Generation of tolerogenic dendritic cells from *Fcgr2b* deficient lupus-prone mice for the model of SLE therapy

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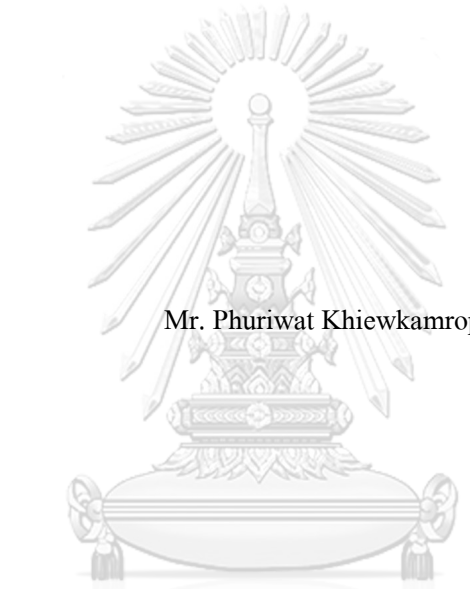

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การสร้างโทเรอโรจีนิกเดนไดรติกเซลล์จากหนูเมาส์ลูปัสที่ไม่มียีน *Fcgr2b* สำหรับเป็นแบบจำลองในการรักษาโรคเอสแอลอี

นายภูริวัฒน์ เขียวคำรพ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ภูริวัฒน์ เขียวคำรพ : การสร้างโทเรอโรจีนิกเดนไดรติกเซลล์จากหนูเมาส์ลูปัสที่ไม่มียีน *Fcgr2b* สำหรับเป็นแบบจำลองในการรักษาโรคเอสแอลอี. (Generation of tolerogenic dendritic cells from *Fcgr2b* deficient lupus-prone mice for the model of SLE therapy) อ.ที่ปรึกษาหลัก : ศ. ดร. พญ.ณัฏฐิยา หิรัญกาญจน์, อ.ที่ปรึกษาร่วม : รศ. ดร. ทญ.พัชรี ฤทธิ์ประจักษ์

Tolerogenic dendritic cells (tolDCs) เป็นหนึ่งในเซลล์ของภูมิคุ้มกันที่มีมาแต่กำเนิด โดยมีฟังก์ชันควบคุมการตอบสนองทางภูมิคุ้มกันควบคุมการทำงานของทีเซลล์ ชนิด (Tregs) ตลอดจนยับยั้งการตอบสนองของภูมิคุ้มกัน ปัจจุบันการรักษาโดยใช้ tolDCs ได้กลายเป็นหนึ่งในแนวทางของการรักษาที่มีแนวโน้มที่ดีสำหรับการปลูกถ่ายอวัยวะและโรคแพ้ภูมิตัวเอง FcgRIIB เป็นหนึ่งในรีเซพเตอร์แบบยับยั้งที่แสดงออกในบีเซลล์ เซลล์ไมอิลอยด์ และเซลล์เดนไดรต์ติค ความผิดปกติของ FcgRIIB ในสัตว์ทดลองพบว่ามีการแสดงอาการของโรคที่คล้ายลูปัสหรือภาวะภูมิคุ้มกันบกพร่องเรื้อรังและอาจส่งผลต่อการควบคุมเซลล์ภูมิคุ้มกันชนิดอื่นๆ ในปัจจุบันมีข้อมูลค่อนข้างจำกัดเกี่ยวกับการเหนี่ยวนำ tolDC ในหนูเมาส์ที่มีข้อบกพร่อง FcgRIIB ดังนั้นในการศึกษานี้เราทำศึกษาการสร้าง tolDC จากเซลล์เดนไดรต์ติคที่ได้มาจากไขกระดูกของหนู Fcgr2b$^{-/-}$ โดยสารที่สามารถเหนี่ยวนำให้ภาวะของ tolDC ได้ เมื่อเปรียบเทียบกับกลุ่มควบคุม (WT) เดนไดรต์ติคที่ได้จากไขกระดูกของหนู Fcgr2b$^{-/-}$ แสดงการกระตุ้นที่สูงขึ้นและมีการผลิตไซโตไคน์ที่มีการอักเสบตามการกระตุ้นของ LPS เมื่อเปรียบเทียบกับกลุ่มควบคุม (WT) โดยสารที่สามารถเหนี่ยวนำให้เกิดภาวะ tolDC ได้นั้น สามารถลดการกระตุ้นและการผลิตไซโตไคน์ของเซลล์เดนไดรต์ติคในหนู Fcgr2b$^{-/-}$ ที่ถูกกระตุ้นด้วย LPS ได้ โดยทั่วไปแล้ว เซลล์เดนไดรต์ติคของหนู Fcgr2b$^{-/-}$ ที่บำบัดด้วยเดกซาเมทาโซนแสดงการลดลงในการผลิตไซโตไคน์ที่เกิดจากการอักเสบและมีการสร้างไซโตไคน์ที่มีประสิทธิภาพในการลดการอักเสบนั้นคือ IL-10 ในการตอบสนองต่อการกระตุ้น LPS นอกจากนี้เดกซาเมทาโซนสามารถเหนี่ยวนำให้ Fcgr2b$^{-/-}$ tolDCs เพิ่มการขยายตัวของ Treg เซลล์ได้ ในปัจจุบันสำหรับการรักษาโรคภูมิคุ้มกันบกพร่องเรื้อรังในร่างกายนั้น Fcgr2b$^{-/-}$ tolDCs เป็นหนึ่งในทางเลือกที่ใช้ในการรักษาและนอกจากนี้เพื่อสร้าง tolDCs ในร่างกายให้สะดวกมากยิ่งขึ้น การประยุกต์ใช้อนุภาคนาโนที่มีการนำส่งเดกซาเมทาโซนนำไปสู่การแสดงออกที่เพิ่มขึ้นอย่างมีนัยสำคัญของ PD-L1 บนเซลล์เดนไดรต์ติค มีการเพิ่มจำนวนของ FoxP3+ Treg และลดระดับแอนติบอดีต่อ double strand DNA ในซีรั่ม โดยสรุป การศึกษานี้แสดงให้เห็นว่าเดกซาเมทาโซนเป็นหนึ่งในยาที่มีประสิทธิภาพที่สามารถกระตุ้นให้เกิด tolDC และนำไปสู่ผลลัพธ์ทางคลินิกที่ดีขึ้นในหนูที่มีแนวโน้มเป็นโรคลูปัส นอกจากนี้สิ่งนี้อาจเป็นประโยชน์สำหรับการรักษาโดยใช้เดนไดรต์ติคเซลล์ในผู้ป่วย SLE โดยที่ไม่ได้จำกัดเฉพาะผู้ป่วยที่มีความผิดปกติบนความหลากหลายของยีน FcgrIIb

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Phuriwat Khiewkamrop : Generation of tolerogenic dendritic cells from *Fcgr2b* deficient lupus-prone mice for the model of SLE therapy. Advisor: Prof. NATTIYA HIRANKARN, M.D. Ph.D Co-advisor: Assoc. Prof. PATCHAREE RITPRAJAK, D.D.S., Ph.D.

Tolerogenic dendritic cells (tolDCs) are DCs with an immunoregulatory function, which can induce regulatory T cells (Tregs) and suppress the immune response. Currently, tolDCs-based treatment has become a promising therapeutic approach for organ transplantation and autoimmune disease. FcgRIIB is an inhibitory receptor widely expressed in B cells, myeloid cells, and DCs. The ablation of FcgRIIB in the murine model shows a spontaneous development of a lupus-like disease and might affect other immune cell regulation. There was limited information on tolDC induction in FcgRIIB defective mice. Thus, in this study, we studied the generation of tolDCs from the bone marrow-derived dendritic cells (BMDCs) of *Fcgr2b*$^{-/-}$ mice by various tolerogenic agents. As expected, when compared with wide-type (WT) control, bone marrow-derived DCs of *Fcgr2b*$^{-/-}$ mice showed higher activation and pro-inflammatory cytokine productionupon LPS stimulation when compared with wide-type (WT) control. Tolerogenic agents can partially reduce the activation and cytokine production of LPS-stimulated *Fcgr2b*$^{-/-}$ BMDCs. Mainly, dexamethasone-treated *Fcgr2b*$^{-/-}$ BMDCs exhibited a notable decrease in pro-inflammatory cytokine production and showed an increase in IL-10 production in response to LPS stimulation. Furthermore, we demonstrated that dexamethasone-conditioned *Fcgr2b*$^{-/-}$ tolDCs could mediate Treg expansion *ex vivo*.

For the *in vivo* treatment of lupus disease, dexamethasone-conditioned *Fcgr2b*$^{-/-}$ tolDCs were adoptively transferred to *Fcgr2b*$^{-/-}$ lupus-prone mice, mediated Foxp3^{+} Treg expansion in the spleens and suppressed anti-double strand DNA autoantibodies in the serum. In addition, to generate tolDCs *in vivo* more conveniently, dexamethasone-loaded nanoparticles (DexaNPs) designed to target DCs were subcutaneously injected into *Fcgr2b*$^{-/-}$ lupus-prone mice. The DexaNPs-treated mice showed a significantly enhanced expression of PD-L1 on CD11c^{+} DCs, increased FoxP3^{+} Treg numbers, and decreased serum anti-double strand DNA autoantibodies.

In conclusion, our study demonstrated that dexamethasone is the most potent drug that can induce tolerogenic DCs and lead to better clinical outcome in *Fcgr2b*$^{-/-}$ lupus-prone mice. This may be helpful for DC-based treatment in SLE patients but not limited to *FcgrIIb* gene polymorphism.

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Figure 16 Immunosuppressive and tolerogenic effects of PDMAEMA-PLGA NPs and dexamethasone-incorporated PDMAEMA-PLGA NPs in *Fcgr2b*$^{-/-}$ BM-cDCs. *Fcgr2b*$^{-/-}$ BM-cDCs were untreated or pre-incubated with blank PDMAEMA-PLGA NPs (55 μg and 110 μg, an equal amount to Dex-NPs containing 2 μM and 4 μM dexamethasone, respectively), dexamethasone (2 and 4 μM), and dexamethasone-incorporated PDMAEMA-PLGA NPs containing 2 and 4 μM dexamethasone for 48 hours. Subsequently, the DCs were stimulated with

0.1 μg/ml of LPS for 24 hours. (A) Live cells and the expression of (B) CD11c, (C) CD40, (D) CD80, (E) CD86, (F) MHC class II, (G) ICOSL, and (H) PD-L1 were assessed by flow cytometry. $n = 5$; $^{n}p \leq 0.05$ compared with the negative control, $^{\dagger}p \leq 0.05$ compared with LPS-stimulated BM-cDCs, $^{*}p \leq 0.05$, $^{**}p \leq 0.001$; (-), negative control (untreated and unstimulated BM-cDCs); LPS, LPS-stimulated BM-cDCs; Dex, dexamethasone; Dex-NPs, dexamethasone-incorporated PDMAEMA-PLGA NPs; MFI, mean fluorescence intensity.....61

Figure 17 Effects of PDMAEMA-PLGA NPs and dexamethasone-incorporated PDMAEMA-PLGA NPs on the cytokine profiles of w *Fcgr2b*$^{-/-}$ BM-cDCs. *Fcgr2b*$^{-/-}$ BM-cDCs were untreated or pre-incubated with blank PDMAEMA-PLGA NPs (55 μg and 110 μg, an equal amount to Dex-NPs containing 2 μM and 4 μM dexamethasone, respectively), dexamethasone (2 and 4 μM), and dexamethasone-incorporated PDMAEMA-PLGA NPs containing 2 and 4 μM dexamethasone for 48 hours. Subsequently, the DCs were stimulated with 0.1 μg/ml of LPS for 24 hours. Culture supernatants were collected and (A) TNF-α, (B) IL-1β, (C) IL-6, (D) IL-23, (E) IL-12 and (F) IL-10 were determined using ELISA. $n = 5$; $^{n}p \leq 0.05$ compared with the negative control, $^{\dagger}p \leq 0.05$ compared with LPS-stimulated BM-cDCs, $^{*}p \leq 0.05$, $^{**}p \leq 0.001$; (-), negative control (untreated and unstimulated BM-cDCs); LPS, LPS-stimulated BM-cDCs; Dex, dexamethasone; Dex-NPs, dexamethasone-incorporated PDMAEMA-PLGA NPs. 63

Figure 18 Direct *in vitro* interaction of dexamethasone-incorporated PDMAEMA-PLGA NPs pretreated BM-cDCs and regulatory T cell. Wild-type BM-cDCs were untreated or pre-incubated with 2 μM dexamethasone, or dexamethasone-incorporated PDMAEMA-PLGA NPs containing 2 μM dexamethasone for 48 hours and DCs were stimulated with 0.1 μg/ml of LPS for 24 hours. Subsequently, the DCs were co-cultured with regulatory T cells isolated by magnetic-activated cell sorting in the presence of soluble anti-mouse CD3 mAb and recombinant mouse IL-2. (A) Cell proliferation was evaluated at 72 hours after coculture and (B) the IL-10 levels in the culture supernatant were measured at 48 hours after the co-culture. $n = 3$; $^{n}p \leq 0.05$ compared with unstimulated regulatory T cell, $^{\dagger}p \leq 0.05$ compared with regulatory T cell alone incubated with soluble anti-CD3 mAbs, $^{*}p \leq 0.05$, $^{**}p \leq 0.001$; Treg (-), unstimulated regulatory T cells; Treg (+), regulatory T cell incubated with soluble anti-mouse CD3 mAb and recombinant mouse IL-2; DCs Treg, LPS-stimulated BM-cDCs cocultured with regulatory T cells; Dex-DCs Treg,

dexamethasone pre-treated BM-cDCs co-cultured with regulatory T cells, Dex-NPs Treg, PDMAEMA-PLGA NPs pre-treated BM-cDCs co-cultured with regulatory T cells, DCs, LPS-stimulated DCs; Dex DCs, dexamethasone pre-treated BM-cDCs, Dex NPs DCs, dexamethasone-incorporated PDMAEMA-PLGA NPs pre-treated BM-cDCs.....65

Figure 19 *In vivo* uptake of nanoparticles by dendritic cells. Phosphate buffer saline or FITC-tagged PDMAEMA-PLGA NPs were subcutaneously administered into wild-type and *Fcgr2b*$^{-/-}$ mice. Seventy-two hours later, (A) The proportion of CD11c^{+}, F4/80^{+}, and CD3^{+} cells in the draining lymph nodes (dLNs) were determined using flow cytometric analysis. (B) The proportions of FITC^{+} cells in CD11c^{+}, F4/80^{+}, and CD3^{+} cells were evaluated by flow cytometric analysis. $n = 5$; *$p \leq 0.05$, ** $p \leq 0.001$; PBS, the control mice that received phosphate buffer saline; FITC-NPs, mice received FITC-tagged PDMAEMA-PLGA NPs.....67

Figure 20 Potent amelioration of lupus disease by dexamethasone-incorporated NPs. The renal damages of wild-type (WT) and *Fcgr2b*$^{-/-}$ mice treated with phosphate buffer solution (PBS) and apoptotic bodies mixed with dexamethasone (Dex) or dexamethasone-incorporated PDMAEMA-PLGA NPs (Dex-NPs) were indicated by (A) the representative of histopathology with H&E staining, (B) the renal tubulointerstitial injury score, and (C) glomerular injury are demonstrated. Serum from all mice was also collected to investigate the alteration of (D) anti-double strand DNA auto-antibodies, (E) IL-6, and (F) creatinine. Proteinuria was measured and represented in urine protein creatinine index (UPCI). $n = 5$; *$p \leq 0.05$ compared with PBS control, ** $p \leq 0.001$ compared with PBS control, $^{\dagger}p \leq 0.05$.....69

Figure 21 Investigation of the DC and T cell population in lupus mice and in vitro restimulation of draining lymph node cells. *Fcgr2b*$^{-/-}$ mice were treated with phosphate buffer solution (PBS) and apoptotic bodies mixed with dexamethasone (Dex) or dexamethasone-incorporated PDMAEMA-PLGA NPs (Dex-NPs). At the end point of treatment, dLNs were collected and (*A*) tolerogenic DC phenotypes and (B) CD4 T cell and Treg population were determined by flow cytometric analysis. (C) The LN cells were restimulated with apoptotic bodies in vitro and the Treg population was evaluated. $n = 5$; *$p \leq 0.05$, ** $p \leq 0.001$. 71

CHAPTER I

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by diverse heterogeneous clinical manifestations involving the vessels, kidneys, skin, or central nervous system (1). Although the factor contributing to and initiating this disease is not precisely known, genetics and environmental factors are involved, which can lead to cumulative organ damage and even mortality (2). The immunological hallmark of SLE is the production of antinuclear antibodies (ANA) that can bind with DNA or RNA, form an immune complex, and deposit in various organs (3). The incidence rate of SLE is significantly higher in females than in males. In addition, the prevalence of SLE has been increasing over time, 100 per 100,000 since 2000, from 40 per 100,000 in the 1970s (4). One factor-mediated SLE development arises from aberrant FcyRIIB function, the unique inhibitor receptor in the FcyRs family. Previous studies have reported that the Asian population represents a significantly higher rate of FcyRIIB dysfunction polymorphism (5). Therefore, treatment validation in SLE patients with FcyRIIB defect is needed. Previous studies have shown that the lack of FcγRIIB in mice models can mediate lupus nephritis after a 32–40-week-old. FcyRIIB is an immunomodulatory inhibitor receptor expressed in myeloid cells and B cells, except T cells (6). The function of these receptors is to reduce immune cells' role through the immunoreceptor tyrosine-based inhibition motif (ITIM)-the dependent inhibitory mechanism and plays important properties in maintaining self-tolerance conditions (7). Previous studies have established FcyRIIB knockout mice (FcyRIIB-/-) by using C57BL/6 mice for the background. FcyRIIB-/- mice significantly increased the level of autoantibodies to double-stranded DNA, elevated immune complex deposition in kidneys, and proteinuria

with higher serum creatinine (8). Other studies also found that the critical role of the FcyRIIB receptor in murine models correlated with the progression of autoimmune skin disease and bullosa acquisita (9). Interestingly, the mice defecting Fcgr2 could not generate tolerance under tolerogenic signal triggered via the mucosal route. This mouse's lack of Fcgr2 genes shows an inability to develop tolerance on DCs in mucosa-draining lymph nodes (10). Therefore, we questioned whether or not the FcγRIIB defect would be the barrier to systemic tolDC induction in SLE patients**.** Currently, corticosteroids are potent anti-inflammatory agents and the best immunosuppressive drug, widely used to lessen the severity of autoimmune disease, graft rejection, or allergic immune response (11). However, treating autoimmune diseases, especially SLE, using corticosteroids has many serious side effects, such as neurological disorders, endocrinopathies, metabolic changes, or osteoporosis. Therefore, specific immune tolerance induction might prevent serious side effects from the long-term use of immunosuppressive drugs. Previous studies have found that dexamethasone, rapamycin, vitamin D3 (VitD3), or natural product from andrographolide shown potentially mediated tolerogenic DCs through reduction of the co-stimulatory molecule, MHC class I or II via suppressed multi-upper signaling cascade (12, 13). Therefore, we aim to explore these tolerogenic agents in FcyRIIB-/- mice both in vitro and in vivo. Moreover, to improve the efficacy of treatment and diminish non-specific immunosuppressive drugs, a nanoparticle-based drug delivery system is the candidate alternative approach for target-orientated delivery of precision medicine to generate tolDCs to self-antigen in vivo directly (14). Now, various biopolymers are widely used in gene delivery systems, poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) is a hydrophilic cationic vinyl-based polymer, that can form a multi-complex with DNA by electrostatic interaction. Unfortunately, the utilization benefit of PDMAEMA nanoparticles (NPs) for medical applications has been limited, and has potential problem of in vivo degradability. Recent studies have tried to combine PDMAEMA and other polymers, including PDMAEMA-coated iron oxide nanocubes. It can reduce the

inflammatory response and low cytotoxicity in DCs and macrophages (15). In addition, Poly(D, L-lactide-coglycolide) (PLGA) is another biocompatible thermoplastic polyester with aliphatic degradable which has been approved by the US Food and Drug Administration (FDA) for delivery of biomolecules as well as many drugs. Current studies have synthesized PLGA-based nanoparticles (NPs) to deliver antigens to DCs and macrophages. These NPs produced low cytotoxicity and were biocompatible in DCs but it mainly generated immunogenic DC (16). In this study, we aim to study the copolymer of PDMAEMA and PLGA to generate tolDC in vivo.

In summary, we aim to generate and develop an alternative way of generating tolerogenic DCs and mediated antigen-specific immune tolerance under FcyRIIB-deficient lupus-prone mice. Furthermore, our *in vitro* and *in vivo* results would give insights into treatment options using autologous tolDCs from autoimmune diseases with FcgRIIB polymorphism and strengthen the critical role of tolDCs in the treatment of autoimmune diseases

CHAPTER II

OBJECTIVE

Research question

1. Do BMDCs from FcγRIIB deficient mice become tolerogenic under tolerogenic DC-inducing conditions?
2. Are tolerogenic DCs generated from FcγRIIB deficient mice able to suppress autoimmune reactions in FcγRIIB deficient lupus mice?

Objective

1. To evaluate the tolerogenic phenotype of DCs derived from *Fcgr2b*$^{-/-}$ bone marrows under the *in vitro* tolerogenic DC-inducing condition.
2. To determine the immunosuppressive function of tolerogenic DCs generated from *Fcgr2b*$^{-/-}$ bone marrows in FcγRIIB deficient lupus mice.
3. To investigate the therapeutic potential of tolerogenic drug-loaded nanoparticles in FcγRIIB deficient lupus mice *in vivo*.

Conceptual framework

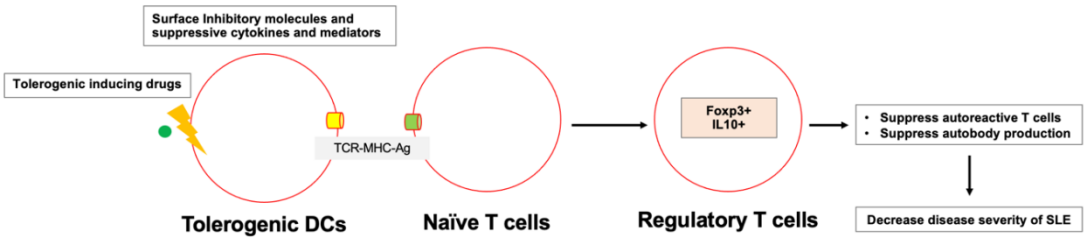

Figure 1 The illustration of conceptual framework

Experimental design

Objective 1: To evaluate the tolerogenic phenotype of DCs derived from *Fcgr2b*$^{-/-}$ bone marrows under the *in vitro* tolerogenic DC-inducing condition.

Experimental design

1.1 Assess the background immunogenicity of BMDCs derived from *Fcgr2b*$^{-/-}$ and wide type (WT) bone marrows in response to various toll-like receptor ligands (TLRLs)

Fcgr2b$^{-/-}$ BMDCs of and WT BMDCs were stimulated with lipopolysaccharides (LPS, TLR4L), Imiquimod (TLR7L), CpGODN 1668 (TLR9L) for 24 hours. The supernatant was collected, and the levels of IFN-γ, IL-1β, IL-4, IL-6, IL-10, IL-12, IL-23, TGF-β and TNF-α were measured by ELISA. The cells were harvested, and the expression of the DC marker, CD11c, and the activation markers, including MHC class II, CD40, CD80, and CD86, was determined by flow cytometric analysis.

1.2 Determination of the tolerogenic phenotypes of BMDCs derived from FcγRIIB knockout and WT bone marrows

To induce tolBMDCs, *Fcgr2b*$^{-/-}$ and WT BMDCs were treated with rapamycin, vitamin D3, dexamethasone, and andrographolide for 24 hours. Then, the supernatant was collected, and the levels of IFN-γ, IL-1β, IL-4, IL-6, IL-10, IL-12, IL-23, TGF-β and TNF-α were measured by ELISA. The cells were harvested, and the expression of the DC marker (CD11c), the DC activation markers (MHC class II, CD40, CD80, CD86), and the DC tolerogenic markers (ICOSL and PD-L1) were determined by flow cytometric analysis.

1.3 Examination of the tolerogenic phenotypes of *Fcgr2b*$^{-/-}$ and WT tolBMDCs after stimulation with LPS

TolBMDCs were induced as described in 1.2, and the DCs were stimulated with LPS on day 6. Twenty-four hours after stimulation, the supernatants were collected as well as the levels of IFN-γ, IL-1β, IL-4, IL-6, IL-10, IL-12, IL-23, TGF-β and TNF-α were measured by ELISA. In addition, the cells were also harvested for the determination of the expression of the DC marker (CD11c), the DC activation markers (MHC class II, CD40, CD80, CD86), and the DC tolerogenic markers (ICOSL and PD-L1) by flow cytometric analysis.

Remark: The most vigorous tolerogenic condition was selected from *in vitro* experiment for further *in vivo* induction of tolBMDCs in the subsequent experiments.

Objective 2: To determine the immunosuppressive function of tolerogenic DCs generated from *Fcgr2b*$^{-/-}$ bone marrows in FcγRIIB deficient lupus mice.

Experimental design

2.1 Investigation of the immunosuppressive function of tolBMDCs in antigen-specific T cell inhibition and regulatory T cell expansion.

Antigen-specific T cells were isolated from the draining lymph node (dLNs) of WT mice after chicken oval albumin (OVA) subcutaneous immunization. The antigen-specific T

cells were co-cultured with unstimulated or LPS-stimulated *Fcgr2b*$^{-/-}$ and WT tolBMDCs pulsed with OVA. T cell proliferation was determined by MTS and CSFE, and the numbers of regulatory T cells ($CD3^+CD4^+FoxP3^+$) were assessed by flow cytometric analysis. In parallel, the production of IFN-γ, IL-4, IL-10, IL-17A, and TGF-β in the culture supernatant was determined by ELISA.

2.2 *In vivo* experiment using adoptive transfer of *Fcgr2b*$^{-/-}$ and WT tolDCs into FcγRIIB deficient lupus mice.

An adoptive transfer experiment was conducted to assess the potential use of tolDCs pulsed with apoptotic bodies in the lupus mice model. Briefly, the onset of lupus was determined by the detection of anti-double strand DNA antibody level in the serum of *Fcgr2b*$^{-/-}$ mice (16 weeks old of *Fcgr2b*$^{-/-}$ mice shows the early onset of lupus). Recipient mice were divided into three groups as follows:

Group 1 Adoptive transfer with PBS

Group 2 Adoptive transfer with LPS-stimulated BMDCs

Group 3 Adoptive transfer with LPS stimulated tolBMDCs

BMDCs were pulsed with apoptotic bodies and were intravenously administrated to the mouse tail vein once a week for four consecutive weeks. The serum was collected one day before adoptive transfer and once a week after adoptive transfer for four consecutive weeks to measure anti-double strand DNA antibodies. Mice were euthanized four weeks after the first adoptive transfer, and lymph nodes (LNs) and spleens were collected.

- The levels of serum anti-double strand DNA antibodies were measured.

- Regulatory T cell populations were observed in both LNs and spleens by identifying $CD3^{+}CD4^{+}$ T cells that express FoxP3, PD-1, and IL-10. The data was analyzed by flow cytometry.

Objective 3: To investigate the therapeutic potential of tolerogenic drug-loaded nanoparticles in FcγRIIB deficient lupus mice *in vivo*.

Experimental design

Evaluating the potential of tolerogenic drug-loaded nanoparticles in mediating the *in vivo* tolDCs and regulatory T cell expansion in FcγRIIB deficient lupus mice.

FcγRIIB deficient lupus mice (mice with the lupus onset) were subcutaneously administrated with tolerogenic drug-loaded nanoparticles and apoptotic bodies once a week for four consecutive weeks. In addition, FcγRIIB deficient lupus mice received PBS or dexamethasone subcutaneously for the control groups.

The serum was collected one day before and four weeks after the first administration of the nanoparticles.

Mice were euthanized four weeks after the administration of the nanoparticles, and lymph nodes (LNs), spleens, and kidneys were collected.

- The levels of serum anti-double strand DNA antibodies were measured.

- The kidney histology was observed for lupus nephritis.

- The level of Serum creatinine and Urine creatinine index were measured

- The level of Pro-inflammatory cytokine (Il-6) in serum was evaluated

- The expression of tolerogenic surface markers, ICOSL and PD-L1, on LN DCs and splenic DCs were characterized by flow cytometric analyses.

- Regulatory T cell populations were observed in both LNs and spleens by identifying $CD3^+CD4^+$ T cells that express FoxP3, CD25, PD-1, and IL-10. The data were analyzed by flow cytometry.

- A part of LN cells and splenocytes were *ex vivo* re-stimulated with apoptotic bodies for 24 hours, and then $CD3^+CD4^+FoxP3^+CD25^+$ regulatory T cells will be analyzed by flow cytometry.

CHAPTER III

LITERATURE REVIEW

Systemic lupus erythematosus (SLE)

SLE is a systemic autoimmune disorder that causes debilitating diseases and can increase the risk of premature death worldwide (8). SLE is more prevalent in the Asian population, of 30-50 per 100,000 (17). Women show a higher incidence rate of SLE than men, particularly during puberty and menopause (18). The female-to-male ratio of SLE in children is 3 to 1 and alters to 9 to 1 during puberty and menopause (19). Both genetic factors and environmental triggers contribute to the etiology of SLE (8). Loss of T and B cell tolerance to self-antigen, aberrant immune response, high production of autoantibody, immune complex deposition, and multi-organ involvement are the significant characteristics of SLE (20). SLE patients may present with various clinical manifestations, including arthritis, serositis, rashes, cytopenias, kidney disease, or neurological aberrant (8).

The pathophysiology of SLE is associated with a higher autoimmune reaction, resulting in an inappropriate immune response to nucleic acid-containing cellular particles (21). As a result of reductive clearance of dying cells, the increase of apoptotic cells is found higher in serum, lymphoid organs, and inflamed tissue in SLE patients (22). In addition, nucleic acid-containing immune complex or nucleic acid enriched in endogenous retrotransposon sequences are the sources of potential stimuli for stimulation of nucleic acid-responsive endosomal toll-like receptors (TLRs) and TLR-independent nucleic acid sensors (8). The antigen-antibody immune complexes (IC) stimulate endosomal TLRs, especially TLR7 and TLR9, presented in immune cells. In addition, autoantibodies specific to RNA-binding proteins such as Sm, Ro, or La can stimulate the TLR-dependent pathway, particularly the TLR7 pathway. The signal transduction of TLR activation can induce the

expression of multiple types of I IFN-inducible genes in peripheral blood, which subsequently affects tissue damage and the development of the disease in SLE patients (23). In addition, current data suggest that the expression of sensors of cytoplasmic nucleic acid such as retinoic acid-inducible gene 1 (RIG-1), melanoma differentiation-associated protein 5 (MDA5), or cyclic GMP-AMP synthase (cGAS), which is a TLR-independent pathway, may also contribute to pathogenesis in SLE patients (24, 25). Type I IFN is released from plasmacytoid dendritic cells (pDCs), as well as other cell types, such as epithelial cells, might be involved in amplifying the signaling of IFN (8). In addition, IFN gene expression in peripheral blood is associated with neutrophil extracellular traps (NETs) (26). In general, NETs are networks of extracellular fibers that comprise DNA and pro-inflammatory proteins managed by neutrophils. NETs might facilitate the transfer of DNA-containing immune complexes to the intracellular endosomes with TLR expression, resulting in increased production of type I IFN through pDCs. T and B cells are essential in SLE pathogenesis (27). T cells isolated from SLE patients showed a high expression and persistence of CD40 ligand (CD40L) than healthy people, and this overexpressed CD40L resulted in the potentiated activation and differentiation of B cells (28). CD4+ T cells are recognized as the strongest drivers of B cell differentiation (29). Increased expression of lymphocyte function-associated antigen 1 (LFA1) led to the increased upregulation of the IFN-regulated gene and T cell proliferation (30). T follicular helper cells are the specific T cell population in SLE patients which can generate differentiation of pathogenic autoantibody-producing B cells (31). In addition, the increment of CD8+ T cells with memory phenotype is correlated with poor prognosis in SLE, together with multi-tissue damage (31). Some studies found the decreased expansion of regulatory T cells (Treg) but over-proliferation of T helper 17 (Th17), which led to the promotion of inflammation in SLE patients (32). In the case of B cells in SLE patients, SLE patients showed aberrant B cell regulation by increasing the production of autoantibodies (8). Dysregulation of B cell function and the clearance and inflammatory response to the

immune complex is the main characteristic of SLE. Moreover, circulating plasmablasts are the source of anti-dsDNA antibodies that correlate with the disease activity (33). The presence of differentiation factors (BAFF and IL-21) or helper T cells can contribute to B cell differentiation, survival, and proliferation, promoting autoimmunity (34). Many SLE patient-associated genetic variants encoding several proteins related to B cell function, e.g., phosphatases, kinases, and adaptor molecules, including PTPN22, BLK, or BANK, promote self-reactive B cells or antigen-mediated B cell activation (35). Moreover, memory B cells in lupus-prone mice and SLE patients exhibited decreased expression of the inhibitory Fc receptor (FCGR2B) together with an altered level of cytokine production (36).

FcγRIIB and SLE

Many immune cells express Fc gamma receptors (FcγRs) that bind to the FC portion of IgG (37). The activation of FcγRs by immune complexes can promote or regulate the inflammatory response. Most FcγRs are activating receptors, including the high-affinity receptor, FcγRI, and low-affinity family receptors, FcγRIIA, FcγRIIC, and FcγRIIIA in humans, and FcγRIII in mice (38). FcγRIIB plays an essential role in inhibiting immune response. FcγRIIB is expressed on B cells and cross-linked to the B cell receptors (BCR), leading to an elevation of the B cells activation threshold and a decrease in antibody production (39). In addition, other immune cells such as DCs, macrophages, neutrophils, basophils, and mast cells also express FcγRIIB (37). Activation of FcγRIIB in these cells inhibits the activation of FcγRs, suppressing pro-inflammatory cytokine release and phagocytosis (40). Inhibition of activating signal is the primary function of FcγRIIB, which is achieved via the ligation of FcγRIIB to the BCR or activating FcγRs by immune complexes (37). After the co-ligation of these receptors and Src-family kinase, Lyn leads to phosphorylation of the cytoplasmic domain ITIM. Subsequent binding of SH2 domain-containing inositol phosphatase (SHPs), especially SHP1, promoted dephosphorylation

downstream targets and decreased activation of a signaling cascade to inhibit signal of activation (41). When FcγRIIB on B cells is activated, it will lead to an increased threshold of B cell activation and a decrease in B cell-mediated antigen presentation to T cells via ITIM-dependent cascade (37). Immune synapse formation can be interrupted from FcγRIIB, which is significant in an early stage of B cell activation (42). Previous studies reported that FcγRIIB-deficient mice show a high antibody level in response to T cell-dependent antigens (43). In contrast, overexpression of FcγRIIB in B cells decreased T cell-dependent antigen response (44). Aberrant expression of FCGRIIB is associated with human susceptibility to many autoimmune diseases (38). Various previous studies have demonstrated a correlation between FcγRIIB and SLE, including the genetically modified mice (Fc γRIIB deficient mice). C57BL/6 mice with defective expression of FcγRIIB spontaneously develop hypergammaglobulinemia, immune complex, and an autoantibody-mediated disease resembling SLE (38, 43). Restoring the expression of FcγRIIB through transferring FcγRIIB-expressing retrovirus to bone marrow cells of (NZB × NZW) F1 mice (a spontaneous SLE model) can protect against autoimmune onset (45). In addition, B cells after transgenic overexpression of FcγRIIB can reduce SLE progression in MRL-*lpr* mice (46). In humans, some SLE patients have a genetic polymorphism in the FCGR2B gene, which results in decreased function (47). The risk allele of Fcgr2b changes I (Isoleucine) to T (Threonine) at position 232 (Fcgr2b-232T/T) on the transmembrane region. The percentage of Fcgr2b-232T/T in the Thai SLE population is 15.2% compared to 7.3% in healthy individuals (48). In summary, FcγRIIB regulates and maintains B cell tolerance; thus, the dysregulation of *FCGR2B* gene expression may result in the development of SLE pathogenesis (49).

Current therapy in SLE

Systemic lupus erythematosus shows high heterogeneity with diverse clinical manifestations and autoantibodies in serum targeting DNA and/or RNA (50). Currently,

immunosuppressive drugs, such as high doses of glucocorticoid and immunosuppressive drugs, are the first-line treatment drugs for moderate-to-severe diseases. However, after treatment with immunosuppressive medication, the condition in some SLE patients remains persistent and active, and the patients suffer from the adverse effects of the drug (51).

The first biologic agent approved by the Food and Drug Administration (FDA) for the treatment of SLE is Belimumab (a recombinant human monoclonal antibody against BAFF) (52). B cells are the main target of the developed biological agents for SLE treatment. Anti-dsDNA autoantibodies produced by autoreactive B are biomarkers of the disease activity. The immune complexes formed by anti-dsDNA autoantibodies deposit in tissue or organs, leading to exacerbated inflammation and multiple organ damage (53). The B cell subset can present antigens to autoreactive T cells and induce T cell activation and differentiation (54). Besides, a mature B cell in the marginal zone can produce a high level of anti-dsDNA autoantibodies in a T-cell-independent manner (55). B cell activating factor (BAFF) is a B cell survival factor that can modulate B cell differentiation, maturation, and antibody production (56). In SLE patients' blood circulation, a high BAFF level was correlated with the disease activity. This correlation may be resulted in the production of anti-dsDNA autoantibodies by the BAFF-activated marginal zone B cells (57). Treatment with Belimumab targeting BAFF requires a lower dose of glucocorticoid, which helps to increase the quality of life of SLE patients (52). Many biological drugs for SLE are still under development, such as those that block co-stimulatory molecules, including CD28, CD40L, and ICOS/ICOSL (51). Target treatment to cytokine or cytokine receptors, especially IL-10, IFN-α, IFN-γ are also of interest (51). In 2021, Anifrolumab, a fully human IgG1κ monoclonal antibody with the ability to bind to IFNAR, was the second biologic drug approved for treatment in SLE (58).

One approach for a novel therapy for autoimmune diseases is to induce specific tolerance and restore self-tolerance but spare the normal immune responses to pathogens. There were several approaches to generate specific regulatory T cells, e.g., low-dose IL-2, adoptive transfer of Treg, mesenchymal stem cells, and the manipulation of dendritic cells (59). MRL/LPR lupus mouse model with DC-ablation demonstrated the decreased expansion of autoreactive T cells, decreased differentiation of plasmablast, and reduced production of anti-dsDNA antibodies (60). As a result, DCs become a target for treating SLE patients.

Dendritic cell phenotype in SLE

Dendritic cells (DCs) are members of cells in the innate immune system, which play an essential role in the initiation, perpetuation, and amplification of SLE disease (21). Various studies have revealed the alteration of phenotype, function, and frequency of DCs population in SLE patients when compared to healthy people (61). During the progression of SLE in lupus-prone mice, the high infiltration of conventional DCs (cDCs) and pDCs were found in spleens and kidneys. Consistently, in patients with SLE, a higher accumulation of cDCs and pDCs in the affected tissue was correlated with tissue inflammation and damage (62). Therefore, the accumulation of DCs in the organs may promote tissue inflammation and poor prognosis in SLE. The stimuli, such as apoptotic cells or nucleic acid-containing immune complexes, activate pDCs through the engagement of TLR7, TLR9, or RIG-1-like receptors (RLRs) and potentiate the production of type I IFN (63, 64). The serum of SLE patients and spleens of lupus-prone mice showed a high level of type I IFN was associated with the increased activated pDCs (65, 66). Previous studies have reported that depletion of anti-dsDNA and glomerulonephritis result from deficient type I IFN in the lupus mouse model (67). In addition, pDC depletion in lupus-prone mice before disease onset led to the impairment of autoreactive T and B cells, consequently resulting in reduced autoantibodies, diminished multi-organs pathogenesis, and decreased type I IFN expression (21). A high mobility group box one protein (HMGB1) is a ubiquitous nuclear protein passively released

by necrotic cells that can activate the NF-kB signaling pathway via the ligation to TLRs or Receptors for Advanced Glycation Endproducts (RAGE) (68). The existence of HMGB1 at a high level in the serum of SLE patients was correlated with the increased level of type I IFN (69).

Conventional DCs (cDCs) plays a significant role in SLE pathogenesis and onset. Previous studies used human monocyte-derived DCs (MoDCs) and murine bone marrow-derived DCs (BMDCs) for investigating and understanding the function of cDCs in SLE. MoDCs showed an increased maturation and high production of IL-6, a significant cytokine for Th17 polarization, after activation with apoptotic cells (21). Similarly, BMDCs stimulated with apoptotic cells were increased in capacity to mediate Th17 response (70). In addition, the adoptive transfer of splenic DCs from aged lupus mice into young mice led to B cell hyperactivation, a high level of autoantibody production, an increase in DC activation, and Th1 differentiation (71). Dendritic cells derived from peripheral blood monocytes of SLE patients exhibited the spontaneous overexpression of a co-stimulatory molecule such as CD86. They were hyperresponsive to the activation signal, which led to an increased level of IL-6 (72). Conversely, the expression of inhibitory molecules such as immunoglobulin-like transcript (ILT) 3 and ILT4, which have a significant role in DC tolerogenic, was reduced in the circulating cDCs and pDCs from SLE patients (73). Leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) inhibited DC differentiation and activation and reduced the expression in SLE patients, subsequently promoting the loss of pDC's immunoregulatory function (74).

General information on dendritic cells

Dendritic cells (DCs) are innate immune cells that are considered the most effective antigen-presenting cells (APC) (75). DCs play key roles in coordinating, regulating, and maintaining an adaptive immune response (76). The origin of human dendritic cells is from $CD34^+$ hematopoietic precursor, which differentiates into myeloid precursor (MP) and

lymphoid precursor (LP) (75), as shown in figure 1. MP differentiates into monocyte, macrophages, and DC precursor (MDP) (77), and MDP then differentiates monocyte and common DC precursor (CDP). CDPs differentiate between plasmacytoid DC (pDC) and pre-conventional DCs (pre-cDCs) in the bone marrow. After that, pre-cDCs migrate to lymphoid and non-lymphoid organs and differentiate into lymphoid resident DCs and migratory DCs (20). Lymphoid resident DC and migratory DC are divided into two major subpopulations, cDC1, and cDC2 (75), which express the transcription factors zinc finger and BTB domain containing 46 (Zbtb46) (78). Human cDC1 is characterized by the expression of CD141, c-type lectin CLEC9A, chemokine receptor XCR1, and adhesion molecules CADM1 (79). Murine cDCs are $CD11c^{+}$ $MHCII^{+}$ population. The expression of XCR1 identifies murine cDC1. Lymphoid resident cDC1 has a unique expression of CD8a, while the expression of CD103 defines migratory cDC1. The essential transcription factor (TF) for generating cDC1 are the basic leucine zipper transcriptional factor ATF-like 3 (BATF3) and IFN-regulatory factor 8 (IRF8) (79). cDC1 primes $CD8^{+}$ T cells by cross-presenting antigen on MHC class I which is important in anti-tumor and anti-viral immunity (80). Human cDC2 markers are $CD1a^{+}$ SIRPα $(CD172a)^{+}$ while murine cDC2 markers are $CD11b^{+}$ $SIRP\alpha^{+}$ (81). cDC2 functions as an activator of T helper (Th) 1, 2, and 17. In addition, cDC2 can mediate regulatory T-cell expansion in the intestine and maintain tolerance in the liver (82). The pDCs were differentiated directly from CDP. The dominant characteristic of pDCs is the production of a high amount of IFN-α/β upon PRR stimulation (20, 83, 84). The phenotypic characterization of human and murine pDCs was distinct. In mice, $CD11c^{low}$ $B220^{+}$ BST2 $(CD317)^{+}$ $SiglecH^{+}$ cells are identified as pDCs (85). In humans, the expression of CD11c was absent, but the expression of CD123, BDCA-2 (CD303), and BDCA-4 (CD304) are used as pDC markers (75). However, human and mouse cDC2 express the master transcription factor, E2.2. Monocyte-derived DCs (moDCs) is suggested to be an inflammatory DC. MoDCs are the designation of another heterogenous sub-population of DCs. The first report

found that the production of TNF and iNOS can initiate a population of inflammatory DC, named Tip-Dc (75). The origin and markers of DCs are shown in Figure 2 and Figure 3.

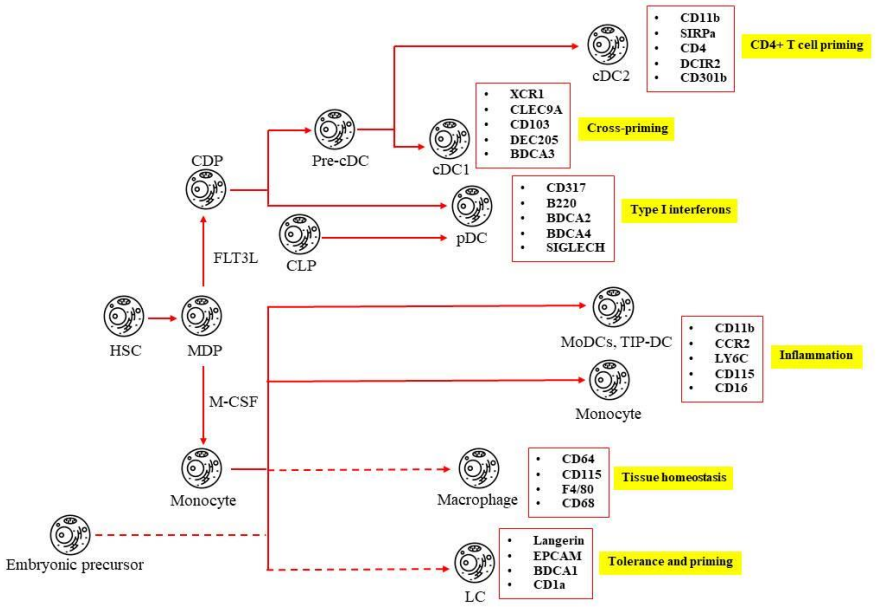

Figure 2 The diagram represents the ontogeny of human DCs

Modified from: Eisenbarth SC. Dendritic cell subsets in T cell programming: location dictates function. Nat Rev Immunol. 2019;19(2):89-103 (77).

DC subset	Developmental origin	Presence in vivo	Main function	Selected surface markers
cDC1s	HSC > CDP > pre-cDC, depend on FLT3L	Lymphoid resident, peripheral tissue, blood	Cross presentation of exogenous antigens on MHCI. Th1 & CD8+ T cell immunity against intracellular pathogens and tumors	M: CD11c+ MHCII+ CD8α+(resident) CD103+(migratory) CD24+ XCR1+ DNGR1/Clec9A+ CD11b-/low
				H: CD11c+ HLA-DR+ BDCA-3/CD141+ XCR1+ DNGR1/Clec9A+ DEC205+ CD1c-
cDC2s			Direct presentation of exogenous antigens on MHCII, Immunogenic CD4+ helper T cells, Treg	M: CD11c+ MHCII+ CD11b+/hi SIRP1α+ CD8α- CD103-
				H: CD11c+ HLA-DR+ CD1c+ SIRP1α+ CD11b+ CD141-inducible CD14+
DN-DCs		Peripheral tissue, blood, spleen	Not well defined, CD4+ and CD8+ T cell priming upon uptake of cell-associated antigen suggestion	M: CD11c+ MHCII+ XCR1-CD103- CD11b- (variation between tissues)
				H: CD11c+ HLA-DR+ CD141-sometimes CD1c+ CD206+
pDCs	HSC > CDP >; depend on FLT3L	Lymphoid resident, blood, lung	Type I Interferon secretion	M: CD11c-low MHCII-low Ly6C+ B220+
				H: CD11c- HLA-DR-low CD123+ CD303+ CD304+
LCs	Yolk-sac macrophage, fetal liver	Epidermis	Apoptotic cell clearance	M: CD11c+ MHCII+ Langerin+ CD11b+/low SIRP1α+ CD24+ EpCAM+ XCR1-
				H: CD11c+/low HLA-DR+ Langerin+ CD1a+ E-Cadherin+ EpCAM+
MoDCs	Blood monocyte depend on GM-CSF + M-CSF	Mainly induced upon inflammation in peripheral tissue	CD8+ T cells, Th1, Th2, Th17 immunity	M: CD11c+ MHCII+ CD11b+ Ly6C+ CD64+ DC-SIGN+ F4/80+ CD14+ (depending on tissue)
				H: CD11c+ HLA-DR+ CD14+ CD141- often DC-SIGN+ CD16+ CD1c+ SIRP1α+ CD11b+

Table 1. Dendritic cell subsets

Modified from: Wculek SK, Khouili SC, Priego E, Heras-Murillo I, Sancho D. Metabolic Control of Dendritic Cell Functions: Digesting Information. Front Immunol. 2019 (75).

Immunogenic and tolerogenic DCs

Immature DCs becomes activated through signal mediated by the engagement of pattern recognition receptors (PRRs) such as TLRs, retinoic acid-inducible gene l-like receptors (RLRs), and c-type lectin receptors (CLRs). After DCs activation by PRRs stimulation, they elevated the expression of chemokine receptors, including C-C chemokine receptor type-7 (CCR-7), leading to migration to the draining lymph nodes. In addition, up-regulation of MHC molecule, co-stimulatory molecule (CD80, CD86, CD40), and pro-inflammatory cytokine production indicates the alteration of DCs from resting-state DCs to mature DCs that can activate T cells in lymph nodes (86). Currently, immunogenic DCs are useful for potentiating robust cellular and humoral activity, which is the major for increasing the vaccine's efficacy in protecting against infectious diseases and cancers (87).

Peripheral tolerance is induced by tolDCs through T cell anergy, regulatory T cell expansion, and deprivation of a pro-inflammatory cytokine level (88). TolDCs maintain immunologic tolerance to prevent hyperactivation of the immune system and the subsequent autoimmune disease (75). TolDCs can be characterized by increased expression of immunomodulatory molecules such as PD-L1 and tolerogenic cytokine production such as IL-10 and TGF-β which can induce Treg activation (89). TolDCs have become promising therapeutic tools for modulating the immune response in an antigen-specific manner. *Ex vivo* induction of tolDCs for adoptive cell therapy is the high interest for autoimmune therapy. Autologous tolDC-based therapy has been recently under clinical trial for the treatment of various autoimmune diseases such as rheumatoid arthritis (RA), type I diabetes, and multiple

sclerosis, as well as the prevention of graft rejection following organ transplantation.

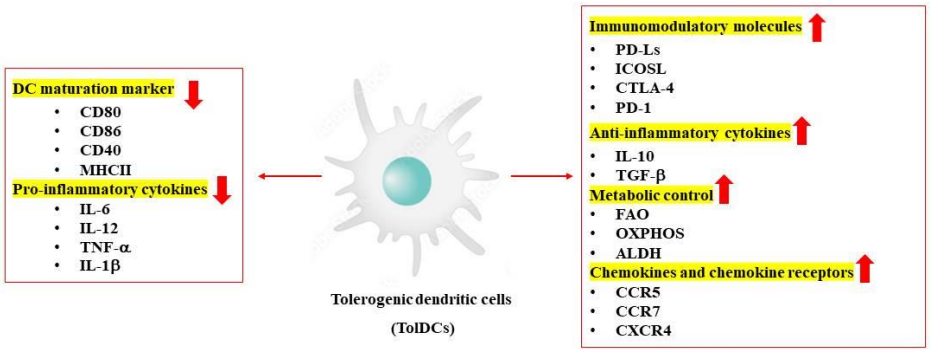

Figure 3 Tolerogenic DCs phenotype

Modified from: Ritprajak P, Kaewraemruaen C, Hirankarn N. Current Paradigms of Tolerogenic Dendritic Cells and Clinical Implications for Systemic Lupus Erythematosus. Cells. 2019 (20).

Ex vivo and *in vivo* induction of tolerogenic DCs

Various substances, including cytokines, lectins, complements, growth factors, hormones, neurotransmitters, vitamins, and immunosuppressive drugs, have been used to induce tolDCs *ex vivo*. IL-10 and TGF-β are frequently utilized for cytokine-modulated tolDCs. Immature moDCs treated with IL-10 exhibited a tolerogenic phenotype and could mediate $CD4^+/CD8^+$ T cell anergy with and induce Treg. TGF-β also can cause tolerogenic APCs and adoptive transfer TGF-β-treated DCs to experiment with autoimmune encephalomyelitis (EAE) mice could limit disease severity through the increased expansion of Treg. Dendritic cells derived from blood monocytes of SLE patients were rendered tolerogenic after co-culture with iC3b-opsonized apoptotic cells (90). Dexamethasone binds

with glucocorticoid receptors and modulates DC immunogenicity, such as antigen presentation or cytokine production (91). Dexamethasone- or vitamin D3- treated MoDCs exhibited tolDCs phenotype with high IL-10 production, and these DCs could downregulate IFN-γ and IL-17 production from memory T cells (92). In addition, vitamin D3 generated tolerogenic DCs with high expression of inhibitory receptors, which could enhance Treg expansion (93). Several natural products have also been used for tolDC induction. Resveratrol, a plant-derived polyphenol, could induce tolerogenic properties in mouse and human MoDC (94). Epigallocatechin gallate (EGCG), a component of green tea catechins with strong biological activity, inhibited the expression of DC maturation markers and increased IL-10 production in human moDCs (95).In addition, curcumin, a major component of turmeric power extracted from rhizomes of *Curcuma long*, could decrease antigen-presenting ability in DCs, promote T cell hyporesponsive, and increase Treg development.

Adoptive transfer of antigen-loaded tolDCs injected into MRL-Fas^{lpr} mice (lupus-prone mice) can ameliorate disease severity and leukocyte infiltration in the kidney (96). The clinical trial of RA patients with the active disease treated with autologous monocyte-derived tolDCs generated by BAY 11-7082 (NF-kB inhibitor) and pulsed with RA-specific antigens (citrullinated peptides) showed the reduction of effector T cells and increased expansion of Treg (97). The advantages of adoptive transfer are practical and low adverse effects due to the ability of DCs to induce an antigen-specific immune response. However, some limitations exist, such as the unstable tolDC phenotype when re-infused under inflammatory conditions and high cost.

Induction of tolDCs *in vivo* is one desirable therapeutic approach for autoimmune disease therapy. However, it is still difficult to selectively target DCs due to DC heterogeneity and the lack of DC-specific molecules for drug delivery. Only a few investigations have demonstrated the in vivo induction of tolDCs. Oral enrofloxacin treatment in mice led to gut dysbiosis and promoted anti-inflammatory bacteria that could mediate the

immunosuppressive environment in the gut by increasing IL-10 and TGF-β levels. Moreover, this anti-inflammatory condition was correlated with the high frequency of DCs with the tolerogenic phenotype (98). Specific antigens for SLE are required for modulated Treg by DCs. Nucleosomal histone is one of the epitopes that can induce tolerance in SLE models. In the spontaneous lupus nephritis model (SNF1 mice) treated with nucleosomal histone peptide, $H4_{16-39}$ showed delayed disease onset with suppressed autoreactive T and B cells (99). In addition, lupus-prone mice were immunized with nucleosomal histone peptide $H4_{71-94}$ can generate tolerogenic phenotype of splenic cDCs and pDCs and increase TGF-β secretion for modulated Treg expansion (100).

Clinical implication of tolerogenic DCs in autoimmune diseases

Loss of tolerance to self-antigen is the central characteristic of autoimmune disease, leading to impaired immune system function, tissue damage, and clinical morbidity and mortality. The Discovery of tolDCs that can modulate Treg expansion leads to the development of an alternative approach for treatment in autoimmune patients. The clinical implications of tolDCs are shown in Figure 3.

Disease/Trial	**Diabetes (62) Pittsburgh**	**Rheumatoid arthritis (RA) Rheumavax (68)**	**RA Newcastle University (73)**	**Crohn's disease (89)**
Clinical Trial ID	NCT00445913	NCT00396812	NCT01352858	2007-003469-42
Cell generation • NF-kB inhibitor • Vitamins • Stimulation • Antigens • Others	- - - With or without Antisense CD40,CD80,CD86	BAY 11-7082 - - Citrullinated peptides	Dexamethasone Vitamin D3 Mono lipid A Synovial fluid	Dex Vitamin A Cytokines -
Cell characterization Sterile/Viable	Passed	Passed	Passed	Passed

CD40	X	V	=	X
CD80	X	V	X	^
CD83	X	X	V	V
CD86	X	=	^	^
IL-10	X	X	^	^
IL-12	V	X	V	V
Therapeutics				
Cell number	1 X 10^7	1 or 5 X 10^6	1, 3 or 10 X 10^6	2, 5 or 10 X 10^6
Injection number	4 injections	1 injection	1 injection	1 Injection
Injection site	Intradermal	Intradermal	Knee joint	Intraperitoneal
Dose number	4, 2 weeks apart	1	1	1 or 3, 2 weeks apart

Table 2 TolDCs treatment in various types of autoimmune disease

Modified from: Phillips BE, Garciafigueroa Y, Trucco M, Giannoukakis N. Clinical Tolerogenic Dendritic Cells: Exploring Therapeutic Impact on Human Autoimmune Disease. Front Immunol. 2017 (101).

Tolerogenic DCs in SLE

The significant characteristics of tolDCs are low co-stimulatory molecule expression, decreased pro-inflammatory cytokine secretion, high co-inhibitory molecule expression, and high immunosuppressive mediator production, which inhibit effector T cell proliferation and mediate Treg expansion (102). Over the past decade, tolDCs have become a promising therapeutic tool due to their ability to regulate the immune response in an antigen-specific manner. However, the utilization of tolDCs for treatment in SLE patients has little information.

Blood monocytes were isolated from SLE patients treated with the combination of dexamethasone and vitamin D, and this treatment could generate IL-10-producing tolDCs with the capability of Treg mediation (92). In addition, the treatment of DCs derived from

monocytes of SLE patients with rosiglitazone and dexamethasone resulted in the reduction of pro-inflammatory cytokines, which indicated their tolerogenic phenotype. In addition, these tolDCs were resistant under apoptotic cell-induced maturation and could diminish $CD4^+$ T cell response (103). Previous studies found that probiotic bacteria are beneficial for treating autoimmune diseases (104). MoDCs from SLE patients were cultured with two probiotic bacteria, *Lactobacillus delbrueckii*, and *L. rhamnosus*. This treatment led to a decrease in the maturation phenotype and generation of tolDCs. The probiotics-treated DCs also showed elevated IL-10 production, enhanced IDO expression, and reduced IL-12 production (104). Adoptive transfer of donor-derived tolDCs could restore the aberrant immune response in RA and type I diabetes (105). Figure 4 summarizes tolDC-based therapy and induction of tolDCs *in vivo*.

Drug/Biologics	**Phenotype of DCs**	**Function of tolDCs**	**Study model**
***Ex vivo* induction of tolDCs**			
Vitamin D + Dexamethasone	High IL-10	Treg induction	In vitro model of moDCs from SLE patients
Rosiglitazone + Dexamethasone	Semi-mature, resistance to maturation	Inhibited CD4+ T cell response	In vitro model of moDCs from SLE patients
***In vivo* induction of tolDCs**			
Tolerogenic nucleosomal peptide	Increased TGF-B	Treg expansion Attenuate autoreactive T & B cells	SNF1 mice
hCDR1	Decreased TNF-a	Treg expansion Attenuate autoreactive T & B cells	BWF1 mice
CTLA4-Ig	Increased IDO	Attenuate autoreactive T & B cells	MRL-Fas^{lpr} mice

Table 3 Implications of tolerogenic DCs in SLE therapy

Modified from: Ritprajak P, Kaewraemruaen C, Hirankarn N. Current Paradigms of Tolerogenic Dendritic Cells and Clinical Implications for Systemic Lupus Erythematosus. Cells. 2019 (20).

Generation of tolDCs and clinical application in Fcgr2b knockout mice

A previous study has reported that mice lacking the *Fcgr2b* gene could not develop tolerance in response to tolerogenic antigens introduced via the mucosal route, and the inability to induce the tolerance in these mice was due to the lack of signaling via FcγRIIB on the DCs in the mucosa draining lymph nodes (LNs) (106). Microarray analysis in *Fcgr2b*$^{-/-}$ BMDCs demonstrated the highly upregulated gene expression profile associated with effective T cell activation, and these results concurred with the enhanced T cell expansion *in vivo*(107). The transcriptome studies of human tolDCs generated by IL-10, TGF-β, and dexamethasone also showed the increase of several vital pathways, including the FcγRIIB (108, 109). Therefore, we questioned whether the FcγRIIB defect would be the barrier to tolDC induction in patients with autoimmune diseases.

Nanoparticles in autoimmune disease

In the new era generation of drug delivery systems for biomedical indication, nanoparticles were generated or engineered to target specific tissues or cells containing high drug loads. Nanoparticles were developed via natural or synthetic materials with previous safety recodes and an average diameter range from 0.1 to 1000X10^{-9} m (110). Nanoparticle of organic or colloidal NPs can be taken up by phagocytic cell of the innate immune system, such as macrophage or DCs. The benefit of NPs can decrease the number of biological agents delivered by 100 to 1000-fold when targeted to specific cells. This decreases the adverse effects as well as the cost. In addition, the delivery of insoluble drugs can be enhanced and their bioavailability can be maximized through improvements in the delivery system.

Meanwhile, combine therapeutic agents with diagnostic, resulting in ‘theranostic’ agents. This concept indicates its appeal in developing immunomodulatory strategy technologies (110).

Nanoparticles are being tested for potential treatment of autoimmune disease due to their versatility in being engineered for three different uses: functioning as carriers for biological agents and small molecule drugs, possessing anti-inflammatory properties, and being able to generate Tolerogenic responce (111). NPs can play a role in polarizing cells to become anti-inflammatory by encapsulating agents. These agents include corticostatin, IL-10, or angiotensin receptor blockers. The administration of biological agents, such as tumor necrosis factor antagonists, resulted in a reduction of inflammatory arthritis. Their size, biodistribution, and route of administration determine the effect of NPs. In general, Particles smaller than 6 nm drain to blood while larger sizes more than 9 nm drain preferentially to the lymphatic system. NPs delivery by intravenous injection targets APC in the spleen and liver. DCs preferentially take up those delivered by subcutaneous injection in draining lymph nodes (dLNs) (110).

A nanoparticle-based drug delivery system offers an alternative solution for the target-orientated delivery of precise medicines to enhance the efficacy of treatment and reduce the side effects of immunosuppressive drugs. Exploiting nanoparticles (NPs) to target multiple DC subsets in situ and harness them into tolerogenic phenotypes represents a promising strategy to mediate antigen-specific immune tolerance in patients with autoimmune disorders (43). In addition, using nanoparticles with immunomodulatory properties can potentially reinforce DC tolerogenic (43). However, to date, few studies are still focusing on nanoparticle-mediated immunomodulation for autoimmune disease therapy.

Poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) is a hydrophilic cationic vinyl-based polymer that has been widely used in gene delivery because it can form a polyplex with DNA by electrostatic interaction. The use of PDMAEMA NPs in medical

applications is limited due to their molecular architecture and lack of *in vivo* degradability (15); however, modified PDMAEMA and the combination of PDMAEMA with other polymers improved the properties for drug delivery applications. We previously fabricated PDMAEMA-coated iron oxide nanocubes, investigated these nanocomposite properties in macrophages and DCs, and found that PDMAEMA-coated iron oxide nanocubes exhibited low cellular toxicity and could suppress the inflammatory response in both cells (16).

Poly(D, L-lactide-coglycolide) (PLGA) is an aliphatic degradable and biocompatible thermoplastic polyester which is one of the best characterized and has been approved by the US Food and Drug Administration (FDA) for the delivery of drugs and biomolecules. Previously, we developed PLGA-based nanoparticles (NPs) for antigen delivery to DCs and demonstrated that PLGA NPs produced low cell toxicity and were biocompatible with DCs; however, PLGA NPs exhibited immunogenic activity that may not be suitable for therapeutic application in autoimmune diseases (112).

In summary, this project aims to generate tolDCs from the bone marrow-derived dendritic cells (BMDCs) of *Fcgr2b* deficient mice by various tolerogenic agents and to test the properties and functions of these tolDCs *in vitro* and *in vivo* via adoptive transfer experiments. In addition, the *in vivo* targeting of DCs in the in vivo targeting DCs by tolerogenic drug-loaded nanocomposites was explored. Our study will benefit DC-based therapy in SLE patients, especially in patients with *FcgrIIb* gene polymorphism.

CHAPTER IV

Materials and methods

Animals

Female *Fcgr2b*$^{-/-}$ mice in the C57BL/6 background were obtained from Dr. Silvia Bolland (NIAID, NIH, Bethesda, MD) and have been bred and housed in the Animal Facility Center, Faculty of Medicine, Chulalongkorn University. Female C57BL/6 mice were purchased from Nomura Siam International Co, Ltd., Bangkok, Thailand. The mice at 5-7 weeks of age were used for the *in vitro* experiments, and the mice at 4-6 months of age were used for the *in vivo* experiments. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, Chulalongkorn University (protocol approval numbers 018/2562 and 006/2563).

Generation of bone marrow-derived dendritic cells (BMDCs) and induction of tolerogenic phenotype

Bone marrow cells were collected from mouse femurs and tibias. The red blood cells (RBC) were lysed with RBC lysis buffer, and the cells were washed with RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 100 unit/ml penicillin and 100 μg/ml streptomycin. Bone marrow cells were seeded into 24-well plates at the cell numbers of 1×10^6 cells/ml/well and were cultured in RPMI 1640 media containing 10% FBS, 0.2 mM of Glutamax, 100 unit/ml of penicillin, and 100 μg/ml of streptomycin, in the present of 10 ng/ml of recombinant mouse GM-CSF and 10 ng/ml of recombinant mouse IL-4. Half of the cultured media was replaced with fresh media every 2 days.

To generate tolDCs, 10 μg/ml of Rapamycin (Sigma-Aldrich, MO, USA) and 10^{-8} M of vitamin D3 were treated on day 2 and day 4 of BMDC culture and 1 μM of dexamethasone and 10 μM of andrographolide will be added on day 5 of the BMDC culture.

BMDC stimulation

On day 6 of the BMDC culture, the untreated BMDCs and tolDcs were stimulated with 1 μg/ml of lipopolysaccharide (LPS), 2 μg/ml imiquimod (Invivogen, CA, USA), or 1 μM CpG 1668 (Invivogen) for 24 hours. In addition, the supernatant of BMDC culture was collected and evaluated for cytokine production by ELISA. The cells were harvested for flow cytometric analyses of the expression of CD11c, CD40, CD80, CD86, MHC Class II, and ICOSL.

OVA immunization

On day 0, WT mice were immunized with 20 μg of chicken oval albumin (OVA, Sigma-Aldrich) in Complete Freund's adjuvant (CFA) (100 μl of emulsion) via subcutaneous route at the scruff of the neck. On day 7, the mice received second subcutaneous immunization with 20 μg OVA in Incomplete Freund's adjuvant (IFA) (100 ul of emulsion). Finally, on day 14, the mice were sacrificed, and the skin-draining lymph nodes (dLNs) were collected for T cell experiments.

***Ex vivo* T cells proliferation assay**

The dLNs of OVA-immunized WT mice were excised and digested with 300 units/ml of collagenase IV (Invitrogen/ThermoFisher Scientific) and 10 units/ml of DNase I (Invitrogen/ThermoFisher Scientific) at 37°C for 45 minutes. Then the cells were washed, and $CD3^+$ T cells were negatively isolated by a magnetic cell sorter (mouse Pan T cell isolation kit, Miltenyi Biotec). Finally, the purity of $CD3^+$ T cells was evaluated by flow cytometric analysis.

The control BMDCs (untreated BMDCs) and tolBMDCs from WT and *Fcgr2b*$^{-/-}$ mice were stimulated with 1 μg/ml of LPS for 2 hours. Then the DCs were pulsed with 500 μg/ml of OVA (in the presence of LPS condition) for 22 hours. Next, the BMDCs were washed twice with culture media, and 4×10^4 cells of BMDCs were co-cultured with 2×10^5 cells of T cells (DC/T ratio of 1:5) in a 96-well plate in 200 μl of complete RPMI media (RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 100 unit/ml penicillin and 100 μg/ml streptomycin) for 72 hours. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (Promega, WI, USA) were added to the cell culture at the last 4 hours of incubation time. The cell proliferation was measured by spectrophotometry at the absorbance of 450 nm.

To trace T cell proliferation, the OVA-immunized $CD3^+$ T cells were labeled with a CSFE cell division Tracer kit (Biolegend, CA, USA) following the manufacturer's instruction. After that, the labeled T cells were co-cultured with BMDCs for 72 h. Finally, the cells were collected, and the CSFE signal and the T cell markers were assessed by flow cytometry.

Ex vivo T cell re-stimulation

$CD3^+$ T cells were isolated from the dLNs of OVA-immunized WT mice, as described above. The control BMDCs (untreated BMDCs) and tolBMDCs from WT and *Fcgr2b*$^{-/-}$ mice were stimulated with 1 μg/ml of LPS for 2 hours, and then the DCs were pulsed with 500 μg/ml OVA (in the presence of LPS). Subsequently, the BMDCs were washed twice with culture media, and 4×10^4 cells of BMDCs were co-cultured with 2×10^5 cells of T cells (1:5 ratio of DC: T) in a 96-well plate in 200 μl of complete RPMI media for 72 hours. The culture media were collected and evaluated for cytokine production by ELISA, and the cells were harvested for flow cytometric analyses of T cell populations.

Flow cytometric analysis

Cells were resuspended in staining buffer (1% FBS and 0.1% sodium azide in 1X PBS) and blocked with anti-mouse CD16/32 (Clone: 93) for 15 minutes at 4^{o}C before staining with the fluorochrome-conjugated monoclonal antibodies. Next, the cells were stained with the fluorochrome-conjugated monoclonal antibodies for 20 minutes at 4^{o}C, and the cells were then washed with staining buffer 2 times. Finally, the stained cells were resuspended in a staining buffer.

The fluorochrome-conjugated monoclonal antibodies used in this work were as follows: anti-mouse CD11c (N418 clone), anti-mouse CD80 (16-10A1 clone), anti-mouse CD86 (GL-1 clone), anti-mouse CD40 (3/23 clone), anti-mouse MHC class II (M5/114.15.2 clone), anti-mouse inducible T-cell costimulatory ligand (ICOSL) (HK 5.3 clone), anti-mouse PD-L1 (10F.9G2), and anti-mouse PD-1(29F.1A12). All antibodies were purchased from Biolegend (CA, USA).

A mouse regulatory T cell staining kit containing antibodies against mouse CD3, CD4, CD25, and FoxP3 and an isotype-matched control antibody were used to identify regulatory T cells.

The stained cell was assessed by flow cytometer (BD Bioscience, NJ, USA), and the data were analyzed by FlowJo software (BD, NJ, USA).

Enzyme-linked immunosorbent assay (ELISA)

Cytokine profiles of BMDCs and T cells were determined by a commercial sandwich ELISA kit for mouse TNF-α, IL-1β, IL-6, IL-12p70, IFN-γ, IL-4, IL-17A, IL-10, IL-23, and TGF-β following manufacturer's instruction. Briefly, the capture antibodies were added to ELISA microplates, and the plates were incubated at 4^{o}C overnight. Next, the plates were blocked for 1 hour at room temperature, and the culture supernatant was added and incubated

for 2 hours at room temperature. Subsequently, the detection antibodies were added and incubated for 1 hour at room temperature. Avidin-HRP was then added and incubated for 30 minutes at room temperature. The plates were washed 3-4 times between each step. Finally, the TMB substrate was added, and the reaction was stopped by 2N H_2SO_4. The absorbance was measured at 450 nm using a microplate reader.

TolDCs adoptive transfer to FcγRIIB deficient lupus mice

FcγRIIB deficient lupus mice at 16-24 weeks of age were utilized for this experiment because the mice spontaneously develop lupus disease at this age (113). The animals were divided into three groups; control (PBS injection), untreated BMDCs, and tolBMDCs (N = 5)

After Fransen's protocol, apoptotic bodies were prepared by centrifugation method (114). Briefly, spleens were collected from the 6-month age of FcγRIIB deficient lupus mice. The spleens were mechanically disrupted, and RBC was lysed. The splenocytes were washed two times with PBS. Then, 2 x 10^6 cells/ml of the splenocytes were treated with 1 μM of staurosporine (apoptotic inducer) for 24 hours. The treated splenocytes were centrifuged at 300 g at room temperature for 5 minutes. The supernatant was collected and subsequently centrifuged at 2000 g at room temperature for 20 minutes. At this step, the supernatant was removed, and the pellet of the apoptotic bodies was collected and resuspended in PBS. The Bradford protein assay determined the number of apoptotic bodies.

Fcgr2b$^{-/-}$ and WT tolBMDCs were stimulated with LPS and pulsed with 10 μg/ml of apoptotic bodies for 24 hours. BMDCs at the cell numbers of 5×10^5 cells in 100 μl of PBS will be intravenously injected into the mice. The mouse serum was collected 1 day before and once a week after the BMDC transfer for 4 consecutive weeks to detect anti-double strand DNA antibody levels. The animals were euthanized 4 weeks after the first adoptive transfer, and lymph nodes (LNs) and spleens were collected. Regulatory T cell populations were

observed in both LNs and spleens by flow cytometric analysis of the markers of CD3, CD4, FoxP3, CD25, PD-1, and IL-10.

Nanoparticle experiment

The tolerogenic drug was loaded into PLGA (poly (lactic-co-glycolic acid)) nanoparticles at the ratio of 100 μg of nanoparticles to 1 mg of the tolerogenic drug. Twenty micrograms of the tolerogenic drug-loaded nanoparticles in 100 μl of PBS and 20 μg of apoptotic bodies in 100 μl of PBS were subcutaneously administrated into the scruff of the neck of FcγRIIB deficient lupus mice (16-week-old mice with the lupus onset) once a week for 4 consecutive weeks. For the control groups, FcγRIIB deficient lupus mice subcutaneously receive PBS or 4 mg/kg of the tolerogenic drug.

The serum was collected 1 day before and 4 weeks after the first administration of the nanoparticles. Mice were euthanized 4 weeks after the administration of the nanoparticles, and lymph nodes (LNs), spleens, and kidneys were collected.

Lymph node cells and splenocytes were prepared by mechanical disruption. RBCs were lysed, and the cells were washed 2 times with PBS. The expression of tolerogenic surface markers, ICOSL and PD-L1, on LN DCs and splenic DCs and the expression of CD3, CD4, FoxP3, CD25, PD-1, and IL-10 on T cells were assessed by flow cytometric analyses.

A part of LN cells and splenocytes (2×10^6 cells/ml) were cultured in 500 μl of complete RPMI media and *ex vivo* re-stimulated with 10 μg/ml of apoptotic bodies for 24 hours and then $CD3^+CD4^+FoxP3^+CD25^+$ regulatory T cells were analyzed by flow cytometry.

Measurement of anti-double strand DNA

The ELISA microplates were coated with 10 μg/ml of UltraPure™ Calf Thymus DNA solution (Invitrogen, Thermofisher) overnight at 4°C, and the plates were blocked with 1% bovine serum albumin (BSA) in 1X PBS for 1 hour at room temperature. Next, mouse serum was added to the plates and incubated for 1 hour at room temperature with shaking. Then, the diluted 1:4000 HRP-conjugated goat anti-mouse IgG monoclonal antibody (Biolegend) was added and incubated for 30 minutes at room temperature. The plates were washed 4-5 times between each step. Finally, the TMB substrate was added, and the reaction was stopped by 2N H_2SO_4. The absorbance was measured at 450 nm using a microplate reader.

Histology

Kidneys were fixed with 10% neutral buffered formalin and embedded in paraffin. The paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin (H&E). All histological specimens were visualized under a bright field microscopy and imaging processor with 40X and 100x stage objectives. The photographs were taken from 3 fields of each sample.

Two blinded pathologists performed the histologic assessment, and lupus nephritis will be classified following the International Society of Nephrology/Renal Pathology Society 2003 (115) as shown in the below table.

Class I	Minimal mesangial LN
Class II	Mesangial proliferative LN
Class III	Focal LN* (<50% of glomeruli) III (A): active lesions

	III (A/C): active and chronic lesions III (C): chronic lesions
Class IV	Diffuse LN* (≥50% of glomeruli) Diffuse segmental (IV-S) or global (IV-G) LN IV (A): active lesions IV (A/C): active and chronic lesions IV (C): chronic lesions
Class V	Membranous LN
Class VI	Advanced sclerosing LN (≥90% globally sclerosed glomeruli without residual activity)

Table 4 the histologic assessment and lupus nephritis

LN, lupus nephritis.

*Indicate the proportion of glomeruli with active and with sclerotic lesions.

*Indicate the proportion of glomeruli with fibrinoid necrosis and with cellular crescents.

*Indicate and grade (mild, moderate, and severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis, or other vascular lesions.

*Class V may occur in combination with III or IV in which case both will be diagnosed.

Statistical analysis

Statistical analyses were performed using SPSS software. Student's *t*-test will be used to compare two groups, and One-way ANOVA with Tukey's HSD Post Hoc test will be used to compare more than two groups. For lupus nephritis scoring, the non-parametric Kruskal–Wallis test was used. Significant differences will be considered when $p < 0.05$.

CHAPTER V

RESULTS

Fcgr2b$^{-/-}$ BMDCs exhibited hyperactivation phenotype in response to TLR-L stimulation

It has been demonstrated that FcγRIIB deficiency leads to loss of immune tolerance and hyperactivation of immune cells (43, 116); therefore, we experimented to confirm whether *Fcgr2b*$^{-/-}$ BMDCs exhibited hyperinflammatory profile in response to inflammatory stimuli. TLR-4, TLR-7, and TLR-9 are critical in recognizing the stimuli produced by tissue damage or cell apoptosis and contribute to the aggravation and persistence of autoimmune responses (117, 118). Accordingly, we stimulated BMDCs from wild-type (WT) and *Fcgr2b*$^{-/-}$ mice with lipopolysaccharide (LPS, TLR-4L), imiquimod (TLR-7L), and CpG-ODN (TLR-9L) and characterized the BMDC phenotype by flow cytometric analyses of the maturation markers, CD80, CD86, MHC class II, and CD40 (Figure 4). Next, the percentages and mean fluorescence intensity (MFI) of the maturation markers were evaluated using dot plots and histogram analyses, respectively (Figure 4). In comparison to the unstimulated WT BMDCs, the unstimulated *Fcgr2b*$^{-/-}$ BMDCs did not display any apparent change in the percentage of CD80^{+}, CD86^{+}, MHC class II^{+} and CD40^{+} cells (Figure 4, left panel), as well as the expression level of these molecules (Figure 4, right panel). LPS (TLR-4L), imiquimod (TLR-7L), and CpG-ODN (TLR-9L) stimulation induced the increased expression of the maturation marker on both WT and *Fcgr2b*$^{-/-}$ BMDCs (Figure 4). CD80 expression on *Fcgr2b*$^{-/-}$ BMDCs was not different from the expression on WT BMDCs (Figure 4A). On the other hand, *Fcgr2b*$^{-/-}$ BMDCs showed significantly increased percentages of CD86^{+} and MHC class II^{+} cells and exhibited the high upregulation of these two molecules in response to LPS (TLR-4L) (Figure 4B-C). In addition, CD86 expression on *Fcgr2b*$^{-/-}$ BMDCs was higher than on

WT BMDCs when the cells were exposed to imiquimod (TLR-7L) (Figure 4B). The percentages of $CD40^{+}$ cells in *Fcgr2b*$^{-/-}$ BMDCs were markedly elevated upon LPS, imiquimod, and CpG (TLR-9L) stimulation, and the expression level of CD40 on *Fcgr2b*$^{-/-}$ BMDCs was highly and partially upregulated when the cells were stimulated with LPS and CpG, respectively (Figure 4D). As expected, the data initially indicated that the loss of the inhibitory function of FcγRIIB led to the hyperactivation of BMDCs in response to inflammatory stimuli, primarily via the ligation of TLR4, a typical TLR pathway in conventional DCs (119, 120). Therefore, we choose LPS (TLR4 ligand) for the subsequent experiment.

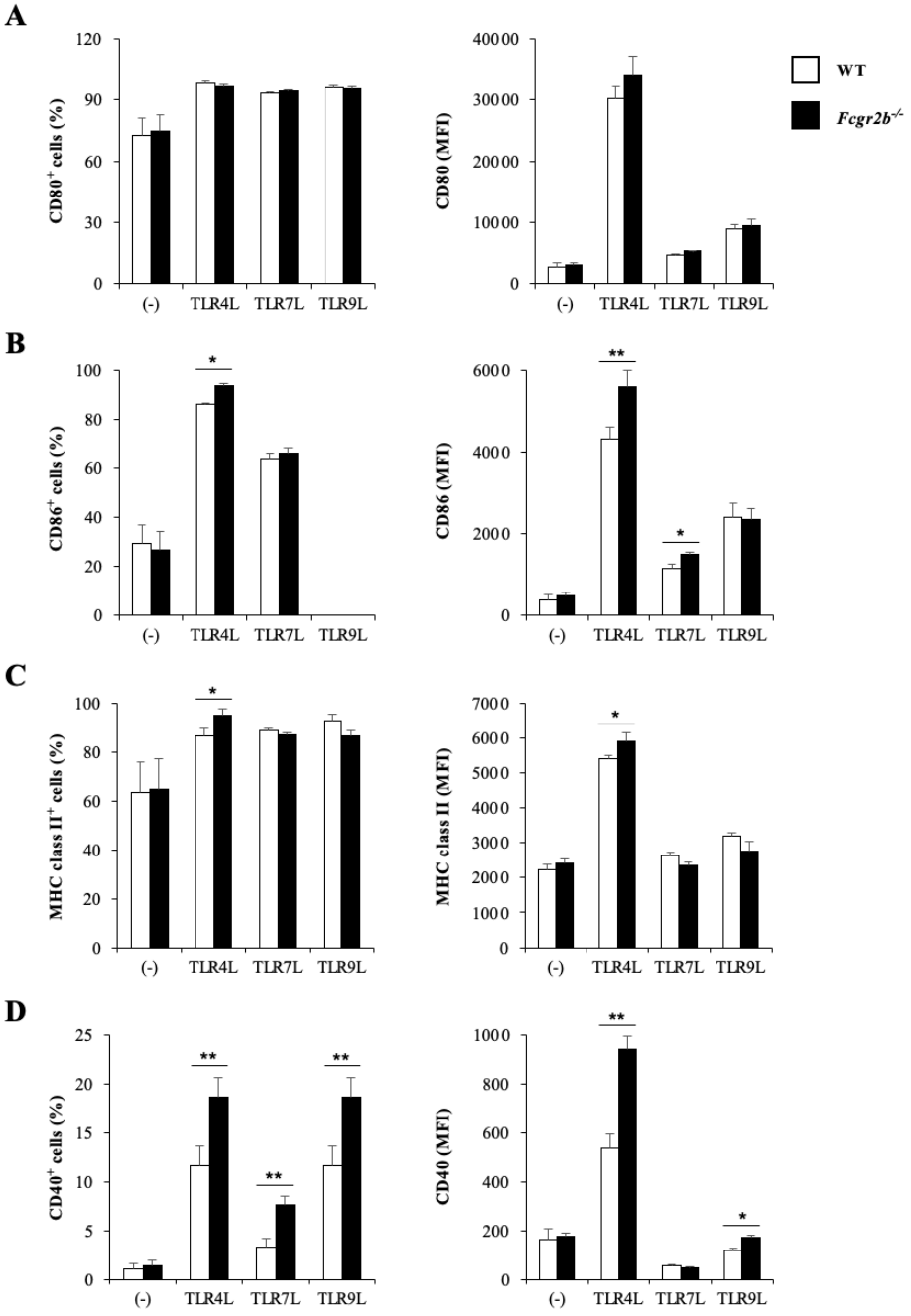

Figure 4 The co-stimulatory molecules from $CD11c^+$ between wild type and deficient FcGRIIB mice under various stimuli conditions (TLR4L, TLR7L, TLR9L). The data are shown as mean ± SD of triplicate experiments. The asterisks (*, **, and ***) indicate *p-value* ($p < 0.05$, $p < 0.01$, and $p < 0.001$), respectively.

Treatment of immature *Fcgr2b*$^{-/-}$ BMDCs with various pharmacologic agents induced differential tolerogenic properties under strong inflammatory stimuli.

In the clinical setting of tolDC-based immunotherapy, the *ex-vivo* generated tolDCs are transfused into the bloodstream of autoimmune disease individuals, and the cells must encounter inflammatory stimuli in the patient body. Therefore, the generation of tolDCs under inflammatory conditions is crucial for successful clinical treatment. As *Fcgr2b*$^{-/-}$ BMDCs displayed hyperactivation phenotypes (Figure 4), we examined the phenotype of tolDCs generated from *Fcgr2b*$^{-/-}$ BMDCs by stimulating the cells with LPS. BMDCs from WT and *Fcgr2b*$^{-/-}$ mice were treated with andrographolide, dexamethasone, rapamycin, and vitamin D, and the BMDCs were subsequently stimulated with LPS (Figure 5). The expression of the co-signaling molecules (CD80, CD86, CD40, and ICOSL) and MHC class II was assessed with flow cytometric analyses. Upon LPS stimulation, unstimulated *Fcgr2b*$^{-/-}$ BMDCs showed significantly increased expression of CD86, MHC class II, CD40, and ICOSL compared to unstimulated WT BMDCs (Figure 4 and Figure 5). Generally, a similar expression level of each molecule was observed in the tolDCs from *Fcgr2b*$^{-/-}$ mice compared to the tolDCs from WT mice (Figure 4). No drug could reduce CD80 expression on both WT and *Fcgr2b*$^{-/-}$ BMDCs when stimulating the cells with LPS. All drugs remarkably downmodulated CD86 and MHC class II expression on both LPS-stimulated WT and *Fcgr2b*$^{-/-}$ BMDCs. Dexamethasone treatment decreased the expression of CD40 on LPS-stimulated WT and *Fcgr2b*$^{-/-}$ BMDCs. Meanwhile, rapamycin and vitamin D3 did not affect CD40 expression on LPS-stimulated WT BMDCs, but these drugs could abrogate CD40 expression on LPS-stimulated *Fcgr2b*$^{-/-}$ BMDCs. Dexamethasone and rapamycin strikingly upregulated ICOSL expression on LPS-stimulated WT and *Fcgr2b*$^{-/-}$ BMDCs, while vitamin D3 enhanced the moderate level of ICOSL only on *Fcgr2b*$^{-/-}$ BMDCs. On the contrary, andrographolide did not

alter the expression of CD40 and ICOSL on LPS-stimulated BMDCs from both WT and *Fcgr2b*$^{-/-}$ mice.

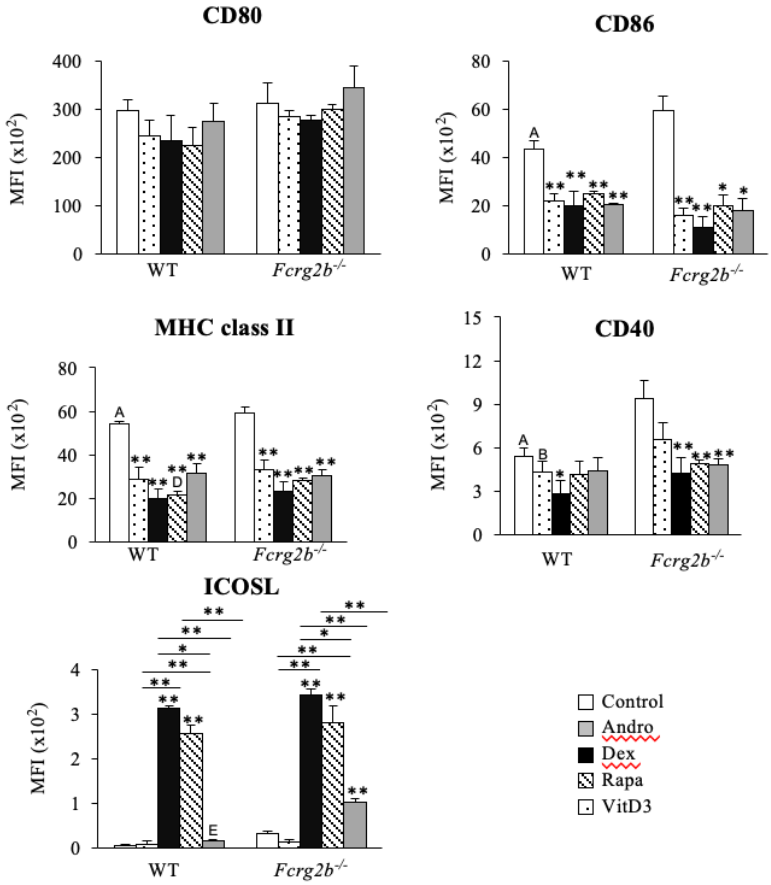

Figure 5. The co-stimulatory molecules from CD11c$^+$ between wild type and deficient FcGRIIB mice in LPS stimulation. The BMDCs with LPS stimulation as shown in MFI. The data are shown as mean ± SD of triplicate experiments. The asterisks (*, **, and ***) indicate *p-value* ($p < 0.05$, $p < 0.01$, and $p < 0.001$), respectively.

Treatment of immature *Fcgr2b*$^{-/-}$ BMDCs with various pharmacologic agents induced differential pro and anti-inflammatory profiles under strong inflammatory stimuli.

The cytokine profiles of the BMDCs were also observed by ELISA**.** Consistent with the hyperactivation phenotypes of *Fcgr2b*$^{-/-}$ BMDCs, LPS-stimulated *Fcgr2b*$^{-/-}$ BMDCs produced high levels of several proinflammatory cytokines, including TNF-α, IL-6, IL-23, IL-12, and IL-4 when compared to LPS-stimulated WT BMDCs (Figure 6). We could observe more clear effects of the pharmacologic drugs on the cytokine productions in LPS-stimulated BMDCs (Figure 6). Andrographolide and dexamethasone potentially reduced TNF-α, IL-6, IL-23, IL-12, IFN-γ, and IL-4 production in WT and *Fcgr2b*$^{-/-}$ BMDCs upon LPS-stimulation, while rapamycin and vitamin D showed lesser effects on the production of these cytokines. Dexamethasone displayed the most significant inhibitory effects on all proinflammatory cytokine production. It was only the pharmacologic agent that could reduce IL-1β production and promote the high IL-10 production in LPS-stimulated BMDCs from both WT and *Fcgr2b*$^{-/-}$ mice.

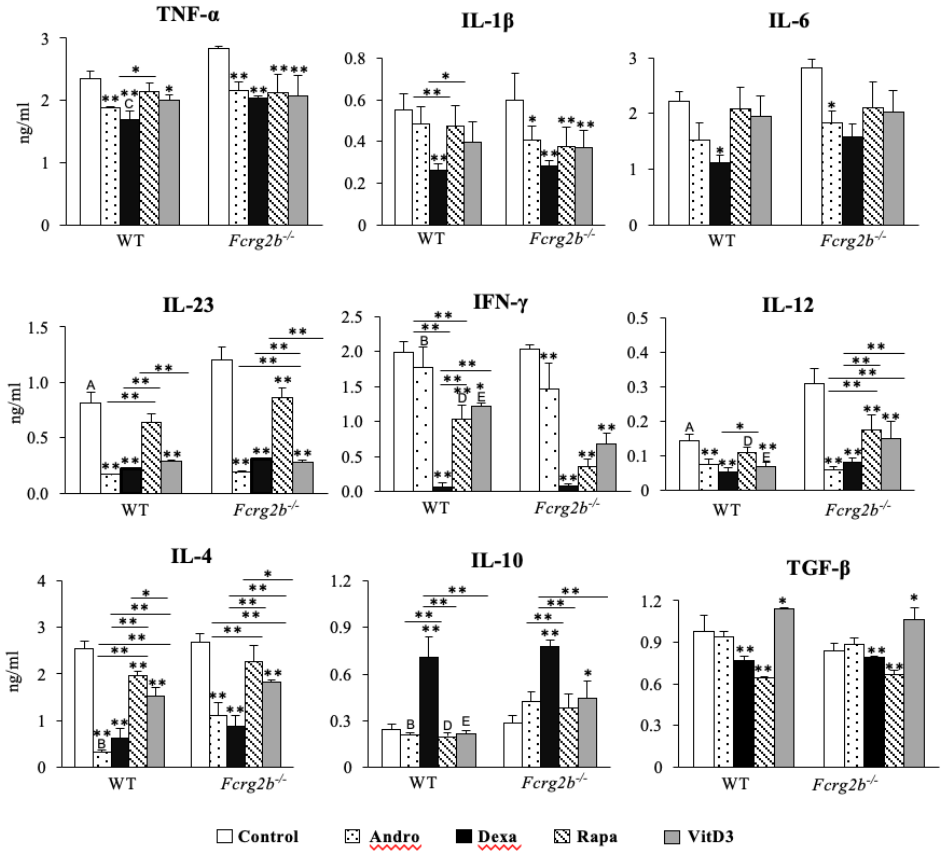

Figure 6 The cytokine production between wild type and deficient FcGRIIB mice in LPS stimulation. The data are shown as mean ± SD of triplicate experiments. The asterisks (*, **, and ***) indicate *p-value* ($p < 0.05$, $p < 0.01$, and $p < 0.001$), respectively.

The ability of Dex-tolDCs to induce OVA-Treg cells and decrease OVA-specific Effector T cells

As dexamethasone displayed the most remarkable ability to induce tolerogenic phenotypes in both WT and *Fcgr2b*$^{-/-}$ BMDCs, we next investigate the functional properties of dexamethasone-treated *Fcgr2b*$^{-/-}$ BMDCs on OVA-specific T cell response as a model. Mice were subcutaneously immunized with ovalbumin (OVA) in CFA and IFA, and T cells were isolated from the lymph nodes of the mice. Dexamethasone-treated BMDCs and untreated-BMDCs from WT and *Fcgr2b*$^{-/-}$ mice were stimulated with 1 µg/ml LPS for 2

hours, and the DCs were pulsed with 500 μg/ml OVA (in the presence of the LPS) for 22 hours. Subsequently, the BMDCs were co-cultured with OVA-immunized T cells (1:5 ratio of DC: T) for 72 hours. The cell proliferation was assessed by MTS and CFSE assay (Figure 7A and 7B, respectively). Dexamethasone-treated WT and *Fcgr2b*$^{-/-}$ BMDCs induced less T cell proliferation than the untreated BMDCs in both proliferation systems. The capability of dexamethasone-treated BMDCs in regulatory T-cell induction was also observed (Figure 7C). Dexamethasone-treated WT and *Fcgr2b*$^{-/-}$ BMDCs enhanced the regulatory T cell expansion compared to the untreated BMDCs (Figure 7C).

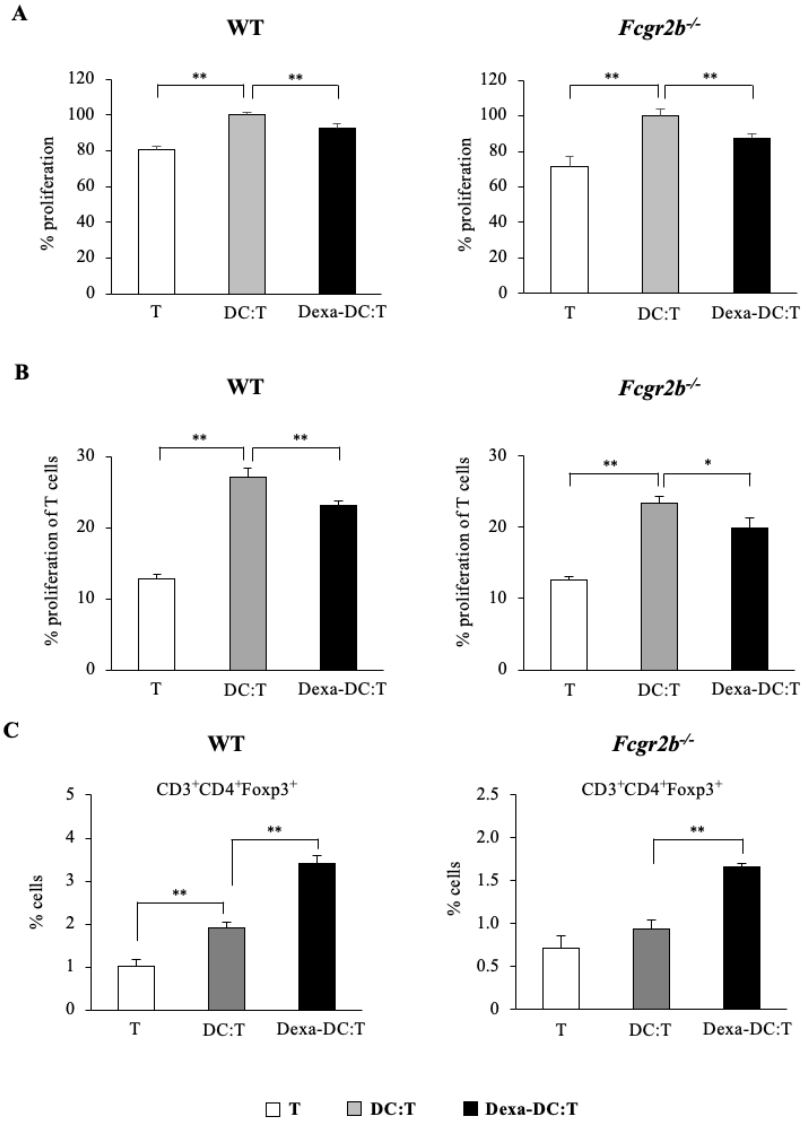

Figure 7 BMDCs function on OVA-T cells *in vitro*. Purified $CD3^+$ T cells were isolated from OVA-immunized both mice (wild type and deficient FcGRIIB) and then co-cultured with either control or dexamethasone-treated BMDCs pulsed with OVA for 72h. Cell viabilities were determined by MTS assay (a), CFSE-tagged T-cell proliferation (b), and $CD4^+CD25^+Foxp3^+$ Treg cells (c) were investigated by flow cytometry analysis (c). The data are shown as mean ± SD of triplicate experiments. The asterisks (*, **, and ***) indicate *p-value* ($p < 0.05$, $p < 0.01$, and $p < 0.001$), respectively.

The effect of Dex-tolDCs on pro and anti-inflammatory cytokines in DC: T cells co-culture

Following the above results, Dex-tolDCs inhibited T cell proliferation. In contrast, Dex-tolDCs increased Treg expansion. Previous studies have reported that co-culture between tolDCs and naïve CD3+ T cells reduces Th1/Th17-mediated IFN-y and IL-17A more than T cells cultured with mDCs. This study aims to evaluate cytokines from the T cell subpopulations (Th1, Th2, Th17, and Treg) after co-culture Dex-tolDCs and T cells. T cells co-cultured with dexamethasone-treated WT and Fcgr2b-/- BMDCs produced lower levels of IFN-Y (Figure 8A), IL-17 (Figure 8B), and IL-4 (Figure 8C) but had higher levels of IL-10 and TGF-B (Figure 8D, E).

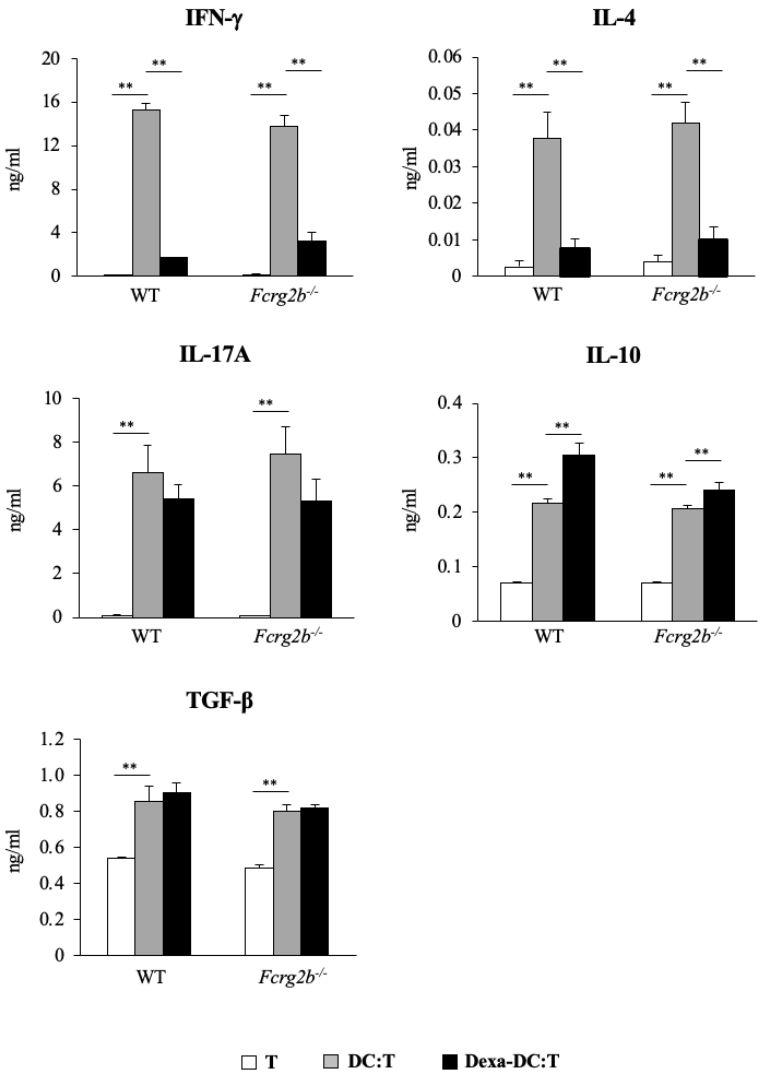

Figure 8 BMDCs function on OVA-T cells *in vitro*. Purified $CD3^+$ T cells were isolated from OVA-immunized both mice (wild type and deficient FcGRIIB) and then co-cultured with either control or dexamethasone-treated BMDCs pulsed with OVA for 72h. Cytokine production was investigated by ELISA. The data are shown as mean ± SD of triplicate experiments. The asterisks (*, **, and ***) indicate *p-value* ($p < 0.05$, $p < 0.01$, and $p < 0.001$), respectively.

Transfer Dex-tolDCs on the tolerogenic phenotype of DCs in secondary lymphoid organs of Fcgr2b-/- mice

To investigate the tolerogenic phenotype of DCs in lymph nodes and spleen of Fcgr2b-/- mice after adoptive transfer of dexamethasone generated tolDCs. After sacrificing the mice, lymph nodes and spleen were collected, and isolated lymphocytes and splenocytes were. Cells were stained with tolerogenic marker antibodies and detected by flow cytometry. In figure 9A, we found that Dex-tolDCs significantly increase the number of CD11c+ more than mature DC and PBS. In addition, the results found that tolerogenic surface markers ICOSL and PD-L1 were significantly higher in DCs in splenocytes compared with mature DCs injection (Figure 9B-C). DCs in lymphocytes show a higher level of PD-L1 but no alteration in ICOSL (Figure 9B-C). In general, the adoptive transfer of tolDCs generated a higher tolerogenic phenotype in the spleen than the lymph node, as expected in systemic injection.

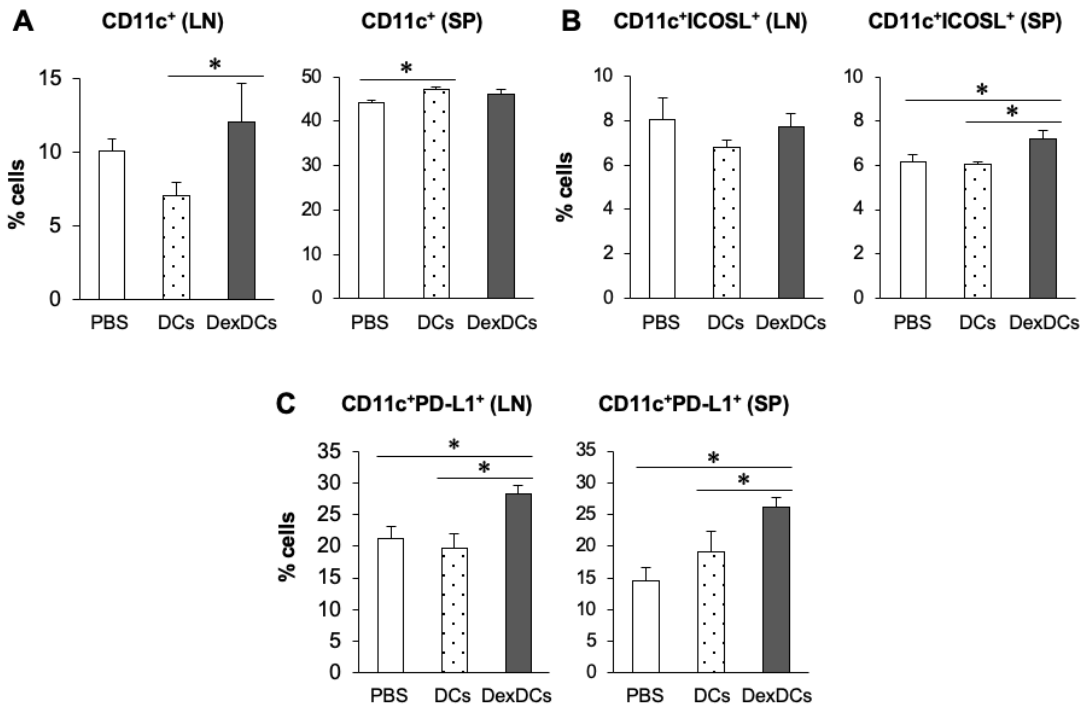

Figure 9 The numbers of tolDCs phenotype markers (ICOSL, PD-L1) were higher after tolerogenic dendritic cell (tolDC) transference in splenocytes. Determination of ICOSL and PD-L1 from spleen and lymph node from Fcgr2b -/- mice. The data are shown as mean ± SD of triplicate experiments. The asterisks (*, **, and ***) indicate *p-value* ($p < 0.05$, $p < 0.01$, and $p < 0.001$), respectively.

The effects of Dex-tolDCs on T cell-mediated immunity in Fcgr2b-/- mice

We examined the Treg population to determine immune status after the adoptive transfer Dex-tolDCs. After euthanizing mice, each group's lymphocytes and splenocytes were isolated, plated, and re-stimulated with apoptotic bodies in complete RPMI for 24 h. Cells were harvested, and subpopulations were determined by flow cytometry. The Treg population (CD4+FoxP3+, CD4+CD25+FoxP3+) in the spleen from the Dex-tolDCs group was significantly higher than the mDCs and PBS groups (Figure 10A-B). At the same time, the Treg-mediated IL10+ subpopulation (CD4+FoxP3+IL10+) in the spleen from Dex-tolDCs groups show significantly higher than control groups (Figure 10D). In contrast, lymphocyte

cells of Dex-tolDCs groups show a Treg subpopulation and Treg-mediated IL10+ trend like that observed for splenocytes (Figure 10A, 10B, 10D). Therefore, these results suggested that Dex-tolDCs can promote *in vivo* Treg expansion.

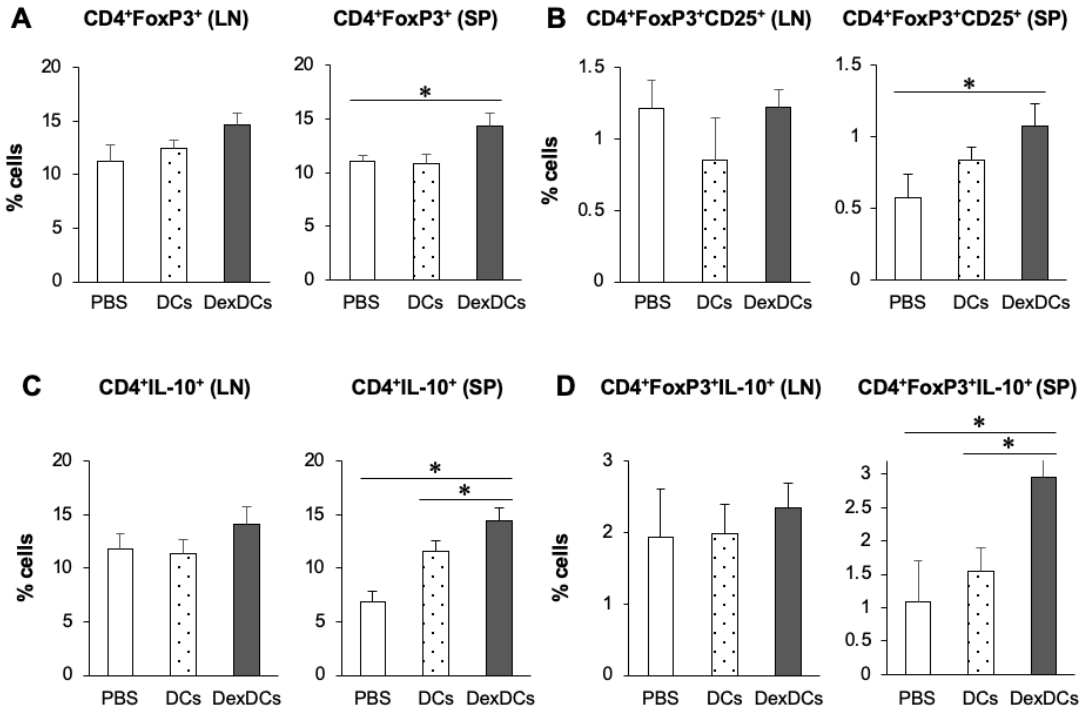

Figure 10 Regulatory T cells and regulatory T cell-mediated IL-10 secretion was higher after tolerogenic dendritic cell (tolDC) transference in splenocytes. Determination of $CD4^+$ $CD25^+$ $Foxp3^+$ cells and $CD4^+FoxP3^+IL10^+$ from spleen and lymph node from Fcgr2b -/- mice. The data are shown as mean ± SD of triplicate experiments. The asterisks (*, **, and ***) indicate *p-value* ($p < 0.05$, $p < 0.01$, and $p < 0.001$), respectively.

Adoptive transfer of antigen-load Dex-tolDCs ameliorates clinical manifestation in Fcgr2b-/- mice

To evaluate whether Dex-tolDCs can improve the clinical representation of lupus-prone mice models, Dex-tolDCs with antigen were injected into *Fcgr2b-/-* mice once a week for 4 times after the onset of the disease development. To determine disease severity, mice of

all groups were evaluated by determining the serum levels of anti-ds DNA antibodies after the end of the experiment. Interestingly, *Fcgr2b-/-* mice treated with Dex-tolDCs improved their clinical manifestation by reducing anti-ds DNA abs level compared with mDCs and PBS-treated mice (Figure 11). Therefore, we showed that antigen-load Dex-tolDCs could affect autoantibody levels as a marker for clinical manifestation in *Fcgr2b-/-* mice.

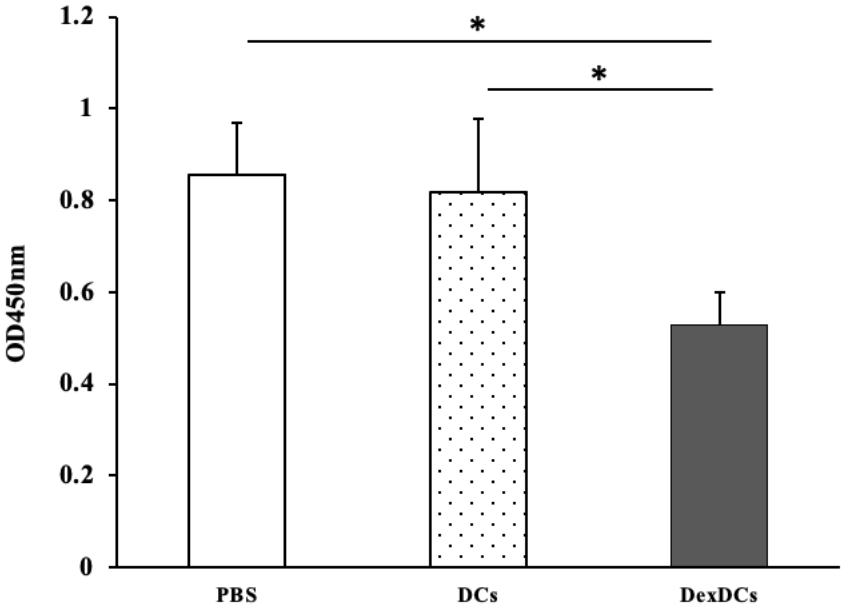

Figure 11 *Fcgr2b -/-* mice treated with dexamethasone-generated tolerogenic dendritic cells (tolDCs) have fewer anti-ds DNA autoantibodies. OD at 450 anti-ds DNA antibodies was determined in sera from Fcgr2b-/- mice. The data are shown as mean ± SD of triplicate experiments. The asterisks (*, **, and ***) indicate *p-value* ($p < 0.05$, $p < 0.01$, and $p < 0.001$), respectively.

Analysis structure of the nanoparticle

A single emulsion/solvent evaporation method was used to synthesize PLGA NPs and PDMAEMA-PLGA NPs, which was adapted from the preparation of PLGA NPs in recent work. In the present studies, we isolated the size of a particle at 500 nm through a centrifuge.

Previous studies have demonstrated that particle size at the 500 nm appropriated range accumulates at the inner side of DCs. (Figure 12A) The size of particles represented in spherical shape including PLGA NPs at 477.30 + 43.28 nm and PDMAEMA-PLGA NPs at 536.61 + 50.10 nm following SEM analysis. In addition, in figure 12B, a Graphic picture demonstrated cationic polymer of PDMAEMA and dexamethasone-loaded were attached to PLGA NPs using electrostatic interaction so that PDMAEMA and dexamethasone were mostly covered on the surface of PLGA NPs. In collaboration with our results, the copolymer of PDMAEMA and PLGA provided a sustained release profile although the drug was adsorbed onto the surface of the NPs. This slow-release rate of Dex-NPs may be explained by the binding of dexamethasone to the NPs via electrostatic interactions.

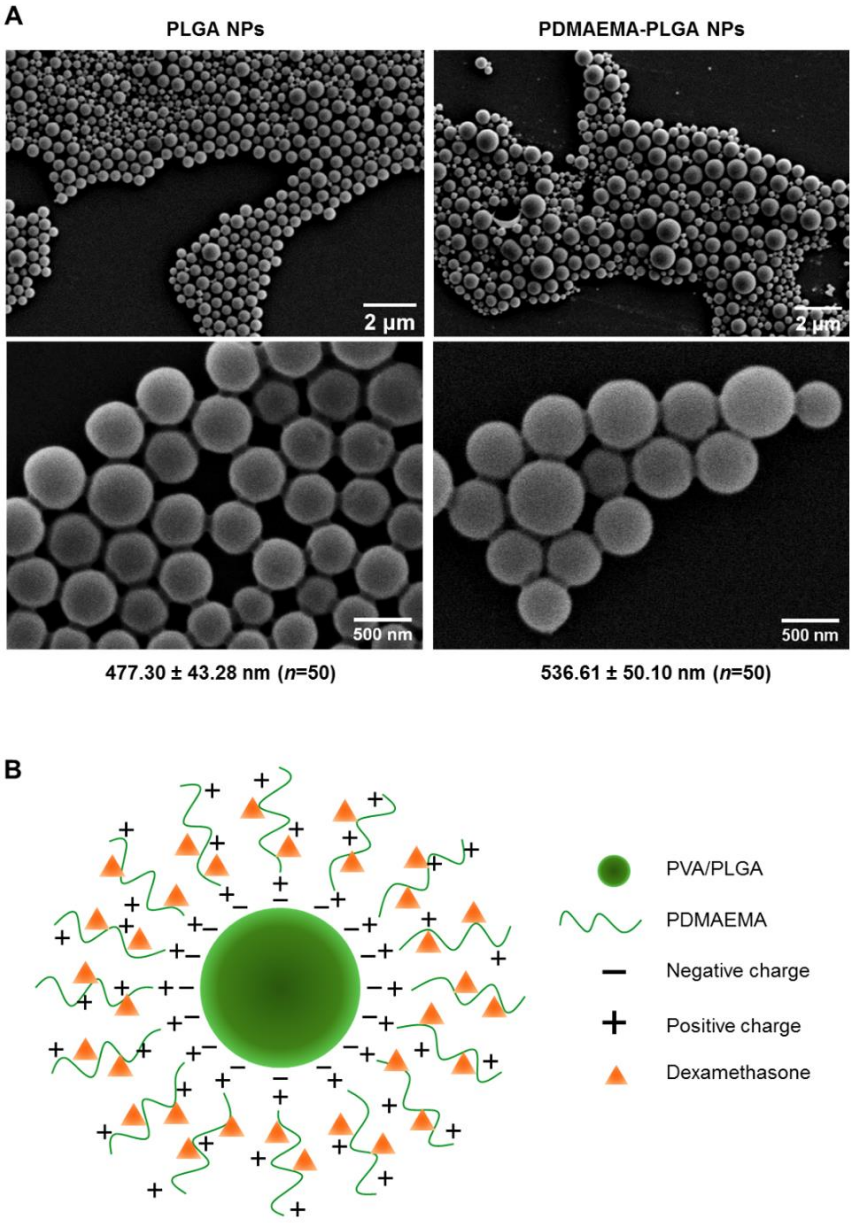

Figure 12 Characterizations of the synthesized nanoparticles. (A) Two magnifications of scanning electron microscopy (SEM) images of PLGA NPs and PDMAEMA-PLGA NPs. The numbers indicated the average size of the NPs (mean ± SD) from 50 particles. (B) Illustration of dexamethasone-incorporated PDMAEMA-PLGA NPs.

Stability and drug releasing of dexamethasone-loaded PDMAEMA-PLGA NPs

In this study, we investigated the stability and drug release properties of PDMAEMA-PLGA NPs (Dex-NPs). UV-VIS spectroscopy was used to evaluate the optical property of independent dexamethasone released from NPs. The suspension of blank PDMAEMA-PLGA NPs and DexNPs was incubated at 4 C and 37 C for 28 days around 5-6% from 7 to 28 days and weekly measured drug release (Figure 12). At 4 C, Dex-NPs were shown high stability together with a low level of free dexamethasone released from NPs around 5-6% from 7 to 28 days (Figure 13A). In the meantime, under 37 C incubation Dex-NPs were released faster at 21 to 28 days but they slowly released at every week of incubation (Figure 13B). Therefore, our results have shown that the combination between PDMAEMA and PLGA supported the sustained release profile of dexamethasone although the drug was adsorbed onto the NP's surface.

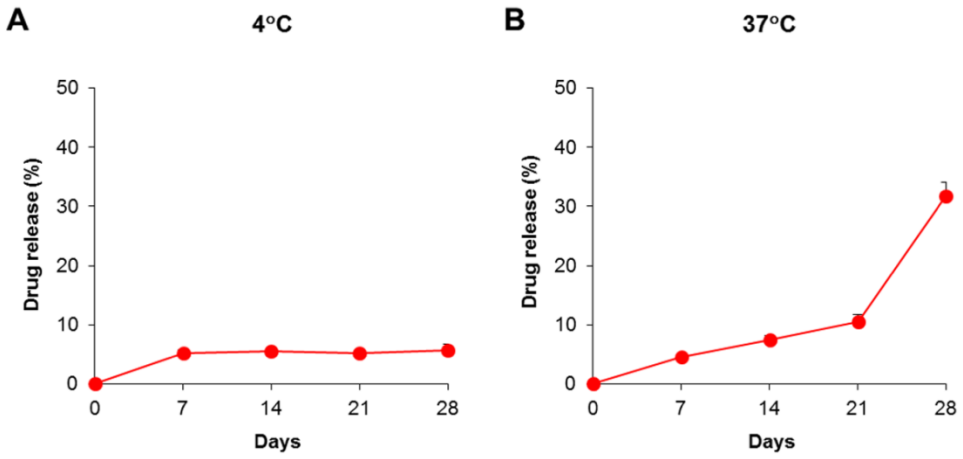

Figure 13 Stability and drug release profiles of dexamethasone-incorporated NPs. The suspension of PDMAEMA-PLGA NPs and dexamethasone-incorporated PDMAEMA-PLGA NPs were incubated at (A) 4°C and (B) 37°C in dark for 28 days. The supernatants were collected on days 0, 7, 14, and 28 for the measurement of free dexamethasone with a UV-VIS

spectrophotometer. The percentages of the drug release were calculated as described in Materials and Methods. The experiments were conducted in triplicate.

Dex-NPs triggered immunosuppressive activities and increase tolerogenic effects of dexamethasone on wide-type BM-cDCs

In general, the major DC population is conventional DCs (cDCs) which FMS-like tyrosine kinase 3 ligands (FLT3L) are importantly required for DCs development. However, GM-CSF is also necessary for the full development of cDCs1 as well as cDCs2. Therefore, to evaluate the effect of NPs on the cDCs population, in vitro cDCs were performed by using FLT3L and GM-CSF.

To determine the ability of Dex-NPs and PDMAEMA-PLGA NPs to mediate tolerogenic induction in cDCs. BM-cDCs from WT mice were added Dex-NPs containing 0.25, 0.5, 1, and 2 uM dexamethasone and blank PDMAEMA-PLGA NPs, 1 and 2 uM of dexamethasone for 48 h. After incubation, cells were stimulated with 0.1 ug/ml of LPS for 24 h and determined DC activation by using flow cytometer analysis (Figure 14). First, we determined live cells that identify by using forward scatter (FSC) and side scatter (SSC) plots. After treatment, each cell in the experimental groups was not altered in several live cells which represents that blank PDMAEMA-PLGA NPs, dexamethasone, and Dex-NPs did not mediate the cytotoxicity of cDCs (Figure 14A).

Consequently, the expression levels of the DC marker, CD11c (Figure 14B), and the DC immunogenic surface markers, CD40, CD80, CD86, and MHCII (Figure 14C-F) were evaluated using histogram analyses. BM-cDCs were stimulated with LPS, leading to highly increased expression of CD11c, CD40, CD80, CD86, and MHCII compare with unstimulated BM-cDCs. The effects of blank PDMAEMA-PLGA NPs, dexamethasone alone, and various doses of Dex-NPs were significantly decreased expression of CD11c, CD40, CD80, CD86, and MHCII under LPS-induced immunogenic response. However, all immunogenic markers

were significantly suppressed from the immunosuppressive effect of blank PDMAEMA-PLGA NPs and Dex-NPs more than the effect of dexamethasone

Next, we also observed the tolerogenic effect of blank PDMAEMA-PLGA NPs and Dex-NPs on the expression of a co-inhibitory molecule, ICOSL, and PD-L1 (Figure 14G, 14H). In the case of ICOSL expression, BM-cDCs after being stimulated with LPS slightly decreased expression of ICOSL. In addition, pretreatment BM-cDCs with blank PDMAEMA-PLGA NPs could not alter the expression of ICOSL. At the same time, pretreatment with 1 uM of dexamethasone slightly increased ICOSL expression but low expression after receiving 2 uM of dexamethasone. For Dex-NPs can maintain ICOSL expression under a concentration of dexamethasone less than 1 uM. In contrast, the expression of PD-L1 show significantly higher after being stimulated with LPS compare with unstimulated BM-cDCs. Dex-NPs containing 1 uM and 2 uM dexamethasone potentially up-regulated PD-L1 expression compared with dexamethasone alone and blank PDMAEMA-PLGA NPs under LPS stimulation.

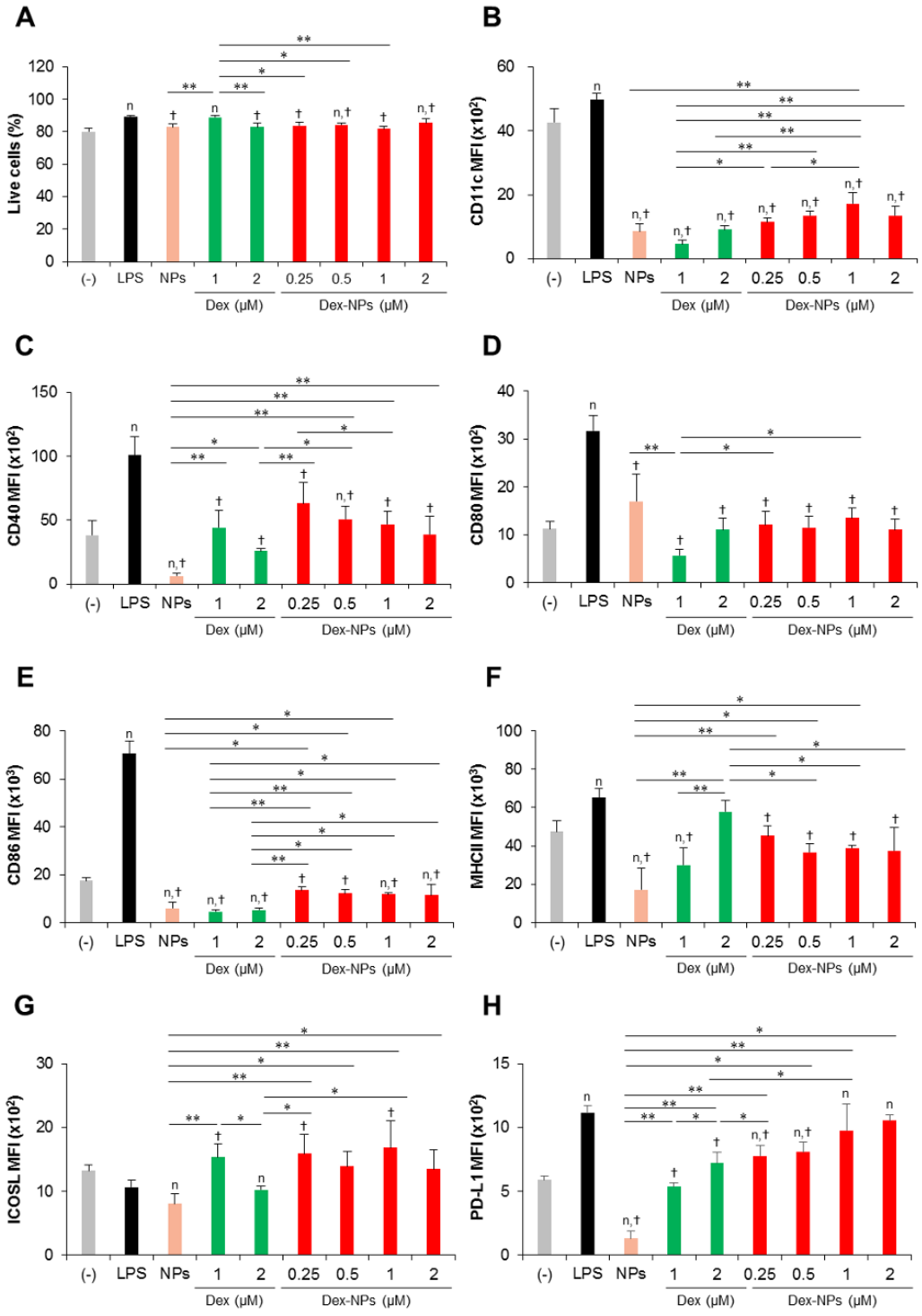

Figure 14 Immunomodulatory effects of PDMAEMA-PLGA NPs and dexamethasone-incorporated PDMAEMA-PLGA NPs on the maturation of wild-type BM-cDCs. Wild-type BM-cDCs were pre-incubated with blank PDMAEMA-PLGA NPs (55 μg, an equal amount

to Dex-NPs containing 2 μM dexamethasone), dexamethasone (1 and 2 μM), and dexamethasone-incorporated PDMAEMA-PLGA NPs containing 1 and 2 μM dexamethasone for 48 hours. Subsequently, the DCs were stimulated with 0.1 μg/ml of LPS for 24 hours. (A) Live cells and the expression of (B) CD11c, (C) CD40, (D) CD80, (E) CD86, (F) MHC class II, (G) ICOSL, and (H) PD-L1 were assessed by flow cytometry. $n = 5$; $^{n}\ p \leq 0.05$ compared with the negative control, $^{\dagger}p \leq 0.05$ compared with LPS-stimulated BM-cDCs, $*p \leq 0.05$, $**\ p \leq 0.001$; (-), negative control (untreated and unstimulated BM-cDCs); LPS, LPS-stimulated BM-cDCs; Dex, dexamethasone; Dex-NPs, dexamethasone-incorporated PDMAEMA-PLGA NPs.

Dex-NPs exhibited pro and anti-inflammatory cytokines on wide-type BM-cDCs

To determine cytokine profiles of BM-cDCs after treatment with various agents. Increment of high levels of inflammatory cytokines after stimulated with LPS including TNF-a, IL-1B, IL-6, IL-23, and IL12 as well as moderate levels of the anti-inflammatory cytokine, IL-10. After pretreatment with blank PDMAEMA-PLGA NPs, all doses of dexamethasone, and Dex-NPs reduced all inflammatory cytokines under LPS stimulation. The suppression of Dex-NPs was comparable to dexamethasone, but blank NPs have shown a less suppressive effect on cytokine production. Following the results of blank NPs and Dex-NPs containing 0.25 – 1 uM dexamethasone increased IL-10 production under LPS activation. Interestingly, DexNPs containing 2 uM dexamethasone had significantly higher levels of IL-10 production compared with all experiment groups. In contrast, pretreatment with dexamethasone alone was unable to initiate the production of IL10.

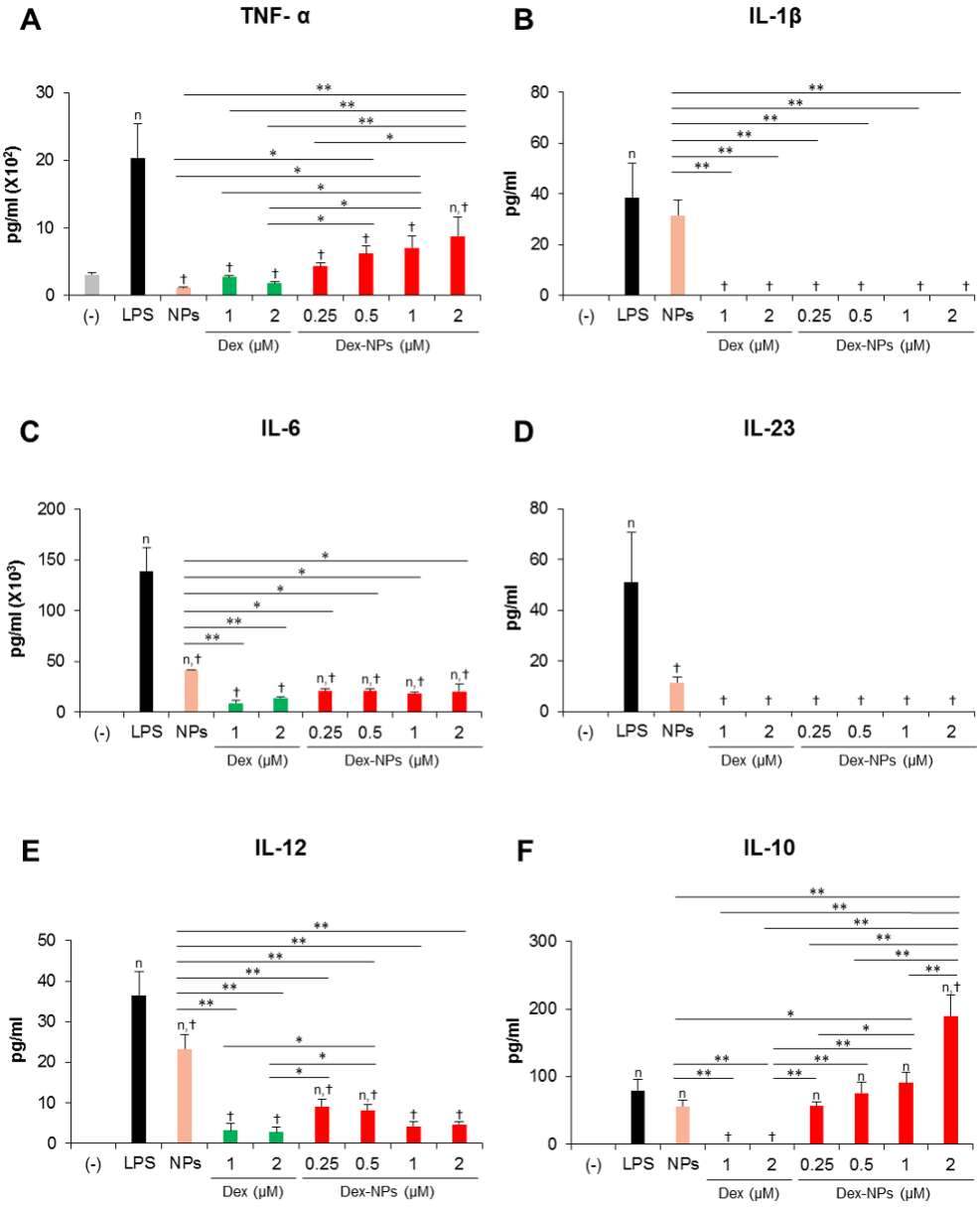

Figure 15 Effects of PDMAEMA-PLGA NPs and dexamethasone-incorporated PDMAEMA-PLGA NPs on the production of inflammatory and anti-inflammatory of wild-type BM-cDCs. Wild-type BM-cDCs were untreated or pre-incubated with blank PDMAEMA-PLGA NPs (55 μg, an equal amount to Dex-NPs containing 2 μM dexamethasone), dexamethasone (1 and 2 μM), and dexamethasone-incorporated PDMAEMA-PLGA NPs containing 1 and 2 μM dexamethasone for 48 hours. Subsequently, the DCs were stimulated with 0.1 μg/ml of LPS

for 24 hours. Culture supernatants were collected and (A) TNF-α, (B) IL-1β, (C) IL-6, (D) IL-23, (E) IL-12 and (F) IL-10 were determined using ELISA. $n = 5$; ^{n} $p \leq 0.05$ compared with the negative control, $^{\dagger}p \leq 0.05$ compared with LPS-stimulated BM-cDCs, $*p \leq 0.05$, $**$ $p \leq 0.001$; (-), negative control (untreated and unstimulated BM-cDCs); LPS, LPS-stimulated BM-cDCs; Dex, dexamethasone; Dex-NPs, dexamethasone-incorporated PDMAEMA-PLGA NPs; MFI, mean fluorescence intensity.

Dex-NPs altered the hyperactivity function of BM-cDCs from FcgRIIB-/- mice to tolerogenic cDCs

Following the literature review of the function of FcgRIIB receptors, its wide expression on B cells and myeloid cells, especially DCs. Previous studies have reported that mice deficient in FcgRIIB expression can develop spontaneous lupus disease. In addition, specific deletion of FcgRIIB in DCs of mice mediated hyperactivation and increased susceptibility to autoimmune disease.

Therefore, we evaluated whether our NPs can decrease immunogenic hyperactivation of Fcgr2b -/- BM-cDCs and turn into tolerogenic phenotype. After treatment BM-cDCs by blank NPs, all doses of dexamethasone, and all doses of Dex-NPs under LPS stimulation found that dexamethasone alone decreases live cells compared with negative control (Figure 16A). Similarly, the above results of WT-BM-cDCs, Blank NPs, all doses of dexamethasone, and Dex-NPs decreased the expression level of CD11c, CD40, CD80, CD86, and MHCII (Figure 16B-F). Meanwhile, Fcgr2b-/- BM-cDCs were stimulated with LPS not to alter the expression level of ICOSL but to increase the PD-L1 level. Next, blank NPs inhibited the expression of ICOSL and PD-L1. At the same time, all doses of Dex-NPs increased higher levels of ICOSL expression but at 4 uM dexamethasone-loaded NPs mediated higher

expression of PD-L1 (Figure 16G-H) compare with negative control and LPS stimulation.

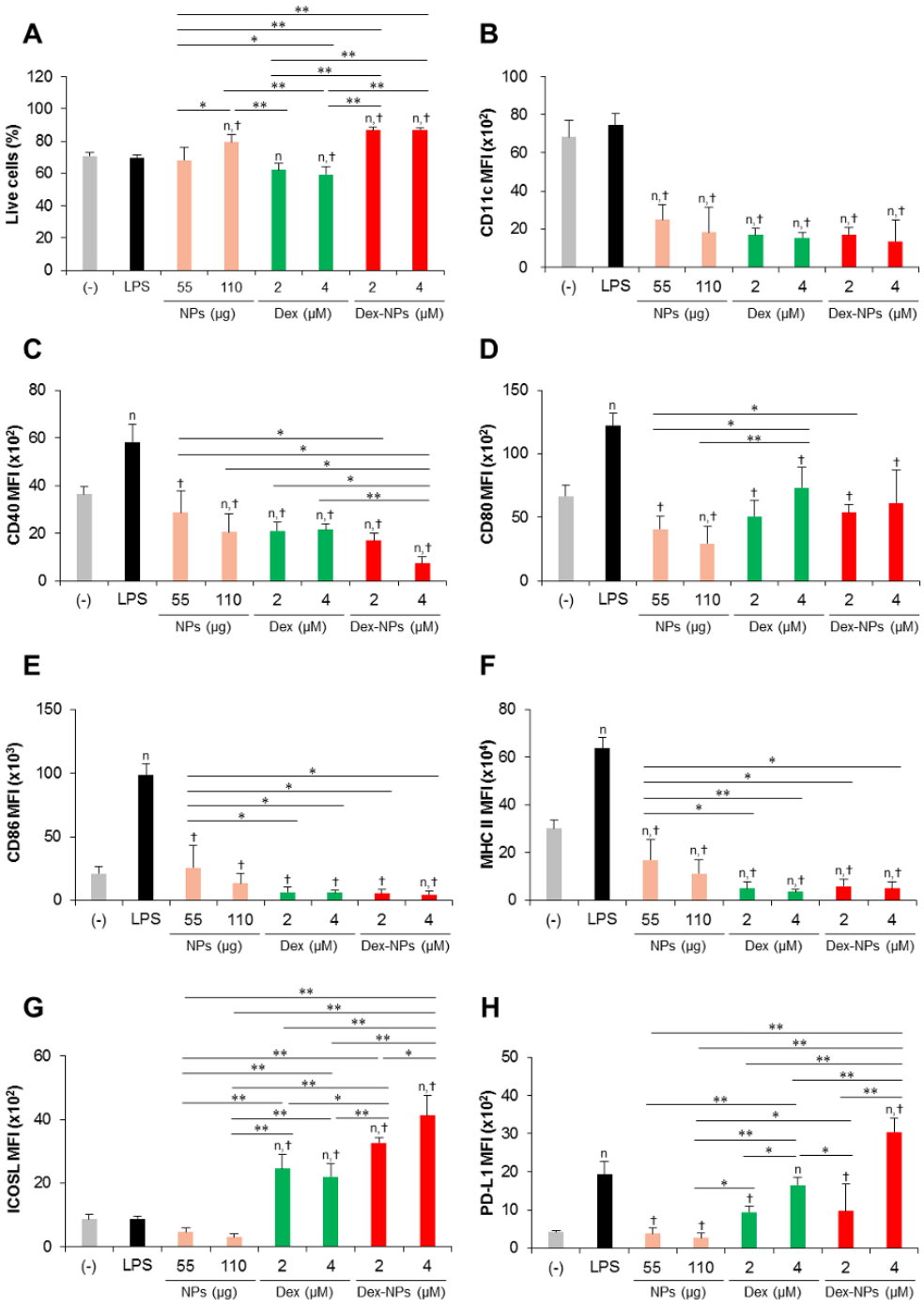

Figure 16 Immunosuppressive and tolerogenic effects of PDMAEMA-PLGA NPs and dexamethasone-incorporated PDMAEMA-PLGA NPs in *Fcgr2b*$^{-/-}$ BM-cDCs. *Fcgr2b*$^{-/-}$ BM-cDCs were untreated or pre-incubated with blank PDMAEMA-PLGA NPs (55 μg and 110

μg, an equal amount to Dex-NPs containing 2 μM and 4 μM dexamethasone, respectively), dexamethasone (2 and 4 μM), and dexamethasone-incorporated PDMAEMA-PLGA NPs containing 2 and 4 μM dexamethasone for 48 hours. Subsequently, the DCs were stimulated with 0.1 μg/ml of LPS for 24 hours. (A) Live cells and the expression of (B) CD11c, (C) CD40, (D) CD80, (E) CD86, (F) MHC class II, (G) ICOSL, and (H) PD-L1 were assessed by flow cytometry. $n = 5$; ^{n} $p \leq 0.05$ compared with the negative control, $^{\dagger}p \leq 0.05$ compared with LPS-stimulated BM-cDCs, $^{*}p \leq 0.05$, ** $p \leq 0.001$; (-), negative control (untreated and unstimulated BM-cDCs); LPS, LPS-stimulated BM-cDCs; Dex, dexamethasone; Dex-NPs, dexamethasone-incorporated PDMAEMA-PLGA NPs; MFI, mean fluorescence intensity.

Dex-NPs altered inflammatory cytokine profiles of BM-cDCs from FcgRIIB-/- mice to tolerogenic cDCs

Production of inflammatory cytokines under LPS stimulation was significantly decreased when Fcgr2b -/- BM-cDCs were treated with blank NPs, all doses of dexamethasone, and Dex-NPs. From the results, Dex-NPs at 2 uM dexamethasone enhance IL-10 production more than 4 uM dexamethasone. However, Blank NPs partially inhibited IL-6 production. Dexamethasone alone interfered with IL-10 production from Fcgr2b-/- BM-cDCs under LPS response (Figure 17).

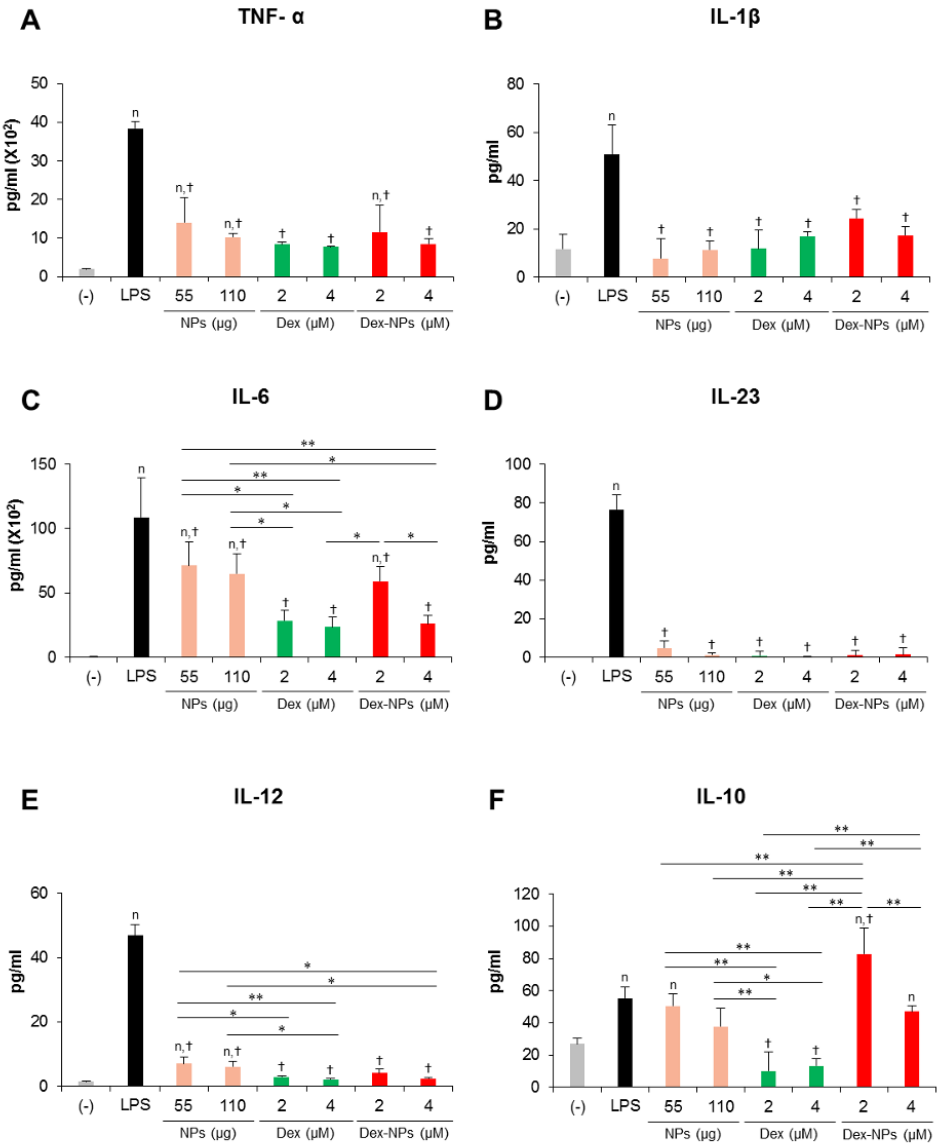

Figure 17 Effects of PDMAEMA-PLGA NPs and dexamethasone-incorporated PDMAEMA-PLGA NPs on the cytokine profiles of w *Fcgr2b*$^{-/-}$ BM-cDCs. *Fcgr2b*$^{-/-}$ BM-cDCs were untreated or pre-incubated with blank PDMAEMA-PLGA NPs (55 μg and 110 μg, an equal amount to Dex-NPs containing 2 μM and 4 μM dexamethasone, respectively), dexamethasone (2 and 4 μM), and dexamethasone-incorporated PDMAEMA-PLGA NPs containing 2 and 4 μM dexamethasone for 48 hours. Subsequently, the DCs were stimulated with 0.1 μg/ml of LPS for 24 hours. Culture supernatants were collected and (A) TNF-α, (B)

IL-1β, (C) IL-6, (D) IL-23, (E) IL-12 and (F) IL-10 were determined using ELISA. $n = 5$; $^{n}p \leq 0.05$ compared with the negative control, $^{\dagger}p \leq 0.05$ compared with LPS-stimulated BM-cDCs, $^{*}p \leq 0.05$, $^{**}p \leq 0.001$; (-), negative control (untreated and unstimulated BM-cDCs); LPS, LPS-stimulated BM-cDCs; Dex, dexamethasone; Dex-NPs, dexamethasone-incorporated PDMAEMA-PLGA NPs.

The effect of Dex-NPs on BM-cDCs from FcgRIIB-/- mice to generate regulatory T cell expansion *in vivo*

The main feature of tolerogenic DCs is to generate regulatory T cell (Treg) expansion and function, thereby mediating tolerance. So, we aim to examine the ability of Dex-NPs treated BM-cDCs in Treg proliferation and function. BM-cDCs were treated with 2 uM dexamethasone and Dex-NPs with 2 uM dexamethasone and then activated with LPS for 24 h. Next, BM-cDCs were harvested and co-cultured with naïve Treg in the presence of soluble anti-CD3 mAb and recombinant IL-2. After 72 h of incubation, Treg proliferation was measured by MTS assay. For the control of this experiment, Treg alone was cultured with soluble anti-CD3 and rmIL-2 to ensure that the signal of soluble anti-CD3 mAb and rmIL-2 was suitable. The results found that dexamethasone and Dex-NPs-treated BM-cDCs enhance Treg proliferation when compare with LPS-stimulated BM-cDCs. In addition, Dex-NPs increased Treg expansion to a significantly higher more than BM-cDCs treated with dexamethasone alone (Figure 18A).

The mechanism of Treg-generated immune inhibition is dependent on the production of IL-10. So, we determined IL-10 production from Treg in this in vitro DC/Treg co-culture system. We used BM-cDCs stimulated with LPS and BM-cDCs treated with dexamethasone and Dex-NPs culture in parallel as the control because we measure IL-10 from the supernatant, IL-10 may be produced from DCs. The results have shown that IL-10 from control groups of DCs was very low level as well as Treg alone. At the same time, BM-cDCs

stimulated LPS were unable to mediate Treg cells to produce the level of IL-10 production but BM-cDCs treated with dexamethasone and Dex-NPs significantly mediated Treg to produce IL-10 level (Figure 18B).

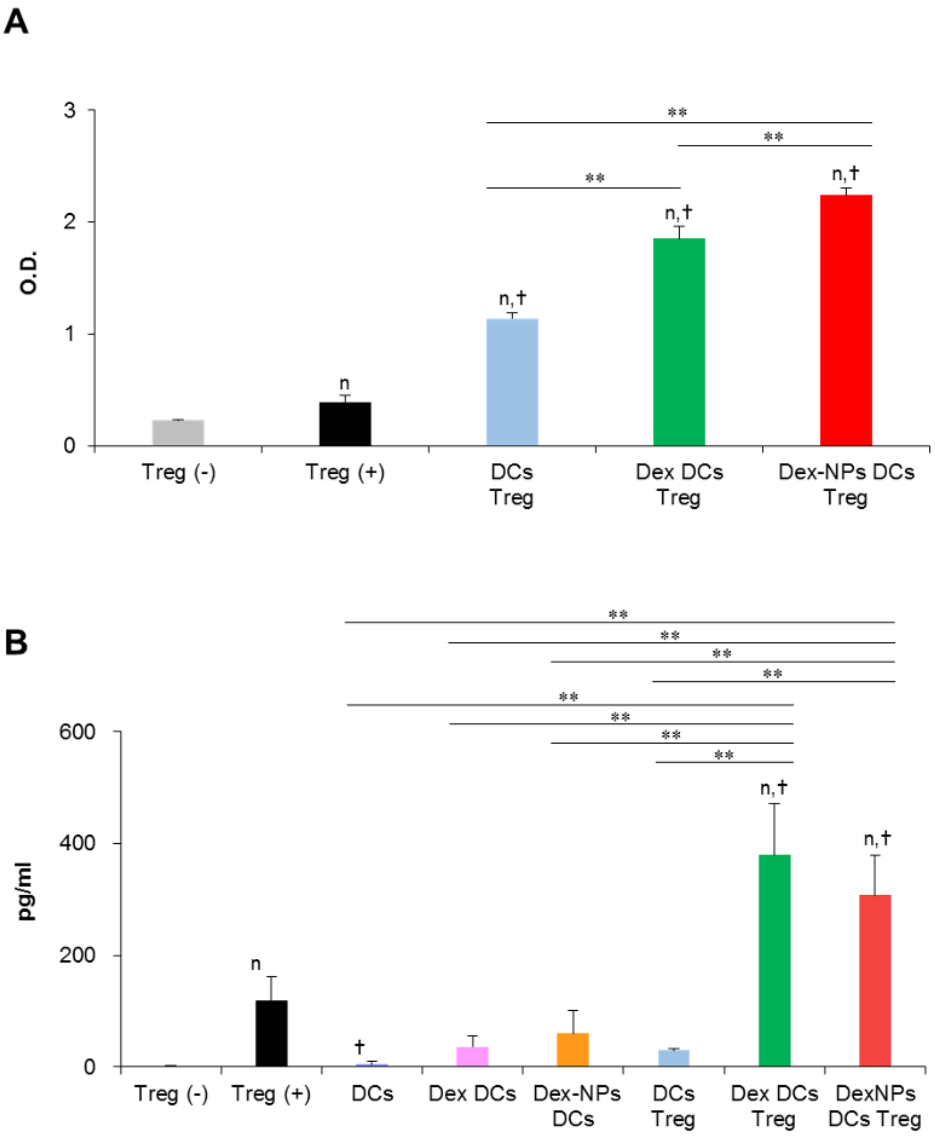

Figure 18 Direct *in vitro* interaction of dexamethasone-incorporated PDMAEMA-PLGA NPs pretreated BM-cDCs and regulatory T cell. Wild-type BM-cDCs were untreated or pre-incubated with 2 μM dexamethasone, or dexamethasone-incorporated PDMAEMA-PLGA NPs containing 2 μM dexamethasone for 48 hours and DCs were stimulated with 0.1 μg/ml of

LPS for 24 hours. Subsequently, the DCs were co-cultured with regulatory T cells isolated by magnetic-activated cell sorting in the presence of soluble anti-mouse CD3 mAb and recombinant mouse IL-2. (A) Cell proliferation was evaluated at 72 hours after coculture and (**B**) the IL-10 levels in the culture supernatant were measured at 48 hours after the co-culture. $n = 3$; ^{n} $p \leq 0.05$ compared with unstimulated regulatory T cell, $^{\dagger}p \leq 0.05$ compared with regulatory T cell alone incubated with soluble anti-CD3 mAbs, $*p \leq 0.05$, $** p \leq 0.001$; Treg (-), unstimulated regulatory T cells; Treg (+), regulatory T cell incubated with soluble anti-mouse CD3 mAb and recombinant mouse IL-2; DCs Treg, LPS-stimulated BM-cDCs cocultured with regulatory T cells; Dex-DCs Treg, dexamethasone pre-treated BM-cDCs co-cultured with regulatory T cells, Dex-NPs Treg, PDMAEMA-PLGA NPs pre-treated BM-cDCs co-cultured with regulatory T cells, DCs, LPS-stimulated DCs; Dex DCs, dexamethasone pre-treated BM-cDCs, Dex NPs DCs, dexamethasone-incorporated PDMAEMA-PLGA NPs pre-treated BM-cDCs.

Dex-NPs were actively captured by dendritic cells *in vivo*

To monitor the in vivo uptake of PDMAEMA-PLGA NPs by DCs, FITC was tagged on the surface of PDMAEMA-PLGA NPs (FITC-NPs), and these FITC NPs were injected into WT mice via subcutaneous injection. After 3 days, skin draining LNs (dLNs) with FITC+ cells were analyzed along with DC, Macrophage, and T cells represent in marker CD11c, F4/80, and CD3, respectively. WT and Fcgr2b-/- mice that received FITC-NPs found a significantly higher percentage of CD11c+ and F4/80+ cells except for CD3+ cells, when compared with PBS injection, represent the migration of DCs and macrophages from the periphery to dLNs (Figure 19A). Following figure 16B, in both WT and Fcgr2b-/- mice, the number of FITC+CD11c+ cells increased while the number of FITC+F4/80+ cells increase moderately. However, the population of FITc+F4/80+ cells was detectable, the number of this population was smaller than the number of FITC+CD11c+ cells. These results indicated that the NPs were highly captured and transported by DC and, to a lesser extent, macrophages.

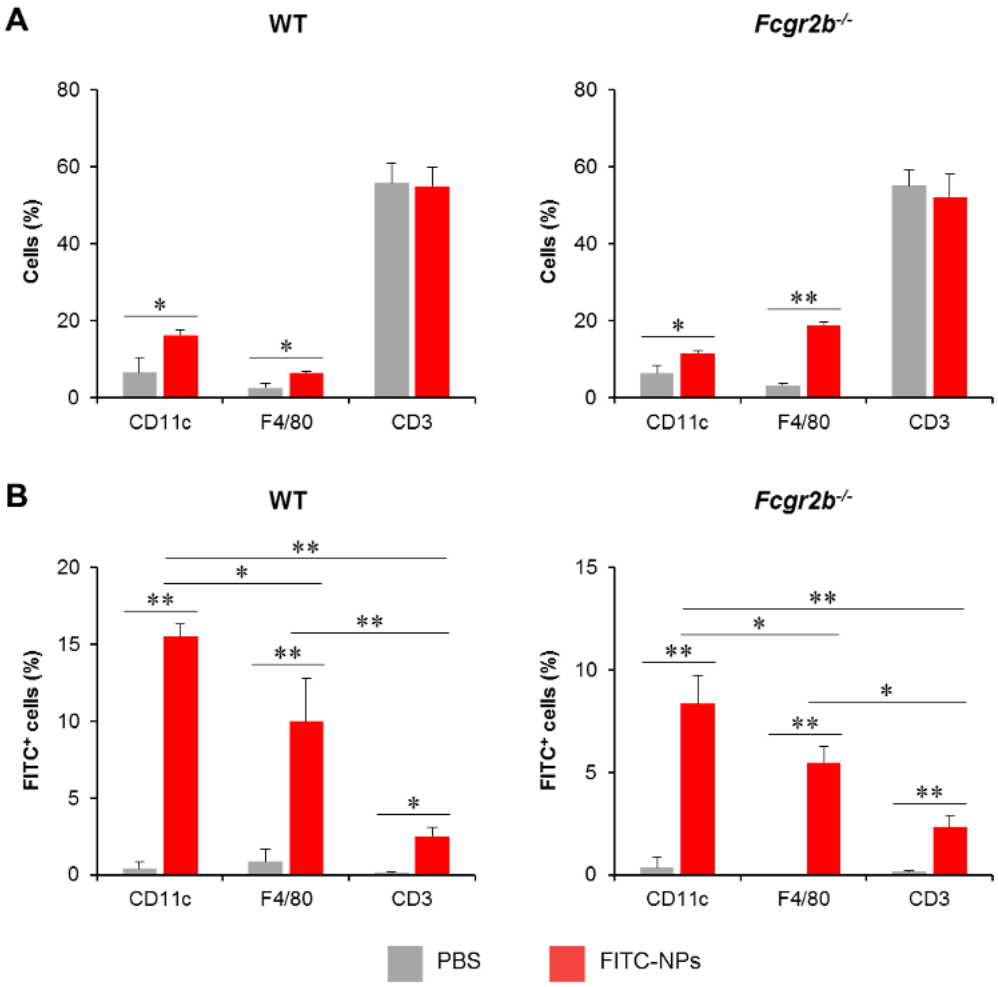

Figure 19 *In vivo* uptake of nanoparticles by dendritic cells. Phosphate buffer saline or FITC-tagged PDMAEMA-PLGA NPs were subcutaneously administered into wild-type and *Fcgr2b*$^{-/-}$ mice. Seventy-two hours later, (A) The proportion of CD11c^{+}, F4/80^{+}, and CD3^{+} cells in the draining lymph nodes (dLNs) were determined using flow cytometric analysis. (B) The proportions of FITC^{+} cells in CD11c^{+}, F4/80^{+}, and CD3^{+} cells were evaluated by flow cytometric analysis. n = 5; *$p \leq 0.05$, ** $p \leq 0.001$; PBS, the control mice that received phosphate buffer saline; FITC-NPs, mice received FITC-tagged PDMAEMA-PLGA NPs.

Dex-NPs ameliorated lupus severity disease in Fcgr2b-/- mice

Next, we determined the therapeutic potential of Dex-NPs in a mouse model. After the onset of lupus in Fcgr2b-/- mice developed around 16-24 weeks old, we subcutaneously injected with PBS, or dexamethasone alone and Dex-NPs pulsed with antigens (apoptotic bodies) weekly for 4 consecutive weeks and investigated the alteration of kidney histopathology, anti-ds DNA antibodies in serum, serum IL-6, serum creatinine, and urine protein/creatinine index (Figure 20). Control *Fcgr2b*$^{-/-}$ mice that received PBS showed lupus nephritis as indicated by renal tubulointerstitial injury scores based on tubular vacuolization and increased infiltration of interstitial cells (Figure 20A, second row, and Figure 20B). Likewise, the prominent glomerular lesion of lupus nephritis, a pathognomonic lesion of the disease indicated by the percentage of glomeruli with moderate to severe mesangial expansion, was detectable in *Fcgr2b*$^{-/-}$ control mice (Figure 20A, second row and Figure 20B). Of interest, *Fcgr2b*$^{-/-}$ lupus-prone mice treated with dexamethasone or Dex-NPs exhibited less severe renal interstitial inflammation and lower glomeruli with mesangial expansion (Figure 20A, third and fourth rows, and Figure 20B-C). Consistently, anti-dsDNA antibodies, serum IL-6, and serum creatinine levels were substantially decreased in mice treated with dexamethasone or Dex-NPs (Figure 20D-F). In addition, dexamethasone, and Dex-NP treatment significantly reduced proteinuria (urine protein creatinine index; UPCI) in *Fcgr2b*$^{-/-}$ lupus-prone mice (Figure 20G). As a result, Dex-NP therapy improved the outcomes of lupus, and its therapeutic effects were comparable to those of dexamethasone. In this study, we aimed to induce tolerogenic DCs in situ using immunomodulatory NPs that incorporate dexamethasone for the treatment of SLE. Since DCs are a major population that resides in the skin, NP administration via the subcutaneous route may provide precise delivery to DCs. Our data also suggested that PDMAEMA-PLGA NPs had a high efficiency to enter skin DCs, therefore, *in vivo* Dex-NP delivery may specifically target DCs. Our findings demonstrated

that the DC-targeted therapy with Dex-NPs produced favorable therapeutic effects in the lupus-prone mouse model as same as the non-targeted therapy with dexamethasone.

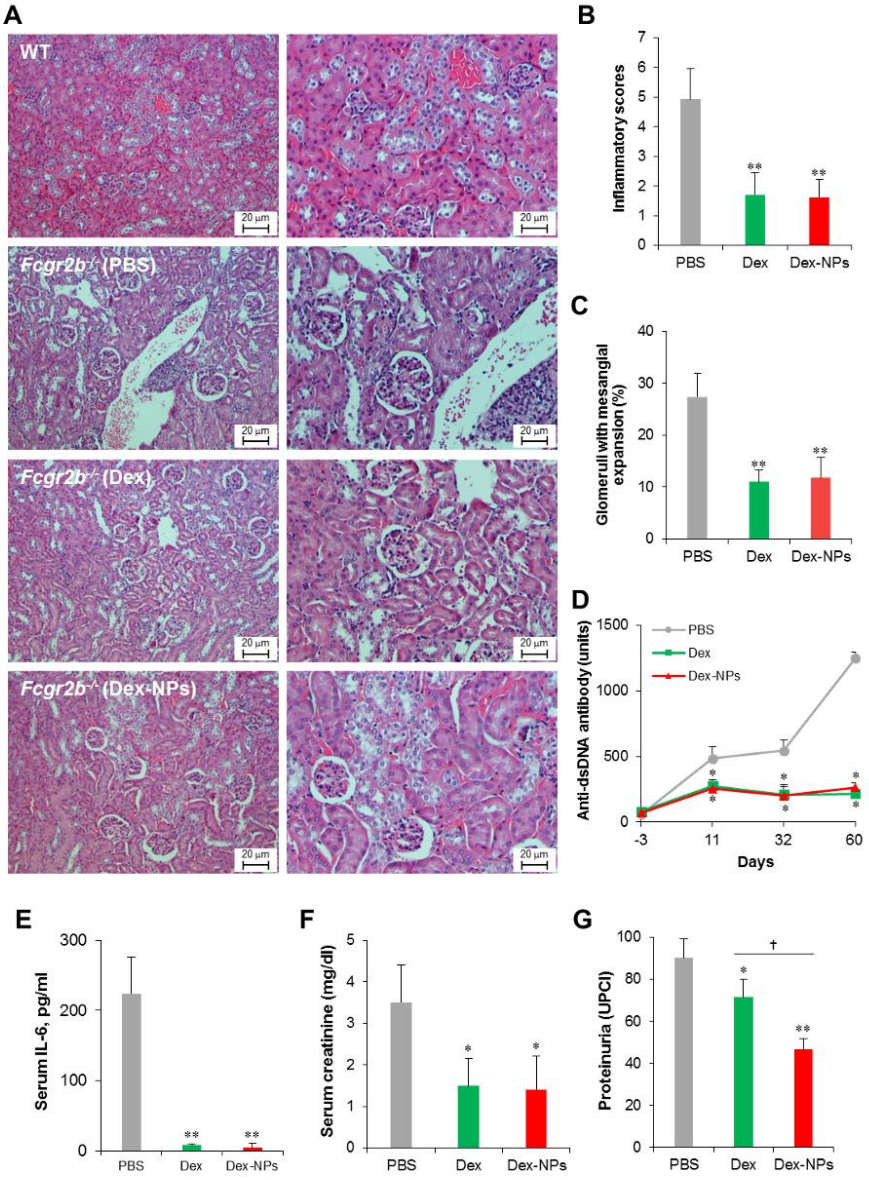

Figure 20 Potent amelioration of lupus disease by dexamethasone-incorporated NPs. The renal damages of wild-type (WT) and *Fcgr2b*$^{-/-}$ mice treated with phosphate buffer solution (PBS) and apoptotic bodies mixed with dexamethasone (Dex) or dexamethasone-incorporated PDMAEMA-PLGA NPs (Dex-NPs) were indicated by (A) the representative of histopathology with H&E staining, (B) the renal tubulointerstitial injury score, and (C)

glomerular injury are demonstrated. Serum from all mice was also collected to investigate the alteration of (D) anti-double strand DNA auto-antibodies, (E) IL-6, and (F) creatinine. Proteinuria was measured and represented in urine protein creatinine index (UPCI). $n = 5$; *$p \leq 0.05$ compared with PBS control, ** $p \leq 0.001$ compared with PBS control, $^{\dagger}p \leq 0.05$.

Dex-NPs increased expression of ICOSL and PD-L1 in DCs and enhanced regulatory T cell expansion in Fcgr2b-/- mice

To further validate the superior therapeutic efficacy of Dex-NPs, the alterations of DC phenotypes and the Treg population were investigated. *Fcgr2b*$^{-/-}$ lupus-prone mice were administered with PBS and the antigens were mixed with dexamethasone or Dex-NPs as described above and the dLNs were collected for the analysis of DCs and T cells by flow cytometry. Compared to PBS control mice, administration of dexamethasone and Dex-NPs did not alter the proportion of $CD11c^{+}$ cells (Figure 21A, right panel), but both treatments significantly increased the number of $CD11c^{+}ICOSL^{+}$ and $CD11c^{+}PD\text{-}L1^{+}$ cells (Figure 21A, middle, and left panel), indicating the successful induction of tolerogenic DCs in situ. The numbers of $CD3^{+}CD4^{+}$ cells were also not affected by treatment with dexamethasone and Dex-NP (Figure 21B, right panel). Of interest, Dex-NPs, but not dexamethasone, mediated the expansion of $CD3^{+}CD4^{+}Foxp3^{+}$ Tregs and $CD3^{+}CD4^{+}Foxp3^{+}CD25^{+}$ activated Tregs in *Fcgr2b*$^{-/-}$ lupus-prone mice (Figure 21B, middle and left panel).

Parallelly, we performed the *in vitro* restimulation assay to determine antigen-specific responses. LN cells from the dLNs of *Fcgr2b*$^{-/-}$ lupus-prone mice treated with PBS, dexamethasone, and Dex-NPs as described above were re-stimulated with apoptotic bodies and Treg populations were evaluated by flow cytometric analyzes. Consistent with the *in vivo* observation, only Dex-NP treatment enhanced the number of $CD3^{+}CD4^{+}Foxp3^{+}$ and $CD3^{+}CD4^{+}Foxp3^{+}CD25^{+}$ cells in response to re-exposure of a specific antigen (Fig. 21C, right and middle panel). In this experiment, we also observed the production of IL-10 by

$CD4^+$ T cells and found that dexamethasone and Dex-NP treatment showed a similarly elevated level of the $CD3^+CD4^+IL\text{-}10^+$ population (Fig. 21C, left panel).

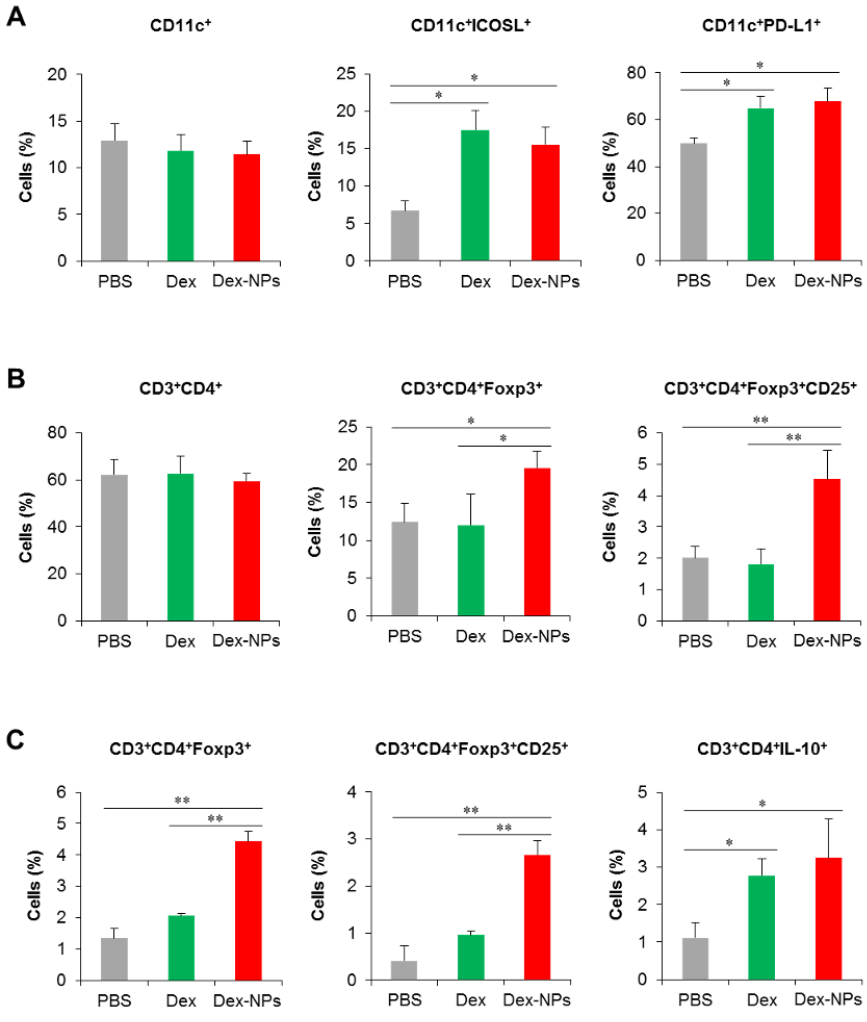

Figure 21 Investigation of the DC and T cell population in lupus mice and in vitro restimulation of draining lymph node cells. *Fcgr2b*$^{-/-}$ mice were treated with phosphate buffer solution (PBS) and apoptotic bodies mixed with dexamethasone (Dex) or dexamethasone-incorporated PDMAEMA-PLGA NPs (Dex-NPs). At the end point of treatment, dLNs were collected and (*A*) tolerogenic DC phenotypes and (B) CD4 T cell and Treg population were determined by flow cytometric analysis. (C) The LN cells were restimulated with apoptotic bodies in vitro and the Treg population was evaluated. $n = 5$; *$p \leq 0.05$, ** $p \leq 0.001$.

CHAPTER VI

DISCUSSION

Either immunity or tolerance to specific antigens, including self-antigen mainly promoted via the DCs activation (121). The manipulation of DCs phenotypes can be applied to alter the aberrant immune responses to self-antigen tolerance (122). The application of tolDCs as a therapeutic option has been tested in various animal models and clinical trials with promising results (20). The characteristics and properties of tolDCs can vary depending on the tolDC-inducing protocol (123, 124). Moreover, the phenotypic and functional features of tolDCs required for effective therapy may differ based on the pathogenesis of distinct autoimmune diseases (101, 124). Previous studies have tested the potential of tolDCs generated from dexamethasone and rosiglitazone in a model of systemic lupus mice model including MRL-Fas^{lpr} and NZM2410 mice (96). However, limited data on the induction of tolDCs are available in FcGRIIb-deficient mice, one report suggested an inability to induce tolerance in FcGRIIb-deficient mice via the mucosal route. They suggested that this defect is via the function of dendritic cells since FcGRIIb is a main regulatory molecule. Since FcGRIIb polymorphism is an important genetic susceptibility in SLE, particularly in Asians (5), we would like to explore the generation of tolDC in FcGRIIb-deficient mice, which will be helpful in our patients in the future.

In this study, we evaluated the induction of tolDCs in wildtype and FcGRIIb-deficient BMDCs using well-known reagents, including dexamethasone, rapamycin, vitamin D3, and andrographolide. We also assessed the differences between DC activation under various stimuli, including LPS (via TLR4), imiquimod (via TLR7), and CpG (via TLR9). Typically, tolDCs have downregulated expression of T cell-activating molecules such as CD40, CD80,

and CD86, resulting in an inability to prime and activate T cells (125). Moreover, the enriched expression of immune checkpoint molecules, such as inducible T-cell co-stimulator ligand (ICOSL), on tolDCs enables these cells to inhibit effector T cells and trigger Treg induction. Other phenotypic features of tolDCs that mediate immune tolerance are decreased production of pro-inflammatory cytokines such as IL-12, IL-1B, TNF-a, IFN-Y, and IL-23 and increased production of anti-inflammatory cytokines such as IL-10 (126). Based on a previous report that mice lacking the FCGRIIB gene had a defect in tolerance induction via the mucosal route (127), we first hypothesized that these mice might have some difficulties generating tolDC from BMDC compared to control mice. However, our result revealed that this was not the case. All pharmacologic agents can comparably induce tolDC in both mice. In general, we could demonstrate that BMDC from FcGRIIb deficient mice could be induced to tolDC phenotypes comparable to the control mice. This finding suggests that the mechanism of tolerance induction via pharmacological agents might differ from mucosal tolerance induction. It is also possible that these pharmacologic agents are strong tolerogenic induction and can overcome the deficiency of FCGRIIB.

Dexamethasone is a potent reagent to generate potent tolDCs from mouse bone marrow cells and human monocytes when used with GM-CSF and IL-4 (91, 96, 128). Our results confirmed the previous report that tolDCs generated with dexamethasone express very low levels of MHC class II molecules, costimulatory molecules (91, 96, 128, 129). These tolerogenic phenotypes of BMDCs by dexamethasone could reduce the pro-inflammatory cytokine levels. Like other reports, dexamethasone induces mouse BMDC to be tolDC and produce high IL-10 (130). In addition, the inhibitory molecules, ICOSL, were significantly increased in Dex-tolDCs, like the result of Lee and colleagues (128).

Rapamycin inhibited the mammalian target of rapamycin (mTOR) which involved cell growth and survival. The inhibition of mTOR by rapamycin could decrease

immunostimulatory molecules of LPS-stimulated moDCs by NF-κB signaling (131). In addition, rapamycin-treated-DC expressed lower levels of MHC II and CD40 and decreased the production of IL-6 and IL-10 in response to LPS. Moreover, rapamycin-treated DCs could promote CD4+ Foxp3+ Treg in mice models (13). In our results, we found that Rapamycin inhibited the expression of CD40, CD80, and MHCII but did not alter the change expression of CD86. At the same time, tolerogenic marker ICOSL was increased but less than dexamethasone and did not produce IL-10 production.

Vitamin D3 induced tolerogenic phenotypes in both mouse and human DCs by reduction of costimulatory molecules (CD40, CD80, and CD86) and IL-12p70 but increased IL-10 production levels (132). 1,25(OH)2D3, which is vitamin D3 form, could interfere with the transcription factors such as NF-κB and MAP kinases, leading to the cascade signaling to activate the anti-inflammatory effects (133). Vitamin D3 inhibited the maturation of DC phenotypes by reducing CD83 as an activation marker in human monocyte-derived DCs during LPS-induced activation. The inhibitory effects were shown in reducing up-regulation of the costimulatory molecules and MHC class II (12) and induction of Treg cells (134). Moreover, tolDCs generated by vitamin D3 could induce Treg cells and modulate the delayed graft-versus-host disease (135). For the results of VitD3, the expression of immunogenic markers was significantly decreased except for CD86 under LPS stimulation. Meanwhile, ICOSL expression and IL-10 production do not alter after treatment with VitD3.

Andrographolide could inhibit NF-κB activation and promote the tolerogenic properties of DCs. This drug inhibited NF-kB activity to reduce SLE susceptibility in FcgammaRIIb-deficient mice (136). Furthermore, this agent can impair DC maturation and let to reduce antigen-specific T cells in EAE mice (137). In the present study, we found that andrographolide decreased the expression of the immunogenic surface marker but did not

alter CD86 expression. In the case of tolerogenic markers, andrographolide mediated moderate expression of ICOSL but did not produce IL-10 production.

In summary, our findings are like previous studies to generate tolDCs from various models of mice. However, dexamethasone, the most potent stimulus could induce more potential tolerogenic of BMDCs from both mice than other pharmaceutical agents in this study.

We also confirmed that dexamethasone-treated DCs could increase Treg expansion in the DC-T cell co-culture system compared with untreated DCs. After co-culture between dexamethasone-treated DCs from FcGRIIb-deficient mice or control mice and OVA-T cells from WT mice, T cell proliferation was significantly decreased when compared with untreated DCs condition. After we were confident that tolDCs induced in our FcGRIIb-deficient mice were functional, we tested the effect in suppressing clinical manifestation by adoptive transfer experiment.

Previous studies have generated tolDCs via dexamethasone combined with rosiglitazone and CoPP. Their tolDCs were loaded with histone and can reduce the severity and progression of SLE through the reduction of anti-DNA antibodies NZM2410 lupus mice model suggesting that the induction of tolerance to nuclear antigen can be used to treat SLE in the animal model (96). In this study, we generated tolDCs from dexamethasone and pulsed them with specific self-antigens (apoptotic bodies). These tolDCs were adoptive transfer to FcgammaRIIb-deficient mice, and as expected, we can demonstrate the reduction of anti-dsDNA antibodies as well as increase tolerogenic phenotype via increased expression of ICOSL and PD-L1 in DCs from the spleen. In addition, the adoptive transfer of dexamethasone-generated tolDCs can increase Treg expansion (CD4+FoxP3+) and Treg-mediated IL-10 secretion. Previous studies have demonstrated that immunization with nucleosomal histone peptide epitope, H471–94, skewed splenic DCs and pDCs toward

tolerogenic phenotypes, capable of substantial TGF-β production, which fostered Treg expansion and Th17 contraction in lupus-prone mice (138). However, due to the complication of the adoptive therapy experiment, we only conducted a limited study. We did not assay for other effector cells and extensive clinical parameters. This preliminary study revealed a possibility of the induction or modulation of the differentiation of Treg responses in a regulatory defect mouse model and decreased anti-dsDNA antibodies.

Adoptive cell-based therapy using tolDC or Treg is an active field of research as a novel treatment to induce specific tolerance to treat or even prevent autoimmune diseases (139). However, cell-based therapy is a complicated procedure with a high cost and requires a GMP facility, thus limiting the broad access to treatment in the general patient population. There is also a limitation regarding the type of self-antigens used in cell generation. Therefore, non-cell-based therapies are being developed to target DC in the natural environment within the patients. However, it is still difficult to selectively target DCs due to DC heterogeneity and lack of DC-specific molecules for drug delivery. Various methods to target DC based on their cell surface receptors have been explored e.g., Clec9A, DCIR2, DEC205, and Langerin (140). This study explores the novel approach using nano-particle-loaded dexamethasone that preferentially targets DCs as a delivery. This method is simple without the use of specific antibodies.

Dexamethasone is a standard treatment in several autoimmune diseases, including SLE. One of the mechanisms of dexamethasone is likely the generation of tolDCs and Treg *in vivo* (141). However, systemic dexamethasone has various adverse effects. Therefore, the new administration of dexamethasone by targeting DC will likely be more effective as immunomodulation and reduce the systemic side effect of dexamethasone (142).

To enhance the treatment's power and diminish non-immunosuppressive drugs' side effects, drug delivery systems based on nanoparticles are applied as an alternative solution for

the target delivery of precision medicine. Previous studies exploiting nanoparticles to target multiple DCs subset and mediated tolerogenic phenotype represents a promising strategy to mediate antigen-specific immune tolerance in autoimmune patients (14). Most studies have synthesized PLGA-NPs by single emulsion/solvent evaporation method, but in this study, we modified the method from PLGA-NPs synthesis to generate PDMAEMA-PLGA-NPs (16). Because PDMAEMA usage is limited by the lack of biodegradability in *vivo* model (143), since PDMAEMA has an immune suppressive effect, if not degradable, it might cause immunosuppression and increase the risk of infection or tumorigenesis. Previous studies have reported that PLGA is a biocompatible and biodegradable polymer (144). PLGA is hydrolyzed to lactic acid and glycolic acids in the body and eliminated from the body via the citric acid cycle. However, PLGA mainly mediates immunogenic DC function and is inappropriate for the autoimmune disease model. Therefore, in this study, we aimed to combine both polymers leading to decrease concentration and improved biodegradable properties of PDMAEMA. We also aim to use the inhibitory effect of PDMAEMA to reduce the immunogenic properties of PLGA. Based on our result, our combination can induce tolDCs and lead to clinical improvement.

The appropriate size of NPs for active internalization by DCs has been reported around 500 nm (145). It has been shown previously that DC can be targeted based on particle size. Previous studies have demonstrated using the injected subcutaneously nanoparticle-conjugated with red fluorescent at size 20 and 500 nm on the footpad of WT and DT mice (Depletion of DCs). They found that the popliteal lymph node of WT mice showed the intensity of red fluorescent at 20 and 500 nm of nanoparticle; in contrast, the popliteal lymph node of DT mice showed a low intensity of red fluorescent at 500 nm. Interestingly, DT mice can represent the red fluorescent intensity of 20 nm nanoparticles. Therefore, 500 nm might be specific to the DC population, and the smaller size of the nanoparticle might be specific to

other phagocytic cells, such as macrophages. In our present work, the particles at 500 nm were proven to target the DC population specifically.

We found that Dex-NPs is shown to have high stability at low temperature, which is suitable for extended storage. In contrast, at 37 C, drugs were released faster at 21 to 28 days of incubation than NPs kept at low temperatures. Previous studies have reported that dexamethasone-loaded PLGA NPs released free dexamethasone at 5-60% of *in vitro* study systems (146). However, there is no direct evidence to define the release of dexamethasone from PDMAEMA NPs.

Interestingly, our data blank NPs have the immunosuppressive effect on the activation of DCs with pro-inflammatory cytokine but do not alter tolerogenic phenotype in BM-cDCs. In the case of Dex-NPs, it improved the tolerogenic induction ability of both PDMAEMA-PLGA NPs and dexamethasone, the ability to induce ICOSL and PD-L1 expression, and the high IL-10 production. However, there is no direct evidence for the effect of dexamethasone on the expression of ICOSL and PD-L1 in DC. A few reports in other cell types demonstrated dexamethasone's down-modulation of ICOSL and PD-L1 (147). Our data also showed that dexamethasone interfered with the expression of PD-L1 (all doses of dexamethasone) and ICOSL expression (2 μM dexamethasone).

In addition, our PDMAEMA-PLGA NPs produced a higher result in the induction of PD-L1 expression. Previously, dexamethasone-incorporated PLGA NPs could not induce PD-L1 expression on DCs (148). Usually, high IL-10 production was detected in GM-CSF-derived BM-DCs treated with dexamethasone. However, dexamethasone could not cause IL-10 production in the BM-cDC system, which indicated the different characteristics of both DCs. Our study in the cDC system demonstrated that PDMAEMA-PLGA NPs could induce IL-10 production in BM-cDCs, and dexamethasone into PDMAEMA-PLGA NPs further enhanced IL-10 production. Furthermore, a previous report revealed that IL-10 production *in*

vivo increased by treatment with a low dose of dexamethasone (149). Therefore, it is likely that the slow release of drugs controlled by PDMAEMA-PLGA NPs may lead to a lower amount of dexamethasone, which consequently affects enhanced IL-10 production in BM-cDCs.

Next, In the case of BM-cDCs from *Fcgr2b-/-* mice, blank NPs and Dex-NPs decreased the hyperactivation of these DCs by reducing the expression of immunogenic markers and inflammatory cytokines production. Furthermore, Dex-NPs efficiently altered *Fcgr2b*$^{-/-}$ BM-cDCs from immunogenic to tolerogenic phenotypes by enhancing the expression of ICOSL and PD-L1 and the production of IL-10. Over the past decades, several pharmaceutical agents have been used to generate tolerogenic DCs derived from monocytes obtained from patients with autoimmune diseases, such as SLE and rheumatoid arthritis. However, combinations of dexamethasone with other agents are mainly used because dexamethasone alone is ineffective in switching hyperactive phenotypes of patient DCs to tolerogenic phenotypes (150). Our study demonstrated the improved efficacy of dexamethasone by PDMAEMA-PLGA NPs; therefore, the delivery of PDMAEMA-PLGA NP may offer an alternative strategy to modulate the aberrant DC functions of DCs *in vivo*.

DCs expressing ICOSL and IL-10 induced Treg expansion with potent inhibitory function through high IL-10 production (151). PD-L1 expression in DC is required for the development and immunosuppressive function of Treg (152). However, the BM-cDCs pretreated with dexamethasone did not produce IL-10; the coexpression of ICOSL and PD-L1 in company with immature phenotypes (low expression of immunogenic molecules) may be these mechanisms that lead to expansion and IL-10 production of Treg. The pretreated BM-cDCs with Dex-NPs also exhibited the immature phenotype, but the DCs expressed ICOSL and high IL-10 PD-L1 along with increased production, possibly resulting in Treg expansion. It is noteworthy that Dex-NP pretreated BM-cDCs could mediate Treg expansion better than

dexamethasone pretreated BM-cDCs. This result may be due to the difference in IL-10 production induced by dexamethasone and Dex-NPs since the induction of Treg differentiation by DCs required IL-10 signaling.

In this study, we aimed to induce tolerogenic DCs in situ using immunomodulatory NPs incorporating dexamethasone to treat SLE. Since DCs constitute a significant population in the skin, NP administration via the subcutaneous route may provide precise delivery to DCs. Our data also suggested that PDMAEMA-PLGA NPs had high efficiency in entering skin DCs; therefore, *in vivo* Dex-NP delivery may specifically target DCs. Our findings demonstrated that the DC-targeted therapy with Dex-NPs produced favorable therapeutic effects in the lupus-prone mouse model, similar to the non-targeted treatment with dexamethasone. Interestingly, our result showed increased uptake of nanoparticles in the CD3 cells as well; we hypothesized that it might be due to gamma delta T cells. These cells have a unique characteristic that can phagocytose foreign antigens (153). Therefore, this population of T cells might be engulfed in nanoparticles and migratory to the lymph node. However, further characterization needed to be done to confirm this hypothesis.

Although we did not investigate the dexamethasone toxicity, the intravenous administration of dexamethasone-incorporated polymeric NPs in MLR/lpr lupus mouse model endorsed that treatment with dexamethasone-incorporated polymeric NPs exhibited lower cytotoxicity when compared to conventional treatment with dexamethasone (154). The comparative treatment effects were observed in traditional dexamethasone therapy, which broadly affected several cells, including other immune cells and parenchymal cells in several organs, and Dex-NP therapy, which mainly selectively targeted DCs (and partially macrophages) (16). These data suggested the importance of innate immune cells, especially DCs, in lupus disease, which is a disease of aberrant functions of the innate and adaptive immune cells. The specific interference of DC functions, which control the fate of adaptive

immune responses through T helper cells, potentially attenuated lupus nephritis (reduced anti-dsDNA, proteinuria, and renal injury). Therefore, the use of anti-inflammatory effects against all cells in the body by conventional dexamethasone therapy, which in turn causes serious complications, may be immoderate. Therefore, DC-targeted therapy in lupus may be an interesting new treatment with fewer steroidal complications.

To further validate the superior therapeutic efficacy of Dex-NPs, the alterations of DC phenotypes and the Treg population were investigated. The *in vitro* restimulation assay substantiated that the increased FoxP3^{+} Tregs in lupus mice treated with Dex-NPs were induced in an antigen-specific manner, and this Treg expansion was probably driven by DCs since the direct *in vitro* interaction between the BM-cDCs pretreated with Dex-NPs and Treg led to the substantial Treg expansion. The observation of the tolerogenic phenotypes of DCs in *Fcgr2b*$^{-/-}$ mice revealed similar abilities of dexamethasone and Dex-NPs in the induction of ICOSL and PD-L1 expression; however, the outcomes of Treg responses were different. The substantial number of Tregs in Dex-NPs treated lupus mice may primarily depend on IL-10 production by DCs since Dex-NPs, but not dexamethasone, induced the high IL-10 production in both WT and *Fcgr2b*$^{-/-}$ DCs. As mentioned above, IL-10 production by DCs is essential for the development and expansion of FoxP3^{+} Tregs (155).

As the balance between immune effectors and regulators is critical for immune homeostasis and the control of autoimmunity, either exacerbated effector responses or insufficient immune regulation can lead to inflammatory diseases and autoimmune disorders (155). Thus, approaches to increasing the number and function of Treg cells can provide significant benefits to patients with autoimmune conditions. Tolerogenic DCs promote central and peripheral tolerance through various mechanisms, including Treg induction (20). Here, we demonstrate the successful reprogramming of *Fcgr2b*$^{-/-}$ DCs to tolerogenic phenotypes both *in vitro* and *in vivo* by exploiting PDMAEMA-PLGA NPs incorporated with

dexamethasone. The use of the PDMAEMA-PLGA NP platform possibly allowed the precise targeting of DCs, and the controlled release of PDMAEMA-PLGA NPs can enable the tolerogenic induction properties of dexamethasone. This selective targeting of DCs promoted antigen-specific immune tolerance via the induction of Treg expansion and consequently led to a potent amelioration of lupus disease.

Previous studies found that Fcgr2b polymorphism with SLE in the Thai population is around 15%, which did not represent the majority populations of SLE (1). This research generated tolDCs under Fcgr2b knockout mice by using dexamethasone alone or dexamethasone-incorporated PDMAEMA-PLGA NPs. The next question is whether this method can be applied to other mice models with different genetic defects. Based on previous studies, Dexamethasone has been used to generate tolDCs successfully in various models. For example, dexamethasone in combination with HO-1 inducer (HO-1 or rosiglitazone generated tolDCs leads to a decrease in the clinical score of lupus mice model (MRL-Fas^{lpr}) and suppressed anti-histone antibodies in NZM2410-chronic lupus mice (96). The following example was dexamethasone loaded with self-antigen (MOG) into microparticle-suppressed disease-associated cytokine in the multiple sclerosis model (156). Moreover, polymeric dexamethasone attenuated lupus nephritis in MRL/lpr mouse model without cytotoxicity (154). Therefore, we propose that Dex-NPs should be able to generate tolDCs in other models of lupus or autoimmune diseases. First, however, we need to further explore this approach's success rate in clinical manifestation *in vivo*

APPENDIX

Cell culture

Media for cell culture

1. RPMI (no L-glutamine)
2. 10% Fetal bovine serum
3. 2 mM Glutamax
4. 1% Penicillin-streptravidin
5. 10 ng/ml GM-CSF
6. 10 ng/ml IL-4
7. 200 ng/ml FLT3L

Tolerogenic inducing agents

1. Dexamethasone
2. Rapamycin
3. Vitamin D3
4. Andrographolide

Flow cytometer staining

Dry	Stock Conc.	Final Conc.	1 Reaction
CD11c+ (APC)	0.2 mg/ml	1.25 ug/ml	0.6 ul
CD40 (PE/cy7)	0.2 mg/ml	2.5 ug/ml	1.25 ul
CD80 (FITC)	0.5 mg/ml	5 ug/ml	1 ul
CD86 (PE)	0.2 mg/ml	1.25 mg/ml	0.6 ul
MHCII (APC/Cy7)	0.2 mg/ml	0.625 ug/ml	0.3 ul
ICOSL (PE)	0.2 mg/ml	4 ug/ml	2 ul
PDL-1 (PerCP/cy5.5)	0.2 mg/ml	4 ug/ml	2 ul

FACS buffer : 1X dPBS, 1% FBS, 0.1% sodium axide

ELISA

Wash buffer:

Chemical	ปริมาณที่ต้องใช้
1. 1X PBS	6000 ml
2. Tween-20	3 ml

Assay diluent:

Chemical	ปริมาณที่ต้องใช้
1. BSA	10 g

Antibody conditions:

Solution	Stock Conc.	Final Conc.
Capture IL6	0.5 mg/ml	2 ug/ml
Detection IL6	0.5 mg/ml	1 ug/ml
Capture TNFa	0.5 mg/ml	2 ug/ml
Detection TNFa	0.5 mg/ml	0.5 ug/ml
Capture IL-1B	200X	1X
Detection IL-1B	200X	1X
Capture IL10	200X	1X
Detection IL10	200X	1X
Capture IL12	0.5 mg/ml	2 ug/ml
Detection IL12	0.5 mg/ml	0.5 ug/ml
Capture IL23	250X	1X
Detection IL23	250X	1X
HRP-Avidin	1000X	1X
High sensitivity TMB		

Treg isolation

MS column and Treg isolation kit

Degas buffer: 1X dPBS, 0.5% BSA, 2 mM EDTA

Soluble anti-CD3 antibody, rmIL-2

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