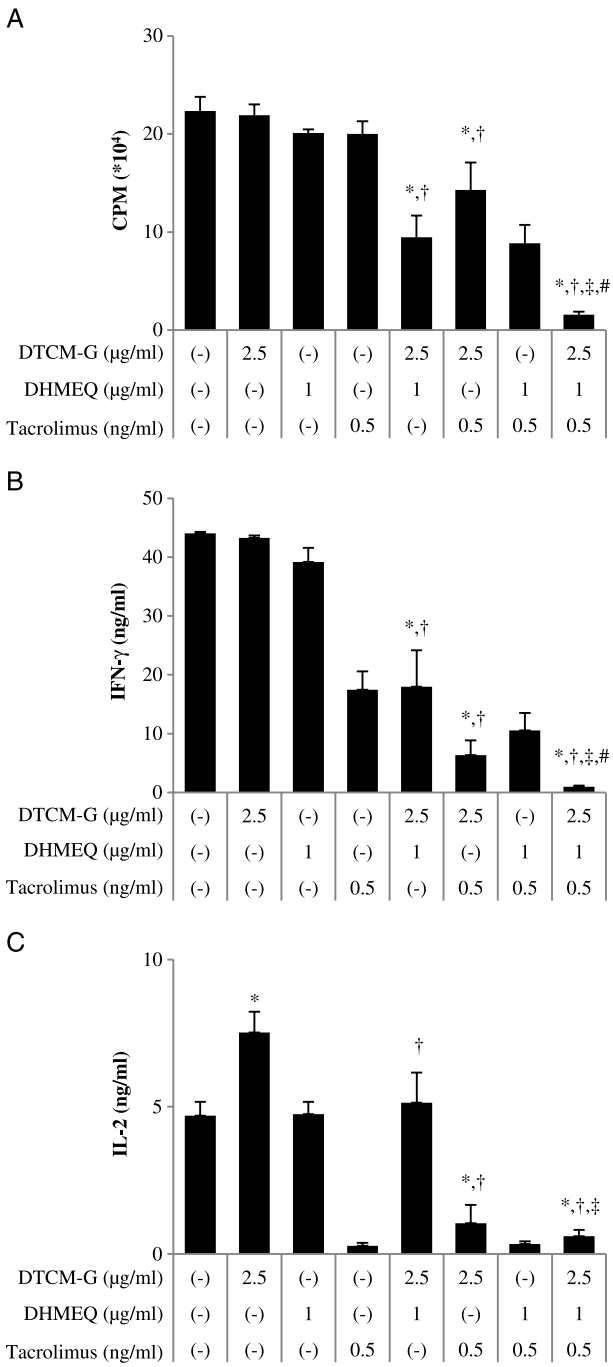

FIGURE 4. Combination with DHMEQ and tacrolimus enhances the immunosuppressive effects of DTCM-G in vitro. B6 mouse T cells were stimulated by αCD3+αCD28 mAbs in the presence or absence of DTCM-G (5 μg/mL), DHMEQ (1 μg/mL), and tacrolimus (0.5 ng/mL) for 48 hr. A, proliferation was examined by counting [^{3}H]thymidine uptake. The protein levels of IFN-γ (B) or IL-2 (C) in culture supernatants were measured by ELISA. Each column represents the mean±SEM of three independent experiments. *P<0.05 vs. control; $^{\dagger}P$<0.05 vs. DTCM-G; $^{\ddagger}P$<0.05 vs. DHMEQ; $^{\#}P$<0.05 vs. tacrolimus. CPM, counts per minute; DHMEQ, dehydroxymethylepoxyquinomicin; DTCM-G, 3-[(dodecylthiocarbonyl)methyl]-glutarimide; ELISA, enzyme-linked immunosorbentassay; IFN, interferon; IL, interleukin; mAb, monoclonal antibody.

through the T-cell receptor, whereby costimulatory molecules then activate transcriptional regulators such as NFAT, NF-κB, and AP-1. This process results in the expression of IL-2 and the IL-2R, inducing cell cycle progression from the G_0 to the G_1 phase. The second phase involves autocrine and paracrine responses to IL-2, which drives cell cycle entry into the S phase, thus leading T cells to clonal expansion and acquisition of effector function (*25–27*). The PI3K/Akt/mTOR pathway plays an important role in the latter phase (*20*). Indeed, the mTOR inhibitor rapamycin has been shown to inhibit the later IL-2–driven responses and cell cycle progression through S phase, although mTOR does not affect IL-2 production or IL-2R expression after stimulation in murine T cells (*28–31*). Through the current in vitro study, we found that the novel compound DTCM-G does not abrogate IL-2 production or IL-2Rα (CD25) expression but that it strongly inhibits G_1/S transition of the cell proliferation cycle (Fig. 1). Similar to rapamycin, these results indicate that DTCM-G chiefly regulates T-cell proliferation at a stage of post–IL-2 production. Immunoblotting assays further revealed that DTCM-G suppresses phosphorylation of p70s6K but not of Akt, mTOR, or PDK1 (Fig. 2). The p70s6K, serine/threonine kinase S6K, is one of the important downstream targets of mTOR, as well as the eukaryotic initiation factor 4E-binding protein 1, and mediates cell survival and proliferation (*32, 33*). In the presence of proliferative stimuli, S6K is phosphorylated and activated by mTOR. In turn, S6K phosphorylates the 40S ribosomal protein S6 (*33, 34*), which itself plays a role in protein synthesis (*31*). Because rapamycin indirectly suppresses p70s6K through mTOR inhibition and results in T-cell proliferation with a minimal alteration of IL-2 production (*35, 36*), it appears likely that our findings of T-cell inhibition by DTCM-G are similar to the effects of rapamycin on T cells. Given this similarity, we hypothesized that DTCM-G inhibits T-cell function by targeting the IL-2 receptor signaling pathway. However, the precise molecular target of DTCM-G has not been conclusively identified.

We also found that DTCM-G inhibited nuclear c-Jun activation in T cells (Fig. 2A). In the previous study, we demonstrated that DTCM-G inhibited nitric oxide production and the expression of inducible nitric oxide synthase and cyclooxygenase-2 in LPS-induced murine RAW264.7 cells. These effects seem likely to be associated via inhibition of nuclear translocation and intracellular phosphorylation of c-Jun, but not the upstream kinase activity of intracellular mitogen-activated protein kinases including p38 mitogen-activated protein kinase, extracellular signal-regulated kinase, and c-Jun NH_2-terminal kinase, nor the nuclear activation of NF-κB (*19*). Although c-Jun phosphorylation was also suppressed (~20% inhibition) on activation in T cells, we did not find clear evidence that T-cell inhibition or the immunosuppressive effect was due to this mechanism. Previously, it has been demonstrated that AP-1 is a key transcription factor involved in IL-2 production (*10*) and that AP-1 has an important ability to form complexes with NFAT or NF-κB to cooperate in the regulating of IL-2 production (*37*). However, Chen et al. (*38*) indicated that stimulation of *c-jun*$^{-/-}$ T cells results in normal levels of IL-2Rα expression, IL-2 secretion, and proliferation. In addition, they suggested that c-Jun is a minor component of the AP-1 complexes in normal T cells

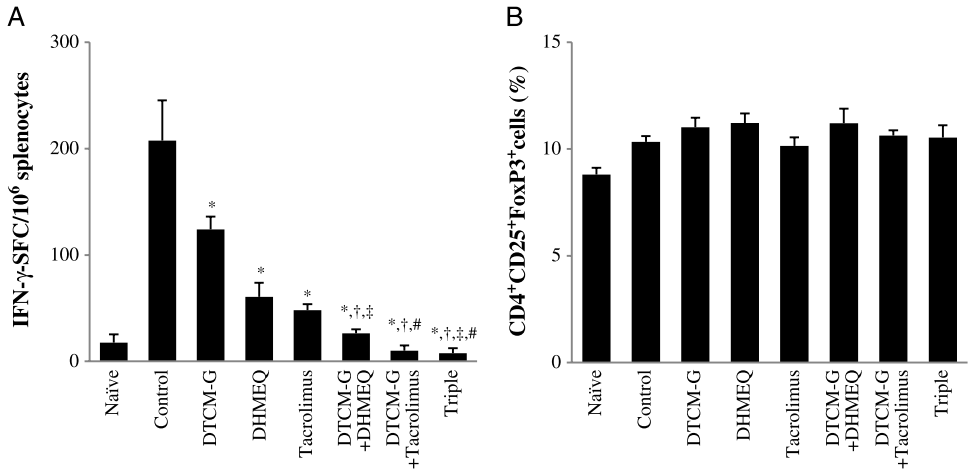

FIGURE 5. Combination of DTCM-G plus DHMEQ and tacrolimus shows amplified immunosuppressive effects in vivo. The combination effect in vivo of DTCM-G plus DHMEQ was examined in a BALB/c to B6 mouse HTx. Recipients were given CMC (control vehicle), DTCM-G (20 mg/kg), DHMEQ (10 mg/kg), and tacrolimus (1.5 mg/kg) intraperitoneally for 14 days after transplantation. Recipient mice were sacrificed 10 days after HTx, and spleen cells were prepared for analysis of the immune response. A, frequency of alloreactive IFN-γ–producing cells. Splenocytes (5×10^5 cells per well) from transplanted mice were cultured with irradiated BALB/c mouse splenocytes (5×10^5 cells per well). SFC numbers were counted by KS enzyme-linked immunospot reader after 24-hr culture. Each column represents the mean±SEM of three or four independent experiments. *P<0.05 vs. control; †P<0.05 vs. DTCM-G; ‡P<0.05 vs. DHMEQ; #P<0.05 vs. tacrolimus. B, splenic $CD4^+CD25^+Foxp3^+$ T-cell population in the transplant recipients measured by flow cytometric analysis. Each column represents the mean±SEM of three or four independent experiments. DHMEQ, dehydroxymethylepoxyquinomicin; DTCM-G, 3-[(dodecylthiocarbonyl)methyl]-glutarimide; HTx, heart transplantation; IFN, interferon; IL, interleukin; SFC, spot-forming cells.

and that most Jun proteins in the AP-1 complexes of normal T cells are JunB and JunD (*38*). These results support our finding that c-Jun inhibition by DTCM-G did not lead to inhibition of IL-2 production. We thus consider that the inhibitory effect of DTCM-G on nuclear c-Jun activation is not a major contributor to the immunosuppressive effects that we report in this study.

We have also demonstrated that triple combination treatment vigorously suppressed in vitro proliferation and IFN-γ production in mouse activated T cells (Fig. 3). We reported previously that DHMEQ inhibits the nuclear translocation of NF-κB in T cells (*15*). Tacrolimus inhibits phosphorylation of calcineurin, which leads to activation of NFAT in T cells (*4*). Combination treatment of DHMEQ and tacrolimus has been shown to markedly inhibit IL-2 production in T cells (*15*). However, we demonstrated that DTCM-G does not inhibit the nuclear activation of NF-κB or NFAT but suppresses p70s6K phosphorylation that leads to inhibition of T-cell activation at the second phase. Accordingly, DHMEQ and tacrolimus inhibit IL-2 and IL-2Rα expression, and DTCM-G inhibits the subsequent cascade activated via autocrine IL-2. Because each drug exerts immunosuppressive effects via different mechanisms of action, we believe that the combination of these three drugs inhibited proliferation and IFN-γ production in activated T cells without antagonizing or interfering with the effects of each other.

Although our study focused on investigating the effects of these three drugs on T cells, the effects of these agents on dendritic cells (DCs) may also be important in facilitating immunosuppression. As shown in Figure 3A, the required IC_{50} value of DHMEQ for inhibiting T-cell proliferation was up to four times higher than that necessary to inhibit MLR. These findings are corroborated by the previous reports that DHMEQ suppresses the up-regulation of surface maturation markers CD40, CD80, CD86, and human leukocyte antigen-DR and proinflammatory cytokines such as IL-6, IL-12p70, and tumor necrosis factor-α in LPS-stimulated human monocyte-derived DCs, and pretreatment with DHMEQ in DCs impairs the Th1-cell response (*39, 40*). Then again, the concentrations of tacrolimus needed to inhibit MLR and T-cell proliferation were almost equal in our experiments (Fig. 3B). However, high concentrations of tacrolimus have been shown to inhibit DC function in LPS-stimulated human monocyte-derived DCs (*41, 42*). In addition, tacrolimus may also induce tolerogenic DCs in vivo in a similar manner to that of the small-molecule compound, norcantharidin, which inhibits phosphorylation of calcineurin phosphatase. The resultant activity of cellular calcineurin changes DCs to differentiate toward a tolerogenic phenotype through calcineurin phosphatase inhibition (*43*). Taken together, we do not deny the possibility that inhibitory effects of DHMEQ and tacrolimus on not only T cells but also on DCs contribute to the potent immunosuppressive effect in combination with DTCM-G. In contrast, DTCM-G alone seems to have limited influence on DCs. This was indicated by the fact that suppression of both MLR and T-cell proliferation by DTCM-G was comparable at an equivalent concentration (Fig. 1A). Additionally, DTCM-G did not suppress surface CD40, CD86, and major histocompatibility complex class II ($I\text{-}A^b$) expression levels in B6 mouse bone marrow–derived DCs after LPS stimulation (data not shown). However,

further studies are necessary to elucidate the effects of DTCM-G on other immune cell types including natural killer and natural killer T cells.

We demonstrated that triple combination therapy in vivo permitted long-term allograft survival (Table 1). In our earlier study (*19*), we showed that treatment with DTCM-G reduces cellular infiltration of transplant allografts, but the potency of DTCM-G monotherapy for preventing acute rejection was modest, and this effect did not persist after drug cessation. In addition, the effect of DTCM-G was saturated at a dose of more than 40 mg/kg per day (*19*). In the present study, combination therapy with a dual-drug regimen of DTCM-G, DHMEQ, or tacrolimus improved graft survival time (Table 1) that was accompanied by an inhibition of alloreactive immune responses in vivo without drug-related side effects (Fig. 5A) (*15, 16*). Furthermore, triple combination therapy exhibited a more robust immunosuppressive effect than dual-drug treatment (Table 1; Fig. 5A). Additionally, the splenic $CD4^+CD25^+Foxp3^+$ T-cell population was not significantly increased irrespective of treatment (Fig. 5B). We believe the mechanism of action for this potent triple combination therapy immunosuppressive effect in vivo is likely due to a robust inhibition of effector T-cell function, rather than increasing regulatory T cells, similar to the combinatorial effect achieved by tacrolimus and *Isatis tinctoria* L. (*44*). These findings suggest that the combination of agents with different target molecules or mechanisms of action complements and enhances each inhibitory effect on T cells, consequently leading to a powerful immunosuppressive effect even in in vivo.

In conclusion, DTCM-G is a novel anti-inflammatory agent that suppresses the secondary phase of T-cell activation and is likely associated with the suppression of p70s6K phosphorylation. These findings may provide new insight into the immunosuppressive mechanisms of DTCM-G in addition to the findings of inhibiting nuclear AP-1 activation in murine macrophages, as reported previously. Finally, DTCM-G may be a promising candidate for immunosuppression after organ transplantation, especially in combination with other immunosuppressants that work via different mechanisms of action.

MATERIALS AND METHODS

Animals

Male B6 and BALB/c (H-2^d) mice were purchased from SLC (Shizuoka, Japan). Animals were maintained in a specific pathogen-free facility and used at 9 to 12 weeks of age. All experiments were approved by the Institutional Animal Care Committee and conducted under the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University.

Chemical Compounds and Reagents

DTCM-G was synthesized as reported before (*18*) and either dissolved in 0.1% dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO) for in vitro studies or dissolved in 4% dimethylsulfoxide and suspended in 0.5% methylcellulose (CMC; Shin-Etsu Chemical Industry, Tokyo, Japan) for in vivo experiments. Tacrolimus (Asteras Pharmaceutical, Osaka, Japan) and DHMEQ were used as described previously (*15*). mAbs against mouse CD3 (145-2C11), CD4 (RM4-5), CD25 (7D4), CD28 (37.51), and CD69 (H1.2F3) were purchased from BD Biosciences (San Jose, CA). Antibodies against phospho-Akt (Ser473), Akt, phospho-mTOR, mTOR, phospho-PDK1, PDK1, phospho-p70s6K (Thr421/424), p70s6K, β-actin, and horseradish peroxidase–conjugated anti-rabbit IgG were purchased from Cell Signaling Technology (Beverly, MA).

Proliferation Assays

Purified B6 T cells (5×10^5 per well), enriched to more than 90% purity by cell suspension filtering through a nylon-wool mesh column (R&D Systems, Minneapolis, MN), were stimulated by 1 μg/mL αCD28 and 1 μg/mL plate-bound αCD3 mAbs for 48 hr. Irradiated (30 Gy, 137Cs) BALB/c splenocytes (5×10^5 per well) were cocultured with B6 splenocytes (5×10^5 per well) for 72 hr for MLR. Cells were cultured in complete RPMI 1640 medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum, and 50 μM 2-mercaptoethanol in an atmosphere of 37°C and 5% carbon dioxide plus air. Cells were pulsed with [^{3}H]thymidine (1 μCi/well) 8 hr before analysis of incorporation with a β-counter (Perkin-Elmer, Boston, MA).

Flow Cytometric Analysis

Cells were stained with isotype control mAbs or specific mAbs against CD4, CD69, and CD25 for surface marker staining. Intracellular staining for Foxp3 was performed according to the manufacturer's instructions (eBioscience, San Diego, CA). Cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). For each analysis, 30,000 $CD4^+$ lymphocytes were acquired.

Cytokine Measurement

IL-2 and IFN-γ levels in culture supernatant were measured by ELISA using a cytokine assay kit (R&D Systems). All measurements were performed in duplicate. IFN-γ production by lymphocytes obtained from transplant recipients was examined by enzyme-linked immunospot assay as described previously (*15*).

Cell Cycle Analysis

Cell cycle analysis was performed using a bromodeoxyuridine flow kit (BD Biosciences) as described previously (*45*). Cells were stained with fluorescein isothiocyanate–conjugated anti-bromodeoxyuridine antibody and 7-amino-actinomycin D before analysis by flow cytometry.

Nuclear Protein Extraction and Quantification of Transcription Factor Activity

Nuclear protein was harvested from 2×10^7 B6 mouse T cells according to the manufacturer's instructions (nuclear extract kit; Active Motif, Carlsbad, CA). DNA binding activity of nuclear extracts containing NF-κB, NFATc1, and AP-1 was examined using the TransAM kit (Active Motif) (*46*).

Immunoblotting

Cells were lysed with radioimmunoprecipitation assay buffer as described previously (*47*). Protein (30 μg) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel and transferred to nitrocellulose (Invitrogen, Carlsbad, CA). The membrane was blocked with 5% dry milk and 0.1% Tween (Sigma-Aldrich) in phosphate-buffered saline and incubated with primary antibody followed by horseradish peroxidase–conjugated secondary antibody. Bands were detected by enhanced chemiluminescence.

Cardiac Transplantation and Treatment Protocol

Intraabdominal heterotopic cardiac transplantation was performed as described previously (*48*). Cardiac graft beating was monitored by daily palpation through the recipient's abdominal wall. Rejection was defined as the time of cessation of graft beating as confirmed by direct inspection and histologic examination. Recipient animals were given DTCM-G (20 mg/kg), DHMEQ (10 mg/kg), tacrolimus (1.5 mg/kg), or control vehicle (0.5% CMC) intraperitoneally starting from days 0 to 14 (n=6 each).

Statistical Analysis

Graft survival time was plotted using a method based on the Kaplan-Meier estimator, and the log-rank test was applied for comparison. Other results were expressed as mean±standard error of the mean (SEM). Statistical analysis was performed by Student's *t* test for paired observations or analysis of variance together with the Tukey-Kramer post hoc test for multiple comparisons. $P<0.05$ was considered statistically significant.

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