The effects of thalidomide on the immune cells and identification of the immune-modulatory mechanism

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The effects of thalidomide on the immune cells and identification of the immune-modulatory mechanism

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The effects of thalidomide on the immune cells and identification of the immune-modulatory mechanism

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Standard immunosuppressants used in transplantation are effective inhibitors of acute rejection but are accompanied by immunodeficiency complications and nonimmune complications. Thus, future immunosuppressive therapy such as immunomodulation and induction of tolerance is targeted at increasing graft survival and reducing immunosuppression-related complications.

The immune-modulatory 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 combination treatment with DX on immune cells using a murine cardiac allograft transplantation model.

Intraabdominal transplant of cardiac allografts from BALB/c (H-2^d) donors to C57BL/6 (H-2^b) recipients or C57BL/6 donors to BALB/c recipients were performed. After transplantation, mice were injected with TM 100 mg/kg or DX 0.1 mg/kg or a combination of both TM and DX daily by intra-peritoneal route until the time of graft loss.

$CD4^+$ T cells and subsets in the peripheral blood mononuclear cells and spleen were examined and quantified with flow cytometry. The changes in B cells ($CD19^+$) and dendritic cells (DCs, $CD11c^+$) were also analysed. The expression of co-inhibitory and co-stimulatory markers, glucocorticoid-induced TNF receptor-related protein (GITR), and programmed cell death-1 (PD-1) were also quantified by flow cytometry. Serum IL-6 collected at day 7 was measured by enzyme-linked immunosorbent assay (ELISA).

A significant increase in allograft survival was noted in both murine cardiac transplant models. The mean graft survival of the BALB/c donors to C57BL/6 recipients in the untreated group was 6.86 days and 10.0 days in the TM/DX group ($p<0.001$). The mean graft survival of the C57BL/6 donors to BALB/c recipients was 9.0 days in the untreated group and 22.5 days in the TM/DX group ($p<0.001$).

TM showed immune-modulatory features which were enhanced with the complementary combination of DX. The TM/DX treatment affected the $CD4^+$ T cell subsets without inhibiting the total $CD4^+$ T cell population. The $CD4^+FOXP3^+/CD4^+CD44^{hi}$ T cell ratio was increased which indicates the expansion of Treg cells. The increase in tolerogenic DCs ($CD11c^+CD85k^+$) with TM/DX was observed. The inhibition of pro-inflammatory cytokine IL-6 was also observed. TM/DX treatment showed the tendency to suppress co-stimulatory molecule GITR expression while TM-based treatments increased or preserved co-inhibitory molecule PD-1 expression in $CD4^+CD44^+$ and $CD4^+FOXP3^+$ T cells after transplantation.

TM/DX treatment resulted in selective T cell subset changes and the

induction of tolerogenic DCs, inhibition of IL-6, and improved allograft survival. These outcomes suggest the immune-modulating effect of the TM/DX combinatorial treatment. In conclusion, TM/DX combination may be a promising immune-modulatory approach for preventing allograft rejection and improving graft survival by inducing tolerance in transplantation.

Keywords: immunomodulation, thalidomide, dexamethasone, T cells, dendritic cells, GITR, PD-1, heart transplantation

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I. INTRODUCTION

Organ transplantation is the preferred treatment for end-stage organ failure. But 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 that inhibit cytokine synthesis[2]. However, these prominent immunosuppressants have immunodeficiency complications inducing infection and malignancy and nonimmune complications such as nephrotoxicity, cardiovascular, and metabolic risks[3,4]. Future immunosuppressive therapy is targeted at increasing graft survival and reducing immunosuppression-related complications. Current strategies include

developing highly selective immunosuppressive agents, immunomodulation, and induction of tolerance[5-7].

Thalidomide (TM) was prescribed as a sedative and antiemetic for morning sickness in the 1950s. But it was withdrawn from the market in the early 1960s due to its teratogenic complications[8]. TM was recognized as an effective treatment for erythema nodosum leprosum in 1965 and was subsequently researched for other potential therapeutic applications[9,10]. The anti-angiogenic, anti-neoplastic and immunomodulatory features of TM have been reported since[11,12]. TM has been proven clinically effective on myelodysplasia and multiple myeloma (MM)[13,14]. Further clinical studies with TM were performed on selected malignancies and autoimmune diseases[8]. 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 (NF-κB), and cytokine production such as interferon-γ (IFN-γ), chemokines, interleukin (IL)-6, IL-12, and cyclooxygenase-2 [8,15,16].

Corticosteroids are one of the most potent anti-inflammatory agents with immunosuppressive effects[17]. Corticosteroids such as dexamethasone (DX) or prednisolone are associated with decreased cytokine production, lymphocyte proliferation, and changes in cellular trafficking[18,19]. Due to these properties, corticosteroids have been used in the treatment of inflammatory, autoimmune disease, and immunosuppressive protocols for organ transplantation[20]. Side effects involving most major organ systems are associated with long-term corticosteroid therapy[4,21]. Therefore, risk/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 avoid or reduce corticosteroid therapy[22]. Combination therapy of TM and DX has been effective in the treatment of newly diagnosed MM and relapsed myeloma in the clinical field[14,23,24]. TM and prednisolone combination therapy was shown effective for nephritis in lupus-prone mice[25].

Immune cells such as T cells, B cells, macrophages, and dendritic cells (DCs) can participate in graft rejection or promote tolerogenic immune responses[26]. Regulatory T cells (Tregs) play an imperative role in immunologic tolerance[26,27]. Tregs inhibit effector T cell (Teff) proliferation, and promote tolerance through various signals, such as the production of IL-10, transforming growth factor-β (TGF-β), and the inhibition of antigen-presenting cells (APC) function[26,28]. In the clinical state of transplantation, the allograft outcome, rejection, or tolerance often depends on the balance between the Teffs and Tregs [29,30]. Therefore, Tregs have been researched as a prospective target for inducing allograft tolerance[31,32]. DCs are potent APCs, which play an important role in stimulating T cells and initiating primary immune responses[33,34]. DCs have also been found to play a role in central and peripheral tolerance[26,35,36]. DCs tolerize T cells to self-antigens, achieving self-tolerance, and alteration of this system may result in autoimmune diseases[34]. In transplantation, allograft rejection is the result of both innate and adaptive immunity. Because DCs function in both immune responses and control immunity and tolerance, they are an important factor for immunosuppression and immune modulation[37,38].

Previous studies in our lab have suggested that TM has immune-modulating

effects by selectively suppressing $CD4^+$ T cell subsets and changing the expression of selected tumor necrosis factor receptor super families (TNFRSFs), including OX40, 4-1BB, and glucocorticoid-induced TNFR-related protein (GITR)[39]. Co-treatment of TM/DX increased CTLA-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[40,41]. 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 immune-modulatory drug in the transplantation field. The current study investigated the effects of TM, and the combination treatment with DX on immune cells using a murine cardiac allograft transplantation model. Effects on $CD4^+$ T cell subsets, $CD8^+$ T cells, $CD19^+$ B cells, and $CD11c^+$ cells were analysed. The changes in co-stimulatory and co-inhibitory molecules, GITR, and programmed cell death-1 (PD-1) were studied. We also examined the tolerogenic changes in DCs and its part in immune modulation with TM and TM/DX treatment.

II. MATERIALS AND METHODS

1. Mice and reagents

8~9 weeks old male BALB/c (H-2^d) mice and C57BL/6 (H-2^b) mice were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained according to the ethical guidelines of our institution.

PE-Cy7-conjugated anti-mouse CD8, PerCP-Cy5.5-conjugated anti-mouse CD11c, PerCP-Cy5.5-conjugated anti-mouse CD19, PE-conjugated anti-mouse CD85k, APC-conjugated anti-mouse PD-1, FITC-conjugated anti-mouse CD44, PerCP-Cy5.5-conjugated anti-mouse FOXP3, and the Fixation/Permeabilization kit were purchased from eBioscience (San Diego, CA, USA). APC-Cy7-conjugated anti-mouse CD4, PerCP-Cy5.5-conjugated anti-mouse GITR antibodies were purchased from Biolegend (San Diego, CA, USA). TM, DX, and red blood cell (RBC) lysis buffer, Histopaque 1.083 were purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-CD44 antibody and anti-FOXP3 antibody were each obtained from Novus Biologicals (Littleton, CO, USA) and LSBio (Seattle, WA, USA). LSAB kit was purchased from DAKO, Glostrup, Denmark. Mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kit was purchased from BD Bioscience, San Jose, CA, USA.

2. Heterotopic cardiac transplantation and drug treatment

The animals were anesthetized with isoflurane during the entire surgical procedure. Intraabdominal transplant of cardiac allografts from BALB/c (H-2^d) donors to C57BL/6 (H-2^b) recipients or C57BL/6 donors to BALB/c recipients,

were performed as described by Niimi[42]. 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 with TM 100 mg/kg or DX 0.1mg/kg or a combination of both TM and DM daily by intra-peritoneal route until the time of graft loss which was defined as the cessation of a palpable cardiac contraction. Peripheral blood mononuclear cells (PBMC) and splenocytes were collected in BALB/c donors to C57BL/6 recipients on day 7. (Fig. 1)

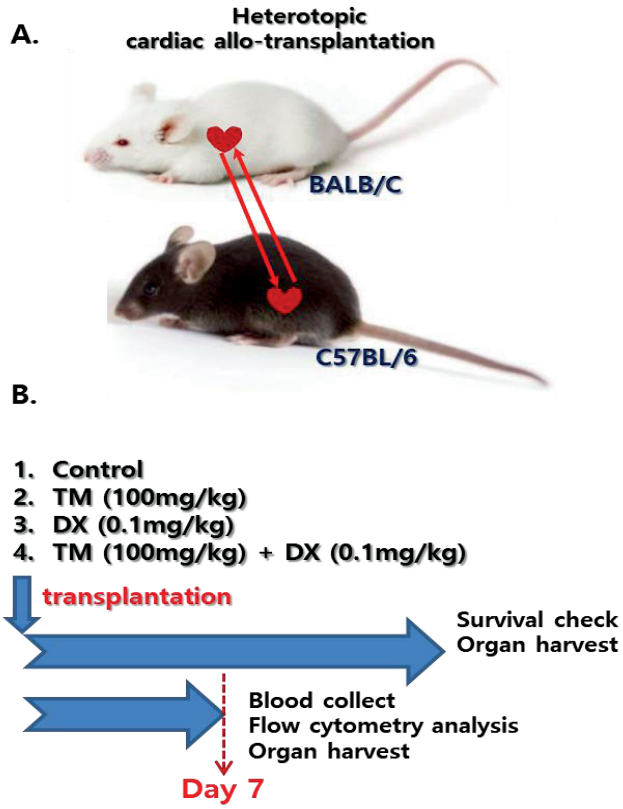

Figure 1. The scheme of murine heterotopic cardiac allotransplantation model. A; Procedures were performed using BALB/c mice for donors and C57BL/6 mice for recipients and vice versa. B; Recipient mice were randomly divided into 4 groups; untreated (control; CTL), thalidomide (TM), dexamethasone (DX), and combinatorial treatment of TM and DX (TM/DX) groups. Injection dose was TM 100 mg/kg and/or DX 0.1mg/kg daily by intra-peritoneal route. Transplanted grafts, blood, and spleens were harvested for histological assessment and isolation of peripheral blood mononuclear cells (PBMC) and splenocytes on day 7.

3. Histology

In the case of BALB/c donors to C57BL/6 recipient models, cardiac allograft specimens were harvested on day 7 and fixed with 10 % phosphate-buffered formalin. Then samples were embedded into paraffin blocks, and cut sections of 5 μm thickness were made. Hematoxylin and eosin (H&E) staining were performed to determine the degree of rejection. We used a revised scoring system[43] for heart allograft rejection in rodent models from previous studies[43,44] based on the modified International Society for Heart and Lung Transplantation (ISHLT) 2004 scoring system[45].

4. Flow cytometry

In order to examine the effects of the drug treatments on immune cells, isolated splenocytes and PBMCs from the BALB/c donors to C57BL/6 recipient model were incubated with the appropriately diluted antibodies for 40 min at 4°C. Activated $CD4^+$ T cells were stained with APC-Cy7-conjugated anti-mouse CD4 and FITC-conjugated anti-mouse CD44, 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-CD8 and FITC-conjugated anti-mouse CD44 antibodies. $CD19^+$ and $CD11c^+$ were used for B cell or dendritic cell 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).

5. *Enzyme-Linked Immunosorbent Assay (ELISA)*

Serum was collected from the BALB/c donors to C57BL/6 recipient model on day 7 and immediately placed in -80 ℃ until measurement. IL-6 levels were measured by ELISA following the manufacturer's protocols (BD Bioscience).

6. *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, and statistical significance was accepted for p values < 0.05.

III. RESULTS

1. Graft survival on cardiac transplantation model

The mean graft survival time of the untreated group (control; CTL) in BALB/c donor to C57BL/6 recipient cardiac graft model was 6.86 ± 0.38 days. Single-drug treatment 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 treatment groups (10.0 ± 0.89 days, p <0.01) (Table 1, Fig. 2A).

In the C57BL/6 donor and BALB/c recipient cardiac graft model, the graft survival of the untreated group was 9.0 ± 2.24 days. The graft survival of DX treatment or combinatorial treatment of TM/DX was 12.3 ± 3.30 or 22.5 ± 7.37 days. TM/DX treatment significantly extended the survival of grafts compared to the untreated group (p <0.01) but also compared to the DX treatment group (p <0.05) (Table 1, Fig. 2B).

Table 1. Mean graft survival time of murine cardiac allotransplantation model

<donor: BALB/c → recipient: C57BL/6>

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	

<donor: C57BL/6 → recipient: BALB/c>

Group	N	Individual graft survival time (days)	Mean graft survival time (days)	
CTL	5	8, 8, 8, 8, 13	9.0±2.24	***
DX	4	10, 10, 12, 17	12.3±3.30	#
TM/DX	4	14, 22, 22, 32	22.5±7.37	

The combinatorial treatment of TM/DX exhibited the longest graft survival compared to the other treatment groups. (** $p<0.01$, *** $p<0.001$ *versus CTL,* # $p<0.05$, ## $p<0.01$ *versus DX*)

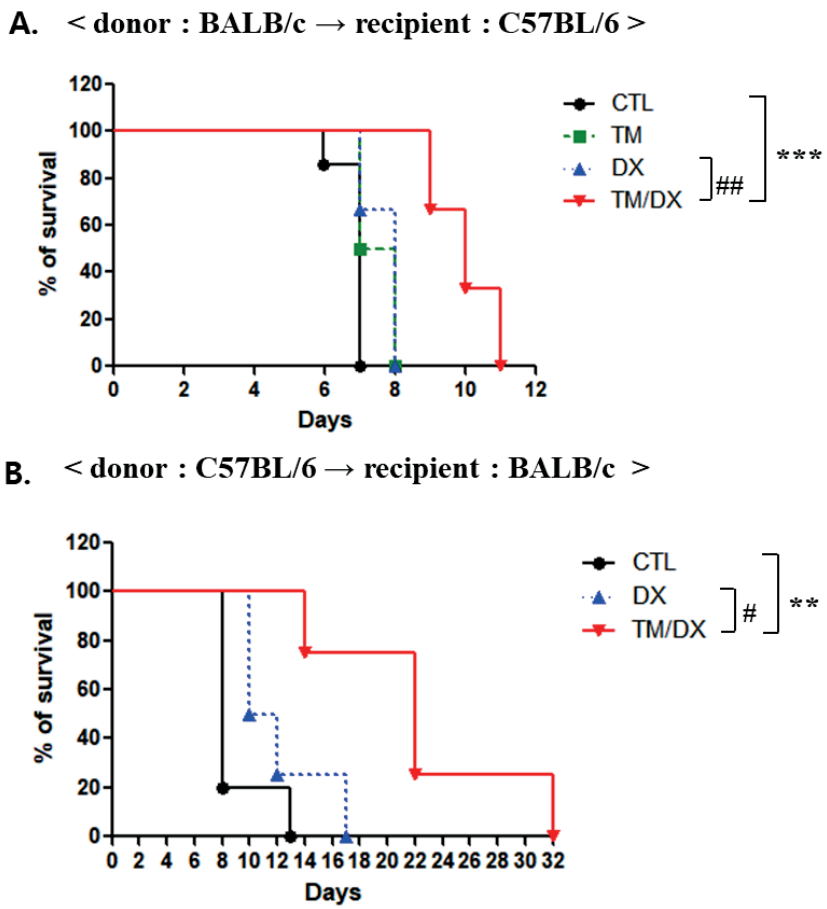

Figure 2. Survival effects of drug treatments on murine heterotopic cardiac allotransplantation model. The combinatorial treatment of TM/DX exhibited the longest graft survival. A; BALB/c donor to C57BL/6 recipients. B; C57BL/6 donors to BALB/c recipients. (** $p<0.01$, *** $p<0.001$ *versus CTL*, # $p<0.05$, ## $p<0.01$ *versus DX*)

2. Histological assessments of rejection grade

Regardless of the survival differences between each treatment group, the rejection grades of BALB/c donors to C57BL/6 recipient model showed no difference in all the groups including the rejection grades of the TM/DX group which showed the longest survival (Table 2).

Table 2. Scoring of rejection grade on murine cardiac allotransplantation model

Group	N	Rejection Grade (R)	mean
CTL	6	3R, 2R, 2R, 3R, 3R, 2R	2.5±0.22
TM	4	3R, 3R, 2R, 3R	2.75±0.25
DX	6	2R, 2R, 3R, 3R, 3R, 3R	2.52±0.21
TM/DX	6	2R, 2R, 2R, 3R, 3R, 2R	2.33±0.21

There was no difference between the treatment groups.

3. *$CD4^+$ 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 than DX treatment (TM/DX; 88.6 ± 2.96 %, DX; 102.9 ± 2.97 %, p <0.001). But splenic $CD4^+$ Teffs showed no difference between the treatment groups.

$CD4^+$ Tregs ($CD4^+FOXP3^+$) showed similar tendencies in 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, combinatorial treatment of TM/DX showed an up-regulating effect of the $CD4^+$ Treg population compared with the untreated group, but also with the DX treatment group (Fig. 3, 4).

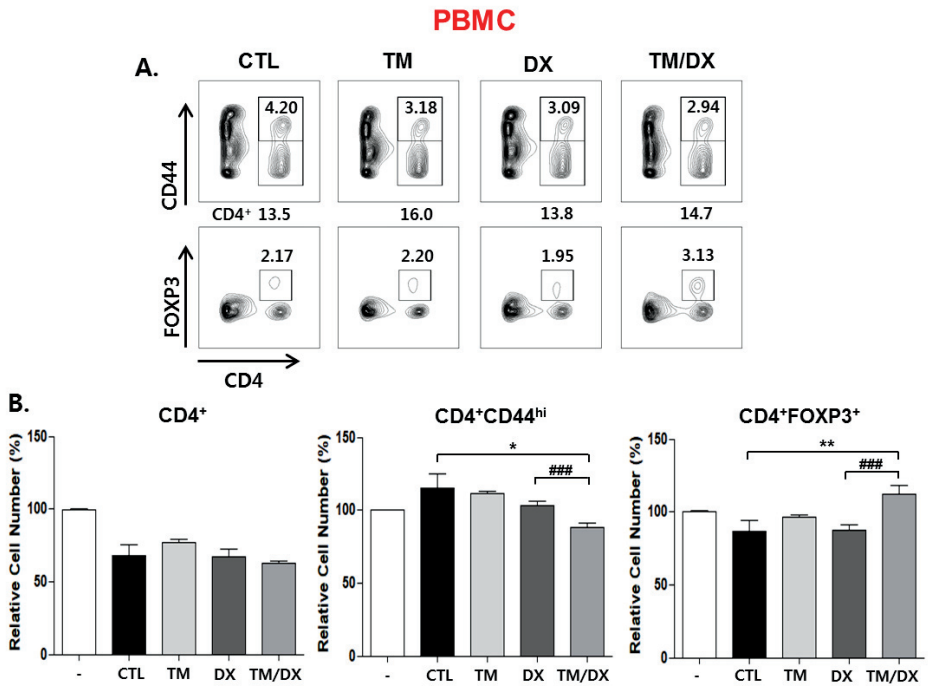

Figure 3. The $CD4^+$ T cell subset changes of PBMC measured by flow cytometry analysis. A; Contour plots of $CD4^+CD44^{hi}$ and $CD4^+FOXP3^+$ T cells. *Representative figures of 5 experiments*. B; Relative cell numbers to the sham control group [(-), (%)], Total $CD4^+$ T cells were consistent, regardless of treatment. $CD4^+CD44^{hi}$ T cells decreased with TM/DX treatment compared to the CTL or DX treatment (CTL versus TM/DX; $p<0.05$, DX versus TM/DX; $p<0.001$). $CD4^+FOXP3^+$ T cells increased with TM/DX treatment (CTL versus TM/DX; $p<0.01$, DX versus TM/DX; $p<0.001$). (* $p<0.05$, ** $p<0.01$ versus CTL, ### $p<0.001$ versus DX treatment)

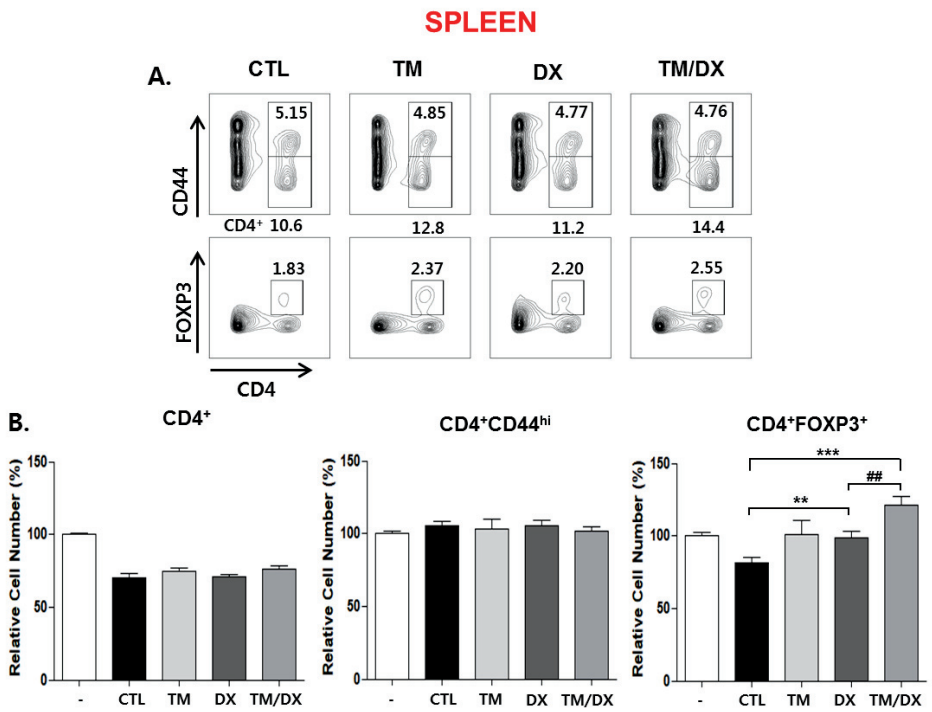

Figure 4. The $CD4^+$ T cell subset changes in the spleen measured by flow cytometry analysis. A; Contour plots of $CD4^+CD44^{hi}$ and $CD4^+FOXP3^+$ T cells. *Representative figures of 5 experiments*. B; Relative cell numbers of sham control group (%), Total $CD4^+$ T cells were consistent, regardless of treatment. $CD4^+CD44^{hi}$ T cells showed no change. $CD4^+FOXP3^+$ T cells increased with TM/DX treatment (CTL versus TM/DX; $p<0.01$, DX versus TM/DX; $p<0.001$). (** $p<0.01$, *** $p<0.001$ versus CTL, ## $p<0.01$ versus DX treatment)

We analysed the ratio of $CD4^+FOXP3^+/CD4^+CD44^{hi}$ T cells in the PBMC and spleen. The ratio decreased after cardiac transplantation (PBMC; 77.6 ± 9.84 %, spleen; 78.0 ± 4.33 %), and recovered 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 combination treatment significantly increased (PBMC; 90.6 ± 4.4 %, spleen; 96.4 ± 4.78 %) the ratio of $CD4^+FOXP3^+$ T cell/$CD4^+CD44^{hi}$ T cell in both sites (Fig. 5).

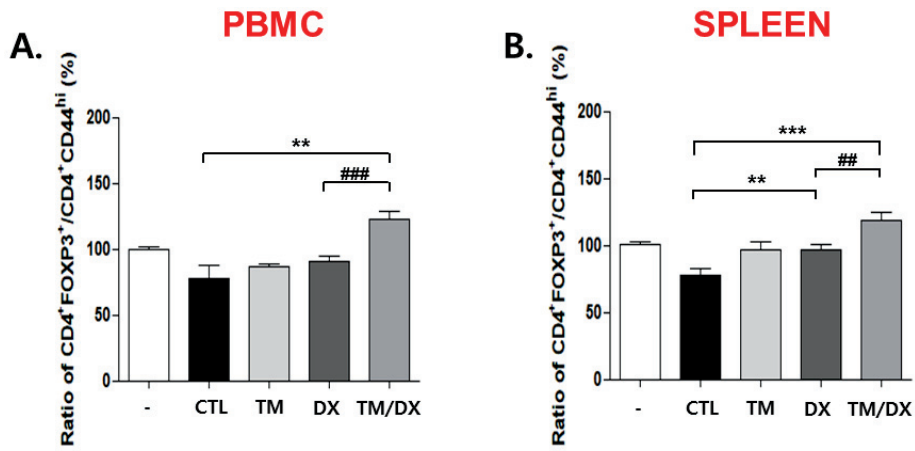

Figure 5. The ratio of $CD4^{+}FOXP3^{+}$ T cells to $CD4^{+}CD44^{hi}$ T cell. A; PBMC, B; spleen. TM/DX combination treatment significantly increased the ratio of $CD4^{+}FOXP3^{+}$ T cell/$CD4^{+}CD44^{hi}$ T cell. (** $p<0.01$, *** $p<0.001$ versus CTL, ## $p<0.01$, ### $p<0.001$ versus DX treatment)

4. $CD8^+$ T cell changes

An increase of activated $CD8^+$ T cells, identified as $CD8^+CD44^+$, was observed in the transplanted groups. In PBMC, TM treatment showed a down-regulating tendency of $CD8^+CD44^+$ T cells compared to DX treated groups. The combinatorial treatment of TM with DX significantly enhanced the manner of down-regulation in the DX treatment group. No significant changes were noted in the spleen (Fig. 6, 7).

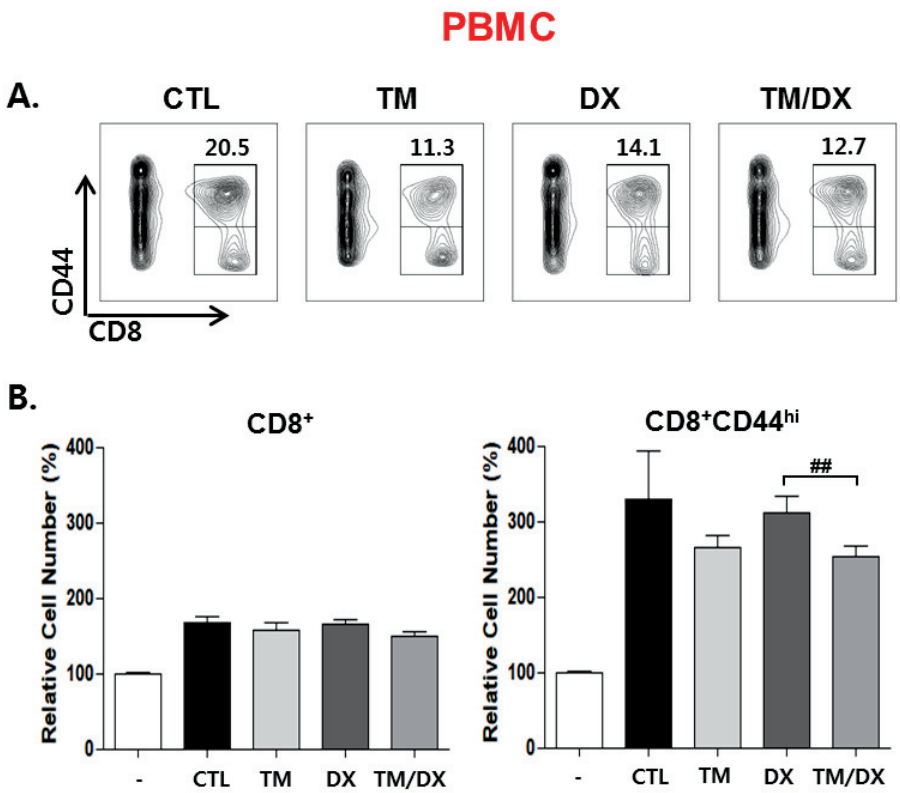

Figure 6. The $CD8^+$ T cell changes in PBMC measured by flow cytometry analysis. A; Contour plots of $CD8^+CD44^{hi}$ T cells. *Representative figures of 5 experiments*. B; Relative cell numbers of sham control group (%). $CD8^+CD44^{hi}$ T cells were decreased by TM/DX treatment compared to DX treatment. Total $CD8^+$ T cells were consistent, regardless of treatment. (## $p<0.01$ versus DX treatment)

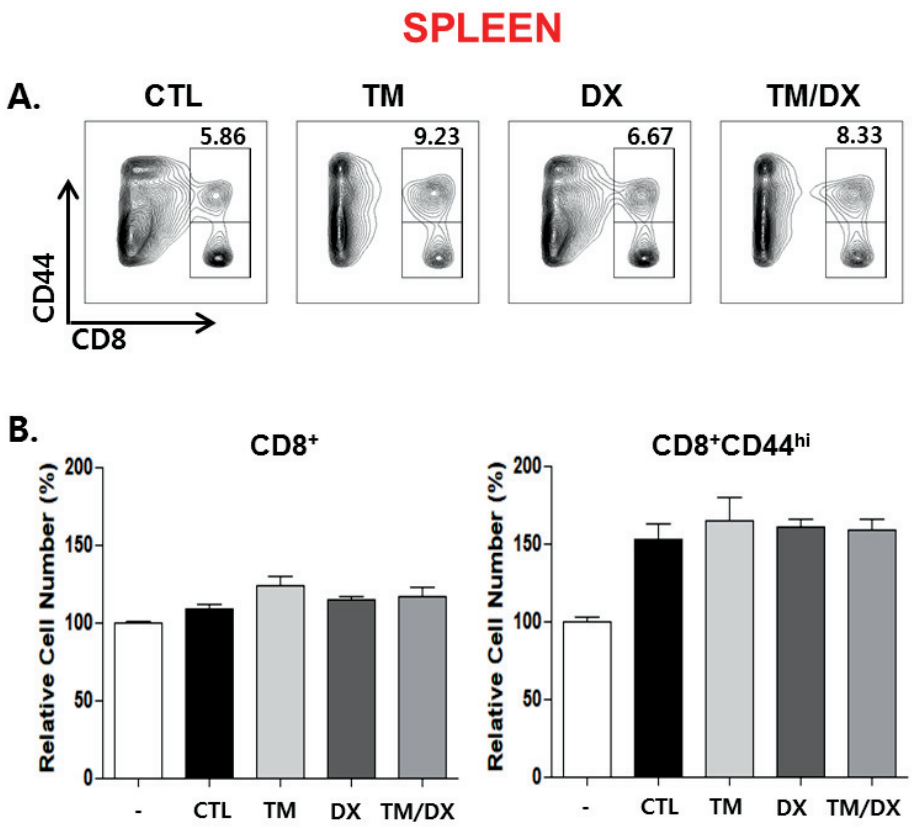

Figure 7. The $CD8^+$ T cell changes in the spleen measured by flow cytometry analysis. A; Contour plots of $CD8^+$ T cells. *Representative figures of 5 experiments*. B; Relative cell numbers of sham control (%). $CD8^+$ B cells were consistent, regardless of treatment.

5. *$CD19^+$ B cell changes*

In contrast to the changes in $CD8^+CD44^+$ T cells, the $CD19^+$ B cells decreased after transplantation in PBMC. DX treatment and combinatorial treatment of TM/DX reduced the B cell population ($p<0.05$). In the TM/DX group, the reduction was stronger than the DX group but showed no significance. $CD19^+$ B cells in the spleen were not affected by any drug treatment (Fig. 8, 9).

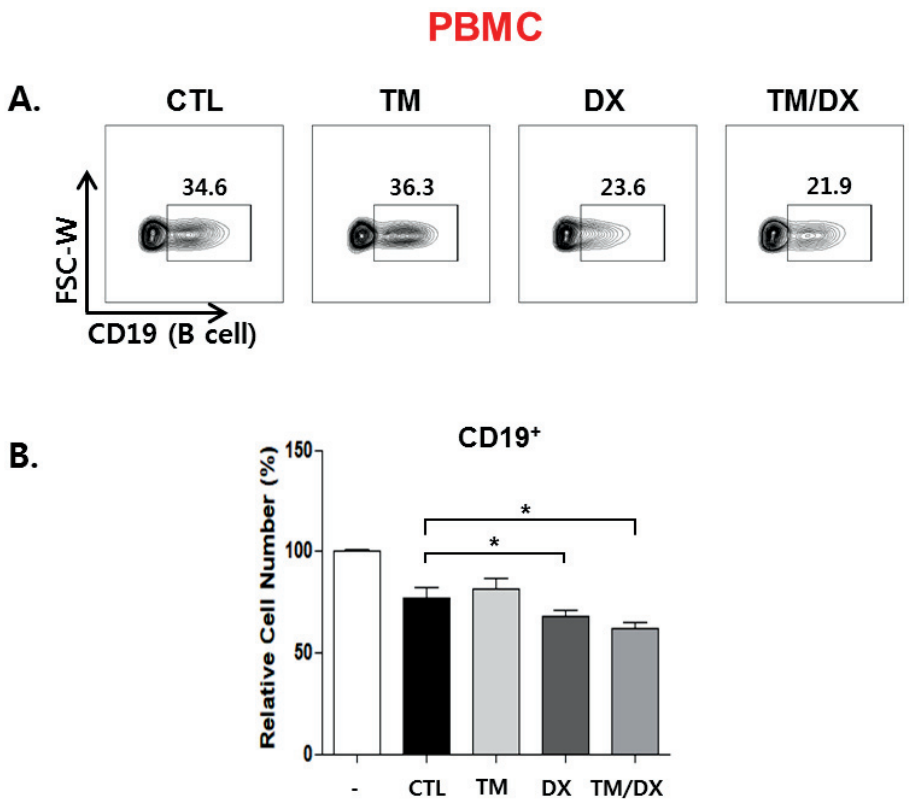

Figure 8. The $CD19^+$ B cell changes in PBMC measured by flow cytometry analysis. A; Contour plots of $CD19^+$ B cells. *Representative figures of 5 experiments.* B; Relative cell numbers of sham control (%). $CD19^+$ B cells were decreased by DX or TM/DX treatment compared to the sham control group (CTL versus DX or TM/DX; $p<0.05$). (* $p<0.05$ versus CTL)

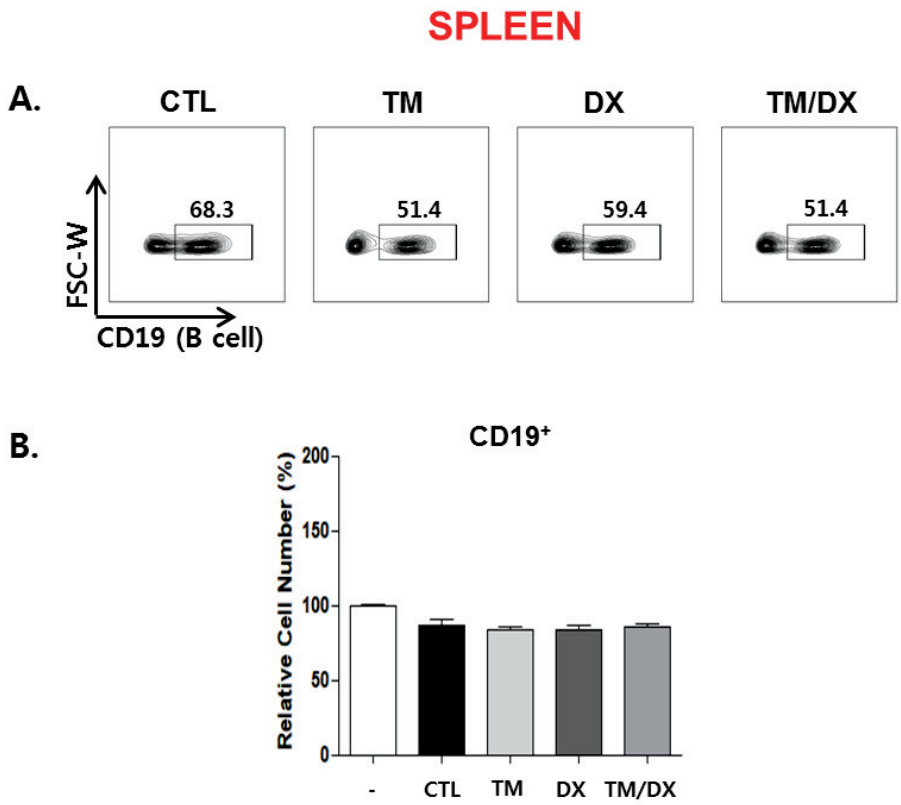

Figure 9. The $CD19^+$ B cell changes in the spleen measured by flow cytometry analysis. A; Contour plots of $CD19^+$ B cells. *Representative figures of 5 experiments.* B; Relative cell numbers of sham control (%). $CD19^+$ B cells were unchanged by drug treatment.

6. $CD11c^+$ 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 the 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.10, 11).

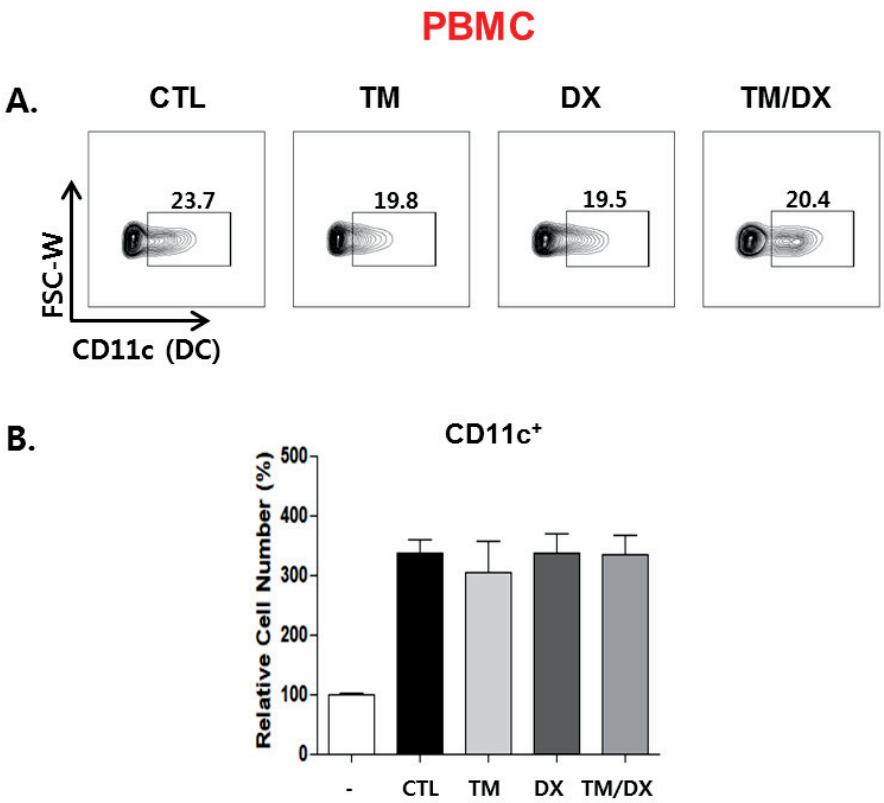

Figure 10. The $CD11c^+$ cell changes in PBMC measured by flow cytometry analysis. A; Contour plots of $CD11c^+$ cells. *Representative figures of 5 experiments.* B; Relative cell numbers of sham control (%). $CD11c^+$ cells were unchanged by drug treatment.

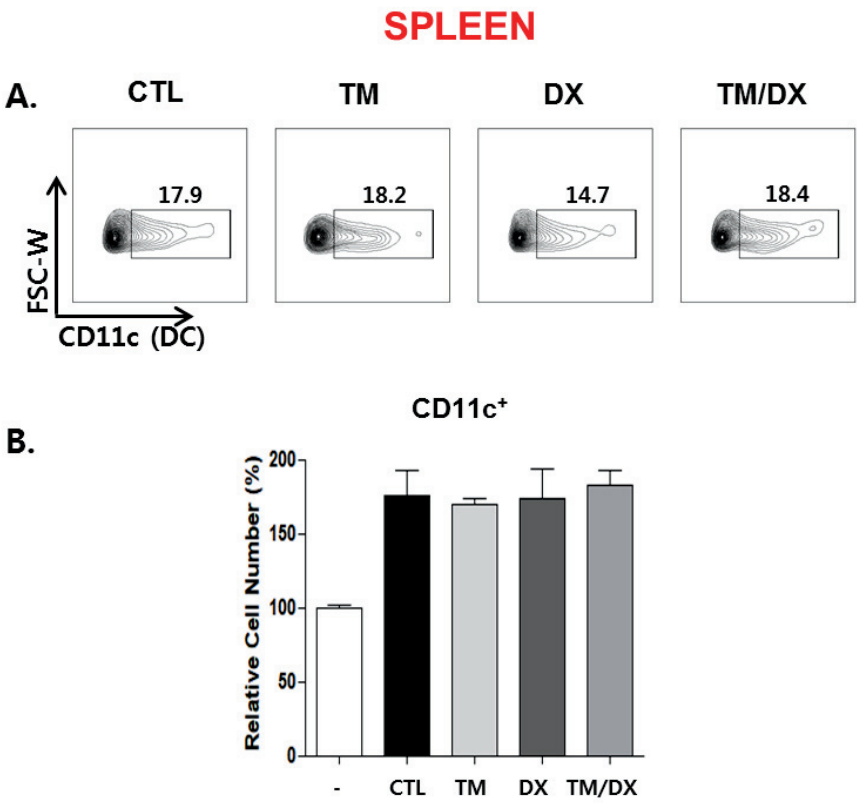

Figure 11. The $CD11c^+$ cell changes in the spleen measured by flow cytometry analysis. A; Contour plots of $CD11c^+$ cells. *Representative figures of 5 experiments.* B; Relative cell numbers of sham control (%). $CD11c^+$ cells were not changed by drug treatment.

7. *$CD11c^+CD85k^+$ cell changes*

The frequencies of $CD11c^+CD85k^+$ cells 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 the spleen ($p<0.05$). However, the cell frequency in PBMC 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 PBMC and spleen (Fig. 12, 13).

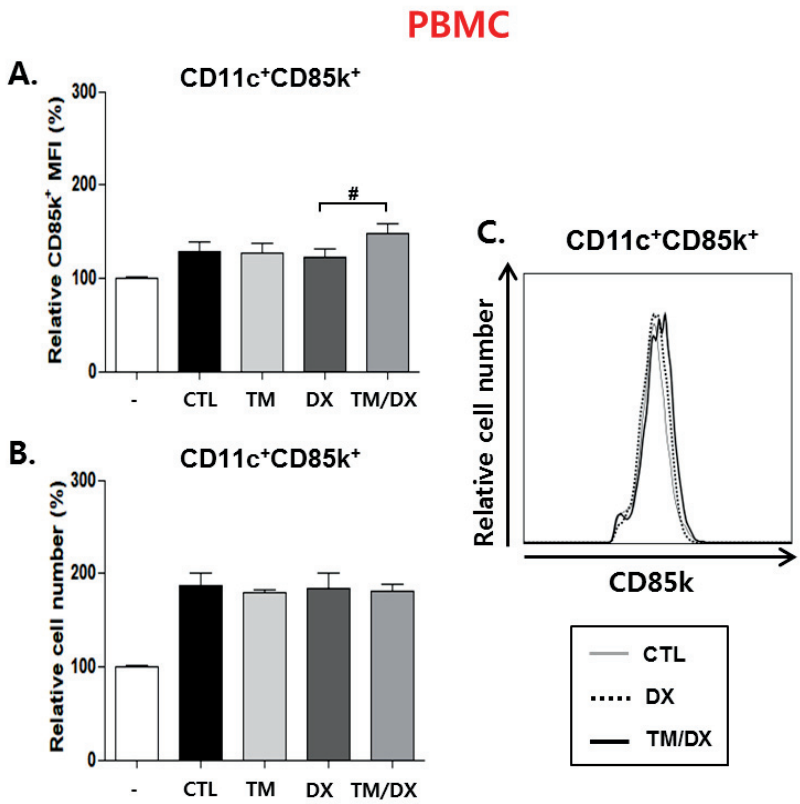

Figure 12. The expressions of CD85k on $CD11c^+$ cell of PBMC measured by flow cytometry analysis. A; Relative $CD85k^+$ median fluorescence intensity (MFI) of sham control group (%). TM/DX treatment increased the MFI on $CD11c^+CD85k^+$ cells compared to the DX treatment. B; Relative cell numbers of sham control group (%). The expressions of CD85k on $CD11c^+$ cells were unchanged by drug treatment. C; Histograms of CD85k expressions on $CD11c^+CD85k^+$ cells. *Representative figures of 5 experiments (*# $p<0.05$ versus DX treatment)

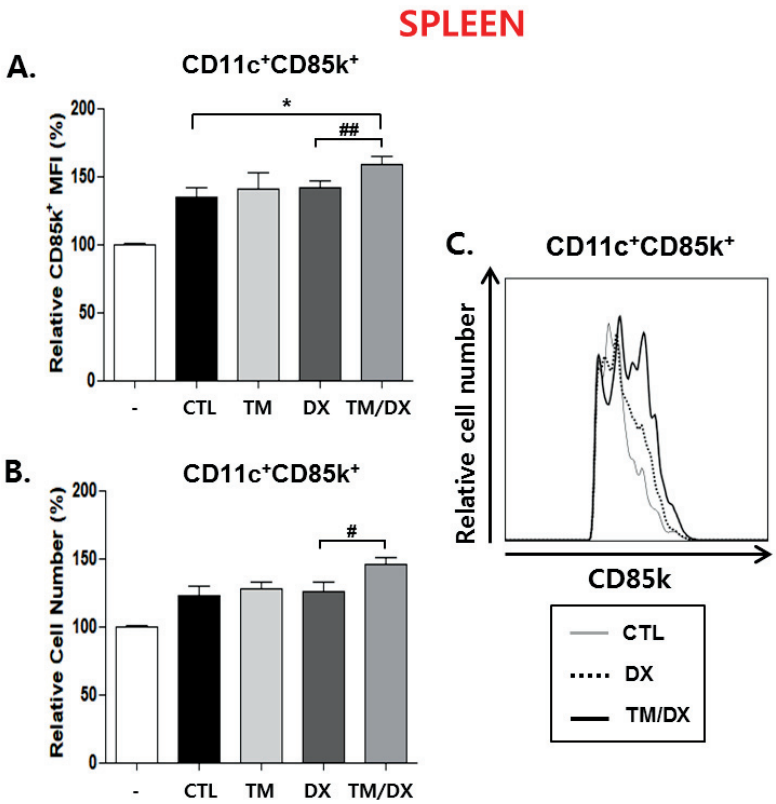

Figure 13. The expressions of CD85k on $CD11c^+$ cells in the spleen measured by flow cytometry analysis. A; Relative $CD85k^+$ MFI of sham control group (%). TM/DX treatment significantly increased the MFI on $CD11c^+CD85k^+$ cells compared to the untreated or DX treatment group. B; Relative cell numbers of sham control group (%). The expressions of CD85k on $CD11c^+$ cells were increased by TM/DX treatment compared to DX treatment. C; Histograms of CD85k expressions on $CD11c^+CD85k^+$ cells. *Representative figures of 5 experiments*. (* $p<0.05$ versus CTL, # $p<0.05$, ## $p<0.01$ versus DX treatment)

8. 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 with DX treatment. DX treatment did not affect the IL-6 levels. ($p<0.01$, Fig. 14)

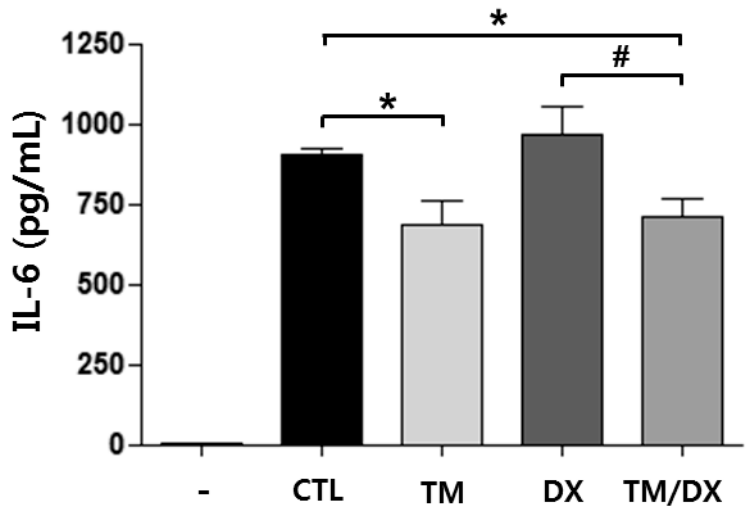

Figure 14. The serum IL-6 levels induced by TM, DX, or TM/DX treatment on the murine cardiac allotransplantation model. The levels of serum IL-6 were down-regulated by TM/DX treatment compared to CTL or DX treatment.
(p<0.05 versus CTL, # p<0.05 versus DX treatment)*

9. *PD-1 and GITR expressions on $CD4^+$ T cells*

TM treatment increased PD-1 expressions compared to the DX treatment group in both PBMC ($p<0.001$) and spleen ($p<0.05$). Moreover, in splenic $CD4^+CD44^+$ cells, combinatorial treatment TM with DX also increased PD-1 expression compared to DX treatment ($p<0.05$).

GITR expressions on $CD4^+CD44^+$ cells were increased by transplantation. TM/DX combination suppressed the increase in PBMC compared to the DX treatment ($p<0.01$). No significant difference was noted between the groups in the spleen. (Fig. 15, 16)

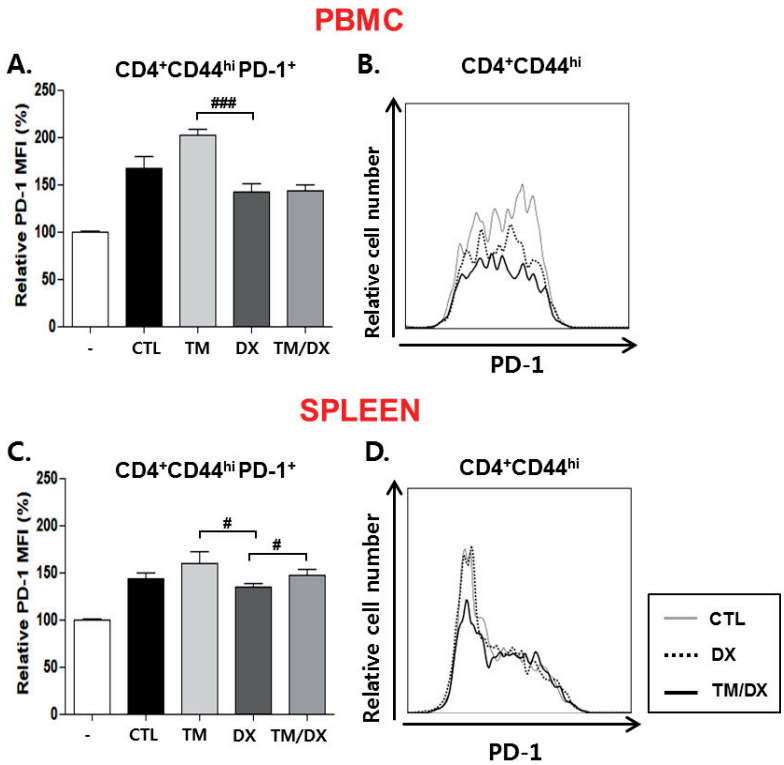

Figure 15. The expressions of PD-1 on $CD4^{+}CD44^{hi}$ T cells measured by flow cytometry analysis. A; Relative PD-1 MFI of sham control group in PBMC (%). TM treatment increased PD-1 expressions on $CD4^{+}CD44^{hi}$ T cells. B; Histograms of PD-1expressions on $CD4^{+}CD44^{hi}$ T cells in PBMC. *Representative figures of 5 experiments.* C; Relative PD-1 MFI of sham control group in the spleen (%). The expressions of PD-1 on $CD4^{+}CD44^{hi}$ T cells increased with TM or TM/DX treatment compared to DX treatment in the spleen. D; Histograms of PD-1 expressions on $CD4^{+}CD44^{hi}$ T cells in the spleen. *Representative figure of 5 experiments. (# $p<0.05$, ### $p<0.001$* versus DX treatment)

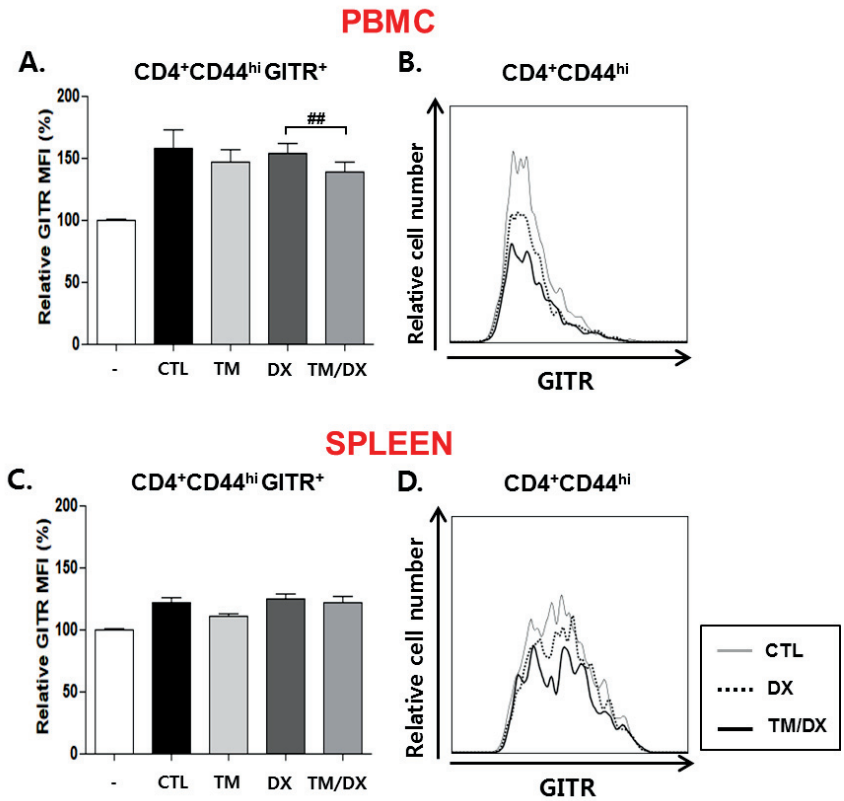

Figure 16. The expressions of GITR on $CD4^{+}CD44^{hi}$ T cells measured by flow cytometry analysis. A; Relative GITR MFI of sham control group in PBMC (%). The expressions of GITR on $CD4^{+}CD44^{hi}$ T cells were further reduced by TM/DX compared to the DX treatment group. B; Histograms of GITR expressions on $CD4^{+}CD44^{hi}$ T cells in PBMC. *Representative figures of 5 experiments*. C; Relative GITR MFI of sham control group in the spleen (%). The expressions of GITR were unaffected by drug treatment. D; Histograms of GITR expressions on $CD4^{+}CD44^{hi}$ T cells in the spleen. *Representative figure of 5 experiments. (*##$p<0.01$ versus DX treatment)

Changes in PD-1 expression on $CD4^+$ Tregs in the spleen were more notable than in PBMC. Up-regulation by TM treatment was observed compared to the untreated ($p<0.05$) or the DX treatment group ($p<0.01$). Comparable responses were shown with the combinatorial treatment of TM/DX, although DX treatment did not affect PD-1 expression on splenic $CD4^+$ Tregs ($p<0.01$).

GITR expressions on $CD4^+$ Tregs were not affected by transplantation in both PBMC and spleen. In PBMC, TM/DX combination treatment also showed no effect on GITR expression compared to the untreated group, while DX treatment increased GITR expression. In the spleen, TM/DX combination treatment reduced the GITR expression compared to both the untreated group and the DX treatment group. (Fig. 17, 18)

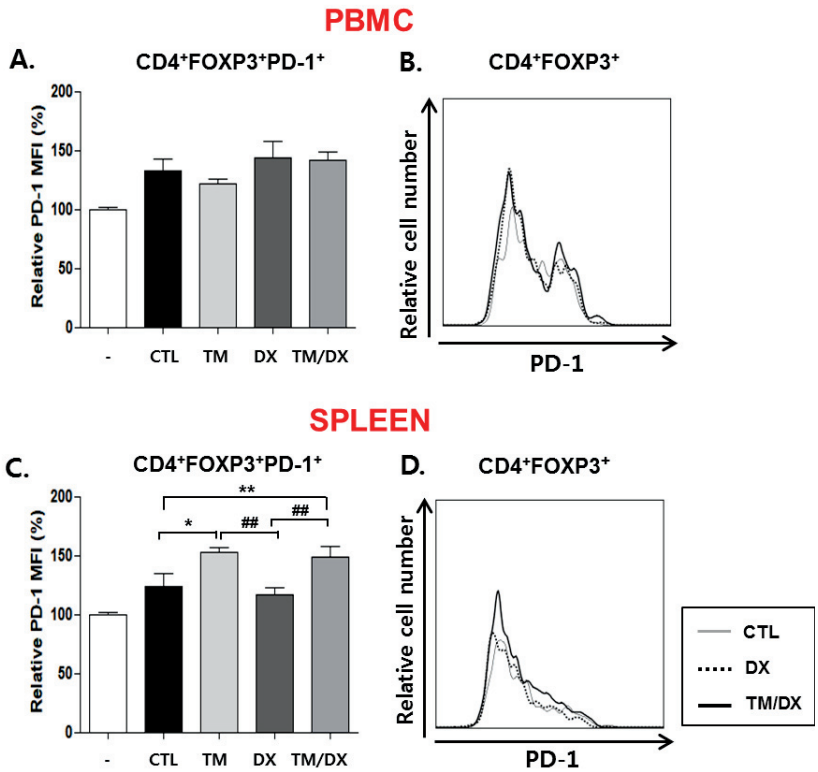

Figure 17. The expressions of PD-1 on $CD4^+FOXP3^+$ T cells measured by flow cytometry analysis. A; Relative PD-1 MFI of sham control group in PBMC (%). The expressions of PD-1 on $CD4^+$ $FOXP3^+$ T cells showed no change. B; Histograms of PD-1 expressions on $CD4^+FOXP3^+$ T cells. *Representative figures of 5 experiments*. C; Relative PD-1 MFI of sham control group in the spleen (%). The expressions of PD-1 on $CD4^+FOXP3^+$ T cells were increased by TM or TM/DX treatment compared to the untreated or DX treatment group. D; Histograms of PD-1expressions on $CD4^+FOXP3^+$ T cells in the spleen. *Representative figures of 5 experiments.* (* $p<0.05$, ** $p<0.01$ versus CTL, ## $p<0.01$ versus DX treatment)

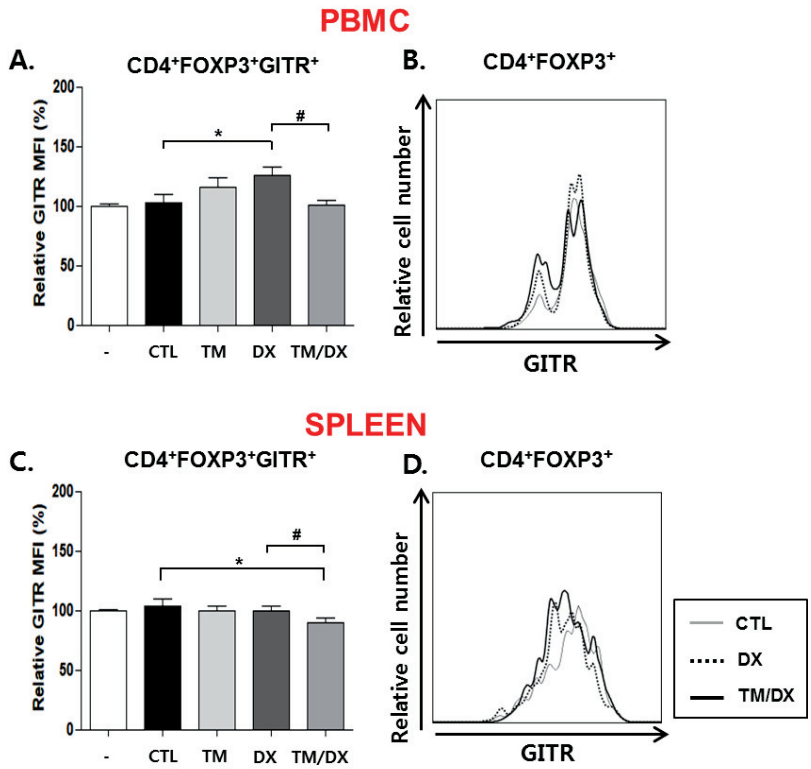

Figure 18. The expressions of GITR on $CD4^+FOXP3^+$ T cells measured by flow cytometry analysis. A; Relative GITR MFI of sham control group in PBMC (%). TM/DX treatment decreased the expressions of GITR on $CD4^+FOXP3^+$ T cells compared to DX treatment ($p<0.05$). *Representative figures of 5 experiments*. B; Histograms of GITR expressions on $CD4^+FOXP3^+$ T cells in PBMC. C; Relative GITR MFI of sham control group in the spleen (%). TM/DX treatment decreased the expressions of GITR on $CD4^+FOXP3^+$ T cells compared to the untreated or DX treatment group. D; Histograms of GITR expressions on $CD4^+FOXP3^+$ T cells in the spleen. *Representative figure of 5 experiments.* (* $p<0.05$ versus CTL, # $p<0.05$ versus DX treatment)

IV. DISCUSSION

Heterotopic murine cardiac transplantation is considered to be the best model to study transplant immunobiology[42,46]. The vascularized allograft has an advantage in studying the mechanism of graft rejection or transplant tolerance compared to the non-vascularized allograft[47]. We have reported the effects of TM and DX on immune cells and their co-stimulatory, co-inhibitory molecules *in vitro* and *in vivo*[39-41]. This study utilizes a murine cardiac transplant model to verify the preceding findings and elucidate the immune-modulating mechanisms of TM.

The untreated BALB/c donor to C57BL/6 recipient model rejected after 6.86 ± 0.38 days, which was compatible with previously reported experiences[42,46]. DX and TM treatment failed to show graft survival benefits. Although TM/DX treatment significantly increased graft survival (10.0±0.89 days, $p<0.001$), it was shorter than we had anticipated. Therefore, to demonstrate the effect of the TM/DX combination treatment in a less immunologic model, we transplanted C57BL/6 donor allografts to BALB/c recipients. The graft survival of the C57BL/6 donor to BALB/c recipient model was 9.0±2.24 days in the untreated group and 22.5±7.37 days in the TM/DX group ($p<0.001$). The survival benefits were reproduced with more significant differences in this model.

As previously described, TM/DX treatment affected $CD4^+$ T cell subsets by down-regulating Teff cells while preserving Treg cells in both *in vitro* and *in vivo*. The results of this present study show similar results in the PBMC analysis, with TM/DX treatment significantly inhibiting $CD4^+$ Teffs ($CD4^+CD44^{hi}$). But

no difference in splenic T cell activity was noted between the treatment groups. $CD4^+$ Tregs ($CD4^+FOXP3^+$) also showed similar results as the preceding study. TM and TM/DX treatment up-regulated the $CD4^+$ Treg population compared with the untreated group and DX treatment group. DX treatment group showed no effect on the Treg population in PBMC and up-regulated the Tregs in the spleen. But combination treatment of TM/DX significantly increased the Treg counts in both sites suggesting a collaborative effect of the two drugs. The $CD4^+$ T cell population showed no difference when comparing the different treatment groups. The proportion of $CD4^+$ Teffs decreased and $CD4^+$ Tregs increased in the TM/DX group in both PBMC and spleen. In the clinical setting, the balance between graft destruction and regulation can be controlled by methods decreasing the activity of Teffs or increasing the activity of Tregs[29]. Therefore, the Treg/Teff ratio may be more crucial than the absolute number of Tregs. Our results show 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. This implies the immune-modulating effect of TM/DX therapy.

T cells are essential in allograft rejection. Both CD4 and CD8 subsets are responsible for allorecognition and allorejection[48]. The activations of $CD8^+$ T cells are traditionally thought to require the participation of $CD4^+$ T cells, but reports have suggested CD8+ may independently initiate rejection[49]. Our results show $CD8^+CD44^{hi}$ T cells increased after transplantation. TM/DX treatment significantly inhibited $CD8^+CD44^{hi}$ T cells compared to the DX treatment group in the PBMC and no significant changes were noted in the spleen between the

treatment groups. Although TM/DX treatment did not significantly inhibit $CD8^+CD44^{hi}$ T cells in both PBMC and the spleen, combination treatment may have some effect on the $CD8^+$ T cell-mediated immune responses.

Ng et al. reported B cells help alloreactive T cell differentiation, proliferation, and survival to generate optimal numbers of functional memory T cells[50]. To demonstrate the effect of TM on B cells and T cell subsets, we also analysed $CD19^+$ B cells in the murine cardiac transplant model. The $CD19^+$ B cells decreased after transplantation and additional decrement was noted with DX and TM/DX treatment in PBMC. TM treatment showed no difference with the untreated group. Therefore, we may assume that mainly DX contributes to the changes in the B cell population. Although the effects on B cells are not fully investigated, reports show B cells were susceptible to apoptosis by glucocorticoid treatments dependent on their stage of differentiation[51]. No significant changes in $CD19^+$ B cells of the spleen were noted, possibly because B cell subset analysis was not performed. T cells and its subset changes were the main objectives of this study, therefore further examination of the B cells was not done.

$CD11c^+$ cells (DCs) increased after transplantation but showed no difference in cell frequency regardless of drug treatment. One of the biomarkers expressed on tolerogenic $CD11c^+$ cells is CD85k (ILT3), an immunoglobulin-like transcripts (ILTs)[52]. On analysis of $CD11c^+CD85k^+$ cells, the MFI of the $CD85k^+$ significantly increased with TM/DX treatment in both PBMC and spleen compared to DX treatment. The enhanced expression of $CD11c^+CD85k^+$ cells with TM/DX combination may indicate the induction of tolerogenic DCs.

The cell number showed no change in the PBMC whereas the $CD11c^+CD85k^+$ cell count increased with TM/DX combination in the spleen. Tregs are developed in the thymus and extrathymic sites such as secondary lymphoid organs (SLO)[53]. Tolerogenic DCs in SLOs promote the differentiation and proliferation of Tregs[34,54,55]. The significant increase in cell frequency and MFI of $CD11c^+CD85k^+$ cells by TM/DX treatment may imply $CD11c^+CD85k^+$ cells homing to the spleen and possibly Treg induction by tolerogenic DCs[56].

IL-6 is a pleiotropic cytokine with pro-inflammatory features which is secreted by most stromal and immune cells[57]. It is a critical cytokine in innate immune response and adaptive immunity[58]. In transplantation, IL-6 plays an important part in cell-mediated rejection, antibody-mediated rejection, and chronic allograft vasculopathy[58]. Our results showed that TM/DX treatment improved allograft survival, increased the proportion of Tregs and tolerogenic DCs, and minimally suppressing B cells, validating our hypothesis of the immune-modulating effect of TM/DX combination. Therefore, we analysed the representative pro-inflammatory cytokine, IL-6 to support our theory. Interestingly, significant inhibition of IL-6 by TM treatment, and TM/DX treatment was shown. According to the literature, DX has been known to inhibit IL-6[17,59]. But DX treatment did not influence the IL-6 level. Our results suggest the decrease in IL-6 production may be attributed to the effect of TM independent of DX. Prelovsek et al. reported high dose DX inhibited the secretion of IL-6 from human muscle[60]. The minimal dose of DX in our study may explain the limited effect on IL-6.

T cells require two signals for activation, antigen-specific signal 1 through T

cell receptor (TCR) and signal 2 through co-stimulatory/co-inhibitory molecules or cytokines[61]. Past *in vitro* and *in vivo* studies in our lab have shown significant changes in co-stimulatory and co-inhibitory markers associated with the immune-modulating effects of TM[39-41]. We analysed the PD-1 and GITR to reproduce the results in the cardiac transplant model and found similar tendencies as the preceding studies. Co-stimulatory molecule GITR is a member of the TNFRSF and promotes T cell proliferation and cytokine production[62]. It is constitutively expressed on Tregs and also up-regulated in activated Teffs cells. GITR signaling appears to either regulate Treg-suppressor function or the sensitivity of Teffs to suppression by Tregs[61,63]. Our results show TM/DX treatment tends to suppress the GITR expression in $CD4^+CD44^+$ and $CD4^+FOXP3^+$ T cells after transplantation. The suppression of GITR may signify the enhancement of the regulatory immune response. Co-inhibitory molecules functions are opposite of the co-stimulatory signals. They inhibit T cell activation and are acknowledged for the induction of transplant tolerance. PD-1 belongs to the Ig superfamily and is expressed on activated $CD4^+$ and $CD8^+$ T cells and also activated B cells, NK cells, and macrophages[61,64]. Treg cells constitutively express PD-1[61]. PD-1 is a checkpoint protein that promotes apoptosis of antigen-specific T cells and reduces apoptosis of Tregs[65]. We noticed TM-based treatments increased or preserved PD-1 expression in $CD4^+CD44^+$ and $CD4^+FOXP3^+$ T cells after transplantation while DX seemed to have a limited effect. The expression of PD-1 suggests the sustained graft protective effects of Treg cells.

This research, however, is subject to some limitations. We have applied a

murine cardiac transplantation model using allografts from BALB/c (H-2^d) donors to C57BL/6 (H-2^b) recipients for our molecular analysis. This model is a fully major histocompatibility complex-mismatched model that induces acute rejection[66]. But BALB/c mice and C57BL/6 mice differ in their immunologic functional architecture and their sensitivity to antigenic stimuli[67]. Accordingly, the graft survivals of allografts from C57BL/6 (H-2^b) donors to BALB/c (H-2^d) recipients were increased compared to the initial BALB/c (H-2^d) donor to C57BL/6 (H-2^b) recipient model. As a result, the allograft survival may have been too short to effectively show the immune-modulatory effects of TM/DX treatment. This acute allograft rejection response may have resulted in similar histopathological changes despite the survival differences in each treatment group. Future studies require a less immunologic murine model which enables the TM/DX treatment to sufficiently exert its immune-modulatory effects. We speculate a chronic rejection model would be more appropriate for further studies.

B cell subset, DC subset analysis, and various pro-inflammatory and anti-inflammatory cytokines must be investigated to further clarify the mechanism of the immune-modulatory effect. To assess the complementary effects of TM/DX treatment, combination with conventional CNI and anti-metabolite therapy is also needed.

V. CONCLUSION

In conclusion, TM shows immune-modulatory features which are enhanced with the complementary combination of DX. We first noted the immune-modulatory effects in nephritis of lupus-prone mice and *in vitro* studies. Further studies with the murine cardiac transplant model resulted in improved graft survival. The TM/DX treatment affects the $CD4^+$ T cell subsets without inhibiting the total $CD4^+$ T cell population, resulting in the expansion of Treg cells. The increase in tolerogenic DCs with TM/DX was observed. The increase of tolerogenic DCs and Tregs may be interrelated but the definite mechanism is yet to be elucidated. The inhibition of pro-inflammatory cytokine IL-6 was also observed. TM/DX treatment showed various evidence of immune-modulatory effects, different from the mechanisms of the standard immunosuppressants, and graft survival benefits in the murine cardiac transplant model. Therefore, we consider TM/DX combination treatment a prospective immune-modulatory approach for preventing allograft rejection and improving graft survival by inducing tolerance in transplantation.

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ABSTRACT(IN KOREAN)

탈리도마이드의 면역세포에 대한 효과 및 면역조절에 대한 기전 규명

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김 수 진

현재 장기이식에서 사용되는 면역억제제는 급성 거부 반응을 효과적으로 억제하지만 다양한 합병증들을 동반한다. 따라서 미래 면역억제 요법에서는 면역조절 혹은 면역관용을 이용하여 이식편의 생존율 증가 및 면역억제제 관련 합병증들을 줄이고자 한다.

Thalidomide(TM)와 dexamethasone(DX)이 *in vitro*와 *in vivo*에서 면역 세포와 co-stimulatory와 co-inhibitory 분자들에 미치는 면역조절 효과에 대한 보고들이 있다. 이 연구에서 쥐 심장이식 모델에서 TM 단독 요법과 TM와 DX의 병합요법이 면역세포에 미치는 영향을 보고자 하였다.

BALB/c ($H\text{-}2^d$) 공여쥐에서 C57BL/6 ($H\text{-}2^b$) 수용쥐로 혹은

C57BL/6 공여쥐에서 BALB/c 수용쥐로 복강내 심장 동종이식을 시행하였다. 이식 수술 이후 이식편의 소실 시기까지 복강내로 TM 100 mg/kg 또는 DX 0.1 mg/kg 또는 TM와 DX 병합요법을 시행하였다.

TM/DX 병합요법을 시행한 쥐 심장이식 모델 두가지 모두에서 이식편의 생존율이 유의하게 증가하였다. BALB/c 공여쥐에서 C57BL/6 수용쥐로 심장이식을 시행한 모델에서 치료를 받지 않은 군의 평균 생존 기간은 6.89일이었으며 TM/DX 병합요법을 투여 받은 군의 평균 생존 기간은 10.0일이었다 (p<0.001). C57BL/6 공여쥐에서 BALB/c 수용쥐로 심장이식을 시행한 모델에서는 치료하지 않은 군의 평균 생존 기간은 9.0일이었으며 TM/DX 병합요법을 시행한 군의 평균 생존 기간은 22.5일이었다 (p<0.001).

TM은 면역조절 특징들이 보였으며 DX와 병합하였을 때 그 효과가 증대되었다. TM/DX 병합요법은 $CD4^+$ T 전체 세포 수에 영향을 미치지 않으면서 $CD4^+$ T 세포 아형에 영향을 끼쳤다. $CD4^+FOXP3^+$/$CD4^+CD44^{hi}$ T 세포 비율이 증가하였다. 이것은 Treg 세포의 증가를 의미한다. TM/DX 병합용법에서 관용 수지상 세포(dendritic cell: DC) ($CD11c^+CD85k^+$)의 증가도 관찰되었다. 염증 유발 사이토카인 IL-6의 억제도

관찰되었다. TM/DX 병합요법은 co-stimulatory 분자인 GITR의 표현을 억제하는 경향을 보였으며 TM 단독 요법은 이식 후 $CD4^+CD44^+$ 와 $CD4^+FOXP3^+$ T 세포에서 co-inhibitory 분자 PD-1의 표현을 증가시키거나 보존시켰다. TM/DX 병합요법에서는 선택적인 T 세포 아형 변화, 관용 DC를 유도, IL-6의 억제 그리고 이식편의 생존율 증가가 관찰되었다. 이러한 결과들은 TM/DX 병합요법의 면역조절 기능을 시사한다. 따라서 TM/DX 병합요법은 면역관용을 유도하여 장기이식에서 이식편의 거부반응 예방 그리고 이식편의 생존율 향상을 시키는 면역조절 요법으로써 가능성이 보인다.

핵심 되는 말: 면역조절, 탈리도마이드, dexamethasone, T 세포, 수지상 세포, GITR, PD-1, 심장이식