



Systemic Immunomodulatory Effects of Combinatorial Treatment of Thalidomide and Dexamethasone on T Cells and Other Immune Cells

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Purpose: In organ transplantation, the need for immune modulation rather than immune suppression has been emphasized. In this study, we investigated whether combinatorial treatments of with thalidomide (TM) and dexamethasone (DX) might be new approaches to induce systemic immunomodulation on T cells and other immune cells that regulate the expression of co-inhibitory molecules.

Materials and Methods: Naïve splenic T cells from C57BL/6 mice were sort-purified and cultured in vitro for CD4⁺ T cell proliferation and regulatory T cell (Treg) conversion in the presence of TM or/and DX. Expression of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1) in proliferated and converted T cells was quantified by flow cytometry. We also quantified in vivo expression of CTLA-4 and PD-1 on splenic CD4⁺ T cells and other immune cells isolated from TM- or/and DX-treated mice. Mixed lymphocytes reactions (MLR) were performed to evaluate the capacity of immune cells in carrying out immune responses.

Results: CTLA-4 expressions in effector T cells in vivo and in Tregs in vivo/vitro significantly increased upon TM/DX combinatorial treatment. Corresponding to increased CTLA-4 expression in T cells, the expression of ligand molecules for CTLA-4 significantly increased in splenic dendritic cells in TM/DX-treated groups. In addition, MLR results demonstrated that splenocytes isolated from TM/DX-treated mice significantly suppressed the proliferation of T cells isolated from other strains.

Conclusion: Based on these results, we suggest that TM/DX combinatorial treatments might be efficient immunomodulatory methods for regulating T cell immunity.

Key Words: Immunomodulation, thalidomide, dexamethasone, CTLA-4 antigen, T-lymphocytes, dendritic cells

INTRODUCTION

Reducing allograft rejection is a critical issue in organ transplan-

tation, and the use of immunosuppressants is nearly inevitable for patients with autoimmune diseases or those undergoing transplantation. However, most immunosuppressants in

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•The authors have no potential conflicts of interest to disclose.

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clinical use deplete lymphocytes, divert lymphocyte traffic, or block lymphocyte response pathways, resulting in diverse side effects that pose serious risks for infection.¹⁻³ Recognizing the need for immune modulation instead of immune suppression, many current studies have focused on developing new methods or drugs that enable immunomodulation.^{4,5} Our previous study introduced thalidomide (TM) and dexamethasone (DX) combinatorial treatment, which regulates specific T cell subsets through activation of co-stimulatory signals, as a new method for immunomodulation.⁶

TM was first prescribed as a sedative, but was quickly withdrawn due to teratogenic effects; however, several studies have reported its anti-inflammatory and anti-angiogenic properties as a potent immunosuppressant.⁷⁻¹¹ Recent studies have revealed that TM exhibits immune-modulating activity by regulating central immune-modulatory molecules and pro-inflammatory cytokines.¹²⁻¹⁷ DX, a synthetic glucocorticoid (GC), is also an effective immunosuppressant that inhibits inflammation and reduces inflammatory cytokine production.¹⁸⁻²⁰ The side effects of GCs, such as impairment of anabolic processes, have limited its clinical use,²¹⁻²³ although administration of small doses of DX as a complementary agent could enhance its immunosuppressive properties. Many studies, including our previous research, have shown that combinatorial therapies involving TM and DX are promising approaches for both treatment of immune-related diseases and post-transplant immunomodulation.^{6,13,24-26} However, the mechanisms underlying the effects of TM and DX combinatorial treatments remain to be elucidated. Our previous study demonstrated that TM and DX combinatorial treatments regulate co-stimulatory signaling induced by CD4⁺ T cells, but how these combinatorial treatments affect co-inhibitory signals and how co-inhibitory signaling between T cells and other immune cells modulate immune responses remain unclear.

Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1) are well-known co-inhibitory regulators of T cell function. PD-1 is expressed primarily on T cells, and by binding to its ligands, programmed death-ligand 1 and 2 (PD-L1 and PD-L2, respectively), PD-1 inhibits T cell expansion and proliferation, and regulates T cell exhaustion.²⁷⁻³⁰ Co-inhibitory signaling via the PD-1 pathway also suppresses previously activated T cells in their effector phase, which regulates T cell tolerance.^{30,31} As ligation of PD-1 facilitates the generation of regulatory T cells (Tregs) and reduces the number of effector T cells (Teffs), PD-1 signaling is a key immunomodulatory pathway. Meanwhile, CTLA-4 plays an important role in the maintenance of T cell homeostasis. By competitively binding to B7 on antigen-presenting cells, ligation of CTLA-4 prevents the co-stimulatory signaling normally provided by ligation of CD28 and B7, resulting in T cell anergy.^{30,32} CTLA-4 is constitutively expressed on Tregs, and ligation of CTLA-4 is thought to be critical for Treg suppression of Teff function in modulation of T cell immune responses.³³

In this study, we examined the effects of TM and DX combinatorial treatments on T cell modulation through co-inhibitory signaling. In addition, we investigated enhanced systemic immunomodulation by examining changes in molecular expressions on immune cells, including dendritic cells (DCs), and by demonstrating their capacity to carry out immune responses upon TM/DX combinatorial treatment.

MATERIALS AND METHODS

Mice and reagents

Seven-week-old male C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained according to national and institutional ethical guidelines. The *in vivo* experimental research proposal was approved by the Committee on Animal Investigation of Yonsei University (IACUC 2018-0199), and *in vivo* experiments were performed in accordance with the Laboratory Animals Manual and the Laboratory Animal Care and Use Committee, edited by the National Research Council of the National Animal Society.

Anti-mouse CD3, anti-mouse CD28, phycoerythrin (PE)-cyanine (Cy)7-conjugated anti-mouse CD8, peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated anti-mouse CD11c, PerCP-Cy5.5-conjugated anti-mouse CD19, fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD44, PerCP-Cy5.5-conjugated anti-mouse CD62 ligand (CD62L), PE-conjugated anti-mouse CD85k, FITC-conjugated anti-mouse forkhead box P3 (FOXP3), PE-conjugated anti-mouse CTLA-4, and allophycocyanin (APC)-conjugated anti-mouse PD-1 antibodies, as well as the fixation/permeabilization kit, were purchased from eBioscience (San Diego, CA, USA). APC-Cy7-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD80, PE-Cy7-conjugated anti-mouse CD86, and APC-conjugated anti-mouse PD-L1 antibodies were purchased from BioLegend (San Diego, CA, USA). Carboxyfluorescein diacetate succinimidyl ester (CFSE; CellTrace™) was obtained from Invitrogen (Carlsbad, CA, USA), and 70- μ m cell strainers were purchased from BD Biosciences (Franklin Lakes, NJ, USA). TM, DX, red blood cell (RBC) lysis buffer, and mitomycin C were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of naïve T cells (Tnaïves)

After harvesting the spleens from 8-week-old male C57BL/6 mice, splenocytes were homogenized using a 70- μ m cell strainer and incubated in RBC lysis buffer for 5 min to obtain a high-purity preparation. Isolated splenocytes were stained with APC-Cy7-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD44, and PerCP-Cy5.5-conjugated anti-mouse CD62L antibodies, and the cells were sorted by gating on CD4⁺, CD44^{lo}, and CD62L^{hi} using a fluorescence-activated cell sorting (FACS) Aria III Cell Sorter (BD Biosciences).

Cell culture

Naïve T cells (Tnaïves) (1.5×10^5) were seeded on 96-well plates with TM (0, 1, or 10 μM), DX (0, 0.1, or 1 nM), or combinations of TM/DX, and incubated for 72 h under the standard culture conditions (37°C, 5% CO_2) for Teff proliferation and Treg differentiation.

For differentiation of Tnaïve to Teff, CFSE-stained Tnaïves were stimulated during the entire culture period with anti-mouse CD3 (5 $\mu\text{g}/\text{mL}$) and anti-mouse CD28 (2 $\mu\text{g}/\text{mL}$) antibodies, which were added to Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum, 100 U/mL of penicillin, 100 $\mu\text{g}/\text{mL}$ of streptomycin (Invitrogen), and 200 mM L-glutamine and 50 mM β -mercaptoethanol (Sigma-Aldrich).

To induce Treg differentiation, Tnaïves were cultured with anti-mouse CD3 (5 $\mu\text{g}/\text{mL}$) and anti-mouse CD28 (2 $\mu\text{g}/\text{mL}$) antibodies, interleukin (IL)-2 (10 ng/mL), and transforming growth factor (TGF)- β 1 (50 ng/mL) under the same conditions in complete RPMI medium as for the Teff proliferation protocol.

In vivo treatment with TM and DX

In order to elucidate the effect of TM/DX treatments on the systemic immune system, changes in the expression of immune molecules on splenic lymphocytes and DCs were examined in vivo using normal C57BL/6 mice treated with TM, DX, or TM/DX combinations. The experimental groups were fed chow containing TM, DX, or TM/DX for 1 week, whereas the control group was fed regular chow. TM and DX were administered at doses of 10 and 1.5 mg/kg body weight per day, respectively, based on our previous research.⁶

Mixed lymphocyte reaction

Mixed lymphocyte reactions (MLR) were performed in order to evaluate the functional and systemic activities of immune cells upon TM and DX combinatorial treatments. Splenocytes isolated from TM- or/and DX-fed normal male C57BL/6 mice were used as stimulators, and sort-purified T cells from normal male BALB/c mice were prepared as responders. T cells for responders were isolated using Pan T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany) according to the manufacturer's instructions. Stimulators were treated with mitomycin C in order to suppress further proliferation, and the treatment of the cells was performed according to the manufacturer's instructions. Responder cells were also labelled with CFSE according to the manufacturer's instructions. After washing, mitomycin C-treated stimulators and CFSE-labelled responders were resuspended in complete RPMI medium as indicated for the Teff proliferation protocol. 4×10^5 stimulator cells/well and 4×10^5 responder cells/well were plated and mixed in round bottom 96-well plates, and maintained for 4 days under standard culture conditions.

Flow cytometry

Cultured T cells from in vitro experiments were collected and

incubated with appropriate diluted antibodies for 40 min at 4°C. In order to detect changes in the expression of co-inhibitory molecules upon drug treatment, surface markers were stained with PE-conjugated anti-mouse CTLA-4 and APC-conjugated anti-mouse PD-1 antibodies. Cells cultured for Teff proliferation were pre-stained with CFSE to determine proliferation levels, and cells cultured for Treg differentiation were fixed and permeabilized using a fixation/permeabilization kit (eBioscience) and then stained intracellularly with FITC-conjugated anti-mouse FOXP3 antibody.

Homogenized splenocytes from in vivo experiments were also incubated with appropriate diluted antibodies for 40 min at 4°C. Both splenic CD4^+ Teffs and Tregs were stained with CTLA-4 and PD-1 antibodies. Splenic CD4^+ Teffs were stained with APC-Cy7-conjugated anti-mouse CD4 and FITC-conjugated anti-mouse CD44, whereas splenic CD4^+ Tregs were fixed/permeabilized after staining with CD4 antibody for intracellular FOXP3 staining. In order to examine the effects of the drug treatments on the functional properties of immune cells, splenic CD8^+ T cells were stained with PE-Cy7-conjugated anti-mouse CD8; B cells were stained with PerCP-Cy5.5-conjugated anti-mouse CD19; and splenic DCs were stained with PerCP-Cy5.5-conjugated anti-mouse CD11c, FITC-conjugated anti-mouse CD80, PE-conjugated anti-mouse CD85k, PE-Cy7-conjugated anti-mouse CD86, and APC-conjugated anti-mouse PD-L1 antibodies. Fixation/permeabilization and staining steps were performed according to the manufacturer's instructions.

Splenocytes from MLR experiments were collected and stained with appropriately diluted APC-Cy7-conjugated anti-mouse CD4 and PE-Cy7-conjugated anti-mouse CD8 antibodies for 40 min at 4°C.

Splenocytes in the form of doublets were eliminated and populations for target subsets were gated with appropriate gating strategies (Supplementary Figs. 1–4, only online).

Flow cytometry was performed using an LSR II, FACS Verse I, or FACS Verse II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software, v10.0.7 (Tree Star, Inc., San Carlos, CA, USA). Expression of the co-inhibitory molecules and functional markers is presented as relative median fluorescence intensity (MFI).

Statistical analysis

The significance of intergroup differences was determined using Student's t-test or one-way analysis of variance. Statistical analyses were conducted using Sigma plot 2.0 (Systat Software Inc., San Jose, CA, USA), and p values < 0.05 were considered statistically significant.

RESULTS

Expression of PD-1 and CTLA-4 on CD4⁺ Teffs and Tregs in vitro

The expression of PD-1 and CTLA-4 on proliferated Teffs did not change significantly upon treatment with either TM (1 or 10 μM), DX (0.1 or 1 nM), or TM/DX combinations (TM 1 μM+DX 0.1 nM, TM 10 μM+DX 0.1 nM, TM 1 μM+DX 1 nM, and TM 10 μM+DX 1 nM) (Fig. 1). The expression of PD-1 on differentiated Tregs also did not change significantly with the combinatorial treatments; however, CTLA-4 expression on Tregs increased significantly with the combinatorial treatments in a

TM dose dependent manner (Fig. 2A and B). The histogram for CTLA-4 on Tregs shown in Fig. 2C indicates a significant right-ward shift with increasing TM dose in the combinatorial treatments.

Expression of PD-1 and CTLA-4 on CD4⁺ Teffs and Tregs in vivo

PD-1 expression on splenic Teffs increased with TM treatment and decreased with DX treatment, but combinatorial administration of TM and DX had no effect on PD-1 expression on Teffs (Fig. 3A). In contrast to in vitro Teff results in which no change was observed, the expression of CTLA-4 on splenic Teffs

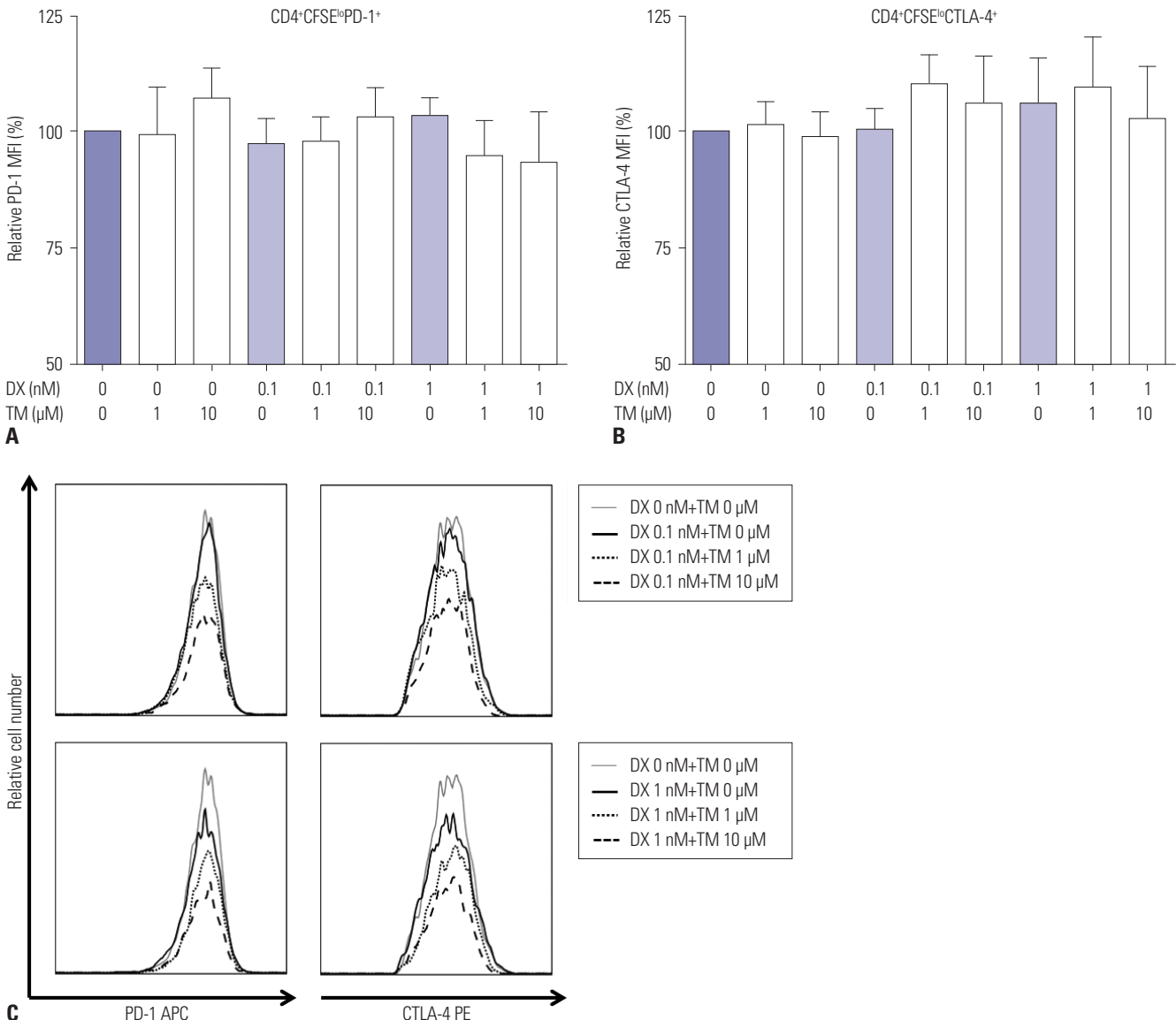


Fig. 1. Expression of inhibitory molecules on proliferated CD4⁺ T cells induced by TM, DX, or TM/DX combinatorial treatment. Tnaïves labelled with CFSE were incubated with anti-CD3 and anti-CD28 antibodies for 72 h for proliferation. Proliferated CD4⁺ T cells were collected, stained with fluorescence-conjugated anti-PD-1 and anti-CTLA-4 antibodies, and analyzed by flow cytometry. Expression of (A) PD-1 (CD279) and (B) CTLA-4 (CD152). (C) Histogram showing changes in expression levels of PD-1 and CTLA-4 upon drug treatments. Changes in protein expression were analyzed by FACS gated on CD4⁺ CFSE⁺ T cells, which were viewed as Teffs by FACS analysis using relative MFI (% of non-treated controls, n=5). TM, thalidomide; DX, dexamethasone; Tnaïves, naïve T cells; CFSE, carboxyfluorescein diacetate succinimidyl ester; FACS, fluorescence-activated cell sorting; MFI, median fluorescence intensity; APC, allophycocyanin.

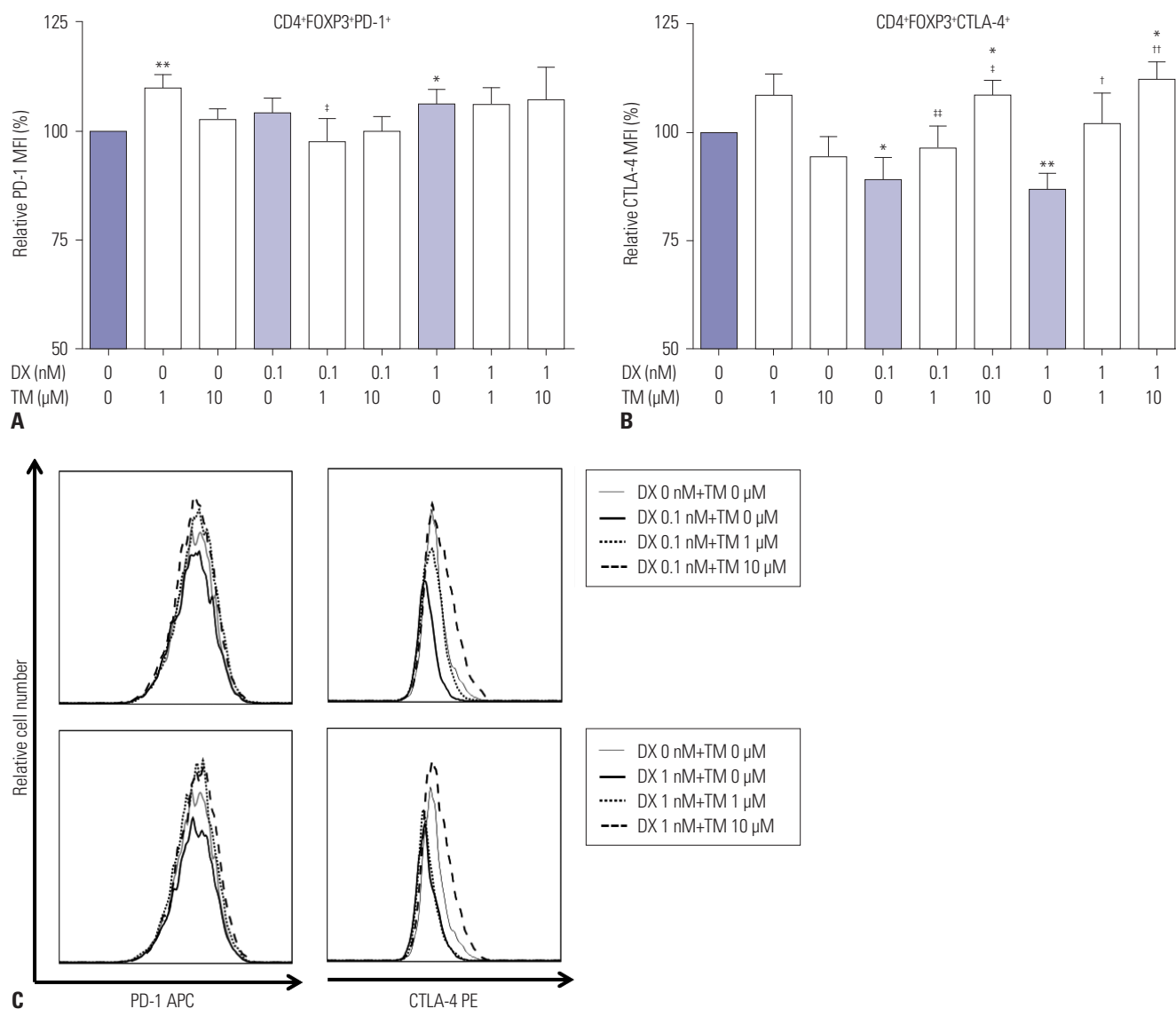


Fig. 2. Expression of inhibitory molecules on differentiated CD4⁺ FOXP3⁺ T cells induced by TM, DX, or TM/DX combinatorial treatment. Tnaïves were incubated with anti-CD3 and anti-CD28 antibodies, TGF- β , and IL-2 for 72 h for differentiation. Differentiated CD4⁺ FOXP3⁺ T cells were collected, stained with fluorescence-conjugated anti-PD-1 and anti-CTLA-4 antibodies, and analyzed by flow cytometry. Expression of (A) PD-1 (CD279) and (B) CTLA-4 (CD152). (C) Histogram showing changes in expression levels of PD-1 and CTLA-4 upon drug treatments. Changes in protein expression were analyzed by FACS using relative MFI (% of non-treated controls, n=5). * p <0.05 and ** p <0.01 compared to non-treated controls; [†] p <0.05 and ^{††} p <0.01 compared to 0.1 nM DX treatment; and [†] p <0.05 and ^{††} p <0.01 compared to 1 nM DX treatment. TM, thalidomide; DX, dexamethasone; Tnaïves, naïve T cells; TGF, transforming growth factor; IL, interleukin; FACS, fluorescence-activated cell sorting; MFI, median fluorescence intensity; APC, allophycocyanin.

increased significantly with TM/DX combinatorial treatment (Fig. 3B). In vivo expression of PD-1 on Tregs differed from its expression in vitro: in vivo expression on splenic Tregs exhibited significant changes in the TM, DX, and TM/DX treatment groups (Fig. 3C). Expression of CTLA-4 on splenic Tregs increased significantly with TM/DX combinatorial treatment, consistent with the in vitro analysis (Fig. 3D).

Changes in populations of splenic immune cells

To obtain a better understanding of the discrepancies between in vivo and in vitro molecular expression of co-inhibitory molecules on Tregs and Tefs, the effects of TM, DX, and TM/DX

combinatorial treatments on other immune cells were examined in vivo. The population of splenic CD8⁺ T cells increased significantly in the DX and TM/DX groups, although the combinatorial effects were attributed only to DX (Fig. 4A and D). In contrast to CD8⁺ T cells, the splenic CD19⁺ B cell population decreased significantly in the DX and TM/DX combinatorial groups (Fig. 4B and D). Interestingly, the population of splenic CD11c⁺ DCs increased significantly upon TM/DX combinatorial administration, while TM or DX treatment did not change the population (Fig. 4C and D).

In addition to cell population expansion, the MFI values of the cell-specific molecular markers were examined to demon-

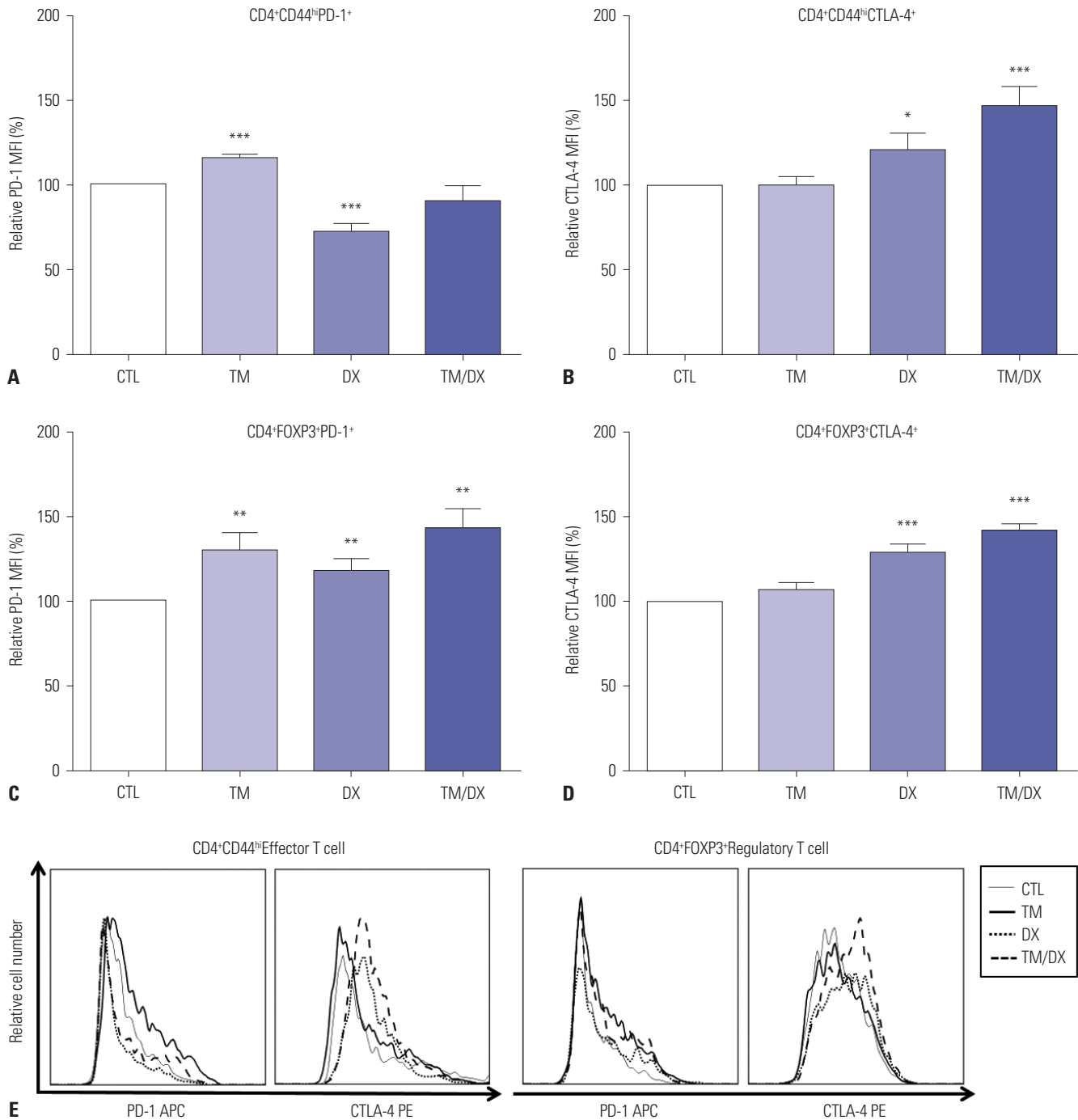


Fig. 3. In vivo expression of inhibitory molecules on effector T cells and regulatory T cells induced by TM, DX, or TM/DX combinatorial treatment. Mice were provided chow containing TM (10 mg/kg), DX (1.5 mg/kg), or TM and DX (10 and 1.5 mg/kg, respectively) daily for 7 days. The spleen was isolated, homogenized into splenocytes, and stained with appropriate antibodies for flow cytometry analysis. (A) PD-1 expression on effector T cells. (B) CTLA-4 expression on effector T cells. (C) PD-1 expression on regulatory T cells. (D) CTLA-4 expression on regulatory T cells. (E) Histogram showing changes in expression levels of PD-1 and CTLA-4 on effector and regulatory T cells upon drug treatments. Changes in protein expression were analyzed by FACS using relative MFI (% of non-treated controls, n=6). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to non-treated controls. CTL, control; TM, thalidomide; DX, dexamethasone; FACS, fluorescence-activated cell sorting; MFI, median fluorescence intensity; APC, allophycocyanin.

strate changes in expression of each marker. While it might be controversial to assert that MFI values directly reflect the function of cells that express certain markers, it is also known that expression levels of CD8, CD19, and CD11c on T cells, B cells, and DCs, respectively, alter significantly when immune respons-

es are required and influence their functional properties.³⁴⁻³⁹

CD8 expression on splenic CD8⁺ T cells increased in both the DX and TM/DX combinatorial treatment groups. Although the population of splenic CD8⁺ T cells decreased with the combinatorial treatments in comparison to DX treatment alone, CD8

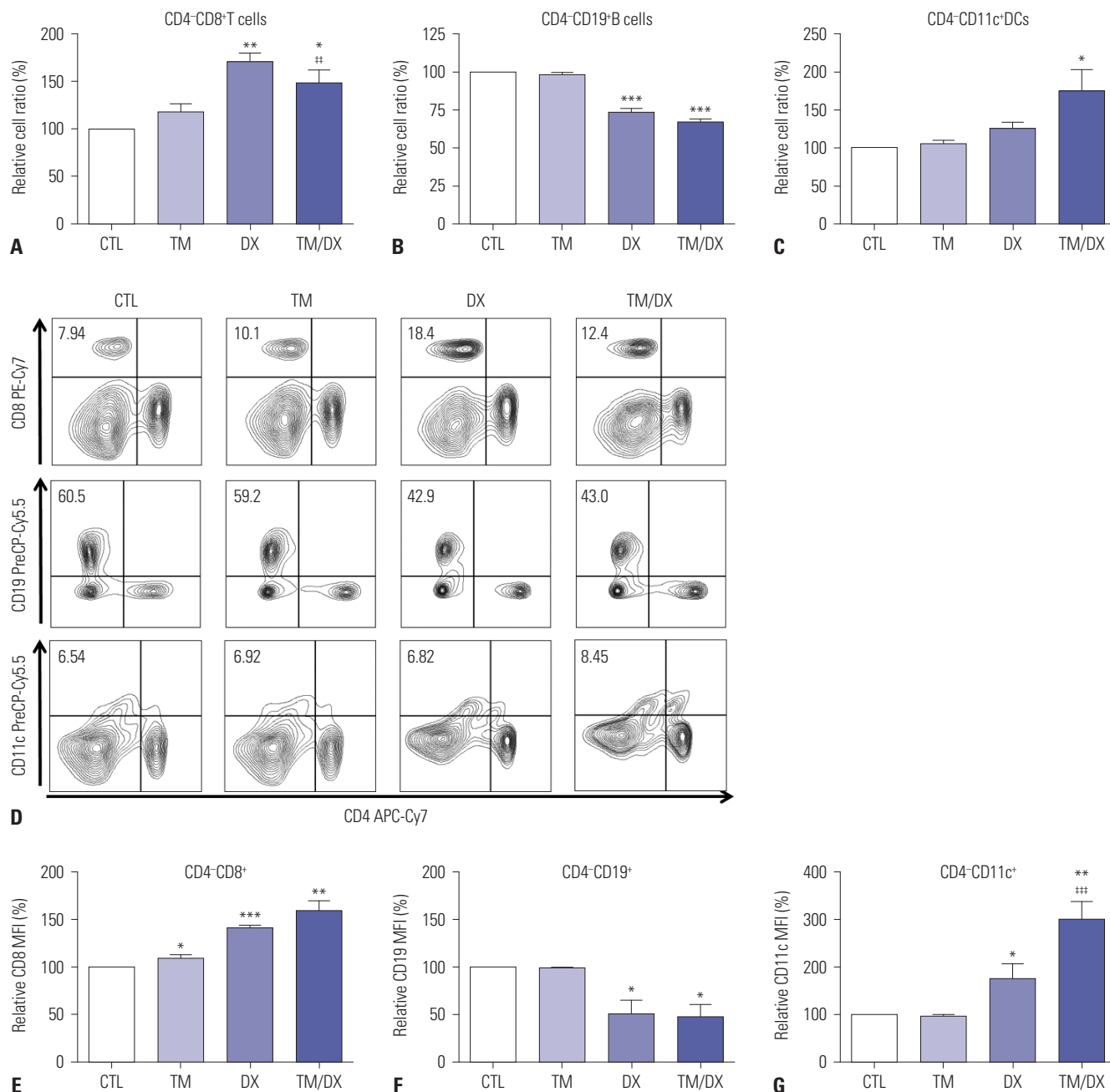


Fig. 4. Changes in splenic immune cells upon TM, DX, or TM/DX combinatorial treatment. In order to characterize systemic effects on inhibitory molecules in effector T cells and regulatory T cells in vivo, the effects of TM, DX, and TM/DX treatments on CD8⁺ T cells, CD19⁺ B cells, and CD11c⁺ DCs were examined. (A) Ratio of CD4⁺ CD8⁺ T cells. (B) Ratio of CD4⁺ CD19⁺ B cells. (C) Ratio of CD4⁺ CD11c⁺ DCs. (D) Contour plots representing population changes in CD8⁺ T cells, CD19⁺ B cells, and CD11c⁺ DCs by flow cytometry analysis. The results shown are representative plots selected from independent experiments. Expression of (E) CD8 on CD4⁺ CD8⁺ T cells, (F) CD19 on CD4⁺ CD19⁺ B cells, and (G) CD11c on CD4⁺ CD11c⁺ DCs analyzed by FACS using relative MFI (% of non-treated controls, n=6). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to non-treated controls, and # $p < 0.01$ and ## $p < 0.001$ compared to DX-treated group. CTL, control; TM, thalidomide; DX, dexamethasone; DC, dendritic cell; FACS, fluorescence-activated cell sorting; MFI, median fluorescence intensity; APC, allophycocyanin.

expression increased slightly in the TM/DX treatment group (Fig. 4E). Consistent with the B cell population results, CD19 expression on splenic B cells decreased significantly with DX and TM/DX treatment (Fig. 4F). Interestingly, the expression of CD11c on splenic DCs did not change with TM treatment but increased with DX treatment; compared to the control group,

the expression of CD11c increased significantly (~3-fold) with TM/DX combinatorial treatment. These results suggest that not only are splenic DC populations increased, but also the functional properties of DCs are significantly affected in a synergistic manner with TM/DX combinatorial treatment (Fig. 4G).

Changes in DC characteristics

Splenic DCs are one of the most-efficient types of antigen-presenting cells, and analysis of changes in splenic DCs characteristics might best systemically explain the observed changes in splenic T lymphocytes upon TM/DX combinatorial treatment. In addition, a significant increase in the molecular expression of the DC marker CD11c better reflected the effects of TM/DX combinatorial treatment, compared with the other immune cell types, including CD8⁺ T cells and B cells. Therefore, splenic

DCs were examined in greater detail in order to systemically elucidate how drug administration affects DC-associated immune responses. The expression of PD-L1, a ligand for PD-1, significantly decreased in splenic DCs in the DX and TM/DX combinatorial treatment groups (Fig. 5A and E). However, the expression of CD80 and CD86, which are two ligands for CTLA-4, significantly increased in splenic DCs in the DX and TM/DX combinatorial treatment groups (Fig. 5B, C, and E).

To determine whether the characteristics of the DCs changed

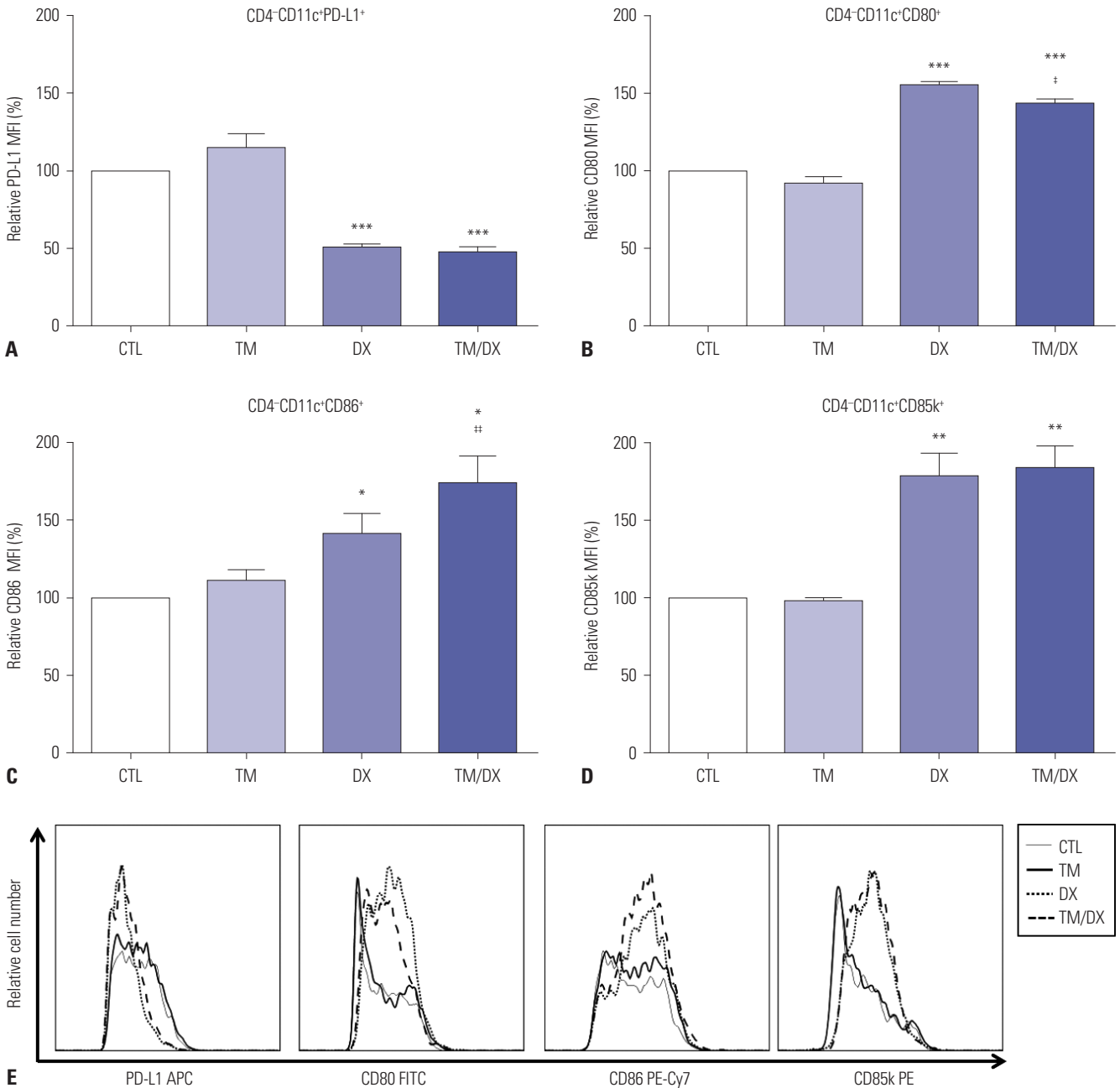


Fig. 5. Changes in DC characteristics. Expression of inhibitory and functional molecules on splenic DCs was examined. Expression of (A) PD-L1, (B) CD80, (C) CD86, and (D) CD85k was analyzed by FACS using relative MFI (% of non-treated controls, n=6). (E) Histogram representing changes in the expression levels of PD-L1, CD80, CD86, and CD85k upon drug treatments. **p*<0.05, ***p*<0.01, and ****p*<0.001 compared to non-treated controls, and †*p*<0.05 and ‡*p*<0.01 compared to DX-treated group. CTL, control; TM, thalidomide; DX, dexamethasone; DC, dendritic cell; FACS, fluorescence-activated cell sorting; MFI, median fluorescence intensity; APC, allophycocyanin; FITC, fluorescein isothiocyanate.

in response to the various treatments, expression of one tolerogenic DC marker, CD85k, was examined. Upon treatment with DX or TM/DX combinations, CD85k expression significantly increased, compared to the control group (Fig. 5D and E).

Functional evaluations of immune cells through MLR
MLR is a reliable T lymphocyte proliferation assay that provides information how unprimed T cells are affected and activated by cell-associated antigens. As the significant increases in the molecular expression of the CTLA-4 ligands CD80 and CD86 in DCs warranted further assessment, since these molecules conduct co-stimulatory signals under certain conditions, we considered that proliferation assay would be helpful to understand overall cell level immunocompetence. Also, the discrepancy in the molecular expression data from *in vitro* and *in vivo* experiments needed more explanation. Therefore, in order to assess the systemic immune responses elicited by the drug treatments, the differences in induction levels of proliferative responses between TM or/and DX groups were observed through MLR.

When responder cells were mixed with stimulator cells isolated from TM-treated or DX-treated mice, the responders did not show changes in proliferation level of CD4⁺ T cells, compared to those mixed with stimulators from control mice. Interestingly, the proliferation level of CD4⁺ T cells from responders decreased significantly when mixed with stimulators from TM/DX combinatorial treated mice (Fig. 6A and C). The proliferation level of CD8⁺ T cells showed no differences between the groups and was not affected when mixed with TM or/and DX treated stimulators (Fig. 6B and C).

DISCUSSION

Most immunosuppressants currently in clinical use can cause serious side effects, and thus, it is important to develop and introduce immunomodulators that function differently than current immunosuppressants. Many studies have reported new methods for immunomodulation that involve regulating a patient's immune system without simply depleting immune cells, and the results of our previous study also suggested that the use of TM/DX combinatorial treatments represents a promising new immunomodulatory approach.

Our previous study reported that TM/DX combinatorial treatment affects Teff and Treg population differently, suppressing Teffs while preserving Tregs, and we found that these effects result from TM/DX-mediated changes in the expression of co-stimulatory molecules on T cells. Co-inhibitory signaling mediated by PD-1 and CTLA-4 plays a critical role in determining the fate of T cells, as they determine whether T cells proliferate, expand, or are exhausted.^{28,30,31} As well as CD4⁺ T cells that conduct co-stimulatory or co-inhibitory signals, other immune cells, including CD8⁺ T cells, B cells, and DCs, play critical roles in

inducing immune responses. This study sought to further enhance understanding of the immunomodulatory effects of TM/DX combinatorial treatment by examining the expression of co-inhibitory molecules on T cells and evaluating its systemic effects on immune cells.

The results in Figs. 1 and 2 show that the expression of other co-inhibitory molecules experienced little change in both Teffs and Tregs *in vitro*, whereas CTLA-4 expression on Tregs increased significantly upon TM/DX combinatorial treatment. Although other factors might substitute in its absence, CTLA-4 still plays critical roles in Treg function, such as eliciting the Treg-suppression activity of Teffs.^{33,40,41} *In vitro* results strengthened our hypothesis that TM/DX combinatorial treatment modulates T cell immunity by enhancing the function of Tregs via the CTLA-4 co-inhibitory pathway. Although other research has shown that the expression of CTLA-4 tends to increase *in vivo* with DX treatment,^{42,43} the decreases in CTLA-4 expression mediated by DX in our *in vitro* experiments can be attributed to the experimental setting, in which systemic indirect influences by other immune cells were absent.

In contrast to the *in vitro* results, more changes in the expression of co-inhibitory molecules on splenic Tregs and Teffs were observed *in vivo* (Fig. 3). CTLA-4 expression in Teffs increased significantly with TM/DX combinatorial treatment. Since the primary role of CTLA-4 in Teffs is to inhibit their activation and diminish their function by blocking the CD28 co-stimulation pathway,^{32,44} increases in CTLA-4 expression in Teffs might result in the regulation of active T cells. In Tregs, both CTLA-4 and PD-1 expression significantly increased upon combinatorial treatment, compared to control. PD-1 signaling promotes Treg development and enhances Treg function,²⁸ and increases in expression of both molecules in Tregs after TM/DX combinatorial treatment might directly further extend Treg development and Teff-suppressing activity, resulting in subsequent modulation of T cell immunity.

In order to understand the discrepancy between the *in vivo* results, which exhibited more distinct changes in co-inhibitory molecule expression, and the *in vitro* results, we examined systemic changes of immune cells upon TM/DX combinatorial treatment *in vivo* and performed MLR. As seen in Fig. 4, while changes in populations and expression of molecular markers of CD8⁺ T cell and CD19⁺ B cell might be attributed to the effect of DX, increases in CD11c⁺ DC populations and increases in the expression of the DC function-associated surface marker CD11c were significantly enhanced in a synergistic manner with TM/DX combinatorial treatment, compared to even DX groups.

Focusing on DCs, we examined the expression of co-inhibitory and functional molecules on splenic DCs to systemically delineate the immune response after drug administration. Fig. 5 shows that the expression of PD-L1 on DCs significantly decreased after TM/DX treatment, which appeared to be inconsistent with previously reported *in vivo* Treg results demonstrating increased PD-1 expression. However, as a complement

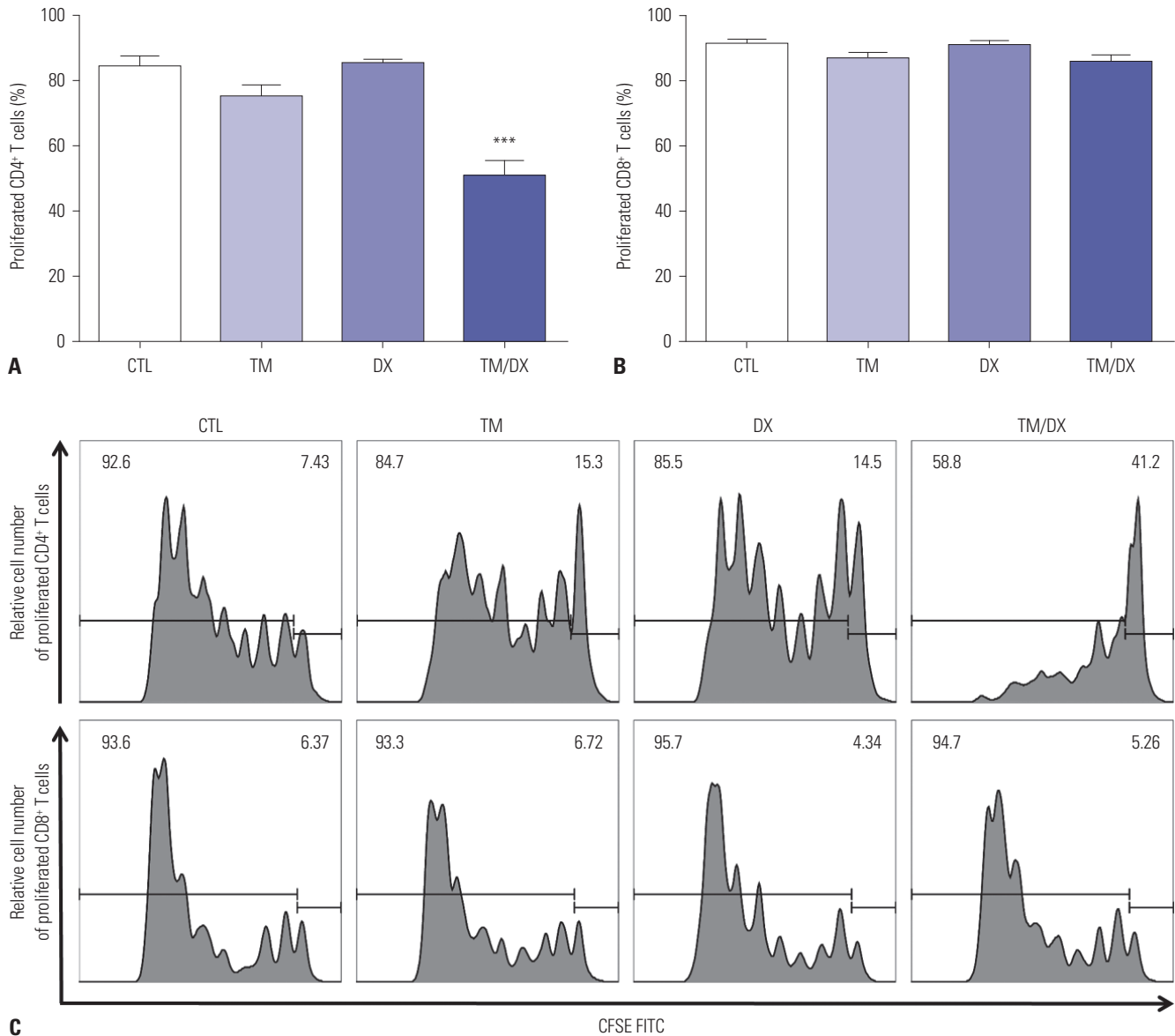


Fig. 6. Suppression of T cell proliferation during mixed lymphocyte reaction experiments. Differences in induction of T cell proliferation between splenocytes isolated from TM or/and DX-treated mice were examined. (A) Ratios of live proliferated CD4⁺ T cells and (B) ratios of live proliferated CD8⁺ T cells (% of non-treated control, n=6). (C) Histogram representing suppressive function of drug-fed stimulators on responders. ****p*<0.001 compared to non-treated controls. CTL, control; TM, thalidomide; DX, dexamethasone; CFSE, carboxyfluorescein diacetate succinimidyl ester; FITC, fluorescein isothiocyanate.

to the decrease in PD-L1 expression, the expression of ligands for CTLA-4, CD80 and CD86, increased significantly upon TM/DX combinatorial treatment, enabling co-inhibitory signaling via the CTLA-4 pathway. The expression of the tolerogenic DC marker CD85k, which is known to bind to one of many human leukocyte antigen classes to increase immune tolerance and blunt immune recognition,^{45,46} significantly increased, and this could suggest that tolerogenic characteristics of DCs were induced by TM/DX treatment.

Fig. 6 further explains the discrepancy between the in vivo and in vitro results and provides clues on systemic immune responses after TM/DX treatments that analysis of molecular expression on splenic DCs could not fully elucidate. The MLR

results highlighted significant suppression of CD4⁺ T cell proliferation by splenocytes only from TM/DX treated mice, but not from those from other groups. This could indicate that TM/DX treated immune cells possess the capacity to inhibit CD4⁺ T cell proliferation, which might be one of the most important abilities an immunomodulator has and which might be strong evidence that TM/DX combinatorial treatment is a clinically promising therapy for patients with autoimmune disease or transplant surgery. Assuming the TM works synergistically to expand the effects of DX, we will conduct research on glucocorticoid-related molecules, such as tumor necrosis factor receptor superfamily, in order to propose potential mechanisms, mainly focusing on very efficient antigen-presenting cells, like DCs.

In conclusion, TM/DX combinatorial treatment increased the expression of co-inhibitory signal-conducting molecules, particularly CTLA-4, in CD4⁺ Tregs and Tregs and also increased the expression of ligand molecules for CTLA-4 on DCs. Moreover, TM/DX combinatorial treatment appeared to influence DCs and other immune cells' characteristics, and their alterations resulted in overall systemic immunomodulation. MLR results clearly demonstrated suppressive functions on T cell proliferation and provided supportive evidence of the systemic immunomodulatory effects of TM/DX. In the future, elucidating the effects of TM/DX combinatorial treatment on several different types of DCs might clarify the mechanisms responsible for the effects of TM/DX on the immune system. Based on our results, we propose TM/DX combinatorial treatment as an efficient immunomodulatory approach for modulating T cell immunity.

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