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The Immunomodulating Effects of Thalidomide and Dexamethasone in a Murine Cardiac Allograft Transplantation Model

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Purpose: The immunomodulatory effects of thalidomide (TM) and dexamethasone (DX) on immune cells and their co-stimulatory, co-inhibitory molecules in vitro and in vivo have been previously reported. The current study investigated the effects of TM and the combinatorial treatment with DX on immune cells using a murine cardiac allograft transplantation model.

Materials and Methods: Intraabdominal transplant of cardiac allografts from BALB/c (H-2^d) donors to C57BL/6 (H-2^b) recipients was performed. After transplantation, mice were injected daily with TM or DX or a combination of both TM and DX (TM/DX) by intraperitoneal route until the time of graft loss. $CD4^+$ T cell subsets and $CD11c^+$ cells in the peripheral blood mononuclear cells and spleen were examined and quantified with flow cytometry. Serum IL-6 levels were measured by enzyme-linked immunosorbent assay on day 7.

Results: The mean graft survivals were 6.86 days in the untreated group, and 10.0 days in the TM/DX group (p<0.001). The TM/DX treatment affected the $CD4^+$ T cell subsets without suppressing the total $CD4^+$ T cell population. The $CD4^+FOXP3^+$/$CD4^+CD44^{hi}$ T cell ratio increased. Increase in cell counts and median fluorescence intensity on $CD11c^+CD85k^+$ with TM/DX were observed. The inhibition of pro-inflammatory cytokine interleukin-6 was also observed.

Conclusion: These outcomes suggest the immunomodulating effect of the TM/DX combinatorial treatment. In conclusion, TM/DX combination may be a promising immunomodulatory approach for preventing allograft rejection and improving graft survival by inducing tolerance in transplantation.

Key Words: Immunomodulation, thalidomide, dexamethasone, T cells, dendritic cells, cardiac transplantation

INTRODUCTION

Organ transplantation is the preferred treatment for end-stage organ failure. However, due to the alloimmune response, the life-long use of immunosuppressants is essential. Currently, combination immunosuppressive therapy is applied to suppress alloimmune responses and minimize the detrimental side effects of immunosuppressants.[1] The standard immunosuppressants are directed at various stages of lymphocyte activation/proliferation, especially T cells, and are often combined with anti-inflammatory drugs to inhibit cytokine synthesis.[2] However, these prominent immunosuppressants have immunodeficiency complications inducing infection, malignancy,

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and nonimmune complications such as nephrotoxicity, cardiovascular, and metabolic risks.[3] Future immunosuppressive therapy is targeted at reducing immunosuppression-related complications and increasing graft survival. Current strategies include developing highly selective immunosuppressive agents, immunomodulation, and induction of tolerance.[4]

Thalidomide (TM) was prescribed as a sedative and antiemetic for morning sickness in the 1950s. However, it was withdrawn from the market in the early 1960s due to its teratogenic complications.[5] TM was recognized as an effective treatment for erythema nodosum leprosum in 1965 and was subsequently researched for other potential therapeutic applications.[6,7] Thereafter, the anti-angiogenic, anti-neoplastic, and immunomodulatory features of TM have been reported.[8] TM has been proven to be clinically effective on myelodysplasia and multiple myeloma (MM).[9] Further clinical studies with TM were performed on selected malignancies and autoimmune diseases.[5] The immunomodulatory effect of TM is attributed to the suppression of tumor necrosis factor (TNF)-α associated anti-inflammatory activity, regulation of nuclear transcription factor-κB, and cytokine production such as interferon-γ, chemokines, interleukin (IL)-6, IL-12, and cyclooxygenase-2.[5,10]

Corticosteroids are one of the most potent anti-inflammatory agents with immunosuppressive effects.[11] Corticosteroids, such as dexamethasone (DX) or prednisolone, are associated with decreased cytokine production, lymphocyte proliferation, and changes in cellular trafficking.[9] Due to these properties, corticosteroids have been used in the treatment of inflammatory, autoimmune disease, and immunosuppressive protocols for organ transplantation.[12] However, there are side effects involving most major organ systems that are associated with long-term corticosteroid therapy.[13,14] Therefore, the risk and benefits must be considered with corticosteroid usage. One strategy to minimize the side effects of corticosteroids is combining more specific anti-inflammatory or immunosuppressive drugs, promoting a synergistic effect to reduce corticosteroid therapy.[15] Combinatorial therapy of TM and DX has been effective in the treatment of newly diagnosed MM and relapsed myeloma in the clinical field.[16,17] TM and prednisolone combinatorial therapy was shown to be effective for nephritis in lupus-prone mice.[18]

Immune cells, such as T cells, B cells, macrophages, and dendritic cells (DCs), can participate in graft rejection or promote tolerogenic immune responses.[19] Regulatory T cells (Tregs) play an imperative role in immunologic tolerance.[19] Tregs inhibit effector T cell (Teffs) proliferation and promote tolerance through various signals, such as the production of IL-10, transforming growth factor (TGF)-β, and inhibition of antigen-presenting cells (APC) function.[19,20] In clinical transplantation, allograft outcome, rejection, or tolerance often depends on the balance between Teffs and Tregs.[21,22] Therefore, Tregs have been researched as a prospective target for inducing allograft tolerance.[23,24] DCs are potent APCs, which play an important role in stimulating T cells and initiating primary immune responses.[25,26] DCs have also been found to play a role in central and peripheral tolerance.[19] DCs tolerize T cells to self-antigens, achieving self-tolerance, and alteration of this system may result in autoimmune diseases.[26] In transplantation, allograft rejection is the result of both innate and adaptive immunity. Since DCs function in both immune responses and control immunity and tolerance, they are an important factor for immunosuppression and immunomodulation.[27]

Previous studies in our group have suggested that TM has immunomodulating effects by selectively suppressing $CD4^+$ T cell subsets and changing the expression of selected TNF receptor super families, including OX40, 4-1BB, and glucocorticoid-induced TNF receptor-related protein.[28] Co-treatment of TM and DX (TM/DX) increased cytotoxic T lymphocyte associated antigen-4 expression in $CD4^+$ Teffs and $CD4^+$ Tregs and increased the corresponding ligands (CD80, CD86) of DCs, suggesting the activation of DC-mediated tolerance effects.[29-31] The competency of TM/DX combinatorial treatments for maintaining a tolerogenic state or immune homeostasis was suggested.

Accordingly, we recognized TM/DX treatment as a prospective immunomodulatory drug in the transplantation field. The current study investigated the effects of TM and the combinatorial treatment with DX on immune cells using a murine cardiac allograft transplantation model. The effects on $CD4^+$ T cell subsets and $CD11c^+$ cells were analysed. We also examined the change of tolerogenic markers on DCs and their part in immunomodulation with TM/DX treatment.

MATERIALS AND METHODS

Mice and reagents

For this study, 8- to 9-week-old male BALB/c ($H\text{-}2^d$) mice and C57BL/6 ($H\text{-}2^b$) mice were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained according to the ethical guidelines of our institution.

The PE-Cy7-conjugated anti-mouse CD8, PerCP-Cy5.5-conjugated anti-mouse CD11c, PE-conjugated anti-mouse CD85k, FITC-conjugated anti-mouse CD44, PerCP-Cy5.5-conjugated anti-mouse FOXP3 antibodies, and the Fixation/Permeabilization kit were purchased from eBioscience (San Diego, CA, USA). APC-Cy7-conjugated anti-mouse CD4 antibodies were purchased from Biolegend (San Diego, CA, USA). TM, DX, and red blood cell lysis buffer, and Histopaque 1.083 were purchased from Sigma-Aldrich (St Louis, MO, USA). Mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kit was purchased from BD Bioscience (San Jose, CA, USA).

Heterotopic cardiac transplantation and drug treatment

The animals were anesthetised with isoflurane during the entire surgical procedure. Intraabdominal transplant of cardiac allografts from BALB/c ($H\text{-}2^d$) donors to C57BL/6 ($H\text{-}2^b$) recipients was performed as described by Niimi.[32] The donor aorta

was anastomosed to the recipient's abdominal aorta, and the donor pulmonary artery was anastomosed to the recipient's adjacent vena cava using standard microvascular techniques with 10–0 nylon suture. Graft function was assessed daily by palpation. After transplantation, mice were injected daily with TM 100 mg/kg or DX 0.1 mg/kg or a combination of both TM and DX by intraperitoneal route until the time of graft loss, which was defined as the cessation of a palpable cardiac contraction.

Flow cytometry

To examine the effects of the drug treatments on immune cells, peripheral blood mononuclear cells (PBMC) and splenocytes were collected from recipient mice on postoperative day 7. Isolated PBMC and splenocytes were incubated with the appropriately diluted antibodies for 40 min at 4°C. Activated $CD4^+$ T cells ($CD4^+$ Teffs) were stained with APC-Cy7-conjugated anti-mouse CD4 and FITC-conjugated anti-mouse CD44 antibodies, whereas $CD4^+$ Tregs were fixed/permeabilized after staining with CD4 antibody for intracellular PerCP-Cy5.5-conjugated anti-mouse FOXP3 staining. Activated $CD8^+$ T cells were stained with PE-Cy7-conjugated anti-mouse CD8 and FITC-conjugated anti-mouse CD44 antibodies. $CD11c^+$ was used for DC markers. Flow cytometry was performed using a FACS Verse I or FACS Verse II flow cytometer (BD Biosciences). Data were analysed using FlowJo software, v10.0.7 (Tree Star, Inc., San Carlos, CA, USA). All experimental groups were compared to a sham control group (negative control group), which performed all surgical issues without a cardiac transplantation. Each experiment was repeated five times in each of the five groups; sham control (-), untreated (CTL), TM, DX, and TM/DX.

ELISA

Serum samples were collected from recipient mice on postoperative day 7 and immediately placed in -80°C until measurement. IL-6 levels were measured by ELISA according to the manufacturer's protocols (BD Bioscience).

Statistical analysis

Data are presented as means±standard error. The significances of experiments or intergroup differences were determined using the one-way ANOVA or Student's t-test. The analysis was conducted with Sigma plot 2.0 (Systat Software Inc., San Jose, CA, USA), and statistical significance was accepted for p values <0.05.

RESULTS

Graft survival on cardiac allograft transplantation model

The mean graft survival time of the untreated group (control; CTL) was 6.86±0.38 days. Single drug treatments of TM (100 mg/kg) or DX (0.1 mg/kg) showed graft survivals of 7.5±0.55 or 7.7±0.52 days, respectively. The combinatorial treatment of TM/DX exhibited the longest graft survival compared to the untreated, TM, and DX treated groups (10.0±0.89 days, p<0.01) (Fig. 1).

A

Group	n	Individual graft survival time (days)	Mean graft survival time (days)
CTL	7	6, 7, 7, 7, 7, 7, 7	6.86±0.38
TM	6	7, 7, 7, 8, 8, 8	7.5±0.55
DX	6	7, 7, 8, 8, 8, 8	7.7±0.52
TM/DX	6	9, 9, 10, 10, 11, 11	10.0±0.89

B

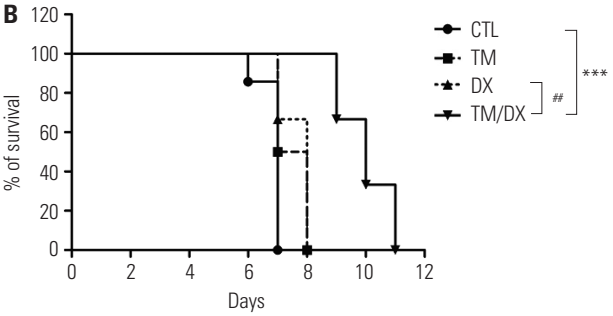

Fig. 1. Survival effects of thalidomide (TM) or dexamethasone (DX) or a combination of both TM and DX (TM/DX) treatments on murine heterotopic cardiac allograft transplantation model. The combinatorial treatment of TM/DX exhibited the longest graft survival. (A) Mean graft survival time (control; CTL). (B) Percentage (%) of survival. Combinatorial treatment of TM/DX exhibited the longest graft survival compared to other treated groups (***p<0.001 vs. CTL, ##p<0.01 vs. DX).

T cell subset change

In the PBMC analysis, $CD4^+CD44^{hi}$ T cells, which indicate $CD4^+$ Teffs, were increased in the untreated cardiac transplant group (115.1±9.56%) compared to the sham control group and were decreased with DX or TM/DX treatment. TM/DX treatment showed higher potency compared to DX treatment (TM/DX, 88.6±2.96%; DX, 102.9±2.97%, p<0.001) (Fig. 2A and C). However, splenic $CD4^+$ Teffs showed no difference between the treatment groups (Fig. 2B and D).

The frequencies of $CD4^+$ Tregs ($CD4^+FOXP3^+$) increased and showed similar tendencies in both PBMC and spleen, which decreased after transplant (PBMC, 86.4±7.81%; spleen, 81.6±3.6%). The cell count recovered with TM treatment or combinatorial treatment of TM/DX. Interestingly, TM/DX treatment showed an up-regulating effect of the $CD4^+$ Treg population compared not only with the untreated group, but also with the DX treatment group (Fig. 2).

The frequencies of $CD8^+CD44^{hi}$ T cells were not influenced by drug treatments in both PBMC and spleen (Supplementary Fig. 1, only online).

We analysed the ratio of $CD4^+FOXP3^+/CD4^+CD44^{hi}$ T cells in PBMC and spleen. The ratio decreased after cardiac transplantation (PBMC, 77.6±9.84%; spleen, 78.0±4.33%), and recov-

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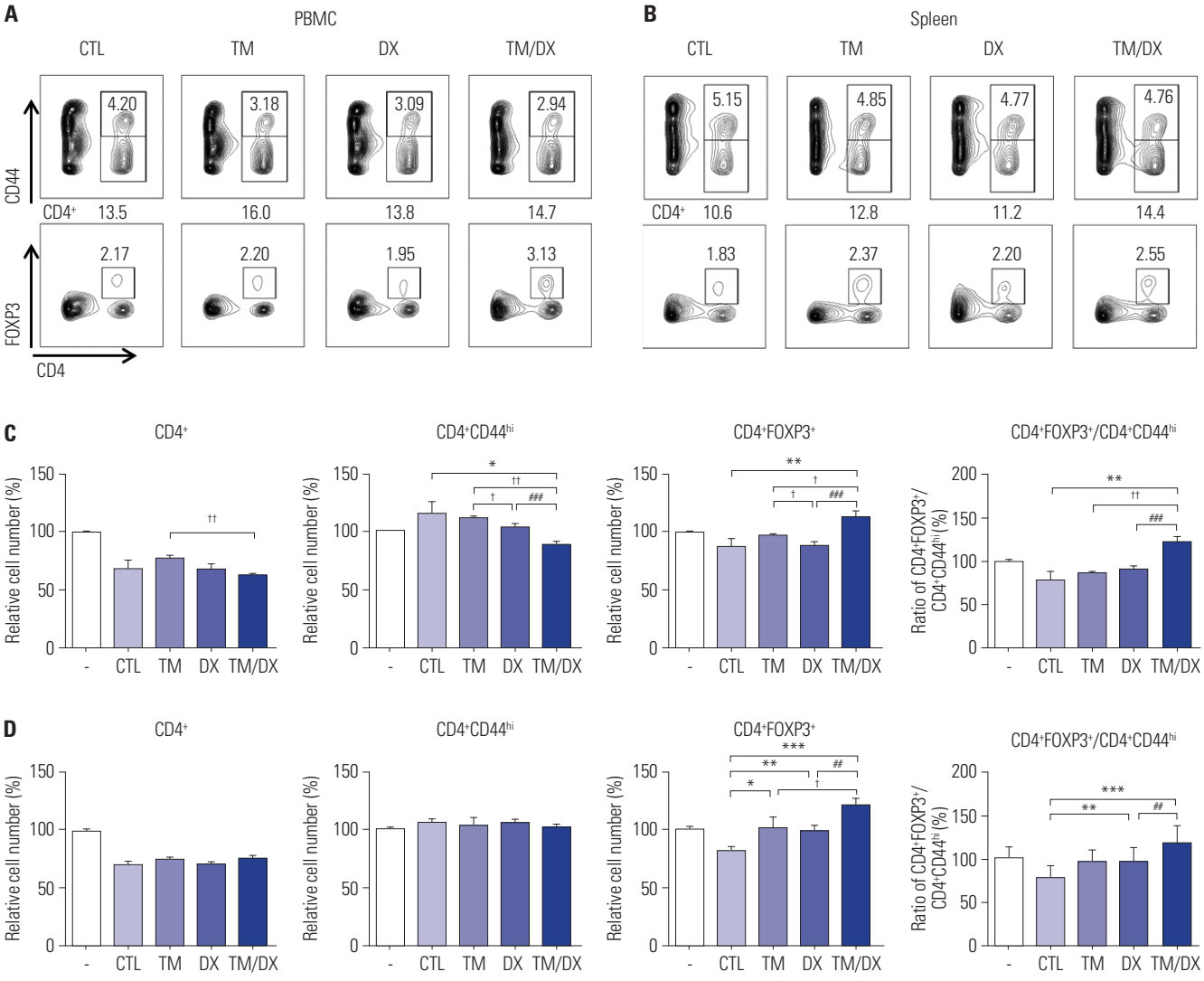

Fig. 2. The CD4+ T cell subset changes and the ratio of CD4+FOXP3+ T cells to CD4+CD44hi T cell of peripheral blood mononuclear cells (PBMC) or spleen measured by flow cytometry analysis. (A and B) Contour plots of CD4+CD44hi and CD4+FOXP3+ T cells (A, PBMC; B, Spleen). Representative figures of five experiments. (C and D) Relative cell numbers to the sham control group [(-), (%)], and the ratio of CD4+FOXP3+ T cells to CD4+CD44hi T cell (C, PBMC; D, Spleen). (A and C) Total CD4+ T cells were consistent, regardless of treatment. CD4+CD44hi T cells decreased with TM/DX treatment compared to the CTL or DX treatment. (B and D) Total CD4+ T cells were consistent, regardless of treatment. CD4+CD44hi T cells showed no change. CD4+FOXP3+ T cells increased with TM/DX treatment. CD4+FOXP3+ T cells increased with TM/DX. TM/DX combinatorial treatment significantly increased the ratio of CD4+FOXP3+ T cell/CD4+CD44hi T cell both PBMC and spleen (*p<0.05, **p<0.01, ***p<0.001 vs. CTL, †p<0.05, ††p<0.01 vs. TM, ##p<0.01, ###p<0.001 vs. DX. Each experiment was repeated five times in each of the five groups). TM, thalidomide; DX, dexamethasone; TM/DX, thalidomide and dexamethasone; CTL, control.

ered with TM (PBMC, 86.6±1.7%; spleen, 97.0±5.7%) or DX (PBMC, 90.6±4.4%; spleen, 96.4±4.78%) treatments. TM/DX combinatorial treatment significantly increased (PBMC, 90.6±4.4%; spleen, 96.4±4.78%) the ratio of CD4+FOXP3+ T cell/CD4+CD44hi T cell in both sites (Fig. 2C and D).

CD11c+ cell changes

The population of CD11c+ cells was significantly increased after transplantation and tended to show a higher increment in PBMC (CTL, 337.9±22.14; TM, 304.5±51.97; DX, 336.0±32.26; and TM/DX, 334.9±33.02) than in spleen (CTL, 154.4±4.43; TM, 169.5±3.79; DX, 150.3±7.66; and TM/DX, 179.0±6.86). However, there were no differences between the untreated and treated groups. These tendencies were similar in both PBMC and spleen (Fig. 3).

CD11c+CD85k+ cell changes

The frequencies of CD11c+CD85k+ cells, expressing a tolerogenic marker of DCs, were increased by transplantation in both PBMC (CTL, 187.1±12.82; TM, 180.2±2.20; DX, 184.5±16.38; and TM/DX, 181.0±7.81) and spleen (CTL, 123.1±6.17; TM, 127.4±5.70; DX, 126.1±6.35; and TM/DX, 145.5±4.96). Combinatorial treatment of TM with DX significantly increased CD11c+CD85k+ cells in spleen (p<0.05). However, the cell frequency in PBMC

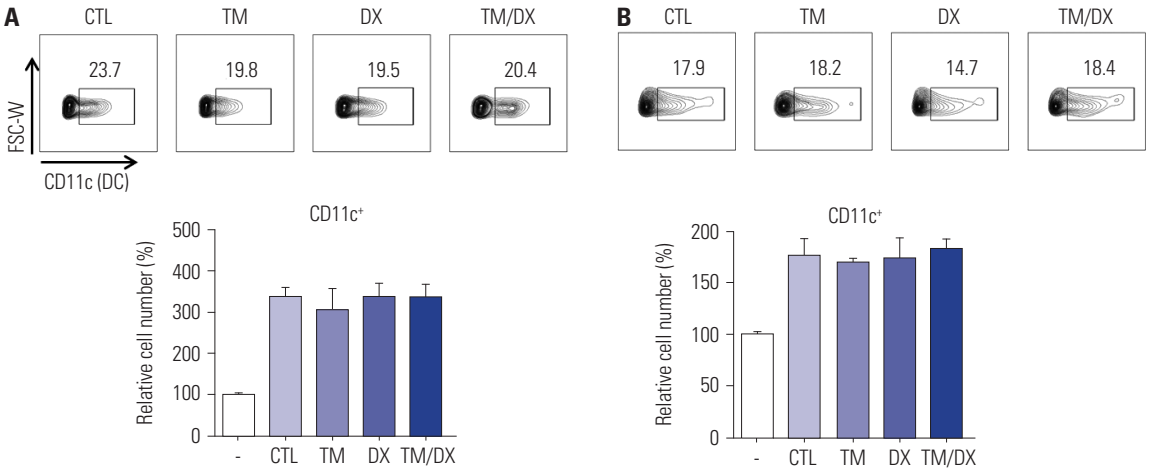

Fig. 3. The CD11c⁺ cell changes of PBMC or spleen measured by flow cytometry analysis. (A) PBMC. (B) Spleen. Contour plots of CD11c⁺ cells and relative cell numbers of sham control group (%). CD11c⁺ cells were unaffected by drug treatment. PBMC, peripheral blood mononuclear cells; TM, thalidomide; DX, dexamethasone; TM/DX, thalidomide and dexamethasone; CTL, control.

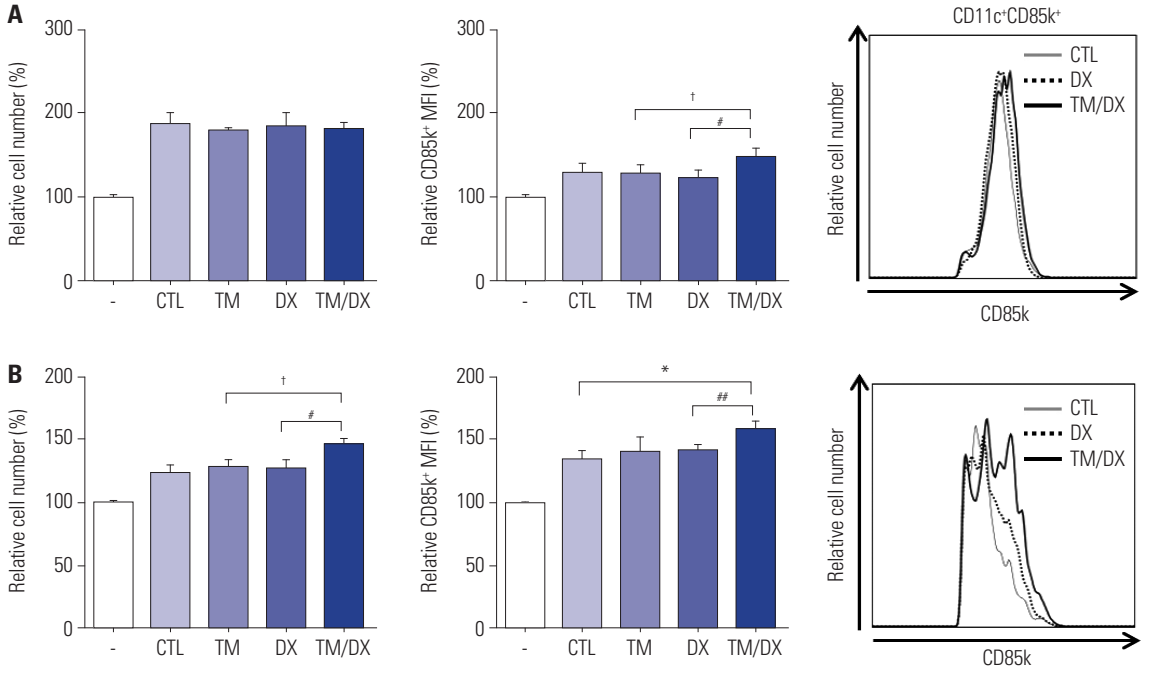

Fig. 4. The expressions of CD85k on CD11c⁺ cell measured by flow cytometry analysis. (A) PBMC. (B) Spleen. Relative cell numbers of CD11c⁺CD85k⁺ cells and relative CD85k⁺ median fluorescence intensity (MFI) on CD11c⁺CD85k⁺ cells of sham control group (%). The expressions of CD85k on CD11c⁺ cells were unaffected by drug treatment (%). TM/DX treatment increased the MFI on CD11c⁺CD85k⁺ cells in contrast to DX treatment. Histograms of CD85k expressions on CD11c⁺CD85k⁺ cells are representative figures of five experiments (*p<0.01 vs. CTL, †p<0.05 vs. TM, #p<0.05 and ##p<0.01 vs. DX). PBMC, peripheral blood mononuclear cells; TM, thalidomide; DX, dexamethasone; TM/DX, thalidomide and dexamethasone; CTL, control.

was not affected. The median fluorescence intensity (MFI) of CD85k⁺ expressions on CD11c⁺ cells were enhanced by TM/DX treatment compared to DX treatment in both PBMC and spleen (Fig. 4).

Serum IL-6 levels

The serum IL-6 levels were significantly down-regulated by TM or TM/DX treatment compared to the untreated CTL group. Moreover, TM/DX also significantly decreased serum IL-6 compared to DX treatment. DX treatment did not affect the IL-6 levels (p<0.05) (Fig. 5).

DISCUSSION

Previously, we reported on the effects of TM and DX on immune cells and their co-stimulatory, co-inhibitory molecules in vitro and in vivo.[28-31] This study utilises a murine cardiac transplant

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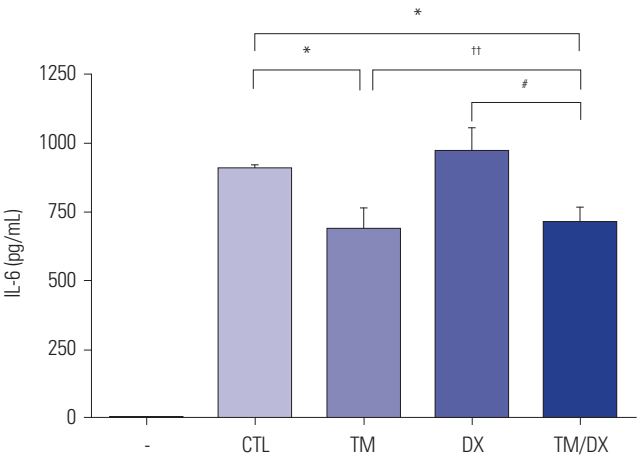

Fig. 5. The serum IL-6 levels induced by TM, DX, or TM/DX treatment on murine cardiac allograft transplantation model. The levels of serum IL-6 were down-regulated by TM/DX treatment more than by CTL or DX treatment (*p<0.05 vs. CTL, $^{\dagger\dagger}p$<0.01 vs. TM, $^{\#}p$<0.05 vs. DX). IL, interleukin; TM, thalidomide; DX, dexamethasone; TM/DX, thalidomide and dexamethasone; CTL, control.

model to verify our preceding findings and elucidate the immunomodulating affinity of TM. As previously described, TM/DX treatment affected $CD4^+$ T cell subsets by down-regulating Teffs while preserving Tregs in both in vitro. And in in vivo setting, Treg population was slightly increased by TM/DX treatment.[29] Fig. 2 shows similar results to our previous reports with TM/DX treatment significantly suppressing Teff counts and increasing Treg, and its tendency was prominent in PBMC. In the clinical setting, the balance between immunological injury and regulation can be controlled by methods decreasing the Teffs or increasing the Tregs.[21] Therefore, the Treg/Teff ratio may be more crucial than the absolute number of Tregs. As shown in Fig. 2, the ratio of $CD4^+FOXP3^+/CD4^+CD44^{hi}$ T cells significantly increased with TM/DX treatment in both PBMC and spleen without total $CD4^+$ T cell depletion. Many immunosuppressive drugs commonly reduce the total number of T cell and also decrease the Treg population.[19] Consequently, increasing the ratio of Tregs without changing the number of $CD4^+$ T cells implies a potent selective immunomodulating effect of TM/DX therapy.

Altering differences in $CD4^+CD44^{hi}$ or $CD4^+FOXP3^+$ T cells by TM/DX in spleen or PBMC may be due to the differences in complex combinations of different immune cell interactions, co-stimulatory molecules, and cytokines in PBMC and spleen. This may have contributed to the diversity of the $CD4^+$ T cell population at each location. Despite the cell population differences, we have demonstrated that the combination treatment of TM/DX significantly increased the ratio of $CD4^+FOXP3^+$ T cell/$CD4^+CD44^{hi}$T cell in both sites.

DCs are the most efficient APCs which determine the fate of T cells. Due to this interrelation, we demonstrated the inhibitory functions of TM/DX treated DCs on T cell proliferation by performing mixed lymphocyte reactions in a previous study.[31] In this mouse cardiac transplantation model, $CD11c^+$ cell population (DCs) increased after transplantation but showed no difference in cell frequency regardless of drug treatment (Fig. 3). However, on analysis of $CD11c^+CD85k^+$ cells (Fig. 4), the MFI of the $CD85k^+$ significantly increased with TM/DX treatment in both PBMC and spleen, in contrast to DX alone, comparable to our previous results.[31] CD85k (ILT3), an immunoglobulin-like transcript (ILT), is one of the biomarkers expressed on tolerogenic $CD11c^+$ cells, and an enhanced expression of $CD11c^+$ cells with TM/DX combination may indicate the induction of tolerogenic characteristics of DCs.[33] Tregs are developed in the thymus and extrathymic sites, such as secondary lymphoid organs (SLOs).[34] Tolerogenic DCs in SLOs promote the differentiation and proliferations of Tregs.[35] The significant increase in cell frequency and MFI of $CD11c^+CD85k^+$ cells by TM/DX treatment may suggest that $CD11c^+CD85k^+$ cells, tolerized DCs, possibly influence the Tregs induction by homing to the spleen. This may be one of the reasons for the increase of Tregs population due to TM/DX treatment.

IL-6 is a pleiotropic cytokine with pro-inflammatory features which is secreted by most stromal and immune cells.[36] It is a critical cytokine in innate immune response and adaptive immunity. In transplantation, IL-6 plays an important role in cell-mediated rejection, antibody-mediated rejection, and chronic allograft vasculopathy.[37] Our results showed that TM/DX treatment improved allograft survival and increased the proportion of Tregs and tolerogenic characteristics of DCs, validating our hypothesis of immunomodulating effect of TM/DX combination. Therefore, we analysed the representative pro-inflammatory cytokine, IL-6, to support our hypothesis (Fig. 5). Interestingly, significant inhibition of IL-6 by TM alone and TM/DX treatment was shown. According to the literature, DX has been known to inhibit IL-6.[11,38] Especially, Prelovsek, et al.[39] reported high dose of DX inhibited the secretion of IL-6 from human muscle. However, in our setting, DX alone did not influence the IL-6 level. This might be due to minimal dose of DX, which also explains the limited effect on IL-6. Therefore, our results suggest that the decrease in IL-6 production may be attributed to the effect of TM independent of DX.

Based on these results, TM/DX combinatorial treatment may affect IL-6 secretion and increase tolerogenic characteristics on DCs, which have the ability to subsequently expand Tregs consistent with immunomodulation, and influence the outcome of graft survival in this mouse cardiac allograft transplantation model. However, DX or TM alone failed to show graft survival benefits (Fig. 1).

Conventional immunosuppressive agents, such as calcineurin inhibitors (CNI) and steroids, are currently some of the most effective immunosuppressants in transplantation in the clinical setting. However, increasing graft survival and reducing the long-term side effects of current immunosuppressants have been major concerns in the transplantation field. These immunosuppressive agents generally lack specificity and broadly suppress the immune cells. Considering our results, the TM/DX combi-

nation treatment shows distinct mechanisms by specifically targeting T cell subsets, such as increasing Tregs or suppressing Teffs. These selective immunomodulatory effects were synergistically increased by combination with DX. Compared to conventional immunosuppressive agents, the TM/DX treatments show a more targeted immunosuppressive effect. Moreover, the minimization of side effects by these immunomodulatory functions is expected, including during long-term treatments.

This research, however, has some limitations. We applied a murine cardiac transplantation model using allografts from BALB/c (H-2^d) donors to C57BL/6 (H-2^b) recipients. This model is a fully major histocompatibility complex-mismatched model that induces acute rejection and results in short-term graft survival.[40] Despite the survival differences in each treatment group, no distinct histopathological differences were observed, possibly due to this acute response (data not shown). Future studies require a less immunologic murine model which enables the TM/DX treatment to sufficiently exert its immunomodulatory effects. We speculate a chronic rejection model would be more appropriate for further studies.

B cells, DC subset analysis, and various pro-inflammatory and anti-inflammatory cytokines must be investigated to further clarify the mechanism of the immunomodulatory effect. Especially, IL-10 and TGF-β must be checked for the evaluation of tolerogenic effect by TM/DX treatment. In addition, histopathological evidence must also be confirmed. Combination of conventional CNI and anti-metabolite therapy is also needed to assess the complementary effects of TM/DX treatment.

In conclusion, TM/DX treatment showed various evidences of immunomodulatory effects, different from the mechanisms of the standard immunosuppressants, and graft survival benefits in the murine cardiac transplant model. Therefore, we consider the TM/DX combinatorial treatment as a prospective immunomodulatory approach for preventing allograft rejection by inducing immunomodulatory effects in transplantation.

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AUTHOR CONTRIBUTIONS

Conceptualization: Beom Seok Kim and Kyu Ha Huh. Data curation: Joon Ye Kim and Hyojung Lee. Formal analysis: Joon Ye Kim, Beom Seok Kim, and Kyu Ha Huh. Funding acquisition: Myoung Soo Kim, Yu Seun Kim, and Kyu Ha Huh. Investigation: Soo Jin Kim, Joon Ye Kim, Eun Jee Kim, Hyojung Lee, and Yuri Cho. Methodology: Soo Jin Kim, Joon Ye Kim, and Beom Seok Kim. Project administration: Beom Seok Kim and Kyu Ha Huh. Resources: Myoung Soo Kim, Yu Seun Kim, and Kyu Ha Huh. Software: Joon Ye Kim. Supervision: Beom Seok Kim and Kyu Ha Huh. Validation: Joon Ye Kim and Beom Seok Kim. Visualization: Joon Ye Kim and Hyojung Lee. Writing—original draft: Soo Jin Kim and Joon Ye Kim. Writing—review & editing: Soo Jin Kim and Joon Ye Kim. Approval of final manuscript: all authors.

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